MARMARA ÜNİVERSİTESİ YAYINEVİ

BASIC AND APPLIED BIOCHEMISTRY

Second Edition



Prof. Dr. Nesrin EMEKLİ

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BASIC AND APPLIED BIOCHEMISTRY

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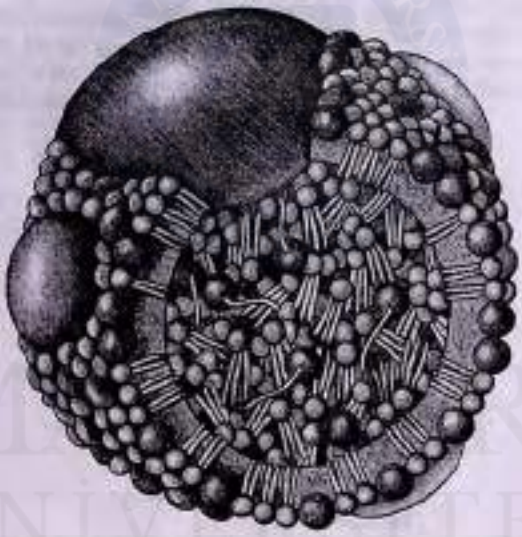

Prof. Dr. Nesrin EMEKLİ

2004
NOBEL TIP KİTABEVLERİ

MARMARA ÜNİVERSİTESİ

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BASIC AND APPLIED BIOCHEMISTRY

Prof. Dr. Nesrin EMEKLİ

ISBN: 975-420-316-8

Bu kitabın 5846 ve 2936 sayılı Fikir ve Sanat Eserleri Yasası Hüktmleri gereğince (kitabın bir bölümünden alıntı yapılamaz, fotokopi yöntemiyle çoğaltılamaz, resim, şekil, şema, grafik vb.ler kopya edilemez) her hakkı yazarına aittir.

NOBEL TIP KİTABEVLERİ LTD. ŞTİ.

Merkez: Millet Cad. 111 Çapa-İstanbul
Tel: (0212) 632 83 33 (PBX)
Fax: (0212) 587 02 17

Şube: Cerrahpaşa Tıp Fakültesi Karşısı
Cerrahpaşa-İstanbul
Tel: (0212) 586 17 58

Şube: Kadıköy Rıhtım Cad. Derya İş Merkezi No. 7
Kadıköy-İstanbul
Tel: (0216) 336 60 08

Ankara: MN Medikal & Nobel Tıp Kitap Sarayı
Halk Sok. No 5. Sıhhiye Ankara
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İzmir: Güven & Nobel Tıp Kitabevleri
SSK İş Hanı, P. 38 Konak-İzmir
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Adnan Kahveci Bulvarı 31/C Adana
Tel: (0322) 233 00 29

Samsun: Nobel Tıp Kitabevi
Uluğazi Mah. 19 Mayıs Bulvarı/Samsun
Tel: (0362) 435 08 03
Fax: (0362) 435 04 90

Düzenleme : Nobel Tıp Kitabevleri Ltd. Şti.
AKAY, M.C.
Baskı / Cilt : Nobel Matbaacılık

T.C.
Marmara Üniversitesi
Dokümantasyon ve Doküman...
Daire Başkanlığı
DEMİRBAŞ NO: M00924G9

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PREFACE TO THE SECOND EDITION

This edition includes lipids in addition to nutrition digestion and absorption, carbohydrates, proteins and hemostatic sistem biochemistry.

A new chapter in this edition is part IV. It contains lipids which are more compleks component in biochemistiry. Accounts are also given of recent advances in relation to lipid and atherosclerosis.

I wish to acknowledge the great patience my husband and my children, understanding and encouragement while I have been writing this book. I would also like to thank Mr Ian Mc Lure a member of our Faculty who assisted with the English text and the Staff of the Marmara University Technical Education Faculty for their help and thanks are also due to the publishers Nobel Company.

Nesrin Emekli

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MARMARA ÜNİVERSİTESİ 1883
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PART I

NUTRITION DIGESTION AND ABSORPTION

A - Nutrients, - what they do and where they are found

Nutrition is one of the most important factors in achieving good health. Good health does not only mean being free of disease; it also means having a sense of vitality and well-being. Nutrition is important for physical fitness and mental alertness. Eating the proper foods will helps one to feel better, think better and work better.

The science of nutrition involves the qualitative and quantitative of the diet necessary to maintain good health. Virtually all components of the diet are needed to maintain life. Metabolic biochemistry provides much of the understanding of modern concepts of nutrition and will be discussed in later chapters of this book. With the progress of science and technology, the cellular role of nutrients has become more clearly delineated and the importance of nutrition, both as a vital component of health and in minimizing the effects of health and in minimizing the effects of disease, has become more apparent.

"Tell me what you eat, and I will tell you what you are" this is a true quotation for the food we eat is responsible for our growth from infancy to adulthood and the constant renewal of our body. The body is continually replacing many of its parts. The outer layer of the skin is shed and renewed; Nails grow for and our entire life, haire grow, falls out, and new hair grows; blood and tissue cells are replaced continually if we eat the right foods or nutrients in the proper quantities, we will have a very good chance of fighting off diseases and staying healthy. In more deprived populations, overt nutritional deficiencies are more wide spread, eg. deficiency in proteins' (kwashiorkor), in vitamins (vitamin A in xerophthalmia), in minerals (iron, giving rise to anemia) and of energy (starvation). On the other hand, in developed societies, some diseases have always been associated with dietary excesses eg. atherosclerosis and coronary heart disease, diabetes, cerebrovascular disease and strokes. Cancer of the breast, stomach, colon, and cirrhosis of the liver may be related to excess or harmful nutrients.

Nutritional requirements for the body are presently definable. Approximately forty dietary components and elements are now recognized

as essential for humans. However additional minerals may be required in such small amounts that is not yet known whether they are essential. Proteins, carbohydrates and lipids are called macronutrients. These are the structural and functional components of the cells and are sources of energy. Vitamins and minerals are called Micronutrients these are components of enzymes or cofactors needed for metabolic reactions.

Recommended dietary allowances (RDA) have been established as goals for good nutrition. RDA's for energy are categorized by age and sex. Energy needs per kg of body weight are highest for the young infant and after age ten they are generally higher for males than females. For young adults, the allowances are set for individuals doing light work; for example, for males 19-22 years of age, the RDA is 2900 400 k cal. Allowances for older groups are set to compensate for a 2 % decrease per-decade in basal metabolic rate (BMR) and for a reduction in activity.

The basal metabolic rate is the standard lowest level of energy consumption. It is determined with the subject at rest but not sleeping, lightly clothed at a comfortable tempereture, and at least 12 hours after his last meal. Thus the BMR represents the energy needed for the functioning of the organs of the body, but not for any external muscular work or the assimilation of food. The units used in the literature for BMR are most commonly kcal per square metre of body surface perhour, although the kj is the preferred unit of energy. The highest Basal Metabolic Rate is observed between 1 to 5 year old children, the lowest by the age of 75. For the average man with a BMR of 40 kcal/m^2/h an a surface area of 1.8 m^2, the daily basal metabolism will be 40 x1.8 x 24 = 1728 kcal. Taking in food increases energy utilisation of the body an amount depending on the nature of the food. This effect is the Specific Dynamic Action (SDA) of the diet, and an average meal of mixed composition in western countries has an SDA of about 10 % of the BMR. Therefore, allowing 10 % for the SDA of the diet, the average BMR for the man mentioned above becomes 1900/kcal.

The caloric value of the various food components is 4.1 kcal/g (17.1 kj/g) for carbohydrates, and 9.3 kcal/g= (39. kj/g) for fats. Fats being more reduced than carbohydrates, requires more oxygen per gram to burn them to carbon dioxide and water, and therefore release more energy per gram. Protein containing amino acid sidechains which are more oxidised than carbohydrates in some cases but more reduced in others. The hydro-

gen atoms of the amino groups of proteins are not oxidised in the body. The caloric value of proteins is equal to that of carbohydrates. In western countries an energy requirement of 2900 kcal/day might well be supplied by 400 g of carbohydrate, 100 g of fat and 100 g of protein. Daily recommendations for calorie allowance are listed in Table 1.

TABLE 1: Recommended energy intake for men and women.

Category	Age (years)	Weight (kg)	Energy Needs (kcal)	
			Mean	Range
Men	23-50	70	2900	2300-3100
Women	23-50	55	2200	1600-2400
Pregnant			+300	
Lactating			+500	

TABLE 2: Approximate calorie expenditure of boys
(Calories per day)

Age, years	Basal metabolism	Very quiet boy	Active boy	Very active boy
0	200			
1	500	750		
2	800	1200	1600	2350
4	900	1400	1860	2800
6	1100	1600	2160	3230
8	1200	1800	2400	3630
10	1300	2000	2640	3950
12	1440	2130	2870	4300
14	1470	2200	2950	4400
15	1550	2300	3100	4620

TABLE 3: Approximate energy expenditure with different activities

Activity	Cal/h	Activity	Cal/h
Dressing and undressing	33	Mental work	7-8
Sitting at rest	11	Mixed carpentry work	180
Standing relaxed	24	Sawing wood	420
Standing stiffly	20-30	Rapid typing	16-40
Walking	180-270	Coal mining (average)	320
Running	500-930	House painting	160
Singing	37	Tailoring	44
Reading aloud	20	Bookbinding	51
Dishwashing	59	Riveting	276
Ironing	59	Cycling	180-300
Sewing	25-30	Swimming	200-700
Sweeping	110	Climbing	200-960
Writing	10-20	Rowing	120-600
Knitting	31	Wrestling	960

Nutrients are divided into six classes: Carbohydrates, fats, proteins, vitamins, minerals and water. Let us examine these individually:

CARBOHYDRATES

The necessity for carbohydretes in the diet is often underestimated by people who are weight-conscious in the belief that they are too fattening. This, however, is only partly true. The main function of carbohydrates in the diet is to provide energy. However they are also used to form many compounds in the body. Plants that contain chlorophyll (The substance that gives them their green color) are the main source of carbohydrates which plants form by using energy from the sun to combine carbon dioxide from the air and water from the soil.

Because carbohydrates are an essential for life, no living creature can exist without eating plants. Even animals that eat only meat get their carbohydrate supply by eating the flesh of animals that live on plants. Animals themselves can synthesize some carbohydrate from fat and protein, but the bulk of animal carbohydrate is derived from plants.

The sugar **glucose** is the most important carbohydrate. Dietary glucose is absorbed into the bloodstream. It is a major fuel of mammalian tissue. Glucose is converted to other, functional highly specific, carbohydrates eg, glycogen for storage (see p.157) ribose in nucleic acids and galactose in the lactose in milk. It is also used in certain complex lipids and in combination with protein as glycoprotein and proteoglycan (see p.151). Diseases associated with carbohydrates include; diabetes mellitus (see p.212), glyrogen storage diseases (see p.164), galactosemia and milk intolerance (see p.202).

Foods supply carbohydrates in three main forms; starches, sugars and cellulose (fiber). Starches and sugars are the main sources of energy for human beings. Cellulose is one of the food materials that cannot be digested. But it is required for optimal health in herbivores. Such as ruminants, fiber (mainly as cellulose) is a major sources of energy after being digested by microorganisms to acetates propionates and butyrates which are absorbed in to the portal vein. Gases such as CH_4, CO_2 and H_2 are also produced.

In humans although cellulose cannot be digested, it provides bulk in the diet. It has beneficial effects of aiding water retention during passage of food along the gut and therefore producing larger, softer faeces. A high-fiber diet is associated with reduced incidences of cancer of the colon, cardiovascular disease, and diabetes merlutus. The more insoluble fibers such as cellulose and lignin found in wheat bran are beneficial with regard to colonic function, whereas the more soluble fibers found in legumes and fruit, eg, gums and pectins, lower blood cholesterol, possibly by binding bile acids and dietary cholesterol. The soluble fibers also slow stomach emptying, and delay the postprandial rise in blood glucose, with consequent reduction in insulin secretion.This effect is beneficial to diabetics.

The entire population of the world obtains 70 percent of its energy from carbohydrates, because most peoples cannot afford foods containing large amounts of the other nutrients, proteins and fats. However in the United States carbohydrates provide 40 to 50 percent of the total food energy. Good sources of carbohydrates are grains (such as corn, oats, wheat, and rice) products made from grain (such as macaroni, breads, noodles, grits), and potatoes, dried beans, and peas. Carbohydrates can also be obtained from other vegetables and from fruits. In vegetables, the

carbohydrates are mainly in the form of starches, in fruits, they are chiefly sugars. Cane, jams, candy and other sweets, honey, molasses and syrups are concentrated sources of sugar in our diet. Meats also contain carbohydrate in the form of glycogen, which is animal storage glulcse. Sucrose, which is common table sugar, is produced mostly from sugar cane and sugar beets. Since this is refined sugar, it contains no vitamins, minerals, or any other food substances. For this reason refined sugar is sometimes called "empty calories" Being just a source of calories. The recomended daily intake of carbohydrate is 50 - 100 g to prevent ketosis and loss of muscle protein. However a balanced diet should contain more polysaccharide (such as starch and glycogen) in order to reduce the amount of fat that would otherwise be required for energy.

FATS

The body changes some fats into compounds called lipids. Although the main nutritional function of carbohydrates is to provide energy for the body, lipids are even more concentrated sources of energy, giving twice as much energy as either carbohydrates or proteins. Yet this is not an essential function, lipids are required as a vehicle for lipid-soluble vitamins and to supply essential fatty acids.

Lipids are composed mainly of fatty acids. Each fatty acid is made up of carbon atoms joined like links in a chain. The carbon chains vary in length, most of them containing 4 to 24 carbon atoms. When every carbon atom in the chain has attached to it as many hydrogen atoms as it can hold, the fatty acid is said to be saturated. When a hydrogen atom is missing from two neighboring carbon atoms, a double bond forms between them, and the fatty acid is said to be unsaturated, A fatty acid that contains more than one such double bond is called polyunsaturated.

Three polyunsaturated fatty acids have been recognized as essential in the diet of some animals: Linoleic acid (18:2), Linolenic acid (18:3), and arachidonic acid (20:4). These acids are found in the lipids of plant and animal food (Figure 1). In humans, arachidonic acid may be formed from linoleic acid and is not essential if sufficient linoleic acid is present in the diet. The most important polyunsaturated fatty acid is therefore linoleic, acid. The body cannot manufacture this acid, so it must be obtained from food. A lack of this fatty acid causes the skin irritation and reddening and abnormalities of the liver to develop, especially in very young children.

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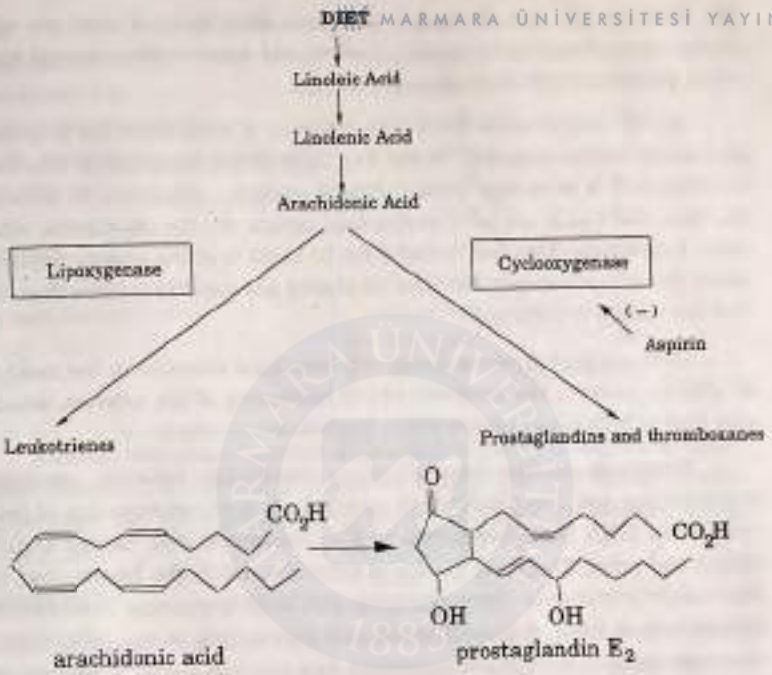

Figure 1 : Fate of essential fatty acid in diet

When young rats are fed on a fat-free diet which is otherwise nutritionally adequate, growth declines and stops, and an eczamatous scaliness of the skin develops. This condition can be prevented by vegetable seed oils or by one of these three unsatunated fatty acids. A principal function of essential fatty acids is to serve as precursors of leukotrienes, prostaglandins, and thrembozanes, which function as local hormones. A dietary intake in which 1-2 % of the total energy requirement is supplied as essential fatty acid prevents clinical deficiency.

Linoleic acid is found in sizable quantities in many plant oils, such as corn, cotton seed, soybean, sesame, safflower, and wheat-germ oils. The difference between a fat and an oil is that an oil is liquid at ordinary temperatures and a fat is solid. Margarine is made from some of these

oils. Walnuts are high in linoleic acid, but other kinds of nuts are not. Poultry fat and fish fat have more linoleic acid than do other animal fats, which rank fairly low as sources of this acid.

As fat can be made from carbohydrate, a lower limit for it in the diet would not be expected. In the Far East fat consumption is very low (as little as 2 % of energy needs) without apparent disability, in spite of the fact that lipids act as transporting agents for the fat-soluble vitamins. In western diets fats account for 20 to 30 % of the energy requirement. But it is recommended that fat should not constitute more than 30 % of the energy requirement.

Over a period of years, lipids can form lipid deposits on the insides of arteries, causing the condition called hardening of the arteries, which may lead to high blood pressure and possible heart attack.

Numerous studies have shown a correlation between coronary heart disease and blood cholesterol particularly from consumption of saturated fat. High fat consumption is also associated with cancer of the breast and colon. The main source of saturated fat in the human diet is the meat of ruminants, dairy products, and hard margarine. Cholesterol is found only in foods of animal origin and particularly in egg yolk. Many scientists believe that polyunsaturated fats are less likely to be turned into lipids and therefore may help to prevent heart attack and high blood pressure.

TABLE 4: Cholesterol content of common measures of selected foods

Food	Amount	Cholesterol, mg
Milk, skim, fluid, or reconstituted dry	1 cup	5
Cottage cheese, uncreamed	1/2 cup	7
Lard	1 tbsp	12
Cream, light table	1 fl oz	20
Cottage cheese, creamed	1/2 cup	24
Cream, half and half	1/4 cup	26
Ice cream, regular, approximately 10% fat	1/2 cup	27
Cheese, Cheddar	1 oz	28
Milk, whole	1 cup	34

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Butter	1 tbsp	35
Oysters, salmon	3 oz, cooked	40
Clams, halibut, tuna	3 oz, cooked	55
Chicken, turkey (light meat)	3 oz,cooked	67
Beef,pork, lobster, chicken, turkey (dark meat)	3 oz, cooked	75
Lamb, veal, crab	3 oz, cooked	85
Shrimp	3 oz, cooked	130
Heart, beef	3 oz, cooked	230
Egg	1 yolk or 1 egg	250
Liver, beef, calf, hog, lamb	3 oz, cooked	370
Kidney	3 oz,cooked	680
Brains	3 oz, raw	More than 1700

Another important function of fats in the diet is to carry the lipid-soluble vitamins, A,D,E and K. Fats also make foods and meals satisfying, because they are digested slowly and delay a feeling of hunger before the next meal.

PROTEINS

Proteins are the basic constituents of every cell in the body. Infact they are the chief tissue constituent. They are fundamental in the formation of muscles, hemoglobin, all hereditary characteristics in body cells, and thousands of enzymes, hormones which regulate chemical processes in the body. Proteins also form antibodies that can prevent diseases caused by foreign proteins (such as bacteria and viruses) that enter the body. Proteins supply energy when the diet does not provide enough carbohydrates and fats. Proteins differ from fats and carbohydrates in that there are clearly definable limits below which consumption should not fall. In western countries proteins suply 10 to 15 % of the caloric value of the diet. There is no specific reserve tissue for protein but permanent loss can occur to all parts of the body, in varying proportions. Such depletion renders the body more sensitive to toxic agents and infection making proteins in the diet very important, especially for infants.

Proteins are broken down into amino acids during digestion. These are then rearranged to make enzymes, hormones, and other special proteins. The proteins in foods are made up of 20 amino acids. The body can make its own supply of more than half of the needed amino acids, but the rest, called essential amino acids, must come ready made from food.

Essential amino acids, whose side-chains contain aromatic and long or branched aliphatic groups, can be synthesised in plants and bacteria. Nutritionally essential amino acids in humans, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine.

Histidine and arginin are semiessential amino acids since their synthesis in the human body is in adequate two other amino acids, cysteine and tyrosine may be formed from the essential amino acids metionine and phenylalanine, respectively.

Nutritionally nonessential amino acis are; glycine alanine, aspartate, glutamate, glutamine, serine, proline, hydroxyprolin, hydroxylysine, asparagin, cystein and tyrosine.

TABLE 5: Amino acids in human nutrition

Essential amino acids	Recommended daily intake, g	Minimum daily intake (men), g	Minimum daily intake (women), g
L-Phenylalanine	2.2	1.10	0.220
L-Methionine	2.2	1.10	0.290
L-Leucine	2.2	1.10	0.620
L-Valine	1.6	0.80	0.650
L-Lysine	1.6	0.80	0.500
L-Isoleucine	1.4	0.70	0.450
L-Threonine	1.0	0.50	0.310
L-Tryptophan	0.5	0.25	0.160

Nonessential amino acids

Glycine	Proline
Alanine	Hydroxyproline
Serine	Citrulline
Cystine	Histidine
Tyrosine	Arginine
Aspartic acid	Norleucine
Glutamic acid	Hydroxyglutamic acid

Nitrogen balance is maintained by the dietary intake of amino acids. Nitrogen is lost in the urine, feces, saliva, skin, hair and nails. The daily recommended diatary allowances of amino acids are given in Table 5 Infants, children and those pregnant, lactating, undergoing tissue repair after injury, recovery from illness and engaged in increased physical activity need more dietary protein. The requirements for infants of essential amino acids in terms of mg/kg body weight per day is 8 to 15 times higher than that for an adult male. Foods vary in their amino acid composition. Animal proteins are called first class proteins" and vegetable proteins have been termed "second class proteins" because vegetable proteins are often deficient in essential amino acids.

The chief sources of protein are meat, poultry, fish, milk, eggs, cheese, beans, peas and nuts. Breads, cereals, vegetables, and fruit contain relatively smaller amounts of protein. In particular, cereal proteins are very deficient in lysine. However legumes, especially soybeans and peanuts provide proteins almost as good as those from animal tissues. Combining a small amount of animal protein-eggs, milk, cheese with cereals added to meat or fish and vegetables will help to provide sufficient protein at a lower cost. Example of tasty combinations are milk and cereal, macaroni and cheese, beans and peas with corn, beans with rice, and peanut butter with wheat as bread. Suitable combinations of vegetable proteins complementing one another in amino acid composition will approach the amino acid composition of animal proteins.

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TABLE 6: Nutritive Values of Some Foods (Adapted from ref 1, 9, 10)

Meat Group	Weight (g)	Water (%)	Calori (kj)	Protein (g)	Carbohyd (g)	Fat (g)	Chol.* (mg)
Veal (Fatty)	100	65	223	18	0	16	90
Mutton (Fatty)	100	56	310	15	0	27	70
Beef (lean)	85	54	245	18	0	19	99
Liver (Veal)	100	70	140	19	4.1	4.7	300
Sausage	100	63	203	20	0	14.0	48
Sausage (garlic)	100	66	179	21	0	11.0	-
Pastrami	100	42	250	30	-	14.0	-
Heart (Veal)	100	76	124	15	1.8	6.0	-
Kidney (Lamb)	100	77	105	16	0.9	3.3	375
Brain (All)	100	79	125	10	0.8	8.6	2000
Salami	100	30	450	24	1.2	38.0	-
Chicken							
White (with skin)	100	68	196	20	0	11.0	67
White (without skin)	100	75	114	23	0	1.7	58
Fish and Shellfish							
Fish (Turbot)	100	71	193	15	0	14	85
Sardine	100	71	160	19	0	8.6	-
Shrimp	100	78	91	18	1.5	0.8	-
Lobster	100	78	91	17	0.5	1.9	-
Meat substitues and fatty seeds							
Beans (white)	100	11	340	22	61.3	1.6	0
Soybean	100	10	403	34	33.5	17.7	0
Broad bean	100	12	338	25	58	1.7	
Lentil	100	11	340	24.7	60	1.1	0
Chickpea	100	10.7	340	20.5	61	4.8	0
Hazelnut	100	5.8	634	12.6	18	62	0
Peanut	100	1.8	582	26.2	20.6	48.7	0
Almond	100	4.7	598	18.6	19.5	54.2	0
Sunflower	100	4.8	560	24	19.9	47.3	0
Sesame	100	5.5	582	18.2	17.6	53.4	0
Pumkin-seed	100	4.0	600	30	10.0	50.0	0
Chestnut (roasted)	100	40.5	245	32	53.0	2.2	0
Walnut	100	3.5	651	14.8	15.8	64.0	0

(-) - represents unknown
(0) - represents no values
Chol : Cholesterol

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TABLE 6: (continue)

	Weight (g)	Water (%)	Calori (kj)	Protein (g)	Carbohyd (g)	Fat (g)	Chol.* (mg)
Vegetable Group							
Beans (green)	125	92	30	2	7	trace	0
Peas	100	78	84	6.3	14.4	0.4	0
Mushroom	100	90	28	2.7	4.4	0.3	0
Spinach	100	91	26	3.2	4.3	0.3	0
Eggplant	100	92	25	1.2	5.6	0.2	0
Potatoes	100	80	76	2.1	17.1	0.5	0
Parsley	100	85	44	3.6	8.5	0.6	0
Lettuce	454	96	80	4.0	13.0	trace	-
Corn	140	74	70	0.3	16	trace	0
Cauliflower	454	96	60	4.0	13	trace	0
Cabbage (white)	100	92	24	1.3	5.4	0.2	0
Cabbage (black)	100	88	46	4.0	5.0	0.3	0
Peppers green	100	93	22	1.2	1.3	0.2	0
Onions (cooked)	210	92	60	3.0	14.0	trace	0
Turnips	155	94	35	1.0	8.0	trace	0
Tomato (raw)	100	93	22	1.1	4.7	0.2	0
Cucumber	50	96	5	trace	2	trace	0
Celery (raw)	40	94	5	trace	2	trace	0
Carrots	100	88	42	1.1	9.7	0.2	0
Artichoke	100	86	53	3.0	7.8	0.2	0
Okra	100	90	36	2.4	7.6	0.3	0
Peppermint	100	83	65	4.0	7.9	1.3	0
Garden rocket	100	90	33	3.0	3.2	0.6	0
Garlic	100	61	137	5.2	30.8	0.2	0
Leek	100	85	52	2.2	11.2	0.3	0
Purslane	100	91	32	2.0	3.8	0.4	0
Milk group							
Butter milk	245	90	90	9	12	trace	7
Whole milk	244	87	160	9	12	9	14
Cheese (Cheddar)	28	37	115	7	1	9	-
Yogurt	245	88	150	7	12	8	13
Milk desserts	265	77	305	14	29	15	-
Ice Cream	133	63	255	6	28	14	-
Milk sweetened (Condensed)	306	27	900	25	166	27	-

TABLE 6: (continue)

	Weight (g)	Water (%)	Calori (kj)	Protein (g)	Carbohyd (g)	Fat (g)	Chol.* (mg)
Fruits							
Apple	150	85	70	Trace	18	Trace	0
Pear	100	83	61	0.7	15.3	0.4	0
Mulberry	100	76	93	0.9	19.5	1.1	0
Strawberry	100	90	37	0.7	8.4	0.5	0
Quince	100	84	57	0.4	15.3	0.1	0
Avocado	100	74	167	2.1	6.3	16.4	0
Kiwi	100	91.5	30	0.3	10	0.1	0
Banana	100	76	85	1.1	22	0.2	0
Grapefruit	100	88	41	0.6	10.6	0.1	0
Orange	100	86	49	1.0	12.2	0.2	0
Lemon	100	90	27	1.1	8.2	0.3	0
Fig (dry)	100	23	274	4.3	69.1	1.3	0
Rock melon	100	90.6	33	0.8	7.7	0.3	0
Watermelon	100	92.6	26	0.5	6.4	0.2	0
Cherries	100	80.4	70	1.3	17.4	0.3	0
Apricot	100	85.3	51	1.0	12.8	0.2	0
Pomegranate	100	82.3	63	0.5	16.0	0.3	0
Plum (dry)	100	28	255	2.1	67.4	0.5	0
Date	100	23	274	2.2	73.0	0.5	0
Grape (dry)	100	18	289	2.5	77.4	0.2	0
Bread and Cereals							
Bread (1 slice)	25	35	65	2	13	1	0
Rice	100	12	363	6.7	80.4	0.4	0

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VITAMINS

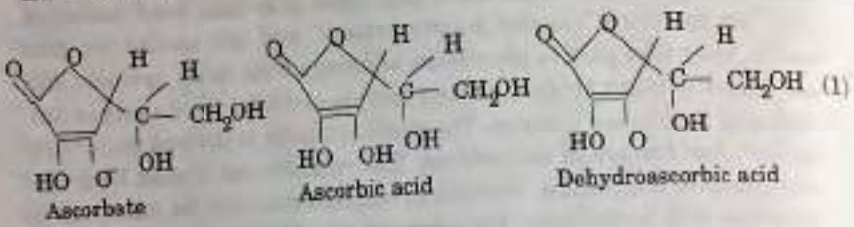
Vitamins are essential as they cannot be synthesized but, in contrast to the essential amino acids, are required in only small amounts. They are therefore "essenial micronutrients" and are needed for good health. They take part in the release of energy from foods, promote normal growth of many kinds of tissues, and are essential for the proper funcioning of nerves and muscles. The name vitamins is derived from "vital amines", but few are in fact amines. The vitamins are a most heterogenoue group of compounds. Vitamin precursors may also be converted to the active form in the body. The vitamins are, divided into water soluble and fatsoluble groups. The following is a summary of the most important vitamins, including some of their functions, and a list of foods that are sources.

TABLE 7: Nomenclature and Grouping of Vitamins

Original designation	Present-day terminology
Vitamin A	Vitamin A
Vitamin B	Thiamin (vitamin B_1, aneurin)
Vitamin B_2	Riboflavin
Pellagra-preventive factor, B_3	Niacin (nicotinic acid) or niacinamide (nicotinamide)
Water-soluble vitamin B complex	Pyridoxine (vitamin B_6)
	Vitamin B_{12}, cyanocobalamine
	Folic acid, folacin
	Pantothenic acid
	Biotin
Vitamin C	Ascorbic acid
Vitamin D	Vitamin D (calciferol)
Vitamin E	Vitamin E (- tocopherol)
Vitamin K	Vitamin K (menadione)

VITAMIN C (Ascorbic acid)

Ascorbic acid undergoes ionization and can act as a reducing agent, being oxidized to dehydroascorbate. (1)



Men and a number of other animals are unable to synthesise ascorbic acid because the absence of the enzyme l-gulunolactone oxidase. The structure of ascorbic acid is reminiscent of glucose and is very important as a donor of reducing equivalents in certain key reactions when vitamin C acts as a donor of reducing equivalents it is oxidized to dehydroascorbic acid. The mechanism of ascorbic acid oxidation is not yet clearly understood, but the following reactions requiring ascorbic acid are well known.

-) Hydroxylation of proline in collagen synthesis. If there is an ascorbic acid deficiency this reaction does not occur and causes scurvy. It is related to defective collagen synthesis. It is indicated by subcutaneous and other hemorrhages, muscle weakness, soft swollen gums, and loose teeth. Is is cured by consumption of fruits and fresh vegetables.

-) bile acid formation

-) Degradation of tyrosine

-) Some hormonal actions

-) Synthesis of epinephrine from tyrosine

-) Absorption of iron

-) Action as a general water - soluble antioxidant, especially in digestive system.

-) An agent in healing wounds

Fruit and green vegetables contain large amounts of vitamin C. Especially citrusfruits - oranges, lemons, grapefruits, fresh strawberries, parsley, are exellent sources of vitamin C. Less important, but still good sources are grape leaf, garden rocket, tomatoes, brussel sprouts, cabbage, cauliflower, and green peppers. Other sources are dark-green leafy vegetables-collards, kale, spinach, and watermelon. Cooking reduces the quantities of most vitamins in foods.

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TABLE 8: Vitamin Values of Foods. (Adepted from ref. 1, 9, 10)

	Vitamin (AIU)	Vitamin (B_1mg)	Vitamin (B_2mg)	Vitamin (B_3mg)	Vitamin (Cmg)
Meat Group					
Veal	-	0.14	0.19	5.5	-
Mutton	-	0.15	0.21	4.9	-
Beaf	30	0.08	0.17	4.71	-
Lamb	-	0.11	0.20	4.5	-
Liver (all)	23500	0.20	2.72	11.4	36
Kidney (Lamb)	690	0.51	2.42	7.4	15
Brain (all)	0	0.23	0.26	4.4	18
Chicken					
white (with skin)	99	0.06	0.09	0.9	1
Fish and shellfish					
Sardine	200	0.05	0.20	5.5	-
Shrimp	-	0.03	0.03	3.2	-
Lobster	-	0.40	0.05	1.5	-
Meat substitutes and fatty seeds					
Bean (white)	0	0.65	0.22	2.4	-
Soybean	80	1.10	0.31	2.2	-
Breadbean	70	0.50	0.30	2.5	-
Lentil	60	0.37	0.22	2.0	-
Chick pea	50	0.31	0.15	2	-
Peanut	-	0.32	0.13	17.1	0
Sesame	-	0.18	0.13	5.4	0
Almond	0	0.24	0.93	3.5	-
Sunflower	50	1.96	0.23	5.4	-
Hazelnut	-	0.46	-	0.9	trace
Pumpkin seed	30	0.20	0.20	0.20	-
Chestnut	24	0.24	0.18	1.34	26
Walnut	30	0.33	0.13	0.9	2
Bread (1 slice, 25g)	trace	0.03	0.02	0.3	trace
Rice	0	0.07	0.03	1.6	0

TABLE 8: (continue)

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Vegetable Group	Vitamin (AIU)	Vitamin (B_1mg)	Vitamin (B_2mg)	Vitamin (B_3mg)	Vitamin (Cmg)
Beans (green)	600	0.08	0.11	0.5	19
Peas	640	0.35	0.14	2.9	27
Mushroom	trace	0.10	0.40	4.2	3
Spinach	8100	0.10	0.20	0.6	51
Eggplant	10	0.05	0.05	0.6	5
Potatoes	trace	0.10	0.04	1.5	20
Parsley	8500	0.12	0.25	1.2	172
Lettuce	970	0.06	0.06	0.3	8
Corn	400	0.15	0.12	1.7	12
Cauliflower	60	0.11	0.10	0.7	78
Cabbage(white)	130	0.05	0.05	0.3	47
" (black)	1580	0.12	0.08	1.1	94
Peppers (green)	420	0.08	0.08	0.5	128
Onions (dry)	40	0.03	0.04	0.2	10
Turnips	trace	0.04	0.07	0.1	36
Tomato (raw)	900	0.06	0.04	0.7	23
Cucumber	trace	0.03	0.04	0.2	11
Celery (raw)	100	0.05	0.06	0.7	8
Carrots	11.000	0.06	0.05	0.06	8
Artichoke	320	0.15	0.05	0.8	5
Okra	520	0.17	0.21	1.0	31
Peppermint	1400	0.13	0.26	1.0	35
Garden rocket	14000	0.18	0.29	-	120
Garlic	trace	0.25	0.08	0.5	15
Leek	40	0.11	0.06	0.5	17
Purslane	2200	0.08	0.10	0.5	30
Grape leaf	17500	0.26	0.03	-	120
Onion green	2000	0.05	0.05	0.4	32

TABLE 8: (continue)

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	Vitamin (AIU)	Vitamin (B_1mg)	Vitamin (B_2mg)	Vitamin (B_3mg)	Vitamin (Cmg)	
Milk Group						
Buttermilk	10	0.10	0.44	0.2	2	
Whole milk	350	0.07	0.41	0.2	2	
Cheese						
Cheddar	1059	0.03	0.38	0.1	0	
Yogurt						
From skimmed milk	170	0.10	0.44	0.2	2	
From whole milk		340	0.07	0.39	0.2	2
Cheese						
(Gravyer)	1219	0.06	0.28	0.1	0	
Fruits						
Apple	90	0.03	0.02	0.1	4	
Pear	20	0.02	0.04	0.1	4	
Mulberry	-	0.06	0.07	0.2	17	
Strawberry	60	0.03	0.07	0.05	59	
Quince	40	0.02	0.03	0.2	15	
Avacado	293	0.11	0.02	1.6	14	
Kiwi fruit	6000	trace	trace	trace	100	
Banana	190	0.05	0.06	0.7	10	
Grapefruit	80	0.04	0.02	0.2	38	
Orange	200	0.10	0.04	0.4	50	
Lemon	20	0.04	0.02	0.1	53	
Fig (Fresh)	80	0.10	0.10	0.7	2	
Melon	280	0.04	0.03	0.6	23	
Watermelon	590	0.03	0.03	0.2	7	
Cherries	110	0.05	0.06	0.4	10	
Apricot (Fresh)	2700	0.03	0.04	0.6	10	
Pomegranate	trace	0.03	0.03	0.3	4	
Plum (Dry)	1600	0.09	0.17	1.6	3	
Date	50	0.09	0.10	2.2	0	
Grapes (Fresh)	100	0.05	0.03	0.3	4	
Peaches	1330	0.02	0.05	1.0	7	

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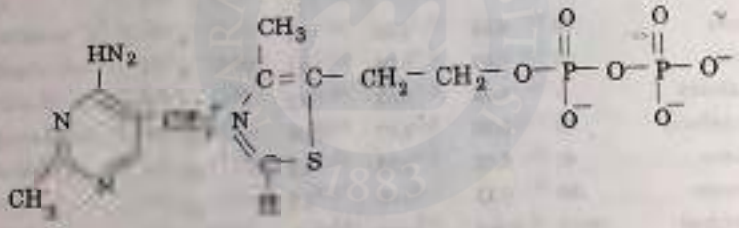
THE B VITAMINS

Common B vitamins are thiamin, riboflavin, niacin, pyridoxine, folic acid, cobalamin and pathotenic acid.Three of these, thiamin, riboflavin and niacin **play a central role in the release of energy from food.** They also help in maintaining normal appetite, good digestion, healthy skin and good nerve function. Meats are the leading source of the B vitamins, although whole grain bread and enriched white bread and cereals supply small but important amounts.

Thiamin

The thiamin molecule comprises two heterocyclic rings, being pyrimidine and a substituted thiazole joined by a methylene bridge(2).



(2)

Thiamin pyrophosphate

TABLE 9: Recommended daily dietary allowances of vitamins

	Age (years)	Weigh (kg)	Height (cm)	Protein (g)	Vitamin A (µg RE)	Vitamin D (µg)	Vitamin E (mg α-TE)	Vitamin K (µg)	Vitamin C (mg)	Thiamin (mg)	Riboflavin (mg)	Niacin mg	Vitamin B_6 (mg)	Folate (µg)	Vitamin B_{12} (µg)
Infants	0.0-0.5	6	60	13	375	7.5	3	5	30	0.3	0.4	5	0.3	25	0.3
	0.5-1.0	9	71	14	375	10	4	10	35	0.4	0.5	6	0.6	35	0.5
Children	1-3	13	90	16	400	10	6	15	40	0.7	0.8	9	1.0	50	0.71
	4-6	20	112	24	500	10	7	20	45	0.9	1.1	12	1.1	75	.0
	7-10	28	132	28	700	10	7	30	45	1.0	1.2	13	1.4	100	1.4
Males	11-14	45	157	45	1000	10	10	45	50	1.3	1.5	17	1.7	150	2.0
	15-18	66	176	59	1000	10	10	65	60	1.5	1.8	20	2.0	200	2.0
	19-24	72	177	58	1000	10	10	70	60	1.5	1.7	19	2.0	200	2.0
	25-50	79	176	63	1000	5	10	80	60	1.5	1.7	19	2.0	200	2.0
	51+	77	173	63	1000	5	10	80	60	1.2	1.4	15	2.0	200	2.0
Females	11-14	46	157	46	800	10	8	45	50	1.1	1.3	15	1.4	150	2.0
	15-18	55	161	44	800	10	8	55	60	1.1	1.3	15	1.5	180	2.0
	19-24	58	164	46	800	10	8	60	60	1.1	1.3	15	1.6	180	2.0
	25-50	63	163	50	800	5	8	65	60	1.1	1.3	15	1.6	180	2.0
	51+	65	160	50	800	5	8	65	60	1.0	1.2	13	1.6	180	2.0
Pregnant				60	800	10	10	65	70	1.5	1.6	17	2.2	400	2.2
Lactating				65	1300	10	12	65	95	1.6	1.8	20	2.1	280	2.6

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An active thiamin is thiamina diphosphate. An ATP dependent thiamin diphosphotransterase present in brain and liver is responsible for the conversion of thiamin to its active form thiamin pyrophosphate (TPP). It also serves as a coenzyme in enzymatic reactions, transfering an activated aldehyde unit. There are two types of such reacitons. The first is oxidative decarboxylation of α- keto acids (eg. pyruvate, α - ketoglutarate and the α - keto analogs of leucine, isoleucine, and valin). The second type are the transketolase reactions.(eg. pentose phosphate pathway) All of these reactions are inhibited in thiamin deficiency.

The thiazole ring participates directly in the oxidaive decarboxylotion of pynivate to acetyl CoA and α - keto glutarate o succinyl CoA. these two reactions are on major metabolic pathways, the first being specific to carbohydrate breakdown, and the latter in the Krebs cycle. (see page...) These reactions occur in a multienzyme complex known as the pyruvate dehydro-genase complex. The oxidation of α - ketoglutarate to succinyl - CoA and CO_2 is catalyzed by an enzyme complex structurally very similar to the pyruvate dehydrogenose complex. A similar oxidative decarboxylation of the α - ketocarboxylic acid derivatives of the branched-chain amino acids utilizes thiamin diphosphate. The role of TPP as a coenzyme in the transketolase reactions is very similar to that described above for the oxidative decarboxylations.

In 1897 Eijkman discovered that the disease Beriberi was produced in military personnel and in chickens fed an rice from which the husks were removed, a cure could be effected by feeding an whole rice. The active factor was crystallised in 1926 and synthesised in 1936. The disease of thiamin deficiency (Beriberi) is very serious, but it is not a disease of starvation. It is commonly found in people eating an ill-balanced diet, rich in partly purified carbohydrates. For thiamin is present in almos all plant and animal tissues commonly used as food. unrefined cereal grains and meat are good sources of the vitamin. Early symptoms of beriberi and (syndrome) include peripheral neuropahy, exhaustion and anorexia lea ding to edema and cardiovascular, neurological and muscular degeneration.

Riboflavin

This yellow compound consists of a heterocyclic isoalloxazine ring attached to the sugar alcohol ribitol. Active riboflavins are flavin adenine mononucleotide (FMN) and flavin adenine dinucleotid (FAD)(3)

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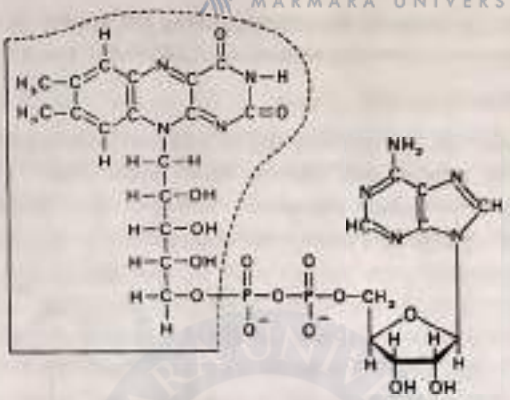


(3)

Flavin adenine dinucleotide (FAD)

These nucleotides serve as prosthetic groups of oxidoreductase enzymes, known as flavoproteins. The prosthetic groups are usually tightly but not covalently bound to their apoproteins. Many flavoprotein enzymes contain one or more metals such as molybdenum and iron so are known as metalloflavoproteins. These enzymes dehyrogenate certain substrates. In their role as coenzymes, flovoproteins undergo reversible reduction of the isoalloxazine ring to yield the reduced forms $FMNH_2$ and FAD H_2. the flavin ring is reversibly reduced in these reactions. Some of the important oxidoreductase and dehidrogenisation reactions in mammalian metabolism are as follows:

-) α - amino acid oxidase in amino acid deamination

-) Succinate dehydrogenase in the citric'c acid cycle

-) A cyl-CoA and the electron-transfering in fatty acid oxidation

-) Aldehyde dehidrogenose in the degradation of aldehydes

-) Xanthine oxidase in purine degredation

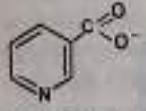
Despite its widespread metabolic functions riboflavin deficiency does not lead to major life threatening conditions. Insufficient riboflavin causes sore lips, tonque, and mouth. and rough, scaly skin. Because riboflavin and the other B vitamins occur together in foods, deficiency of one is usuall complicated by deficiencies of the others. A well-balanced daily

diet supplies 1 to 2 mg of the vitamin, being the suggested daily allowance. Pregnant and Lactating women need additional intake.

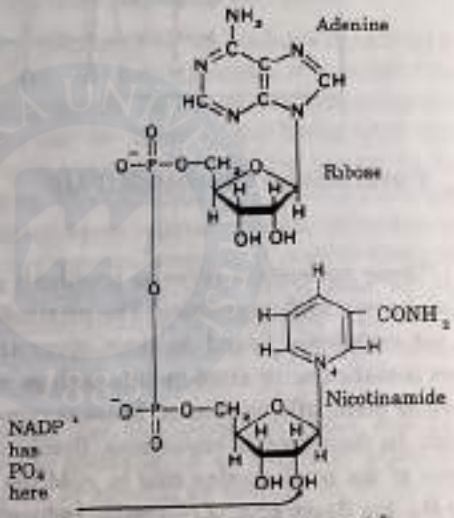
Niacin or Nicotinic Acid

Nicotinic acid is a monocarboxylic acid derivative pyridine (4a) Active niacins are nicotinamide adenine dinucleotide (NAD^+) and nicotinamide adenine dinucleotide phosphate (NADP+) (4b). Nicotinamide becomes reduced in dehydrogenase reactions. (4b).

(4a)



Nicotinic acid



(4b)

Nicotinamide adenine dinucleotide (NAD^+)
NADP is nicotinamide adenine dinucleotide phosphate

the forms of the coenzymes should be written as NAD^+, NADH + H^+, NADP and NADPH = H^+ but the more simple, less corect versions, NAD, $NADH_2$, NADP and $NADPH_2$ are commonly used. Those nucleotides are coenzymes of many oxidoreductase enzymes, and key components of many metabolic pathways affecting carbohydrate, lipid and amino acid metabolism.

-) Generally, NAD - linked dehydrogenases catalyze oxidoreduction reactions in the glycolytic pathway and the citric acid cycle,

-) Whereas NADP. linked dehydrogenases found in the pentose

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phosphate pathway are concerned with other reductive syntheses such as fatty acid sythesis.

A deficiency of niacin causes pellegra, the symptoms of which are inflamed skin, diarrhea, and mental confusion progressing o delirium. Untreated, it can be fatal. Niacin is found widely in most animal and plant foods.

In 1928 the effectiveness of boiled yeast extract in curing pellegra was discovered and extracts of other tissues were exaemined for a heat-stable curative factor. In 1937 nicotinamide from liver extract was shown to cure pellegra in animals. Synthetic nicotinic acid was then quickly introduced for the therapy of human pellegra. The function of nicotinamide as a component of the coenzymes of dehydrogenation had already been demonstrated in 1935 during the study of fermentation and tissue respiration.

The essential amino acid tryptophan can be converted to NAD For every 60 mg of tryptophan, 1 mg equivalent of niacin can be genereted. Niacin deficiency occurs in popilations dependent on corn as the stable food, resulting in the condition known as pellegra. Niacin is in fact present in corn but in a bound, unavailable form. On the other hand, excess dietary leucine can also bring about niacin deficiency by inhibiting the key enzymes in the conversion of tryptophan to NAD+. Other conditions leading to symptoms of pellegra include administration of some drugs such as isoniazid, malignant carcinoid syndrome in which tryptophan metabolism is diverted to serotonin, and Hartnup disease, in which tryptophan absorption is impaired.

The recomended allowance range for niacin is 9-19 niacin equivalents per a day for children and adults. Both the acid and amide forms of niacin are readily absorbed in the intestinal tract. Both compounds are convered to the coenzyme forms in the blood cells, kidneys, brain and liver. There is little storage of niacin.

Pyridoxine and its relatives

The vitamin B_6 group comprises three natural forms: Pyridoxine, (5a) the primany alcohol, is found in foodstuffs together with the corresponding aldehyde (pyridoxal) and amine (pyridoxamine) and all three have similar vitamin activity. (5)

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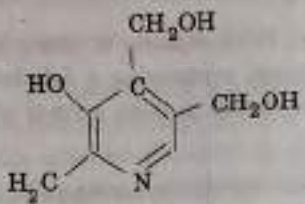
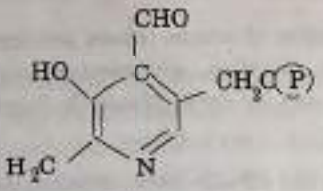


Pyridoxine



Pyridoxal phosphate

(5)

Pyridoxal phosphate is the coenzyme of several enzymes of amino acid metabolism. The active coenzyme is derived from pyridoxol by phosphorylation of the alcohol group. Pyridexol phosphate is involved in:

-) Transamination reactions

-) Decarboxylation of α - amino acids

-) Action on phosphorylase to break down glycogen in glycogenolysis pathway. Muscle phosphorylase may account for as much as 70-80 % of total body vitamin B_6.

Since vitamin B_6 is widely distiributed in animal and plant tissues, no natural deficiency disease has been proved in man. The three B_6 vitamins are readily absorbed by the mucosal cells. Recommended daily allowances with, some margin of safety are 2.2 mg/day for adult males and 2.0 mg/day for a adulf females, with and additional 0.6 mg/day for pregnant or lactating women. Liver, bananas, meat, vegetables and eggs are good sources of vitamin B_6 ingestion of alcohol, chemotherapy and ingestion of vitamin B_6 antagonists such as the tuberculostatic drug isoniazid, hydrazines and hydroxylamines have led to hypovitaminosis B_6. A possible deficiency is recognized in breast fed infants whose mothers are depleted of the vitamin due to long term use of oral contraceptives.

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TABLE 10: Pyridoxine in selected foods
(Micrograms per 100-g edible portion)

Apple	26	Milk, human	3.5-22
Asparagus, canned	30	Milk, evaporated	25-41
Banana	320	Milk, dry	330-820
Barley	330-560	Milk, dry skim	580
Beans, green, canned	32	Molasses, blackstrap	2000-2490
Beef	230-320	Oats, rolled	93-150
Beer	50-60	Onions	63
Beet greens	37	Orange juice, canned	16-31
Brains beef	160	Orange juice, fresh	18-56
Cabbage	120-290	Peaches, canned	16
Cantaloupe	36	Peanuts	300
Carrots, raw	120-220	Peas, fresh	50-190
Cauliflower	20	Peas, canned	46
Cheese	98	Peas, dry	160-330
Cod	340	Pork	330-680
Corn, canned	68	Potato	160-250
Corn, yellow	360570	Raisins	94
Corn grits	200-250	Rice, whole	1030
Cottonseed meal	1310	Rice, whie	340-450
Eggs, fresh	22-48	Rye	300-370
Flounder	100	Salmon, canned	450
Frankfurter	130	Salmon, fresh	590
Grapefruit juice	8-18	Sardines, canned	280
Grapefruit sections	17-24	Soybeans	710-1200
Halibut	110	Spinach, canned	60
Ham	330-580	Strawberries	44
Heart, beef	200-290	Tomatoes, canned	710
Honey	4-27	Tuna, canned	440
Kidney, beef	350-990	Turnips	100
Lamb	250-370	Veal	280-410
Lemon juice	35	Watermelon	33
Lettuce	71	Wheat bran	1380-1570
Liver, beef	600-710	Wheat germ	850-1600
Liver, calf's	300	White flour	380-600
Liver, pork	290-590	Yams	320
Malt extract	540	Yeast, bakers'	620-700
Milk, whole	54-110	Yeast, brewers' dry	4000-5700

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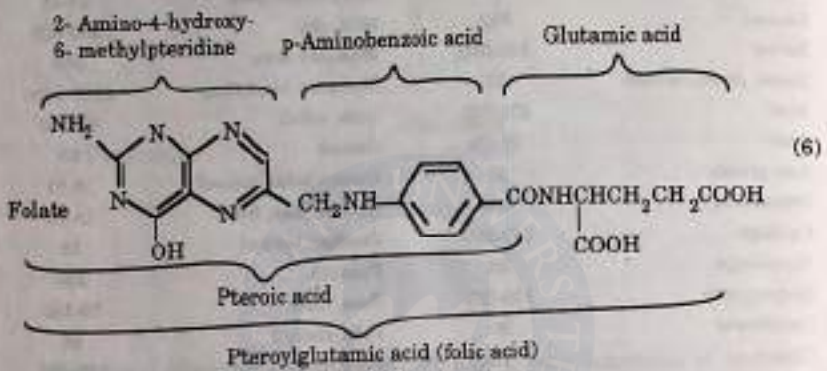
Folic Acid (viamin B_9)

Folic acid, or folate, consists of the base pteridine attached to one molecule each of P-aminobenzoic acid (PABA) and glutamic acid. (6)



(6)

Folate and folic acid are general terms for a family of compounds related to pteric acid. Pteroic acid is composed of a pteridine ring joined to a PABA residue, Pteroic acid, when conjugated with one molecule of L-glutamic acid (pteroly glutamic acid) can be reduced to dihydrofolic acid or tetrahydrofolate. Only the reduced forms are biologically active. Although various forms of folic acid are normally present in human serum and other body fluids, the principal form is N^5-methyltetrahydrofolate.

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TABLE 11 : Dietary sources of folic acid
(Micrograms per 100-g edible portion)

Meat, eggs		Cereals and other grain products	
Beef		Breads	
Round steak	7-17	Cracked wheat	27
Chuck	15	Rye	26
Hamburger	5.0	Vienna	11
Heart	3.1	White	15
Kidney	58	Breakfast cereals	
Liver	290	Cornflakes	5.5
Sweetbreads	22.8	Cornmeal	6.5
Lamb		Corn and soya	60
Stew meat	1.9	Oats, ready to eat	22
Leg	3.3	Oatmeal	30
Liver	280	Wheat bran	100
Pork		Wheat farina	14
Liver	320	Wheat, shredded	28-87
Loin	2.4	Flour	
Ham, smoked	7.8	Cake	66
Sausage	12	Rye	18
Poultry		White, enriched	8.1
Chicken, dark	2.8	Whole wheat	38
Chicken, white	3.1	Grains	
Chicken, liver	380	Barley	50
Turkey	3-15	Corn, yellow	24
Eggs		Oats, white	23-66
Whole	5.1	Rice, brown	32
White	0.6	Rye	34
Yolk	13	Wheat	27-51

Nuts		Milk and cheese	
Almonds	46	Milk	
Brazil nuts	4.5	Buttermilk	11
Coconuts	28	Evaporated milk	0.7
Filberts	67	Cheese	
Peanuts	57	Cheddar	15
Pecans	27	Cottage	21-46
Walnuts	77		

TAPLE 11: (continue)

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Vegetables (fresh)	
Asparagus	89-140
Beans, lima	10-56
Beans, lima, dry	100
Beans, snap	13-56
Beans, navy, dry	130
Beans, wax	15-39
Beets	13
Broccoli	34
Brussels sprouts	27
Cabbage	6-42
Carrots	8
Cauliflower	29
Celery	7.2
Corn, sweet	9-70
Cucumbers	6.7
Eggplant	5-15
Greens	
Beets	20-50
Chicory	30
Endive	27-63
Escarole	26
Kale	50.9
Mustard	17-38
Parsley	43
Spinach	49-110
Swiss chard	32-64
Turnip	83
Watercress	45
Kohlrabi	10
Lentils, dry	99
Lettuce	4-54
Mushrooms	14-29
Okra	24
Onions	
Green, with tops	13
Mature	6-14
Parsnips	8-37
Peas, fresh	5-35
Peas, dry split	22
Peppers, green	4-11
Potatoes, peeled	4-12
Potatoes, whole	2-130
Pumpkin	5-10
Radishes	3-10
Rutabagas	3-7
Soybeans, dry	1,91
Squash	
Acorn	16.7
Crookneck	7-16
Zucchini	11
Sweet potatoes	5-19
Tomatoes	2-16
Turnips	4.3
Fruit	
Apples	0.5
Apricots, fresh	3.6
Apricots, dried	4.7
Avocados	4-67
Bananas	9.6
Berries	
Blackberries	6-18
Blueberries	7.6
Cranberries	1.7
Red raspberries	5.1
Strawberries	5.3
Cantaloupes	3-8
Cherries, Bing	6.5
Dates, dry	25
Figs, fresh	6.7
Figs, dry	7-14
Grapefruit	2.7
Grapes, green	4.5
Grapes, red	4.9
Honeydew melon	4.9
Lemons	7.4
Limes	4.6
Oranges	22-35
Orange juice	26-40
Peaches, yellow	2.3
Pears	2.5
Pineapple	0.8-6
Plums, red	0.6-3
Plums, yellow	1.2
Prunes, dry	5.4
Rhubarb	2.5
Tangerines	7.4
Watermelon	0.6

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Tetrahidro folat (H_4 folate) is the carrier of activated one-carbon units. Some major reactions terahidrofolat are;

-) Conversion of serine to glycine

-) Catabolism of histidine

-) Synthesis of thymidylate, methionine, and purine.

Serine is the major source of one-carbon unit in the form of a methylene group, which it transfers reversibly to H_4 folate to form glycine and N^5, N^{10} - methylene H_4 folate, This molecule plays a central role in one-carbon unit metabolism.

Folic acid and B_{12} metabolism are linked by the reaction that transfers a methyl group from N^5-methyltetrahydro folate to cobalemin. In cases of cobalamin deficiency, folate cannot be recycled back into the functional folate polyglutomate pool. and diffuses out of the cell. Thus, folate deficiency results in megaloblastic anemia and neuropathies. A deficiency of folate may result from the absence of intestinal microorganisms (gut sterilization), poor intestinal absorption (e.g. after surgical resection or in celiac disease or spme) insafficient dietary intake, excessive utilization (as in pregnancy, liver disease, and malignancies) and from the administration of antifolate drug such as methotrexate. The human daily requirement for folate is about 50 μg. The body stores about 5 mg of folae. Interruption of the absorption of folate may therefore result in deficiency within a few months.

Pantothenic Acid

Pantothenic acid is an essential food factor which forms part of coenzyme A which is imporant in fatty acid metobolism group (-SH) can react with a carboxyl group to form a thiol ester (7) this group can in turn react with a carboxyl group form a thioester. Such thiol esters are involved in many transfer reactions involving acyl groups, including acetyl and fatty acyl groups. A thioester is in some ways analogous to the ester formed between a carboxylic acid and an alcohol.

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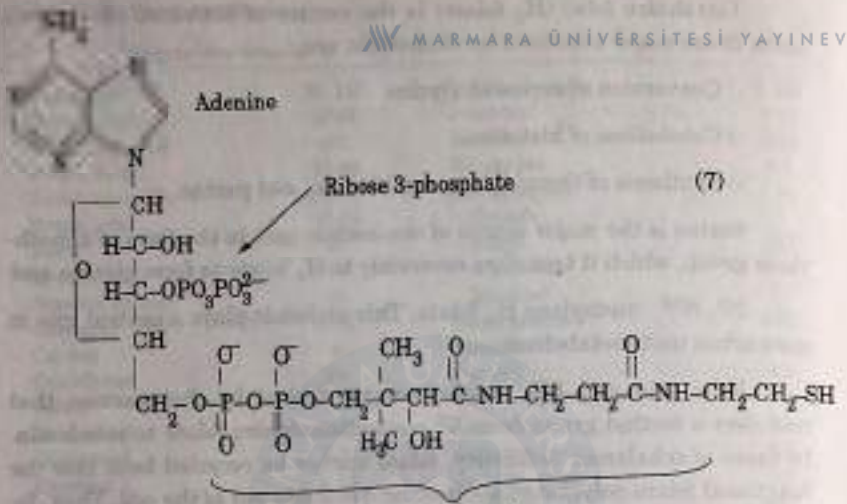

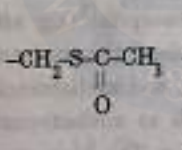

Coenzyme A and Acetyl Coenzyme A

Pantothenic acid was discovered as a growth factor for certain yeast and deficiencies have been produced in a number of animals. No deficiency has been recorded in man, perhaps because of its widespread occurrence in foodstuffs. Good sources of the vitamin are liver, kidney, yeast, egg yolks and fresh vegetables.

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TABLE 12: Pantothenic acid in foods
(Micrograms per 100-g edible portion)

Bananas	300	Oysters	490
Beans, dried lima	830	Peaches	140
Beef, brain	2100-2900	Peanuts, roasted	2500
Beef, heart	2100-2500	Pears	70
Beef, kidney	3400	Peas, fresh	600-1040
Beef, liver	5700-8200	Peas, dried	2800
Beef, muscle	1100	Pineapple	170
Bread, whole wheat	570	Pork, bacon	280-980
Bread, white	400	Pork, ham	340-660
Broccoli	1400	Pork, kiney	3100
Cauliflower	920	Pork, liver	5900-7300
Cheese	350-980	Pork muscle	470-1500
Chicken	530-900	Potatoes, Irish	400-650
Eggs	2700	Potatoes, sweet	940
Lamb	600	Salmon	660-1100
Lamb, kidney	4300	Soybeans	1800
Milk, whole	390	Tomatoes	310
Mushrooms	1700	Veal chop	110-260
Oats	1300	Whey, whole	1300
Onions	140	Wheat, germ	2000
Oranges	340	Wheat, bran	2400

Cobalamin (vitamin B_{12})

Vitamin B_{12} comprises a porphyrin-like ring with cobalt as the central metal atom and a nucleotide structure linked between the cobalt atom and one of the side-chains of the ring system. A Cyanide ion is also linked to the cobalt atom in cyonocobalamin (8).

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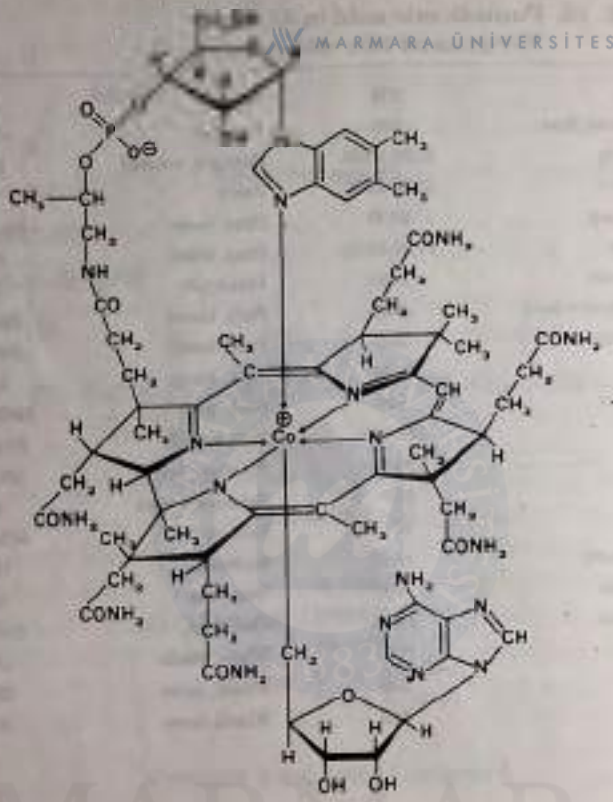

(8)

Structure of the 5,6-dimethylbenzimidazole cobalamide coenzyme

The cobalamin coenzyme is required for the transfer of methyl groups from terehydrofolate. Cyanocobalamin is also called **anti-pernicious anemia factor**, being able to cure pernicious anemia in some individuals. A factor required for the satisfactory absorption of vitamin B_{12} is called **intrinsic factor**. Persons lacking this "intrinsic factor" cannot be cured of pernicious anemia by oral doses of cyanocobalamin, but injection of 1 mg twice a week produce rapid remission of symptoms.

Pernicious anemia occurs when absorption is prevented by lack of intrinsic factor (or by gastrectomy). The vitamin B_{12} deficiency leads to

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impairment of the methionine synthase reaction. Anemia results from impaired DNA synthesis affecting formation of the nucleus of new erythrocytes. This is due to impaired purine and pyrimidine synthesis resulting from tetrahydrofolate deficiency as a consequence of folate being trapped as methyl tetrahydro folate as mentioned under vitamin B_9. In such cases macrocytosis, hypersegmentation of neutrophils, anemia, leukopenia, thrombocytopenia are observed. Irreversible neurologic disorders, weokness, spasticity and paralysis, confusion are other symptems of vitamin B deficiency. Neurologic symptoms may occur wihout any discernible hematological changes. Howeever when hematological changes are observed, they are usually accompanied by some neurologic changes. Homocytinuria and methylmalonic aciduria also occur in these metabolic disturbances. The neurologic disorder associated with vitamin B_{12} deficiency may be secondary to a relative deficiency of methionine.

The active B_{12} coenzymes are methycobalamin and deoxyadenosyl cobalamin. After transport in the blood, free cobalamin is released into the cytosol of cells as hydroxocobalamin.It is either converted in the cytosol to methylcobalamin or enters mitochondria for conversion to 5', deoxyadenosylcobalamin. The known biochemical action of these coenzymes are summarised as follows;

-) Conversion of mehylmalonyl - CoA to succinyl - CoA

-) Conversion of homocysteine to methionine

-) Conversion of methyltetrahydro folate to tetrahydrofolate

TABLE 13: Vitamin B_{12} in foods

(Micrograms per 100-g edible portion)

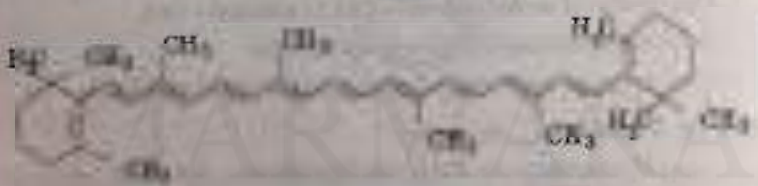
Beans, green	0-0.2	Haddock	0.6
Beef, kidney	18-55	Ham	0.9,1.6
Beef, Liver	31-120	Milk, whole	0.3-0.5
Beef, round	3.4-4.5	Milk,evaporated	0.1-0.3
Beets	0-0.1	milk, powdered, whole	1-2.6
Bread, white	0-0.3	Milk, powdered, skim	2.5-4.0
Bread, whole wheat	0.2-0.4	Oats	0.3
Carrots	0-0.1	peas	0-0.1
Cheese, American	0.6	Scallops	0.7
Cheese, cream	0.2	Sole, fresh fillet	1.3
Cheese, Swiss	0.9	Soybean meal	0.2
Corn, yellow	0.0	Wheat	0.1
Egg, whole	0.3		

Vitamin B_{12} is synthesized by microorganism. It is therefore absent in plants, unless they are contaminated by microorganisms. Dietary sources of vitamin B_{12} are meats, milk o milk products, and eggs. Vitamin B_{12} is taken up by hepatocytes from the plasma ofter digestional absorbtion it is stored within the liver until it is again released into plasma to meet physiological demands. In the liver vitamin B_{12} is found as methylcobalamin, adenosylcobalamin and hydroxocobalamin. Liver is therefore a good source of vitamin B_{12}. A commercial preparation is known as reguirement is in humans, the daily vitamin B_{12} requirement is about 0.5 µg. Normally the body stores about 1 mg which for exceeds the daily metobolic requirement. For this reason symptoms of physiologic vitamin B_{12} deficiency do not become evident for five years or more after dietary deficiency begins.

LİPİD-SOLUBLE VITAMINS

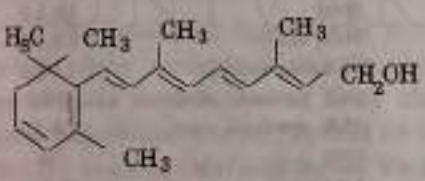
Vitamin A

The natural foms of vitamin A_1, (retinol), Vitamin A_2 (3 - dehydroreinol) and provitamins are all isoprene derivatives. They are apolar hydroptilic molecules (9).

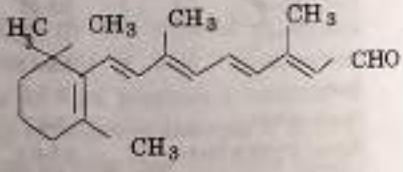


(9)

Carotene



A_1 retinol (all trans)



A_2 dehydroretinol (all trans)

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Vitamin A is a generic term referring to all compounds from animal sources that exhibit he biological activity of vitamin A. Those are retinol acid and retinal, only retinol has full vitamin A acivity, the others fulfilling some, but not all, vitamin A functions. The term retinoids has been used to describe both the natural forms and the synthetic analogs of retinol. Retinoids cannot be synthesized by the body in adequate amaunts and therefore must be supplied by diet. Higher animals are unable to synthesize the β-ionone type ring structare but can derive the aldehyde, retinal from provitamin carotenes and cryptoxanthin in plants. Retinal is then reversibly reduced to retinol.

Retinoids require normal fat absorption. After absorption, they must be transported in the blood like any other apolar lipid.

Any condition affecting the digestion and absorption of the Lipid-soluble vitamins, such as steatorrhea or disorder of the biliary system can lead to vitamin A deficiencies.

Retinol, retinal and retinoic acid each have their own unique biochemical function. Retinol and retinal are interconvertad in the presence of NAD or NADP- requiring dehydrogenases or reductases, present in many tissues. The metobalic function of vitamin A groups are as follows;

-) The best understood physiological function of vitamin A is its role in vision in the form of retinal

Retinal is a component of the visual pigment rhodopsin. Retinol and retinal are involved in a reversible cycle with opsin and rhodopsin, resulting in optic nerve transmission. Rhodopsin occurs in the rod cells of the retina, which are responsible for vision in poor light. The biochemical reaction mentioned above, is accompanied by a conformational change that induces a calcium ion channel in the membrane of the rod cell. The rapid influx of calcium ions triggers a nerve impulse, allowing light to be perceived by the brain.

-) Retinol acts like a steroid hormone. It is necessary for reproduction and growth. It may be involved in the contral of the expression of certain genes. A role in cancer prevent has been described to both vitamin A and B-caroten which known as provitamin A. Some experiments have shown that retinoid administration diminishes the effect of some carcinogens.

-) Vitamin A and provitamin A are necessary for the stabilization of cellular and intracellular membranes and maintenance of the integrity of epithelial tissue. They also have an antioxidant activity.

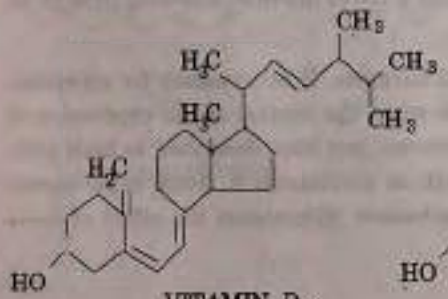
This autioxidant effect of vitamin A is due to the stabilization of organic peroxide free radicals within its conjugated alkyl structure. Since β carotene is effective at low oxygen concentrations it complements the anticoxidant properties of vitamin E, which is effective at higher oxygen concentrations. The antioxidant properties of these two vitamins may well account for their possible anticancer activity.

-) Retinoic acid is involved in glycoprotein synthesis. this may represents, in this way vitamin A is involved in promoting growth and differentiation of tissues.

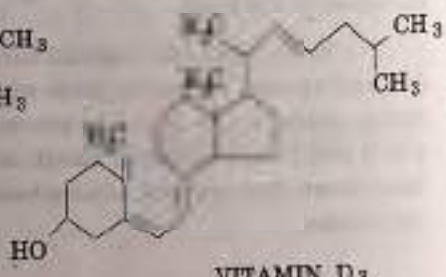
Recommended daily allowances (RDA) of vitamin A, for adult males are 1000 and for females 800 retinol equivalents. One retinol equivalent is 1 μg retinol, or 6 μg B-carotene or 12 μg of other provitamin A carotenoids. In terms of international units (IU), 1 retinol equivalent is 3.33 IU of retinol or 10 IU of B-carotene. Higher levels are recommended to satisfy growth needs of children and adolescents. An additional 200-400 retinol equivalents are recommended for pregrant or lactating women.

Vitamin D

There are two forms of vitamin D. One of them occurs from ergosterol formed by ultraviolet irradiation, which occurs in molds, yeast, and higher plants. It is called vitamin D_2 or ergocalcifenol (10a) The other is called vitamin D_3 or cholecalcifenol (10b). It derives from irradiation of provitamin dahydrocholesterol in our skin. Both of them are steroid-derived compounds. **(10)**



VITAMIN D_2



VITAMIN D_3

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As shown by structures the only chemical difference between the two vitamin and the provitamin forms is in the side chain.

Vitamin D is absorbed by the gastrointestinal tract unless there is fat malabsorption due to lack of bile salts, pancreatic insufficiency, or a defect of intestinal mucosal function. When it is absorbed, the vitamin is bound directly to chylomicrons. Dietary vitamin D_3 or D_2 is taken up from the blood by the liver. Hydroxylation of the Vitamin D_3 - 25 - hydroxylase enzyme yields 25 - hydroxy - D_3 This 25 - hydroxycholecalciferol, which represents the major metabolite of D_3 in plasma, circulates in the blood bound to a vitamin D - binding - α globulin. this is the major form of vitamin D - in the circulatory system and the major storage form in the liver. A significant fraction of 25 - hydroxy D_3 undergoes enterohepatic circulation and disturbances of this process can lead to a deficiency of vitamin D.

In the kidneys, bones and placenta a second hydroxylation occurs at the 1 α - position by the enzyme 25 - hydroxy D_3 1 - hydroxylase which is a mitochondrial enzyme. The product, 1α , 25 - dihydroxy D_3 is also called calcitriol. It acts as a hormone and it plays a central role in calcium and phosphate metabolism. 1,25 - dihydroxy D_3 induces a calcium carrier protein, and so promotes the absorption of calcium at the intestinal mucosa and the reabsorption of calcium from the bone.

25 - hydroxy D_3 can also be hydroxylated at the 24 position by a mitochondrial enzyme present in renal tubules, cartilage, intestine, and placenta. The level of the 24 - 25 dihydroxy D_3 is related to the level of 1,25 - dihydroxy D_3 in serum. (For further deails see p.. 53)

Vitamin D regulates calcium and phosphote metabolism in the body. Fish liver oil have been used a remedy for the bone disorder rickets for morethan 200 years. Experimental rickes in pups has been shown to be a nutritional disorder which responds to a fot- soluble vitamin other evidence indicates that the disease is caused by a lack of sunshine. These views were reconciled when both cod liver oil and irridiation with ulfraviolet lamps proved equally effecive in treating rickets in children. Vitamin d deficiency may result in an increased urinary phosphate excretion and a fall in plasma inorganic phosphae below the normal range of 2.50 to 4.16 µg/dl in adults and 3.6 to 5.9 µg/dl in children. The major roles of vitamin D are:

-) Regulation of calcium phosphate metebolism,

-) A major factor in building strong bones and teeth.

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The administration of vitamin D increases the absorption of calcium and phosphate. Phosphate reabsorption in the renal tubules is also facilitated and the increase in plasma calcium and phosphate promotes mineralization of the bones. Not only vitamin D, but also parathyroid hormone and calcitonin are necessary for the maintenance of plasma calcium and phoshate concentretion. Osteomalacia, is the adult disease corresponding to rickets (in child) in which loss of mineral occurs from the bones. This syndrome may occur in pregnancy, where a mineral supply is insufficient for both fetus and mother. Hypervitaminosis D can be produced by routinely taking excessive vitamin preparations. This may lead to renal stone formation and calcification of organs such as the kidney, lungs, stomach and arteries. Secondary results include polyuria, constipation, vomiting and renal failure.

Not many foods contain vitamin D naturally. Fish liver oil is a good source but is not part of a daily diet. Smaller amounts are found in eggs, butter and liver and some what larger amounts are found in sardines, salmon and tuna. Since vitamin D is not found in most foods and since it

TABLE 14: Vitamin D in foods

(International units/100-g edible portion)

Beefsteak	13	Liver, beef, raw	34
Beet greens	0.2	liver, lamb, raw	18
Butter	92	Liver, pork, raw	44
Cabbage	0.2	Liver, veal	9.6
Carrot tops	3	Mackerel, fresh, raw	1.100
Cheese	33	Milk, whole	4.4
Cod-liver oil	10.000	Milk, vitamin D	44
Corn oil	9	Pilchards, canned	745
Cream	17	Salmon, canned	314
Crisco	9	Sardines, canned	1.380
Egg yolk	265	Shrimp	150
Halibut-liver oil	140.000	Spinach	0.2
Herring, canned	330	Tuna	200-320

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is so important for normal growth of children, synthetic vitamin D is added to almost all the milk sold in some western countries. 400 IU (10 mg) is recommended as the daily allowance from infancy through 18 years of age. As the rate of skeletal growth and calcium needs decrease, the daily allowance are reduced to 5 mg after age 22. An additional 5 mg per a day is recommended for pregnant and lactating women.

TABLE 15: Average composition of human and cow's milk

	Breast milk, %	Cow's milk, %
Water	87.6	87.2
Total solids	12.4	12.8
Casein	0.4	2.7
Lactalbumin	0.4	0.4
Lactoglobulin	0.2	0.2
Total protein	1.1	3.3
Fat	3.5	3.8
Lactose	7.0	4.8
Ash	0.21	0.71
Sodium	0.015	0.058
Potassium	0.055	0.138
Calcium	0.034	0.126
Magnesium	0.004	0.013
Iron	0.00021	0.00015
Chlorine	0.043	0.100
Phosphorus	0.016	0.099
Sulfur	0.014	0.030
Calories per 100 ml	71	69
Vitamins per 100 ml		
Vitamin A	53 µg	34 µg
Carotinoids	27	38
Thiamin	16	42
Riboflavin	43	157
Niacin	172	85
Pyridoxine	11	48
Folic acid	0.18	0.23
,Vitamin B	0.18	0.56
Vitamin C	4.30 mg	1.80
Vitamin D	0.4-10.0	0.3-4.0 IU

Vitamin E

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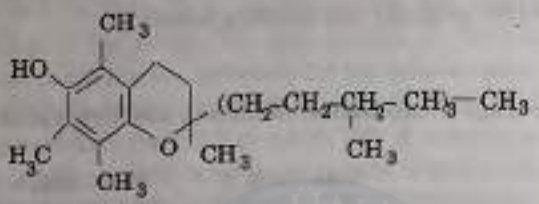
There are various tocopherols which have vitamin E activity in plants; The most active and aboundant is α-tocoherol (11).



(11)

VITAMIN E
∝ - Tocopherol

The biological function of tocopherol is still obscure but they do have antioxidant activity. They prevent the autoxidation of highly unsaturated fatty acid acids, when they are exposed to molecular oxygen. Actually the antioxidant action of tocopherol is effective a high oxygen concentrations. Such oxidation results in the polymerization of unsaturated fatty acids. One of the functions of tocopherol may be to protect highly unsaturated fatty acids in the lipids of biological membranes against the deleterious effects of molecular oxygen. Normally, autoxidation products of unsaturated fats do not occur in the tissues, but in tocopherol deficiency they are detectable in the fat deposits, liver, and other organs. The requirement for vitamin E is increased with greater intake of poly unsaturated fat. Intake of mineral oul exposure to insufficient lipid absorbtion may cause deficiencies of the vitamin E, leading to neurologic disorder. Anemia of the newborn may occur due to a deficiency of vitamin E here may then be a need for supplementation of tocopherol in the diet of pregnant and lactoting women and newborn infants. Anemia may be due to decreosed production of hemeglobin and a shortened erythrocyte life span.

Although fish liver oils are rich sources of viamins A and D, they possess insignificant amounts of vitamin E. wheat germ, sunflower seed, corn and soya been oil are all good sources of vitamin E It is also found in leafy vegetables, legumes, whole grain, liver, milk, butter and eggs. It is

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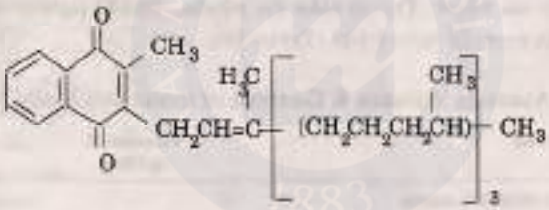
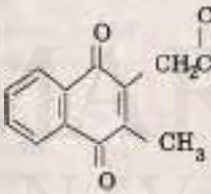
destroyed by commercial cooking and food processing, including deep-freezing. Recommended daily dietary intakes, in -tokopherol equivalents are 10 mg for adult males and 8 mg for adult females and boys 11-14 years old. During pregnancy and lactation, intake should be increased by 2-3 mg. Infants should receive 3 mg per a day during the first half year, and children 4-8 mg per a day.

Vitamin K

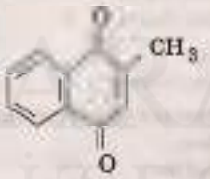
The vitamin K series are the phylloquinones (type K_1) synthesized in plants **(12a)** and the menaquinones. (type K_2) of bacterial origin. **(12b)** Several synthetic analogs and derivatives have been used such as menadione (K) There is no a side-chain substituent at position 3 in menadione. **(12c)**. Vitamin K is necessary for carboxylase reactions.



(12 a) VITAMIN K_1



(12 b) VITAMIN K_2



(12 c) VITAMIN K

The vitamin K- dependent carboxylase reaction occurs in the endoplasmic reticulum of many tissues and requires Co_2, O_2 and glutamic acid. A vitamin K cycle is belived to exist in the endoplormic reticulum of the liver where the quinone form of vitamin K is converted to the hydroquinone by NADH dependent de hydrogenisation reaction. Thus, regeneration of vitamin K occurs (Figure 3) The funtions of vitamin K are summarised as follows;

-) Vitamin K acts as cofactor of the carboxylase to forms γ carboxyglutamate residues.

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-) As γ - Carboxyglutamate residues occur not only in coagulation proteins but also in bone Gla proteins, Mineralization of bone may be regulated by these carboxylated proteins.

The absorption of natural vitamin K from the small in testine is facilitated by bile salts, as are other lipids and they are distributed by the blood stream via the Lymphatics, in chylomicrons. Menadione being water soluble, is absorbed even in the absence of bile salts.

Green leafy vegetables, egg yolk and meats are sources of vitamin K. no specific RDA are presently given for vitamin K, since intestinal bacteria in normal individuals synthesize vitamin K, which are partially absorbed into the blood. The in take for adults, 70-140 µg/day is easily supplied by an average mixed diet (Table: 16).

TABLE 16 : Average Vitamin K Content of Some Ordinary Foods

Food	Vitamin K µg/100 g
Milk and Milk Products	
Butter	30
Cheese	35
Milk (cow)	1
Milk (human)	0.3
Eggs	
Hen (whole)	11
Meat and Meat Products	
Bacon	46
Beef liver	92
Chicken liver	7
Ground beef	7
Ham	15
Pork liver	25
Pork tenderloin	11
Fats	
Beef fat	15
Corn oil	3
Safflower oil	3
Soybean oil	193
Cereals and Grain Products	

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Bread	4
Maize	5
Oats	10
Rice	3
Wheat flour	4
Whole wheat	17
Vegetables	
Asparagus	57
Beans, green	40
Broccoli	175
Cabbage	125
Kale	729
Lettuce	129
Peas, green	29
Potato	1
Pumpkin	2
Spinach	415
Tomato	10
Turnip greens	650
Watercress	80
Fruits	
Applesauce	2
Banana	2
Orange	1
Peach	8
Raisin	6
Strawberry	10
Beverages	
Coffee	38
Cola	2
Tea, black	
Tea, green	712
Tobacco	
Cigarettes	5000

Vitamin K was first recognized as a dietary antihemorrhagic factor the absence of vitamin K from the tissues of animals causes bleeding due to a coagulation defect, due to inadequate synthesis of biologically active procoagulants, vitamin K was shown to be necessary for establishing levels of blood clotting factors II (prothrombin), FVII (proconvertin), FIX (plasma thromboplastin component), FX (Stuart Power) and Protein C

(Autoprotnombur II - anticoagulant), all of these coagulation factors are synthesized in the liver, initially as inactive precursors of proteins. (Figure 2) Vitamin K incorporates $C0_2$ into specific L-glutamyl as inactive precursors of proteins and the other coagulation factors mentioned above. After the carboxylation reaction these inactive factors become active form and coagulation occurs. Generation of the biologically active clotting factors involves the posttranslational modification of glutamate (Glu) residues of the precursor proteins to γ - carboxyglutamate (Gla) residues by a specific vitamin K- dependent carboxylase. These coagulation proteins known as vitamin - K - dependent coagulation proteins (Figure 2). For exemple; prothrombin complex contains 10 of these residues, which allow chelation of calcium in a specific procin - calcium - phospholipid interaction.

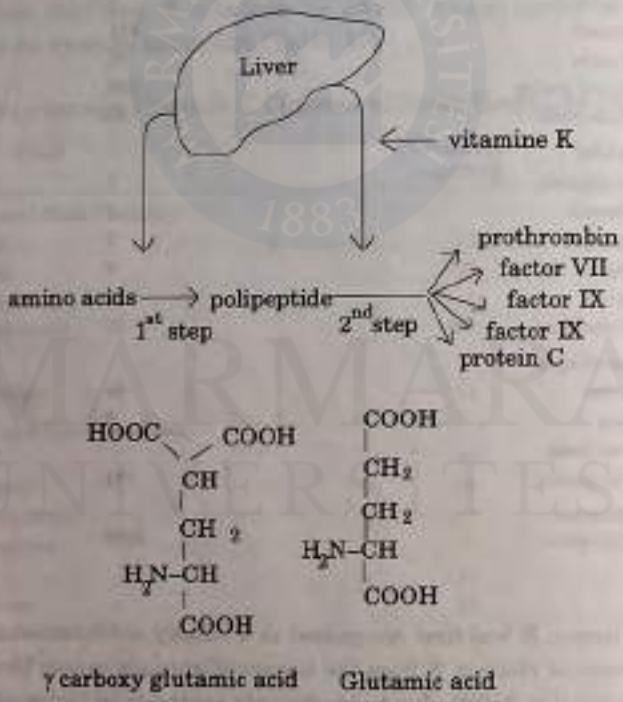

Figure:2 Carboxylation of vitamin K , dependent coagulation factors in the liver. (From- ref. 39)

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Bis - 4 - hydroxycoumarin (dicumarol) the cloting compound from spoiled sweet clover, and synthetic coumarin (warfarin) act as anticoagulants by interfering with the carboxylation of viamin K , dependent coagulation factors. Hence, coumarins are able to suppress the formation of prothrombin and other vitamin K-dependent Ca-binding proteins (Figure 3) The administration of vitamin K will by pass the coumarin-induced inhibition and allow maturation of the Gla-containing factors. Reversal of coumarin inhibition by vitamin K requires 12-24 hours, whereas reversal of he anticoagulant effects of heparin by protamine is almost instantaneous. Coumarin derivatives are known as oral anticoagulants.

Spontanecus internal haemorrhage occuring in young chicks havebeen cured by a fat-soluble component of plant leaves. Cattle poisoned by spoiled sweet clover, which contains the antagonist dicumarol, rats poisoned by warfarin and chickens which are poor at absorbing vitamin K all suffer from intestinal bleeding. The clotting time of the blood is increased and the blood prothrombin level is decreased. Coumarin derivatives are therefore widely used in clinical medicine to prevent clotting in blood vessel, for patient suffening from thrombos.

TABLE 17: Causes of Deficiencies of Vitamin K- Dependent Coagulation Factors

Hemorrhagic disease of the newborn

Dietary inadequacy (low-fat diets, protein-calorie malnutrition)

Total parenteral nutrition

Biliary obstruction (gallstones, stricture, fistulas)

Malabsorption syndromes (cystic fibrosis, sprue, celiac disease, ulcerative colitis, regional ileitis, short-bowel syndrome)

Liver disease

Drug therapy

- **Coumarin anticoagulants and related drugs (warfarin, indanediones, phenprocoumon, hydantoins, salicylates)**
- **Broad-spectrum antibiotics including cephalosporins**
- **Megadoses of vitamins A and E**

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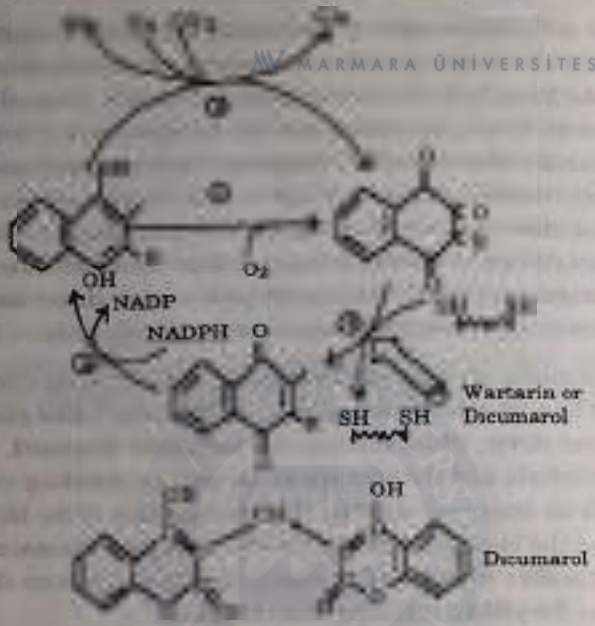

Figure 3: Regeneration of vitamin K in the vitamin K cycle and dicumarol inhibition. (From ref. 45)

In normal individuals, intestinal bacteria synthesize vitamin K which is partially absorbed. Hemorrahgic disease of the newborn can develop readily, since the vitamin K-synthesizing intestinal flora have not become established within the first week postpartum, and early breast milk is low in vitamin K. Prothrombin levels during this period are only about 25 % the adult level. Severe diarrhea and antibiotics being used will readily exacerbate the situation, so that prothrombin levels can drop below 5 % of the adult level and bleeding can occur. The use of high dose vitamin K leads to hemolytic anemia with severe hemolysis and increased biluribin formation.

Several hypotheses have been proposed to explain the action of vitamin K. Originally it was thought that vitamin K was a component of prothrombin. Other ideas proposed more recently suggested that vitamin K regulated;

- mammalian electron transport,
- specific mRNA synthesis and uptake by ribosomes,

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- the posttranlational modification of a precursor peptide by addition of a prosthetic group.

Later, γ-carboxyglutomic acid in a tetrapeptide (Leu-Gla-Gla-Val) is isolated from bovine prothrombin. This new amino acid was absent from the corresponding peptide derived from the abnormal inactive prothrombin present in the plasma of cows that had been fed dicumarol.

TABLE 18 : Properties of the vitamin K dependent coagulation proenzymes

	Factors				Proteins			
Characteristic	**II**	**X**	**IX**	**VII**	**C**	**S**	**M**	**Z**
Plasma concentration (μg/ml)	100	20	3	1	10	1	<1	<1
Molecular weight	72,000	56,000	55,000	46,000	57,000	69,000	50,000	55,000
Carbohydrate percentage	8	13	26	13	8	(+)	(+)	(+)
Number of chains	1	2	1	1	2	1	1	1
Number of Gla residues	10	12	12	10	11	10	(+)	13

These findings strengthened the view that vitamin K was involved in the posttranslational carboxylation of an inactive precurson peptide.

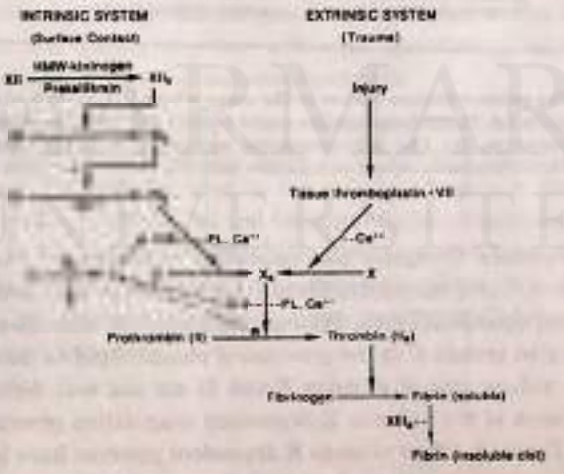

Figure 4: Activation and inactivation of vitamin K dependent coagulation factors. (From ref. 51)

As mentioned above like prothrombin F VII, IX and X are also regulated by vitamin K. More recently, four new vitamin K-dependent proteins containing Gla have been isolated, namely proteins C, S, H and Z. All require Ca^{2+} for activity and contain areas of amino acid homology with the established vitamin K dependent factors. Protein C and S are anticoagulants.

Effect of Autoprotrombin II-A (protein C) on experimental DIC induced animal is shown in figure 5. Protein C is activated by thrombin.

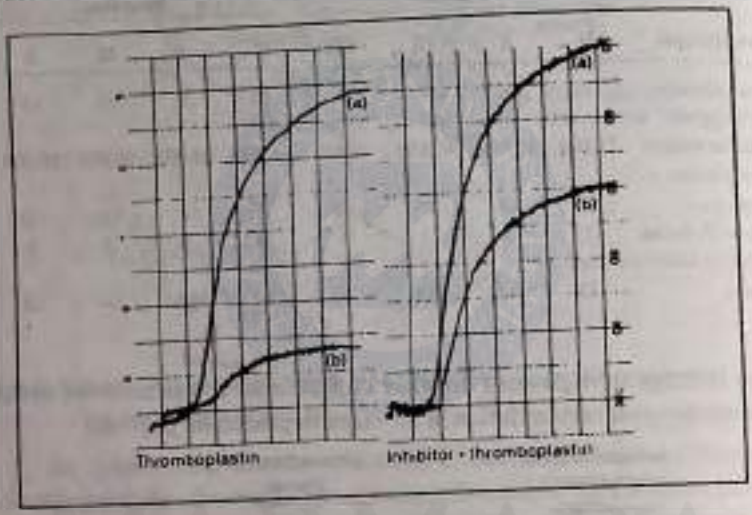

Figure 5: The polymerization curves in the cases where DIC is formed experimentally. (a) The polymerization curve before and 1 h after thromboplastin injection. (b) The polymerization curves value before and 1 h after thromboplastin injection together with the inhibitor. (From ref. 40)

Activated protein C rapidly and selectively inactivates Factors V and VIII. Protein C also stimulates fibrinolysis by release of plasminogen activator from endothelial cells. Proteins enhances the activation of Factor V by activated protein C in the presence of phospholipid as shown in Figure... the biologic role of proteins Z and M are not well defined. Some characteristics of the vitamin K-dependent coagulation proenzymes are shown in Table 18. Other vitamin K-dependent proteins have been found in kidney and bone, but they play no role in coagulation.

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Oral Tissues and Vitamin Deficiencies

Although vitamins have no specific effects on oral tissues but some vitamins show oral manifestation. This may be of value in diagnosing the condition of bleiding, sore and swollen gums. These as noted were some early symptoms of scurvy which is due to vitamin C deficiency. It is possible that a subclinical deficiency of ascorbic acid in some individuals contributes to the gingivitis so commonly found in the general population. In cases of severe vitamin C deficiency, there is a degeneration of the alveolar bone in man, which may result in loss of attachment of the periodontal fibres, and weakening of the periodontal membrane. Loss of support renders the teeth mobile and thus easily extractable. In dentine, the odontoblasts degenerate and the dentine itself may be reduced.

Experimental riboflavine deficiency in volunteers produced a pallor of the lips followed by epithelial denudation, having a red, shiny appearance (cheilosis). Open fissures, many of them infected appeared at the corner of the mouth. Enlargement and inflammation of the tongue (glossitis) are characteristic of ariboflavinosis. Such oral manifestation is observed with niacin deficiency. Although oral lesions have not been attributed to pyridoxine deficiency, a cheilosis has been induced in human volunteers by the pyridoxine antivitamin. Sore and swollen tongues also occurred in some of these cases. These effects were reversed by pyridoxin administration. Some pernicious anemia patients suffer from inflammation of the oral mucosa, together with other symptoms. Oral inflammation disappears after cyanocobalamin treatment.

In vitamin A deficiency, hyperplasia and keratinisation occur in the gingival epithelium and the ducts of the salivary glands Atrophy of odontablast has veen observed in the induced vitamin A deficiency in rats. For normal development and function of odontoblasts depend on vitamin A. In vitamin D deficiency, the thinner enamel than normal and poorly calcified with irregular teeth surfaces have been observed in dogs. Reduced rate of dentine calcification due to vitamin D deficiency have been observed in animals.

NUTRITIONAL REQUIREMENTS AND FUNCTIONS OF MINERALS

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Minerals make up another important food groups. They may be divided into 2 groups;

a) macrominerals, which are required in amounts greater than 100 mg/day

b) Micraminerals or trace elements whick are required in amounts less than 100 mg/day.

Intakes of trace elements are measured in mg or pq/day. Improvments in the sensitivity of analytical methods have allowed the study of ultratrace elements, those present in ng/g amounts or less macro and micro mineral play vital roles in human and animal metcholism. Of course a biochenical basis for the essential function of the element fanctions must be demonstrated. For optimal health there must be homeostatic regulation for avery element. At low nutrient levels, dependent biological functions are impaired, this defining a deficiency state. Very high nutrient levels may result in another impairment or a toxicity state. Each nutrient has its own characteristic curve, The elements currently accepted as essential in animals or humans are shown in a periodic table. (Figure 6). Aluminium, cadmium, lead and mercury, which are trace elements, are consistently present in human tissues but they are considered nonessentiol and can be toxic.

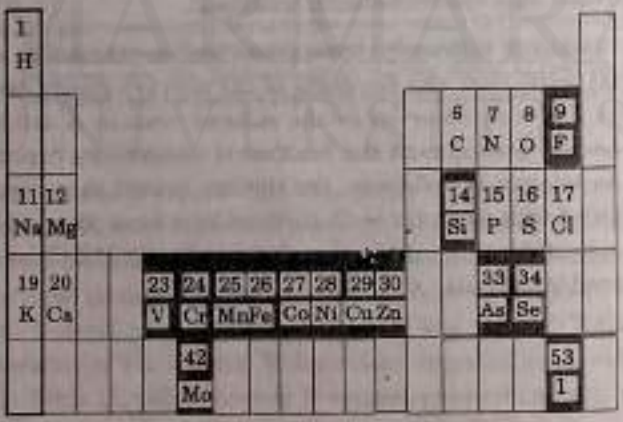

Figure: 6 Elements accepted as essential for human or animal nutrition (trace elements shaded)

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If a diet is balanced between carbohydrates, lipids and protein it will get sufficient amounts of these minerals. Some of the essential elements are discussed in this section i.e. calcium, phosphorus, magnesium, iron, iodine, fluorine, chromium and selenium. where calcium is discussed in details.

TABLE 19: Recommended mineral intake for men and women in different ages

	Age (years	Weight (kg)	Calcium (mg)	Phosphorus (mg)	Magnesium (mg)	Iron (mg)	Zinc (mg)	Iodine (µg)
Infants	0.0-0.5	6	360	240	60	10	3	40
	0.5-1.0	9	540	360	70	15	5	50
Children	1-3	13	800	800	150	15	10	70
	4-6	20	800	800	200	10	10	90
	7-10	28	800	800	250	10	10	120
Males	11-14	45	1200	1200	350	18	15	150
	15-18	66	1200	1200	400	18	15	150
	19-22	70	800	800	350	10	15	150
	23-50	70	800	800	350	10	15	150
	51+	70	800	800	350	10	15	150
Females	11-14	46	1200	1200	300	18	15	150
	15-18	55	1200	1200	300	18	15	150
	19-22	55	800	800	300	18	15	150
	23-50	55	800	800	300	18	15	150
	51+	55	800	700	300	10	15	150
Pregnant			+400	+400	+150		+5	+25
Lactating			+400	+400	+150		+10	+50

TABLE 20: Recommended electrolyte intake for human in different ages

	Age (years)	Electrolytes Sodium (mg)	Potassium (mg)	Chloride (mg)
Infants	0-0.5	115-350	350-925	275-700
	0.5-1	250-750	425-1275	400-1200
Children and	1-3	325-975	550-1650	500-1500
adolescens	4-6	450-1350	775-2325	700-2100
	7-10	600-1800	1000-3000	925-2775
	11+	900-2700	1525-4575	1400-4200
Adults		1100-3300	1875-5625	1700-5100

TABLE: 21 Mineral values of some food (Adapted from ref. 1,9,10)
(per 100 g edible portion)

Meat Group	Ca	Fe	K	Na	Mg	P	Cu	Zn
Veal (fatty)	11.0	2.8	500	80	16	185	0,25	2.8
Mutton (fatty)	9.0	1.1	290	70	14	135	0,13	3.0
Beef (Lean)	8,0	2.2	370	60	21	180	0.08	4.2
Lamb (Lean)	9.0	1.5	-	-	-	-	-	-
Liver (Veal)	8.0	8.8	281	73	16	333	2.8	3.8
Kidney (Lamb)	13.0	7.6	230	200	-	218	-	-
Brain (All)	10.0	2.4	219	125	-	312	-	-
Chicken								
White (with skin)	11.0	0,8	204	65	23	163	0.04	0.93
White (without skin)	12.0	0.7	239	68	27	187	0.04	0.97
Fish and Shell fish								
Sardine	33.0	1.8	-	-	28	215	0.19	0.7
Sprimp	63.0	1.6	220	140	42	166	-	1.5
Lobster	29.0	0.6	-	-	22	183	1.7	1.8
Meat substitues								
Bean	144.0	7.8	1196	19	170	425	0.11	-
Soybean	236.0	8.4	1677	5	264	554	-	-
Broad bean	102.0	7.1	-	-	-	391	-	-
Leatil	79.0	6.8	790	30	80	377	-	3.10
Chick pea	150.0	6.9	797	26	3	331	-	270
peanut	72.0	2.2	701	5	256	407		3.0
sesame	110.0	2.4	-	-	347	592	-	10.2
Almond	234.0	4.7	773	4	270	504	-	-
Sunflower	120.0	7.1	920	30	38	837	1.8	-
Hazelnut	209.0	3.4	704	2.0	184	337	1.4	-
Pumpkin-seed	40.0	10.0	-	-	-	1144	-	-
Chestnut	29.0	0.9	592	2,0	41	107	-	-
Walnut	99.0	3.1	450	2	131	308	1.1	-
Bread (1 slice - 25 g)	22	0.3	24	0.5	-	24	-	-
Rice	24	0.8	92	5	-	94	-	-

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TABLE : 21 (Continue)

Vegetable Group	Ca	Fe	K	Na	Mg	P	Cu	Zn
Beans (green)	56	0.8	243	7	32	44	0,10	0,4
Peas	26	1.9	316	2	35	116	0,24	0,9
Mushroom	6	0.8	414	15	13	116	-	-
Spinach	93	3.1	470	71	88	51	0,26	0,8
Eggplant	12	0.7	214	2	16	36	-	-
Potatoes	7	0.6	407	3	22	53	0,15	-
Lettuce	35	2.0	264	9	10	26	0,04	0,04
Corn (raw)	3	0.7	280	trace	147	111	-	3,9
Cauliflower	25	1.1	296	13	24	56	0,14	-
Cabage (white)	49	0.4	233	20	13	29	0,05	0,4
Cabbage (black)	116	1.6	-	-	-	-	-	-
Peppers (green)	9	0.7	213	13	18	22	0,10	-
Onions (cooked)	27	0.5	157	10	12	36	-	0,3
Turnips	38	0.5	268	48	30	30	-	-
Tomato (raco)	13	0.5	244	3	14	27	0,06	0,2
Cucumber	25	1.1	160	6	11	27	0,06	-
carrots	37	0.7	341	47	23	36	0,08	0,4
Artichoke	50	1.1	860	86	-	176	-	-
Okra	90	0.6	249	3	41	51	0,12	-
Peppermint	755	30.2	-	-	-	-	-	-
Garden rocket	205	9.5	-	6	-	-	-	-
Garlic	29	1.5	529	19	-	202	-	-
Leek	52	1.1	347	5	23	50	0,09	-
Parsle	203	6.2	727	45	41	63	-	-
Parsnane	79	2.5	-	-	-	-	-	-
Squash (Summer)	21	0.8	340	1	16	44	0,05	-
Pepper (powder red)	148	8.0	2014	30		293		
Olive (black)	77	1.6	-	-	-	-		
(green)	90	2.0	-	-	-	-		
Celery	43	0.6	300	100	115	-	0,14	-
Grape Leaf	392	3.9	-	-	-	-	-	-

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TABLE: 21 (Continue)

Milk Group	Ca	Fe	K	Na	Mg	P	Cu	Zn
Butter milk	296	0.1	143	51	19	94	-	0.97
Whole milk	119	0.1	152	49	13	93	0.02	0.38
Cheese				630		512	-	
(Cheddar)	721	0.7	98	47	28	87	0.03	3.11
Yoğurt	111	0.1	132	-	12	-	-	0.59
Milk desserts	297	1.1	-	-		-	0.05	-
Ice cream	194	0.1	-	-	14	-	-	0.5
Fruits								
Apple	8	0.4	110	1	5	10	0.10	0.05
Pear	8	0.3	130	2	7	11	0.10	-
Mulberry	60	1.1	-	-	-	-	-	-
Strawberry	21	1.0	164	1	13	21	0.02	-
Quince	11	0.7	194	4	-	17	0.14	-
Avacado	10	0.6	604	4	45	42	-	-
Kivi	100	3.0	300	-	-	30	-	-
Banana	8	0.7	370	1	33	26	0.09	0.2
Grape fruit	16	0.4	135	1	12	16	0.03	-
Orange	41	0.4	200	1	-	20	-	-
Lemon	26	0.6	138	2	-	16	0.04	-
Grapes (dry)	62	3.5	763	27	35	101	0.020	-
Fig (dry)	126	3.0	640	34	71	77	-	-
Melon	14	0.4	251	12	16	16	0.04	-
Water melon	7	0.5	100	1	8	10	0.07	-
Cherries	22	0.6	191	2	14	19	-	-
Apricot	16	0.5	979	26	62	106	0.11	-
Peaches	9	0.5	202	1	10	19	0.01	0.2
Pomegranata	3	0.3	259	3	-	8	-	0.2
Plum	51	3.9	694	8	405	79	-	-
Date	59	3.0	648	1	58	63		

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- CALCIUM -

Calcium is the most abundant mineral in the body. About 99% of the total body calcium exists as crystalline hydroxy apotite in bones and teeth. Of the 1 to 1,5 kg of calcium in the adult human body, no more than 10g is found in the soft tissues and body fluids. Approximately 5g (125 mmol) of calcium is in the exchangeable calcium pool located mainly on bone-forming surfaces and in soft tissues, where the calcium is readily accessible for physiological funcions. In adults calcium intake and ouput are normally in balance. In infancy and childhood, there is normally a positive balance then occurs. Significant amounts of calcium are normaly found in gastrointestinal secretions. Calcium excreted in feces is derived partly from the diet and partly from the intestinal secretions. The efficiency of calcium absorbtion tends to decrease with age, so increased dietary intakes may be needed in the elderly. the excretion of calcium in the urine varies considerably with the diet, but the relationship is not linear.

Bone is made up of approximately 40% inorganic matarial, 20% organic material and 40 % water. The inorganic material consists mainly of the complex salt hydroxy apatite, (3 Ca) (PO 4) 2Ca (OH)2 but sodium, magnesium floride, carbonate, citrate and chloride as well as other ions are also present. The mineral crystals of bone tend to be deposited at the regions around the ends of he trophocollagen units. Based on purely physicochemical considerations, calcium and phosphate should precipitate from the extracellular fluid when the solubility product (calcium) x (phosphate) is exceeded.

Calcium salts in bone have a mechanical role, but are not metabolicall ynert, there being a constant state of turnover in the skeleton associated with deposition of calcium of sites of bone formation and release at sites of bone resorption. Calcium in bone acts as a reservoir which helps to stabilize the calcium ion in the extracellular fluid. Deposition of bone salt, is not simply, a passive physicochemical process. Rather seems that it involves the removal of the inhibitors of calcification, possibly inorganic phosphates. Alkaline phosphatase which can catalyse the breakdown of inorganic phosphate, may be involved in this procees, because calcification is defective when alkaline phosphatase is deficient, as in hypophosphatasia. Hormonal influences are very important. As was mentioned under vitamine D plasma and intestinal fluid calcium is regulated in man by two hormones; parathormone (PHT) and 1:25. dihydroxy cholecalcife-

rol (1,25) $(OH)_2$ D) How calcium, levels are maintained in the body is shown in figure 7.

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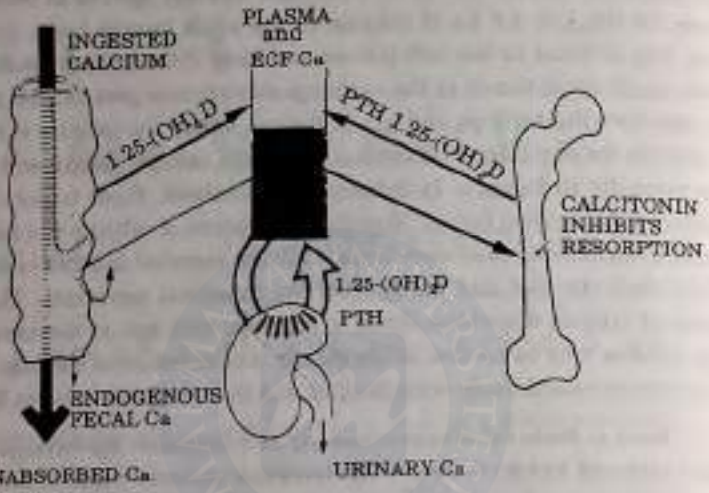

Figure 7: Maintaining calcium homeostasis (From ref. 53)

PTH and 1,25 (OH2) D act to increase plasma calcium, however, calcitonin and katacalsin are calcium-lowering hormones in plasma and extracellular fluid (ECF) Growth hormone, glucocortioids (e.g.cortisol), oestrogens, testosterone and the thyroid hormones (T3 - T4) also exert a minor influence on calcium homeostasis.

The extracellular fluid contains Igr of calcium. Of this, the plasma contains a total of about 300 mg in the average adult male and 225 mg in the female. Calcium exists in plasma in three physicochemical states; protein-bound (45 %), complexed with small diffusible ligands such as citrate, lactate, phosphate (10 %) and ionized (45 %). Eighty per cent of the protein-bound fraction is bound to albumin and 20 % to globulins. The ionized calcium is the physiologicall active from.

In contrast to the large quantity of extracellular calcium only 1 mmol is present in the cytosol (10^{-7}mol/L) This is very low compared to the extracellular calcium (10^{-3} mol/L). Intracellular calcium serves a vital role, in which calcium acts as a secondery messenger. Increased intracellular free calcium is responsible for the cell response regulating cell functions; e.g.:

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- Cell movement or shape change

- Secretion

- Mitosis

- Neuromuscular activity

- Membrane permeability

- Activities of many enzymes (e.g. coagulation proteins), through reversible combination with calmodulin which is a small protein found in all cells, having four calcium-binding sites for each molecule.

- In striated muscle, calcium activates contraction of the myosin fibril through combination with troponin which is a calcium-binding protein similar, if not identical, to calmodulin.

The mean plasma or total serum calcium level for adults is 9.4 mg/dl (8.8-10.2 mg/dl). In ages 4-20 is (9.2-11.Omg/dl). Urinary total calcium excretion depends on dietary intake of calcium. Calcium excretion for 90 % healthy individuals are: Men 300 mg/day; women 250 mg/day and children 6 mg per kg. body weight perday. Assuming a net intake of 700 mg calcium per 24h and net absorption rate of 20-40 %, the fecal calcium excretion rate in healthy adults ranges from 420-560 mg per day.

As was just mentioned calcium is constantly lost from the body through excretion in feces, urine and, to a small extent, sweat. The dietary calcium requirement of humans depends on age, growth, pregnancy and lactation. The minimum dail requirement in adults is about 500 mg/day. The RDA for calcium is 800 mg/day for adults and children above one year of age. An additional 400 mg/day calcium is recommended during adolescence, pregnancy and lactation. Net absorption of ingesed calcium in the healthy adult is about 20-40 % However, in African and eastern countries often only 200 to 300 mg of calcium is ingested per day. Absorption of calcium is facilitated by acidic conditions in the gastrointestinal tract. In neutral and alkaline conditions, the calcium salts of phosphoric acid and phytic acid precipitate. Milk and milk produts are the main sources of calcium. In certain dark-green leafy vegetables (collards kale, mustard greens, turnip greens) are good source of calcium. The calcium level of some nutrients are shown in table 21.

Disease involving calcium metabolism may be classified as 1) Disease associated with hypercalcemia or, 2) Disease associated with hypocalcemia and normocalcemia.

Causes of hypercalcemia

Parathyroid disease (PTH)	:	The great majority of hypercalcemic patient suffer from primary hyperparathyroidism and malignant hyper calcemia
Bone Disease	:	Carcinoma with skeletal metastases results in hyper-calcemia by causing bone resorption. Multiple myeloma Bone reticuloses
Drug - induced	:	Thiazide diuretics
Familial disorder	:	Familial hypocalcuric hypercalcemia.
Excess intake vitamin D or calcium	:	Self medication with vitamin D, during treatment hypocalcemic administration causing over dosage of vitamin D.
Miscellaneous causes	:	Sarcoidosis, Thyrotoxicosis, Cushing's syndrome, hypercalcaemic penostitis..

Causes of hypocalcemia

Parathyroid disease (PTH)	:	Hypoparethyroidism due to deficient secretion of PTH. Pathogenesis of hypoparathroid disease includes congenital aplasia of the parathyroid glands or damage during thyroidectomy or autoimmune destraction of glands. Hypocalcemia may be asymptomatic or associated with tetanus

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Hypoproteinaemia	:	Various causes
Renal Disease	:	Chronic renal failure
Inadequate intake of Calcium and Vitamin D.	:	Dietary deficiency or malabsorption in the intestine. Rickets and osteomalacia occur due to lack of dietary vitamin D, calcium or phosphate or both, or various intestinal malabsorptive diseases. Rickets occurs in growing children, osteomalacia in adults.
Bone disease with normocalcemia	:	Paget's disease; this is a common disorder of bone. It occrs in osteosclerotic and osteolytic form, both associated with increased plasma alkaline phosphate activity. Plasma calcium and phosphate are usually normal. Osteoporesis, this is also very commen disorder. It is not usually positively diagnosed until there is a marked loss in the bone density.
Misellaneous	:	Acute pancreatitis, cystinosis, cytotoxic drugs

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- PHOSPHATE -

The body of a human adult contains 620-grams of phosphorus entirely in the form of phosphate. The blood level in adults is 3-45 mg/dl, in children 4-7 mg/dl. Phosphate is distributed between extracellular and intracellular compartments. Intracellularly, phosphate occurs mainly as a component of phospholipids and phosphoproteins. A small but very important fraction also exists intracellularly, as inorganic phosphate that participates in high energy transfer reactions (ATP) About 85 % of extracellular phosphate ocurs in inorganic form as the mono and di hydrogen forms ($H PO_4^{2-}$, $H_2 PO^-_4$).

The concentration of inorganic phosphate in circulating plasma is influenced by the parathyroid hormone, vitamin D, intestinal absorption, renal function, bone metaholism and nutrition. A combination of increased plasma calcium and decreased fasting plasma phosphate supports a diagnosis of primary hyperpara thyroidism. Some of the physiological functions of phosphate may be summarised as follows;

-) Phosphate in the form of phospholipid is a stuctural component of cell membranes.

-) It participates in many important cell functions such as; ATP, NADP, GTP. phosphorylation of sugar and other molecule and gene replication.

-) Pi (inorganic phosphate) is the substrate for oxidative phosphorilation

-) Glycogen breakdown in the liver and kidneys requires Pi to produce free glucose

-) It is important for bone and teeth structure

Decreased plasma concentration of phosphorus may observed in hyperparathyroidism, hypovitaminosis D (rickets and osteomalacia) malabsorption syndrome, ingestion of antiacids that bind phosphate in the gut, starvation, chronic alcoholism, renal tabular defects, use of thiazide diuretics, acid-base disturbances, and diabetic ketoacidosis. It is accasionally decreased during pregnancy and hypothyroidism.

Increased plasma concentration of phosphorus is found in renal insufficiency, hypoparathyroidism and hypervitaminosis D.

The RDA for phosphorus in mg per day is the same as that for calcium. Phosphorus is present in most foods, as shown in table 21.

Like calcium, phosphorus and magnesium are vitally important minerals. The body needs them for bones and teeth, they also aid in converting foods into energy. Good quantities of magnesium are found in nuts, whole-grain products, dry beans, dry peas and dark-green vegetables. If a diet contains enough protein and calcium, it will probably get enough phosphorus as well.

- IRON -

Iron is needed by the body in relatively small amounts, but these amounts are vital. Iron combines with protein to make hemoglobin in the red cells that carry oxygen from the lungs to body cells and remove the waste product carbon dioxide from them. By carrying oxygen to each cell, iron makes it possible for the cells to obtain energy from food. Only a few food contain much iron. Liver is a particularly food source but it most be remembered that liver is high in cholesterol. Heart and kiney muscle are also good sources of iron, but these are also high in cholesterol. Other sources of iron are learn meats, shell fish, dry beans, dry bean, dry peas, darkreen vegetables, oats, dried fruit, egg yolk (high in cholesterol) and molasses. Whole-grain breads and cereals contain smaller amounts of iron, but because they are eaten fre quently they can be an important source. Other nutrients and iron content is shown in table 19.

Research has shown that the diets of young children, preteen and teenage girls and women of childbearing age are most likely to be iron deficiencit. It is therefore recommended that they eat enough foods containing this mineral.

- IODINE-

Iodine deficiency result in the disease called goiter, a swelling of the thyroid gland. Iodine is found in seafood-fish, clams, oysters, and mussels - and in seeweed. People who live near the coast usually get enough iodine because their diet includes seafood. People living inland however will get enough iodine if it is a constituent of the soil. Where the soil does not contain iodine, people are at risk of devoloping goiter. The

absence of iodine in the environment can be made up for by eating canned or frozenseafood and using iodized salt, which is ordinar table salt with a little potassium iodide added.

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- FLUORINE -

This is one of the essential minerals. It helps to protect teeth from decay. Especially during the years when teeth are being formed, drinking water should contain a proper amount of flourine in the form of natural or added fluoride, which will make teeth more resistant to decay.

Interest in human floride began after the discovery of chronic, endemic floride toxicity in several countries. Initially, it was observed that children with mottled enamel (a sign of floride excess) had very little dental decay. Since than, many clinical and epidemiological studies have demonstrated the anique propert of floride in preventing tooth decay. The exact form of floride treatment that provides the maximal anticariogenic effect and the mechanism of this effect are not definitely known. It is known however that supplemental floride treatment is generally most effective during the period of tooth eruption and immediate there after.

Inorganic floride is readily absorbed in the small intestine and distributed almost entirely to bones and teeth. Floride replaces hydroxyl groups in tooth apatite. Renal excretion is most important for regulation of body floride levels.

- Distribution of Floride In The Body -

According to research the floride concentration in healthy adults serum or plasma is 10-370 ng/ml, 450 ng/ml in erythroctes and 0.2 - 1.9 mg/l in urine the concentrations of floride vary widely, depending on whether or not the water in an area is fluorated. Fluoridated drinking water contributes appreciably to total floride intake. Seafoods and tea contain high levels of floride the estimated safe and adequate diatery intakes of fluoride are listed in table 22.

In young adults from areas with 0.1 p.p.m. in the water supply. The femoral cortex contains about 500 p.p.m. floiride on an ash basis, and 70 years of age, about 2000 p.p.m. As the floride content of water increases, the bone floride increases approximately linearly. It is interesting to nute that the body of an adult can easily contain about 20 gr. The distribution within bone is not uniform. Cancellous bone often contains

more than compact bone. Soft tissues generally contain fluride, usually 0.03 p.p.m. on a dry weight basis.

It was thought that most floride in plasma was bound to albumin and it is present as unbound free ions. The distribution of floride in enamel has been extensively studied. It shows the greatest variation in concentration being high in the immediate surface zone, and then falling sharply towards the amelodentinal junction where there is sometimes a slight rise. A surface zone about 0.05 mm thick can contain about 3000 p.p.m. in extreme cases with 5 p.p.m. floride in the water supply. The outer regions of this zone will have even high concentrations. Some pathological calcification, for example urinary calculi, contain apatite with about 2500 p.p.m. floride.

TABLE 22: Dietary sources of fluorine
(Micrograms per 100-g edible portion)

Almonds	80	Herring, smoked	350
Apples	5-130	Honey	100
Apricots	3-22	Kale	16-300
Bananas	23	Lamb	120
Beans, string	13	Mackerel	2700
Beef	29-200	Mackerel, canned	1200
Round steak	130	Milk	10-55
Beef liver	99	Oats	25
Calf's liver	19	Onions	60
Beets	20	Oranges	7-17
Butter	150	Oysters	65
Cabbage	15	Parsley	80-100
Cantaloupe	30	Peaches	21
Carrots	40	Pears	19
Celery	14	Peas	60
Cheese	160	Pineapple	14
Cherries	25	Plum	21
Chicken	140	Pork	34-98
Chicken, canned	63	Pork, salt	100-3300
Chocolate	50	Potatoes	7-640
Choclate milk	50-200	Radish	80
Codfish	700	Rhubarb	40
Corn	62	Rice	10-67
Cornmeal	22	Salmon, canned	450-900
Crab meat, canned	200	Sardines, canned	730-1600
Cucumber	20	Shrimp, canned	440

Egg		Soybeans	130
Whole	120	Spinach	20-180
White	150	Strawberries	18
Yolk	59	Tea infusion	120-6300
Eggplant	40	Tomatoes	24
Figs	21	Tuna, canned	10
Fish	160	Turnips	30
Frankfurters	170	Veal	90
Gooseberries	11-52	Watercress	160
Grapefruit	36	Wheat	70
Grapes	16	Wheat flour	27-35
Hazelnuts	30	Wheat germ	88-400

Some evidence suggests that low levels of floride intake are associated with osteoporosis and that sodium fluoride and calcium supplementation may stmulate bone formation. Excessive fluoride intake produces mottled or pitted enamel in growing teeth. Chronic fluorosis may produce osteosclerosis, calcification of ligaments and tendons, and crippling deformities such as kyphois, stiffness of the spine and bony exostoses.

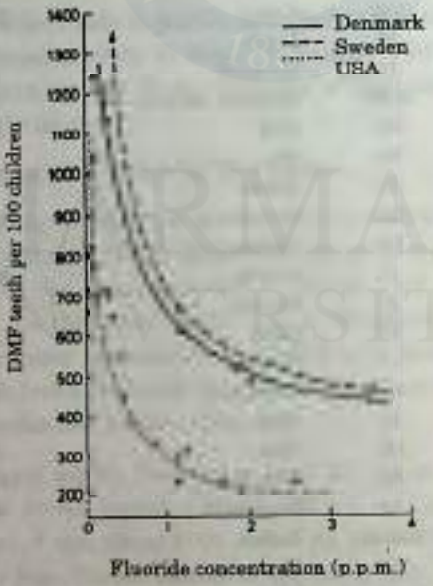

Figure 8: Caries experience in 12-13 years old children from Denmark, Sweden and the USA in relation to concentration of fluoride in water supplies (From ref. 62)

The world Health Organisation (WHO) has always taken a keen interest in the effect of flouridation on dental health. In 1958 a who export committe their produced first report on water fluoridation and concluded that drinking water containing about 1 ppm fluoride had a marked caries-preventive action and that controlled fluoridation of drinking water was a practicable and effective public health measure (WHO, 1958). Water floridation and relation to caries in some countries is shown in figure 8. In a later article, "Fluoridation and dental health" (WHO, 1969), it was stated that as a result of the initial controlled fluoridation studies in America and Canada, further programmes were under way in more than 30 countries and territories serving over 120 milion people. In the USA, the first compulsory fluoridation was introduced by municipal ordinance in various large or mediumsized cities, Including Baltimore, Chicago, Denver, Detroit, Miami, New York, San Francisco, St. Louis and Washington DC. Some of these cities have now been fluoridating their water supplies for 25 to 30 years, during which time dramatic improvements in the dental health of their populations have occurred.

While the constitutionality of community water fluoridation has been clearly established in the USA, its legality in the UK has been subject to question. However Birmingham was fluoridated in 1964 and Wewcastle followed in 1968. Regular fluoridation studies was done between 1975 - 1990 in Britain.

- CHROMIUM -

Chromium is a trace metal essential for glucose and lipid metabolism, being necessary for the action of insulin. Experimental chromium deficiency leads, in rats and squirrel monkeys to diminished glucose tolerance. In rats, chromium deficiency also has led to relatively elevated serum cholesterol levels and to the deposition of aortic plaques. However the feeding of chromium to rats appear to prevent both the formation of aortic plaques and the rise of serum cholesterol with age. A syndrome similar to moderate human atherosclerosis can be therefore reproducad in rats by chronic chromium deficiency. The agent that potentiates the action of insulin has been isolated from brewer's yeast and kidney powder and has been termed glucose tolerance factor (GTF). GTF is an organic low-molecular weight complex containing trivalent chromium. The biosynthesis and stmcture of GTF have not however been elucidated and the regulation of chromium homeostasis is still obscure, Furthermore the form in which most orally absorbed chromum is excreted is unknown.

Trivalent chromium has a low level of toxicity. So an apparently wide margin of safety exists between toxicity and supplementation required to cure any deficieny. Chromium (VI) however is considerably more toxic than Cr (III). Acute exposure may produce allergic reaction, conjunctivitis, nasal septum ulceration, dermatitis, edema, and ulcers. Chronic exposure may produce gastrointestinal symptoms, hepatitis, and lung cancer. Some other signs and symptoms of chromium deficiency are shown in table 23.

TABLE 23: Signs and Symptoms of Chromium Deficiency

Functions	Animals
Impaired glucose tolerance	Human, rat, mouse, squirrel monkey, guinea pig
Elevated circulating insulin	Human, rat
Glycosuria	Human, rat
Fasting hypergiycemia	Human, rat, mouse
Impaired growth	Rat, mouse, turkey
Hypogiycemia	Human
Elevated serum cholesterol an trigiyceridee	Human, rat, mouse
Increased incidence of aortic plaques	Rabbit, rat, mose
Increased aortic intimal plaque areas	Rabbit
Neuropathy	Human
encephalopathy	human
Corneal lesions	Rat, squirrel monkey
Decreased fertility-and sperm count	Rat
Decreased longevity	Rat, mouse
Decreased insulin binding	Human
Decreased insulin receptor number	Human

Estimated adequate and safe dietary intakes of chromium are 50-200 µg/d for adults (see table 25). In the USA, simple addition of 150 µg of Cr (III) to the daily diet improved the glucose tolerance of maturity-onset diabetics and middle-aged and elderly subjects.

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Several studies have reported daily dietary Cr intakes in excess of 100 or even 200 μg, and some studies have reported even higher valus. However, recent well-conrolled studies suggest that dietary Cr intake is approximately 50 μg or less in Finland, Canada, England and the United States. Values for dietary Cr intake for Canada were slightly above 50 μg, while the daily dietary Cr intake for another three countnes was approximately 30 μg. Calculated selected biological activities of food a values are shown in table 24.

TABLE 24: Calculated Chromium Biological Values of Selected Foods. (Edible portion as purchased)

Sample	Relative biological value	Sample	Relative biological value
Yeast, Brewers (dried)	44.88	Peppers, chili (resh)	2.27
Pepper, black	10.21	Wheat bran and midlings	2.21
Liver, calf's	4.52	Vegetarian chicken	2.16
Cheese, American	4.39	Cornmeal, white	2.09
Wheat germ	4.05	Shrimp	2.03
Bread, whole wheat	3.59	Grits	1.97
Cornflakes cereal	3.01	Lobster	1.95
Bread, white	2.99	Mushrooms	1.92
Spaghetti	2.89	Chicken leg	1.89
Beef round	2.89	Haddock	1.86
Wheat grain	2.86	Patent flour	1.86
Butter	2.81	Beer	1.77
Bread,	2.67	Egg white	1.77
Margarine	2.48	Chicken breast	1.75
Oysters	2.43	Vegetarian chaplets	1.72
Cornmeal, yellow	2.35	Skimmed milk	1.59

As indicated in the table above some foods have high chromium content. The range is similar for fruits, vegetables, dairy products, meat, cereal products and negligible dietary cr is dctainable from fish and seafood. Normal cow's milk is reported to contain 5 to 15 ng of Cr per ml,

and cow's colostrum level five times higher. Breast milk has been reported to contain similar levels, but recent reports indicate that breast milk contains approximately 0.3 to 0.4 ng of Cr per ml. Some brands of beer are very good sources of Cr, and a single serving may contain approximately 20 ng or approximately twothirds of the normal dietary Chromium intake. However, some brands of beer contain much lower concentrations of Cr, and the mean Cr intake per serving of beer is approximately 7 % of the minimum suggested safe and adequate intake. Estimated adequate and safe daily dietary intakes is shown in table 25.

Diets high in Cr also appear to be high in correlations of potassium, energy, fat, and sodium. However copper is the only trace element that has correlated chromium compounds are poorly absorbed in animals and humans, with mean Cr absorption of 0.4 to 3 percent or less. There is some evidence that natural complexes in the diet are absorbed better than simple Cr salts. Chromium absorption is rapid, with substantial absorption of radioactively labeled Cr within two hours of Cr ingestion.

Chromium absorption by human subjects consuming normal self-chosen diets is inversely related to dietary intake at levels found in the diet, at a daily intake of 10 µg, Cr. absorption is approximately 2 % and as intake increases to 40 µg, Cr absorption decreases to 0.5 %. At dietary intakes above 40 µg, Cr. absorption appears contant at approximately 0.4 %. The site and mechanism of Cr. absorption have yet to be elucidated.

TABLE 25 : Estimated Adequate and Safe Daily Dietary Intakes Some trace elements

	Age, Years	Copper mg	Manganese, mg	Fluoride, mg	Chromium, mg	Selenium, mg	Molybdenum, mg
Infants	0-0.5	0.5-0.7	0.1-0.5	0.1-0.5	0.01-0.04	0.01-0.04	0.03-0.06
	0.5-1	0.7-1.0	0.7-1.0	0.2-1.0	0.02-0.06	0.02-0.06	0.04-0.08
Children and adolescents	1-3	1.0-1.5	1.0-1.5	0.5-1.5	0.02-0.08	0.02-0.08	0.05-0.10
	4-6	1.5-2.0	1.5-2.0	1.0-2.5	0.03-0.12	0.03-0.12	0.06-0.15
	7-10	2.0-2.5	2.0-3.0	1.5-2.5	0.05-0.20	0.05-0.20	0.10-0.30
	11+	2.0-3.0	2.5-5.0	1.5-2.5	0.05-0.20	0.05-0.20	0.15-0.50
Adults		2.0-3.0	2.5-5.0	1.5-4.0	0.05-0.20	0.05-0.20	0.15-0.50

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- SELENIUM -

Significant advances have occurred in our understanding of the role of selenium in human nutrition and health. This is due to the existence of naturally occurring human selenium deficiencies in some areas of the world. In certain other selenium poor regions, studies on the metabolism and nutritional bioavailability of selenium have been facilitated by the low selenium status of the popllations living there. As a result, research in the selenium field has repidly received much international interest. Selenium is efficiently transfered up the soil-plant-animal-human food chain. So geographical differences in the availability of the selenium in soil for up take by plants, account for most of the variations in the selenium content of foods. The highest and lowest values which have been reported were both in the peoples' Republic of China. The lowest was 7μg/day (resulting in Keshan disease and highest being toxic) was 38000 μg/day.

Less extreme values for low dictary selenium intakes, 28-30 μg/day have been observed in New Zealand and Finland. Intakes in Caracos, Venezuela are somewhat ele vated (218 μg/day) because an important agricultural, production area in that country is located in seleniferous zone.

The human essentiality for selenium is based on its incorporation into the emyme glutathiane peraxidose. Selenium has no other existly confirmed function in humans. Glutathione peroxidose catalyzes the breakdown of hydrogen peroxides and lipid hydropenoxides in body tissues and fluids, thereby protecting against damage to body tissues. High activity of glutathione peroxidose in phagocytic cells, such as leucoytes and macrophapes, helps to protect the phagocytic cell itself from peroxides it generates during oxidative destruction of foreign matter. Reduced glutation activity in platetets has been linked to bleeding disorders. Reduced activity in plasma has been linked to edema due to peraxidation of capillary membranes. Selenium, vitamin E, glutathione, and sulfur containing amino acids all provide some protection against hepatic damage due to oxidative stress, such as occurs in carbon tetrachloride poisoning.

At present, selenium is regarded as a protective factor against oxidatixe stress with special relevance to heart disease and drug metabolism. In addition, the results of numerous animal tumor model studies and several epidemiologic investigations have indicated that selenium

may be a dietary factor that protects against carcinogenesis. During the late 1970 s, reports from endemically selenium deficient areas of China presented two human diseases associated with severe nutritional selenium deficiency. The first was a joxenile cardiomyopathy named Keshan disease and the second a chondrodystrophy named Kaschin-Beck disease the etiologies of these disease have been fully elucidated. Neither disease has been reported outside its endemic areas in Asia. Keshan disease was diagnosed in China as endemic Selenium deficiency. It occurs almost exclusively in rural populations in the mountainous parts of those regions. Affected areas are very low in soil selenium, producing food of extremely low selenium content. Residents of these regions have been reported to have the lowest blood selenium concentrations of any nonhospitalized persons in the world, an avarage of 18 μg/L. Keshan disease is a multifacal myocarditis that occurs primanily in children from weaning to 10 year, and to a lesser extent, among women of childbearing age. It is diagnaosed on the basis of signs of acute or chronic insufficiency of cardiac function, cardiomegal, arythmias and electrocardiographic and radiogrephic abnormlities. Patients may show cardiogenic shock or congestive heart failure. Embolic episodes from cardiac thromboses have also been reported. Although the heart is the organ primarily affected, patients may also show hepatic congestion, mesanteric lymphadenosis, degenemative changes in the disphragm, or pancreatic exocrine dysfunction. In children Keshan disease is most commonly manifested as chronic congestive heart failure with varying degrees of cardiac insufficiency. In some patients, signs and symptoms may occur with sudden onset, whereas in others the effects may be so mild that patients are unaware of the disease.

Dietary intakes of Selenium

Selenium content in foods is directly releated to the protein content and the selenium content in the soil from which the food was produced. For this reason, much of the variation in tre selenium contents of foods is due to large - scale geograplic differences in the amounts and availabilities of soil selenium. Becacese of the many variables involved in human, selenium intake quautitaive estimates attempted for specific population groups have not been precise.

Such studies for the US population indicate that the selenium intakes are highly variable, ie, between 60 and 216 μq per adult per day.

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Little is known about the site and mechanism of gastrointestinal absorption of selenium. Urinary excretion, however appears to correlate well with dietary intake and plays a major role in regulation of selenium homeostasis. In blood, selenium is preferentially taken up by erythrocytes, where as plasma selenium in bound less strongly by protein sulfhydryl groups.

The major source of Selenium in most diets are meats and cereal products, which contribute approximately 50 % and between 25 % and 35 %, respectively, of the total selenium intakes of Americans, In addition, dairy products and eggs provide, 1 % to 20 %, while fruits and vegetables provide less being 5 % of the total Selenium intake therefore, individual patterns of meat consumption can be important determinants of selenium intake, with high-meat diets providing as much as twice the selenium of low-meat diets. The selenium content of some foods is shown below;

TABLE 26: Selenium Content of some food

Meat (Beef)	116	ng/g
Chicken	106	ng/g
Egg	390	ng/g
Milk	12	ng/g
Rise	21	ng/g
Orange	13	ng/g
Peach	3	ng/g
Carrot	2	ng/g
green bean	4	ng/g

A survey of the selenium content of human milk in the United States found an overall mean of 18 µg/L, with 99 % of the 241 subjects from 17 states falling within the range of 7 to 33 µg/L. The level of selenium in mother's, milk varies even more on a worldwide basis maternal selenium status, dependent on the selenium content of the regional food supply, would be expected to influence the selenium content of mother's. Milk, colostrum contains more than twice the selenium concentration of mature mother's milk. Estimates of the quantitative dietary selenium

needs. In man were first published almost 10 years ago by the Food and Nutrition Board of the US National Acedemy of Sciences. The estimated adequate and safe dietary intakes of selenium are listed in table 25.

Excessive dietary selenium intake

The potential for selenium toxicity was first recognized in animals in the 1930s, before the nutritional essentiality of the element was known. There are no well defined cases of human disease due to selenium deficiency other than in China. Yet a number of clinical disorders have been shown to involve glutathione peroxidase deficiency, among them being hemolytic anemia, a platelet disorder, a chronic grenulomatosis and carcinoma, low blood selenium concentrations have been observed in patients with gastrointestinol cancer, during pregnancy, and in proteincalorie malnutrition, whereas high values have been raported in patients with reticuloendothelial neoplasia.

Only a few cases of human exposure to hazardous levels of selenium have been reported. These are ocupational exposure of workers to Selenium-aerasols in copper smelters o selenium-rectifier plants. The accidental ingestion of gun bluing solutions containing appoximately 2% selenious acid ($H_2 SeO_3$) was reported to result in nonfatal selenosis in a 2 years old girl, and to be lethal in two cases; a 3 year old boy and a 52 years old woman. Also, antidandruff shampoos that contain 1 % selenium sulfide ($Se S_2$) could potentially result in acute toxicity if accidentally ingested in large amounts.

THE COMPOSITION OF THE BODY

The chemical composition of man is protein, carbohydrate, fat, water and minerals. Those are listed in table 27. There are variations in people due to use and sex. As shown in table, of the 9 kg of fat not more than about 1 kg is essential, the ramainder represents a store which can be drawn upon in times of need. In obese people this store may be very much larger and form up to 70 per cent of the body weight. Protein is an essential component of the cells, but probably about 2 kg can be lost without serious result. In contrast, the body can be depleted by at most, 200 g of carbohydrate. During starvation the store of the carbohydrate is continually replenished by synthesis from the larger reserves of protein and fat.

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TABLE 27: A normal chemical composition for a man weighting 65 kg.

	kg	%
Protein	11	17
Carbohydrate	1	1.5
Fat	9	13.0
Water	40	61.6
Minerals	4	6.1

An important difference exists between the chemical composition of the fluid within the cells and that of the extracellular fluid which surrounds them. Cell fluid is primarily a solution of potossium ions and extracellular fluid a solution of sodium chloride. The anions within the cell are provided mainly by phosphates, proteins and organic acids in varying proportions. The anions and cations of intracellular and extrecellular fluids are shown in figure 9

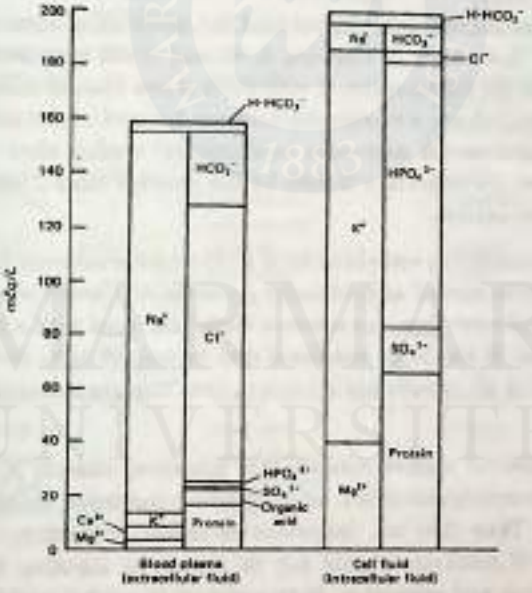

Figure 9: Electrolyte composition of blood plasma and intracellular fluid.

The difference between the concentration of ions inside and outside the cells is only maintained by the expenditure of energy which is provided by the metabolic processes within the cells. Much of the energy expenditure of the resting body is used to maintain this electrolyte equilibrium.

Nutrients and Cancer

Specific dietary factors can clearly suppress certain tumors, while others promote their development. Since 1914 moderately reduced intakes of carbohydrates and protein in animals have been known to reduce the rate of growth and the incidence of tumors. Whether this is true in humans has not been tested. Much less is known about the effects of high dietary intakes of carbohydrates and protein on cancer incidence and enhancing longevity, but the current information is exciting and encouraging. Studies of migrant populations indicate that many cancers have strong environmental and/or dietary factors in their genesis, as migrant cancer patterns have been found to be more like that of the host than that of the home country. Research and public attention have been increasingly focused on the hypothesis that increased dietary intakes of vitamins and micronutrients may help prevent some cancers (Table 28).

Epidemiological data suggest that the incidence of some cancers is lower when foods high in vitamins A, C, and E are consumed and increases when the consumption of such foods is less than optimal. A number of sources indicate a relationshp between diet and cancer incidence in human populations. A numerous experimental studies show that diet and nutrition are important factors in the possible control and prevention of human cancers.

Most cancers (as estimated 80 % to 90 %) have external causes and can therefore be viewed as potentially preventable. Cancer is believed to be the result of more than one external factor combined with a hereditary predisposition. It has been estimated that in men 30 % to 40 % and in women 60 % of all cancers are related to diet. This percentages are very impressive.

A number of studies indicate that selenium, vitamin A, C and E have an anticarcinogenic effect, altering cancer incidence, differentiation and growth. Thus they are important in cancer prevention. Although these kinds of nutrients should not be viewed as curative, they may prove beneficial and protective. More research is needed in this field to decide.

Nutritional anticarcinogenic effects are carried out by:

-) Inhibition of tumor initiation via alteration of cell metabolism

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-) Picking up active forms of carcinogens and preventing them from reaching target sites.

-) Alterations to the body's defense systems

-) Inhibition of a cancer progression once it has been initiated by the alteration.

-) Prevention of gene activation and cellular proliferetion by tumor promotors

TABLE 28: Considerations regarding the use of selenium and vitamins A, E, and C in cancer prevention (From ref. 100)

nutrient	RDA	conservative recommendati for cancer prevention	dietary sources	synthetic supplementation available	levels of possible toxicity	factors possibly increasing requirements
vitamin A	5.00 IU (1,000 g RE)	12.500 IU	green vegetables	yes	limited (chronic intakes above 25.00 IU; acute intakes 300.000-1.000.000 IU)	smoking
vitamin E	10-20 IU (7-13 mg)	200-800 IU	vegetable oils, eggs, whole grain, cereals	yes	negligible (<1,200 IU)	high polyunsaturated fatty acid intake, smog
vitamin C	60 mg	1.000 mg	fruits and vegetables	yes	negligible (1-2 gm)	emotional-environmental stress, oral contraceptives, smoking, diabetes
selenium	none (50-200 µg estimated safe and adequate)	50-200 µg	seafood, kidney, liver, meat, grain; content dependent upon soil selenium content	NR*	significant (>200 µg)	aging, high PUFA intake, smog, heavy metal contamination (high Vitamin E decreases selenium requirement)

*NR = not recommended as safe because of potential severe toxicity with high doses

B - DIGESTION

Very few foods can be used without further processing. These are water, some small saccharides, vitamins, and inorganic salts. Also many drugs can be absorbed and utilized without digestive processes. Most foodstuffs must undergo a series of changes before they can be absorbed and utulized (Table 29). The cooking of food is not absolutely necessary prior to digestion, but it does help since a great number of substances are partially hydrolyzed and protein is denatured. One advantage of heating food is that it destroys parasites and bacteria. Digestion therefore consists of the hydrolysis of biological polymers into small molecules which can be taken up by the gut mucosa.

TABLE 29: Products of digestion of the major components of the diet.

Before Digestion	Products
Starch, glycogen	Glucose
Sucrose	Glucose and fructose
Maltose	Glucose and glucose
Lactose	Glucose and galactose
Protein	Amino acids
Nucleic acids	Phosphoric acid, ribose or deoxyribose and organic bases,
Triglycerides	Glycerol, fatty acids, diglycerides, monoglycerides.
Phosphoglycerides	Diglycerides plus phosphoric acid and organic molecules e.g. choline, etanolamine.

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DIGESTION IN THE MOUTH

Digestion begins in the mouth. Food is broken down into small particles by the process of mastication and at the same time is mixed with saliva. Foods are moistened and lubricated by saliva they can more easily be swallowed. In addition, saliva contains a carbohydrate digesting enzyme called = – amylase. Which hydrolyzes amylose or the amylose-like portions of starch molecules to yield maltose and smaller starch fragments. The optimum pH for amylase is about 7. The length of time for amylase acticity is limited, because when the food passes from the mouth to the stomach it encounters a very acidic environment, which inhibits furher amylase activity.

Nevertheless amylase may continue to act for up to 30 minutes in the stomach, particularly in the centre of a food bolus. About half the dietary starch is hydrolysed to maltose and glucose in the stomach. No carbohydrase are produced by the gastric mucosa, but = – amylase is found in the pancreatic and intestinal juices. The ultimate product of amylase action is 85 % maltose and 15 % glucose. Maltose is split by maltase, sucrose by sucrase, and lactose by lactase. All these enzymes are bound to the membrane of intestinal mucosa .

THE FUNCTION OF SALIVA

Saliva is secreted by the parotid, sublingual and submandibular glands, and by small isolated glands distributed over the oral mucosa. It bathes the oral tissues and is basic to the health status of the oral cavity.

The traditional view of saliva has been that, it is primarily a digestive fluid, initiating the digestion of starch through the secretion of amylase. Breakdown of starch to maltose indeed starts in the mouth but most of the digestion of starch results from the action of pancreatic, not salivary amylase.

The mouth is the portal of entry for a variety of antigens, including numerous microorganisms, into the alimentary and respiratory systems. Normally, these antigens do not cause disease and are flushed away with swallowed saliva into the distal parts of the alimentary tract. Immunologic defense mechanism, particularly Ig A, prevent adherence of microorganisms to mucosal and tooth surfaces by aggregating them and possibly rendering them more susceptible to phagocytosis. Most oral disease are due to an imbalance between oral organisms and the host response.

The most important functions of the salivary secretions therefore are protective in nature, helping to maintain the integrity of the teeth, tongue, and mucous membranes of oral and oropharyngeal areas. When the protective action of saliva is lost, its importance becomes apparent. Malfunction of the salivary glands (because of obstruction, drug effects, irradiation, nerve damage or disease) results in dry mouth or xerostomia. Some of drugs cause xerostamia because of their hyposalivatory side effect. The mucosa becomes dry, rough, and sticky. It bleeds readily and is subject to infection. The tonque becomes red, smooth, limy, and hypersensitive to irritation and loses taste acuity. Caries progress rapidly and extensively, and periodontal disease can become exacerbated.

For these and other reasons, the secretion and composition of aliva are of direct concern to the dentist.

Total salivary fluid produced during a 24 hour period is about 1000 ml. About 90 % of this fluid is derived from the parotid and submaxillary glands (in roughly equal amounts). Abot 5 % from the sublingual glands, and up to 5 % from the minor salivary glands. This is because the unstimulated flow rate of the major salivary glands is less than 0.05 ml/min gland at rest and 0.5 ml/min gland with stimulation. It is apparent then that 80 - 90 % of the daily production of saliva is the result of stimulation. This stimulation is mainly gustotory and masticator and is asscociated with eating. During most of the day and all night, salivary flow is minimal.

As just mentioned the inorganic and organic components of saliva have many important roles. These are summurised as follows:

1- Lubrication and protection: The glycoproteins and mucoids produced by the major and minor salivary glands form a protective coating for the mucous membrane. This coating is a barrier against irritants acting directly on the membrane. It is also a barrier against;

a- Proteolytic and hydrolytic enzymes produced in bacterial plaque

b - Potential carcinogens (smoking, chemicals etc)

c - Desiccation (mouth breathing).

The mucoid layer in oral mucos may be considered ccomparable in some respects to gastric mucin which protects the stomach from the hydrochloric acid which produced in stomach.

2 - Buffering action: Primarly because of its bicarbonate, but also because of its phosphate and amphoteric protein content, saliva has considerable buffer capacity. This protective function occurs in the plaque, directed against acidogenic microorganisms and occasionally on the mucous membrane surface, where acids from foods or regurgitation may occur.

3 - Maintenance of tooth integrity: Saliva functions to maintain the integrity of the tooth in a number of ways;

a - It provides minerals for posteruptive maturation,

b - It provides ions such as calcium and phosphate in sufficient amounts to counteract tooth dissolution by saliva.

c - It forms a film of glycoprotein on the teeth (the pellicle) that may act as a diffusion barier, thus preventing loss of tooth mineral. Saliva contains at least one special peptide or protein that apparently allows saliva to contain supersaturated amounts of calcium and phosphate ions by conteracting nucleation of calcium phospate salts. Thus spontaneous precipitation of calcium phosphate within the saliva and on the tooth surfaces is prevented.

4 - Antibacterial activity: Saliva contains a number of components that can, provide strong defense against bacterial and viral invasion. Presently great interest is focused on secretory IgA. This immunglobulin is effective against a number of viruses and bacteria. The most reasonable explanation is that, bacterial cells coated with secretory IgA have a reduced tendency to adhere to teeth and mucous membrane surfaces. The exact relationship between the production of secretory IgA and dental disease is still unclear. There may be a negative correlation between the accumulated dental caries experience in permanent teeth and IgA production. Conversely, the periodental index appears to be positively correlated. That is, the higher the periodontal index the greater the amount of IgA produced by the parotid salivary gland. obviously, much more work is required to clarify the role of secretory IgA in the oral cavity as it relates to periodontal disease.

COMPOSITION OF SALIVA

Table 30 lists the concentration of parotid and submaxiallary components for saliva. Plasma values are included for comparison. In general

it can be seen that the parotid concentrations are somewhat higher than the submaxiillary-sublingual. The principal exception is calcium. There is important amount of calcium in saliva but no correlation has been found between dental growth and saliva calcium level . Salivary calcium phosphate and floride have a positive influence on remineralization as well as salivary pH and buffer capacity the flourinde content of saliva is very low. The mean concentration being 0.028 mg/100 ml. The range is between 0.025 to 0.045 mg/ml. The relationship between dental caries and salivary elements has been studied by many researcher.. It has been demonstrated that salivary copper, selenium, cadmium and lead increase caries formation. However, salivary floride, strontium molybdenum zinc, manganese, litium, vanadium, calcium, phosphorus and magnesium decrease it. Submaxillary sublingual calcium is approximately twice that of the paratid concentration. Saliva calcium concentration is high and as mentioned above sublaxillary sublingual calcium is approximately twice that of the parotid concentration. electrolytes are actively transported to the saliva but it is not an ultrafiltrate of plasma.

On of 2 % citric acid stimulation applied to the tongue, the salivary flow is increased. The concentrations of sodium, chloride, bicarbonate and calcium also increase, but magnesium phosphate, urea ammonia and uric acid decrease. Potassium concentration is independent of flow rate. Protein increases in the submandibular sublingual secretion but is variable in the paratid. The pH increases with the flow rate.

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TABLE 30: Salivary composition in normal adults (Mean values)

	Parotid	Submaxillary	Plasma
Flow rate (ml/min/gland)	0.7	0.6	-
mEq/L			
Potassium (K^+)	20	17	4
Sodium (Na^+)	23	17	140
Chloride (Cl^-)	23	20	105
Bicarbonate (HCO_3^-)	20	18	27
Calcium ($^{++}$)	2	3.6	5
Magnesium (Mg^{++})	0.2	0.2	2
Phosphorous (HPO_4^-)	6	4,5	2
mg/100 ml			
Urea	15	7	25
Ammonia	0.3	0.2	-
Uric acid	3	2	4
Glucose	1	1	80
Total lipid	2.8	2	500
Cholesterol	1	-	160
Fatty acids	1	-	30
Amino acids	1,5	-	50
Total proteins	250	150	7.35 (Gr)
PH	6.8 - 7.2		7.2-7.4

Glucose is present in very low concentration in saliva but it becomes elevated in diabetes mellitus, lipids and amino acids are also present low concentration in saliva.

Saliva is slightly acidic before secretion into the oral cavity. It becomes slightly alkaline on excretion from the gland because of a loss of

CO_2 (Carbonic acid in solution). Since bicarbonate concentration increases with increasing flow rate, salivary pH becomes elevated at high flow rates.

The major component of parotid saliva is amylase, which is peresent in a number of molecular forms known as isoenzymes. Amylase activity in the submaxillary gland is only about 20 % that in the parotid. There is virtually no amylase in sublingual or minor salivary gland secrations since these glands are almost exclusively mucous.

Recently, electrophoretic techniques have been widely used for analysis of the protein components of human saliva. As seen in figure 10 there are different salivary proteins between the molecular weight 66.000 and 14.300 It has been shown that a linear increase in the amylase levels in children with caries.

Serum proteins can be identified in saliva by immonologic techniques. They are peresent in low concentration and appear to enter the secretion by "leakage". Only albumin is present at any appreciable level being about 1 mg/dl. In salivary gland disease in which ductal integrity is altered (e.g. parotitis or Sjörgen disease), albumin levels are marcedly elevated. The finding that IgG is present in saliva in much lower quantities than IgA supports the observation that the salivary IgA is produced in the gland. In blood the IgG to IgA ration is about 10:1, in saliva it is the reverse. Again compared to that in blood, the concentration of proteins in saliva is extremely low. Some of the saliva proteins are summarized in table 31. Many of saliva proteins are glycocoproteins.

TABLE 31: Proteins of parotid and submaxilary saliva

Parotid	Submaxillary
Amylase (high)	Amylase (low)
Cationic glycoproteins (high)	Cationic glycoprotein (low)
Anionic glycoproteins (low)	Anionic glycoprcteins (high)
Secretory component	Secretory component
Kallikrein	Kallikrein
Lactoperoxidase	Laktoperoxidase
Lactoferrin	Lactoferrin

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Proline rich	High molecular weight glycoproteins (blood group substance)
Basic polypeptides	Phosphoproteins
Histidine rich	-
Secretory IgA	Secretory IgA
Lysosyme (low to moderate)	Lysosyme (high)
Phosphatases, esterases,	Phosphatases, esterases,
β glucorinidase	β - glucuronidase
Ribonucleases (moderate)	Rbonucleases (low)
LDH	
Leakage From Blood	Albumin, IgG, IgM, Lipoprotein and traces of other serum proteins.

Figure 10: SDS - Polyacrylamide electrophoresis of salivary proteins (From ref. 137)

Albumin, IgG and IgM, lipoprotein and traces of other serum proteins, which are responsible for the viscosity and lubricating properties of saliva. The proline rich proteins in human saliva have been classified. They can be divided into acidic, basic and glycocylated proline rich proteins. Acidic proline rich proteins bind calcium with a strength which indicates that they may assist in maintaining the concentration of ionic calcium in saliva. Salivary proteins also play a role in the formation of

dental plaque. It has been observed that saliva proteins change during various diseases. No correlation has been found between df-t index levels and salivary protein levels.

The tyrosine rich protein has also been shown to possess a marked abilitiy to inhibit calcium phesphate precipitation in supersaturated solutions. Some of the acidic proline rich proteins also exhibit this property but to a lesser degree.

In the parotid gland, serous cells produce a major glycoprotein quite different from the mucous cell product. It is cationic (positively charged) nonviscous, of relatively low molecular weight and has an amino acid profile unlike that of mucous glycoproteins.

Acinar cells of both the parotid and the submaxillary gland produce a glycoprotein (of molecular weight about 50.000) known as secretory component. (Table 31) This together with immunoglobulin A (One of the gamma globulins), forms a specific structural entity. The secrotry IgA (produced in exocrine glands) is active on mucosal surfaces. The cells also produce small amounts of lactoferrin, an iron-binding glycoprotein.

The secretory immunoglobulin (IgA) found in saliva is produced by plasma cells in the connective tissue of the glands, particularly around the intralobular ducts. It leaves the plasma cell as a dimeric molecule. IgA occurs as a monomer or as double unit aggregates of the γ-shaped protein molecule (13).



(13)

IgA
(monomer
or dimer)

The two halves of the dimer are held together by a polypeptide known as the "J" chain. The dimer then enters the epithelial cells of the striated duct or possibly the acinar cells. Then it joins with the secretory component (or piece). This component acts to stabilize the molecule and aids in its release into the lumen of the duct. Over 90 % of the IgA in sali-

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va is of the secretory variety. Secretory IgA neutralizes viruses and can act as an antibody to bacterial antigens and probably fold antigens as well. It is relatively resistant to proteolytic enzymes and hence can survive in the oral cavity and the gastrointestinal tract.

The mucous cells of the submaxillary sublingual and minor salivary glands produce blood group substance. These are high molecular weight glycoproteins that have blood group activity. Some parts of the glycoprotein molecule are characterictic for the blood type of the individual.

The antibacterial enzyme, lysozyme, is found in both parotid and submaxiallary secretions. It is formed in basel cells of the striated ducts. Lysosyme is a muramidase, it breaks up bacterial cell walls in the glycopeptide region that contains muramic acid. Lysozyme may act together with other antibacterial systems in saliva (IgA) as a general scavenger of susceptible bacterial cell walls.

Another important component of the salivary antibacterial system is the enzyme sialoperoxidase. Together with hydrogen peroxide and thiocysnate, sialoperoxidase provides defense again to Lactobacilli and cariogenic streptococci. A similar system exists in mother's milk and is considered to he part of the early defence mechanism of the newborn. Comparable peroxidase activity (myeloperoxidase) is part of the antibacterial weaponry of the leukocyte. A system has been introduced for oral use that is purported to generate an ideal level of hydrogen peroxide for endogenous sialoperoxidase activity against streptococcus mutans and other plaque organisms.

Lactoferrin, which was originally discovered in bovine milk, is widely disributed in body fluids in granules of polymorphonuclear leukocytes, lactoferrin also found in salivary secretion and exhibits a bacterial effect on cariogenic streptococci. This iron binding glycoprotein should be an effective antibacterial agent in saliva by with holding iron from facultive and aerobic organism.

A considerable number of other enzymes have been identified in both parotid and submaxillary-sublingual saliva. These include acid and alkaline phosphatases, nonspecific esterases, and kallikrein. The enzyme, lactic acid dehydrogenase (LDH) has been found in parotid saliva but not in submaxillary sublingual saliva.

Secretion Mechanism

Salivary secretion is controlled by a salivary center in the medulla. This center is composed of the superior and the inferior salivary nuclei. Stimulation of flow is generated mainly by unconditioned reflex stimulation and is primarily gustatory (by way of taste buds) and masticatory (through proprioceptors in the periodontal ligement and the muscles of mastication). Conditioned reflexes as well as emotional and phsychic factors have also been shown to affect salivary flow rate.

Neural control of salivary secretion is largely parasympathetic, but there is also a symphathetic component to neural regulation in human salivary glands. Sympathetic control of all salivary glands is maintained through postganglionic fibers from the superior cervical ganglion.

A major factor affecting salivary secretion, espacially in older persons, is the large number of pharmacologic agents that can reduce salivary flow. Many drugs produce dry mouth (xerostoma) as a side effect Commen exaples of these drugs are barbiturates antihypertansive antihistamines, atrophin like agents (for digestive disorders, ulcers, and Parkinson disease) and others.

Other factors that can affect flow rate are: Psychic factors, such as pain or threat of pain, interference with taste perception, gland, size age changes in the gland such as atrophy and fatty and inflammatory infiltrates light deprivation, systemic disease such as hypothyroidsm, diseases of the salivary gland and irradiation of the glands during tumor therapy of the face and neck area.

Digestion in the Stomach

Gastric digestion consists primarlly of proteolytic reactions. The gastric wall secretes gastric juice in response to a number of different stimuli. The thought, smell and teste of food as well as the machanical stimulation brought about by the presence of food in the stomach, are effective stimuli. Gastric juice is characterized by the presence of HCL at a concentration of approximately 0.17 N with a pH of about 0.87.

Protein hyrolysis begins in the stomach with pepsin, which has an acidic pH optimum for activity. Pepsin degrades proteins extensively at specific sites but produces little free amino acids or dipeptide. In the small intestine the large peptides are further split by a series of enzymes

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of two basic types. The endopeptidases hydrolyse peptide linkes other than those of the terminal amino acids, these all being pancreatic in origin. Exopeptidases detach terminal amino acids, and include enzymes specific for dipepides and tripeptides. of these, only carboxypeptidase is pancreatic, the others are found in the inestinal juice. Rennin is a specialised enzyme of the gastric juice which converts the casein of mil to a more soluble product, paracasein. Digestion of proteins continues in the lumen of the small intestine under the action of trypsin and chymotrypsin from the pancreas. These three endopeptidases are secreted as inactive precursers such as pepsinogen, trypsinogen and chymotrypsinogen. Pepsinogen is an inactive form of the enzyme pepsin. In the presence of HCl, pepsinogen undergoes a hydrolytic change with the release of several peptides to form the active proteolytic enzyme pepsin.

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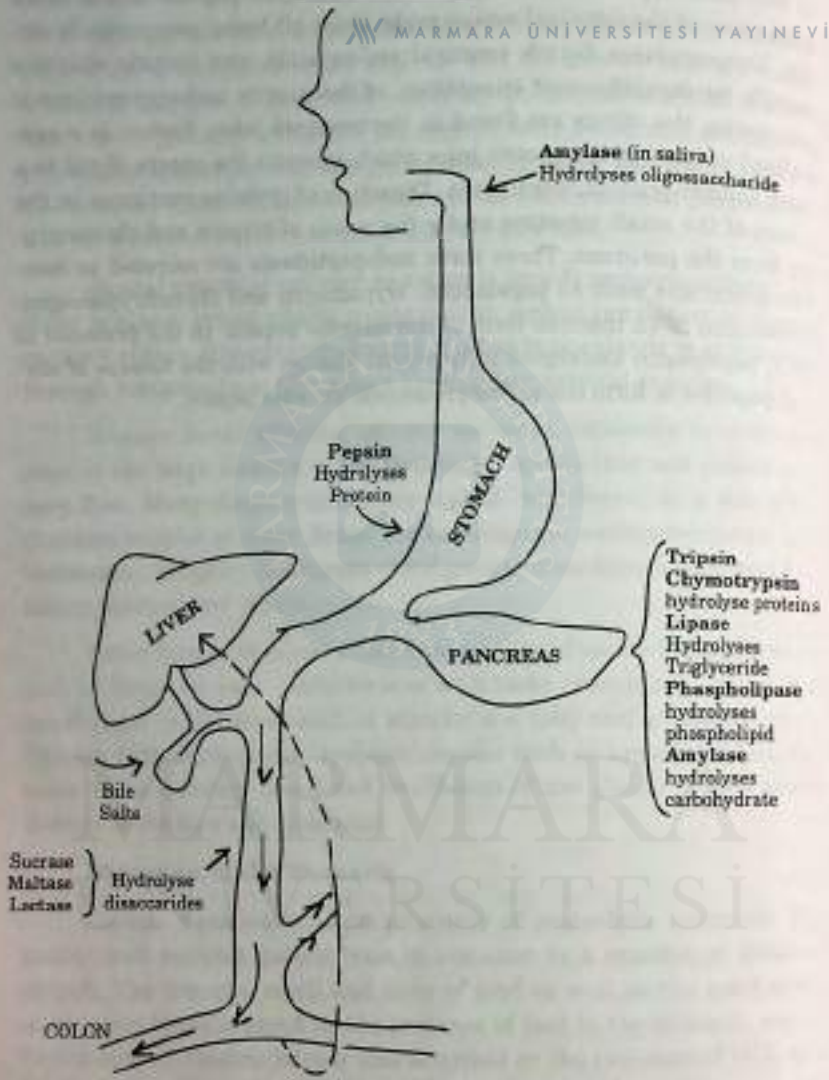

Figure 11: Gastrointestinal tract and digestive enzymes. Before food can be absorbed from the digestive tract it must be processed to convert it to a form suitable for absorption, enzymes released during digestion hydrolyse food constituents. Dotted lines represent enterohepatic circulation.

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$$\text{Pepsinogen} \xrightarrow{\text{HCL}} \text{Pepsin peptides}$$

This reaction is also autocatalytic which means that the product of the reaction, pepsin, is able to convert itself (pepsin) from pepsinogen. Pepsin is an endopeptidase, meaning that it attacks the interior of molecules and not the terminal amino acids. It hydrolyses peptide bonds of the amino groups tryptophan, phenylalanine, and tyrosin. Pepsin atacts a protein molecule only at the sites indicated and does not hydrolyze a protein completely, merely breaks it up into smaller peptide units.

Protein digestion in the gastrointestinal tract occurs as follows:

$$\text{Pepsinogen} \xrightarrow{\text{HCl}} \text{Pepsin A (fundus of the stomach)}$$

$$\text{Pepsinogen} \xrightarrow{\text{HCl}} \text{Pepsin B (pylorus of the stomach)}$$

$$\text{Trypsinogen} \xrightarrow[\text{pH 5.2 - 6.0}]{\text{Enterokinase}} \text{Trypsin}$$

$$\text{Chymotrlpsinogen} \xrightarrow[\text{pH 8.0}]{\text{Tripsin}} \text{Chymotrypsin}$$

$$\text{Procarboxypeptidase} \xrightarrow{\text{Tripsin}} \text{Carboxypeptidase}$$

$$\text{Proelastase} \xrightarrow{\text{Tripsin}} \text{Elastase}$$

The chief cells and parietal cells of the stomach glands secrete gastric juice in response to reflex stimulation and chemical action of gastrin. The required enzymes except pepsinogen are secreted from pancrease glands. The stomach content, or chyme, which is of a thick creamy consistency, is intermittently introduced during digestion into the duodenum through the pyloric velve. The pancreatic and bile ducts open into the duodenum at a point very close to the pylorus. The alkaline content of pancreatic and biliary secrations neutralizes the acid of the chyme and changes the pH of this material to alkaline, this shift of pH being necessary for the activity of the enzymes contained in pancreatic and intestinal juice but inhibiting further action of pepsin.

Analysis of gastric contents:

The analysis of gastric contents for HCl and pepsin activity is often used for diagnostic purposes. Usually gastric contents are collected by placing a tube in the stomach and withdraing specimens at definite inervals. The samples can be basal (that is, those collected without any stimulation) or can be collected following various stimuli. The stimulants commonly used are ethyl alcohol, caffeine, and histamine.

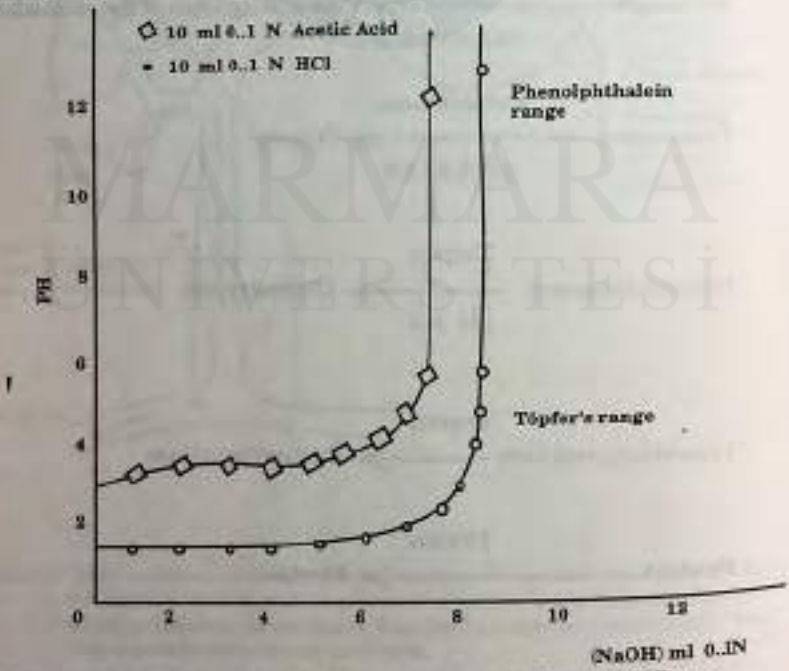

Figure 12: Titration curves for hydrochloric and acetic acids. (From ref. 166)

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The HCl and other acids in gastric contents can most conveniently be quantified by titration procedures. Figure (12) shows the titration curves for HCl and acetic acids. Where is can be seen, pH is plotted against the mililiters of added NaOH. The pH of a HCl solution changes very little until just before the equivalance point, when there is a very fast increase in the pH until the pH becomes alkaline. For organic acids (such as acetic acid) the initial pH is higher and the equivalance point is reached with a gradual change rather then a sharp change. Because of the difference in the behavior of the two types of acids, it is possible to quantitate HCl and strong organic acids when they occur as a mixture. Hydrochloric acid can be measured by titrating to pH 3, which is essentially the end point for the HCl titration. If the titration is continued to pH 8 or 9 the total amount of acid present can be quantitated. In the titrimetric analysis of gastric contents this difference in pH at the equivalance points is utilized to separate the titration values for HCl and other organic acids present. To do this Töpfer's reagent (methyl yellow) a pH indicator, is added to the solution and is titrated until the methyl yellow changes color. Methyl yellow is red at pH of about 3 and changes to yellow at pH 4. When the methyl yellow end point is reached, phenolphthalein is added to the sample and the titration continued until the phenolphthalein end point is reached. The amount of NaOH needed to titrate to the methyl yellow end point is then a measure of the HCl present and the amount of NaOH required to titrate from the beginning to the phenolphthalein end point the is a measure of the total acid present in the sample. Therefore, the number of mililiters of NaOH required to go from the methyl yellow end point to the phenolphthalein end point is a measure of the organic acids present. A pH meter in place of the indicator can also be used for this analysis.

The normal values for gastric acidity depend on whether the sample is a basal sample or one collected flowing stimulation. In addition, the normal values vary depending on the length of time during which the sample is collected. A sample of gastric juice collected under basal conditons from 6.00 PM to 6.00 AM varies from 1 to 90 mEq of HCl per liter with an average of about 30. The volume for such samples can range between 140 and 1.200 ml.

Normal individuals usually produce maximum HCl secretion approximately 40 minutes after histamine stimulation. The concentration

of HCl averages about 90 meq/liter in a 40 minute sample. The production of gastric juice following histamine stimulation averages about 30 ml per minute.

Achlorhydria is a term used to describe gastric juice which contains no HCl, a condition often associated with pernicious anemia. Hypoacidity or hyposecretion of HCl by the gastric wall is commonly observed in cases of carcinoma of the stomach and less commonly with certain other diseases. Hyperacidity or hypersecretion of gastric juice has long been associated with duodenal ulcers. If the gastric contents contain HCl, pepsin activity will be present, if it does not, no pepsin activity will occur.

DIGESTION IN THE SMALL INTESTINE

When food leaves the stomach and enters the small intestine, digestion becomes quite complex. The pH of the intestine is close to neutral. In addition to digestion, absorbtion occurs and the influence of the pencreatic, intestinal wall, and hepatic secretion all become significant. The secretions from there three sources are very effective in neutralizing the high acidity carried from the stomach. The secretions have a pH of about 8 or above and all contain large amounts of sodium bicarbonate, which reacts with the HCl of the stomach.

Pancreatic secretion:

Pancreatic juice contains several enzymes which attack macromolecules. Included among these are three proteolytic enzymes. Trypsin and chymotrypsin are endopeptidases elaborated by the pancreas in inactive forms known as trypsinogen and chymotrypsinogen, respectively. An enzyme secreted by the intestinal mucosa attacks trypsinogen, splits off a peptide, and converts it to the proteolytically active trypsin. Chymotrypsinogen is converted to active chymotrypsin in a somewhat complicated process by the action of trypsin and chymotrypsin. Trypsin attacts peptide linkages which are formed by the carboxyl group of either lysine or arginine, and chymotrypsin attacks peptide bonds formed by the carboxyl group of tyrosine, tryptophan and phenylalanine. The third major proteolytic enzyme from the pancreas is carboxypeptidase. This is a nonspecific enzyme which hydrolyzes peptides, one amino acid at a time, starting from the carboxyl end of the chain.

Pancreatic juice also contains amylase activity. The amylase of pancreatic origin appears to be identical with that of the salivary glands

Amylase attacts starches and hydrolyzes 1,4 links to produce maltose from amylose or straight-chain portions of starch molecules.

Lipase is also found in pancreatic juice. Lipase is an esterase which hydrolyzes triglycerides with long-chain fatty acids first to diglycerides, then monoglycerides, and if the reaction time is long enough, free glycerin is liberated.

Ribonuclease (RNase) in pancreatic juice hydrolyzes RNA to the corresponding nucleotides. DNase (decoxynucleotidase) hydrolyses deoxynucleic acids to the deoxnucleotide level.

The analysis of pancreatic juice obtained by incubation and collected following stimuli is often used for the detection of pancreatic carcinoma. The analysis of amylase and lipase activities and bicarbonate content, are common in this procedure.

Intestinal wall secretion

The intestinal wall secretes enzymes which attack smaller substrate molecules. Included in this secretion is an anzyme called oligo 1-6 glucosidase. This enzyme is able to split the 1-6 links in starch molecules (branch points) and continue starch hydrolysis. In addition, the intestinal wall secretes the enzyme maltase which hydrolyzes maltose to glucose, sucrase which hydrolyzes sucrose to glucose and fructose and lactase which hydrolyzes lactose to its constituent monosaccharides.

Enzymes which hydrolyze proteins are also secreted by the wall of the intestines. Aminopeptidase hydrolyzes polypeptides, one amino acid at a time starting from the amino end. In addition, there are other enzymes which complete the hydrolysis of proteins to the constitment amino acids. also, enzymes which complete the digestion of nucleotides are elaborated by the intestinal wall.

Hepatic Secretion (Bile)

Bile is probably produced continously by the liver and is stored between digestive periods in the gallbladder. Liver bile is green yellow viscous and slimy in character, and is alkaline (pH about 8). Between 500 and 1,000 ml of liver bile is produced each day. While the bile is storad in the bladder it becames concentrated since the bladder wall is able to remove water and some of the salts.

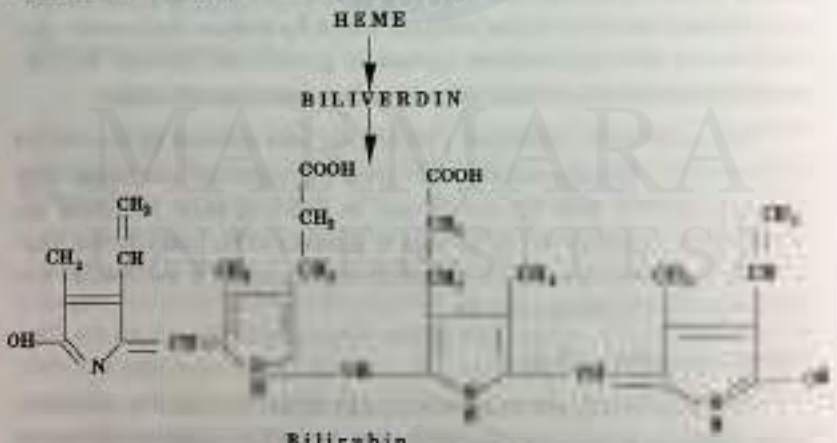
Bile has different function on lipid digestion. In adition to its functions in digestion, the bile is a reservoir of alkali, which helps to neutralize the acid chyme from stomach. The enterohepatic circulation helps to function. Bile is an important vehicle for cholesterol.

Indeed, bile may be considered a unique product. It represents a pathway for the disposal of certain waste products from the organism. It contains no digestive enzymes, and its contribution to digestion consists of certain emulsifying and stimulative agents. The primary constituents of bile and those that will be considered here include bile pigments, bile salts, and cholesterol.

Bile Pigments

Bile pigments derive from hemoglobin. When erythrocytes are destroyed in the reticuloendothelial system hemoglobin is decomposed.

The protein portion, globin, is removed, and the amino acids are returned to the amino acid pool. Iron is removed and the cyclic tetrapyrole labelled to structure heme is released. The removal of one of the methylene bridge opens the cyclic structure and yields first biliverdin and finally the linear tetrapyrole compound bilrubin.



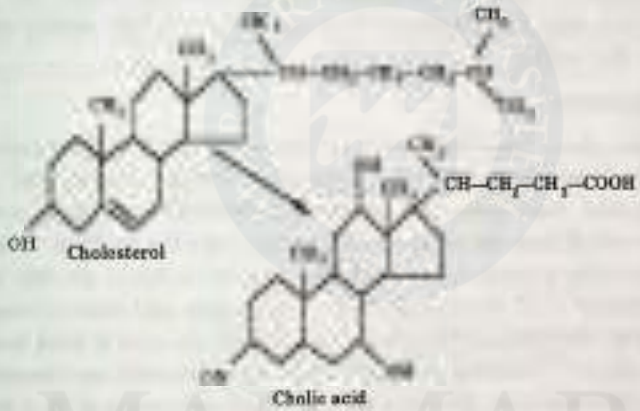
Bilirubin

Bilirubin is then carried to the liver where it is normally conjugated primarily with glucosiduronic acid and then excreted with the bile. Some of the bilirubin is oxidized back to biliverdin, which is secreted with the bile. These two compounds contribute to the color of bile, biliver-

din is green and bilirubin is red-orange. When bilirubin enters the intestinal tract it undergoes a number of metabolic changes and is excreted with the feces in the form of urbilinogen. The brown color of fecal material is due primarily to the presence of urobilinogen. Bilirubin apparently does not contribute to the digestive or absorptive processes. The presence of bilirubin and biliverdin in bile simply represents their pathway of elimination.

Bile salts

The bile salts are cholesterol metabolities. Cholesterol can be degraded to form several acids, one of which is cholic acid as shown below:



The various bile acids prior to secretion are conjugated with amino acids such as glycine to produce glycocholic acid or with aminoethyl sulfonic acid (taurine) to form taurocholic acid. These compounds are, of course, secreted as a salt, and it might be of interest to note that the salt of taurocholic acid indeed a detergent The bile acids do not represent the end product of cholesterol metabolism since much cholesterol is eliminated unchanged in the bile. One of the great dificulties with fat digestion is that fat - splitting enzymes such as lipase are water soluble, whereas fats are water insoluble. The water soluble enzymes and water immiscible lipids must come together before enzymolysis can take place. The soap or detergent action of bile salts emulsifies intestinal contents and allows hydrolysis to occur. Through emulsification they also facilitate absorption by the intestinal wall. Included among these compounds are the fat solu-

ble vitamins D and K. The bile salts are also absorbed and carried to the liver where they stimulate the production of more bile than they themselves are excreted.

Gallstones

In certain individuals, women more commonly than men, the constituents of bile precipitate in the bladder to form gallstones. The stones may occur as a single stone or there may be many of them. When gallstones are examined by cross sectioning, they are found to contain a central nucleus with concentric layers of deposits. Gallstones normally contain cholesterol, the bile pigments, calcium salts (particularly $CaCO_3$) and phosphates. Just why gallstones form in some individuals and not others is not clear, but apparently this is not related to bile concentration levels in the bladder.

Large intestine

Most of the digestible material is absorbed by the small intestine. Undigested material, unabsorbed substances, cellular debris, and certain vegetable fibers pass from the small intestine into the large intestine. Secretions from the large intestinal wall make the medium slightly alkaline and as a result make conditions ideal for bacterial growth. The temperature is 37 C, there is no light, little oxygen, and there is ample material for bacterial growth. About one-fourth to one-half of fecal material is made up of bacteria. Although water and some salts are absorbed from the large intestine, the main purpose of this area seems to be a storage place until the faces can be conveniently evacuated.

ABSORPTION

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Digestive processes are necessary before the nutrients can enter the body. The process by which these nutrients are transported from the lumen of the small intestine into the capilleries is called absorption. Not a great deal is known about the absorption of many nutrients and many other substances. It is known, however, that energy is required to absorb glucose. The absorbption of glucose from the intestine can occur at very low concentration, even though the concentretion in the body is very high. Glucose absorption therefore occure by active transport. Most amino acids absorption also occurs by active transport but the mechanism are yet not at all clear. The brush border the enterocyte contains several transporter system. Membrane of the enterocyte are very active. There are specific carrier proteins for amino acids and sugars. Transport of glucose across the intestinal epithelium is shown in (Figure 13).

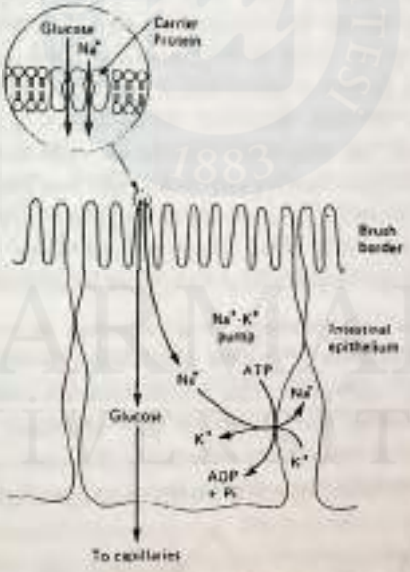


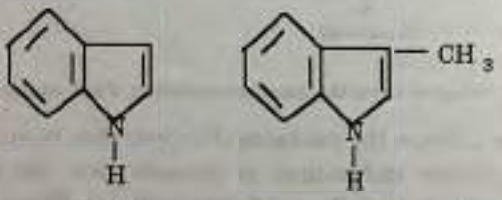
Figure 13 : Glucose transport across the intestinal epithelium (From ref. 24)

As is shown in figure the existance of a carrier has been postulated that binds both glucose and sodium at separate sites and transports them through the plasma membrane of intestinal cell. There glucose is against its concentration gradient so the free energy required for this ac-

tive transport is obtained from ATP linked to a sodium pump that expels Na^+ from the cell in exchange for K^+. There is also an Na^+ independant carrier of glucose and diffusion of glucose is represented by active transport is coupled to the Na^+ - K^+ pump.

Most of the absorption from the digestive tract takes place, via the small intestine, ethyl alcohol and certain other substances (some drugs) can be absorbed vis the mouth and stomach but mast ingested food absorbed from the small intestine. Glucose and other monosaccharides some disaccharides, a mono acylglycerols fatty acid, glycerol, cholesterol, amino acids, peptides, vitamins, electrolytes, iron, calcium and some water are absorped at the jejunum site of the small intestine. Bile acids, vitamin B_{12}, electrolytes and some water are absorbed at the ileum site of the small intestine. The residue pases into the large ingestine. There considerable absorption of water takes place, and the semiliquid intestinal contents gradually become more solid. During this period, considerable bacterial activity occurs. By fermentation and putrefaction the bacteria produce various gases, such as CO_2, methane, hydrogen, nitrogen and hydrogen sulfide, as wall as acetic, lactic, propionic and butyric acids. Intestinal bacteria are also beneficial. In herbivora, whose diet cosists largely of cellulose, the intesitanal bacteria are essential to digestion, for they decompose polysaccharides and make them available for absorption. In humans, the intestinal flora are not as important as in herbivores, but they have some beneficial effect. They make available to the body vitamins K and B_{12} in particular and posibly other β complexes.

Many amino acids undergo decarboxylation as a result of the action of intestinal bacteria to produce toxic amines. Such decarboxylation reactions produce cadaverine from lysine, tyramine from tyrosine, putrescine from ornitine and histamine from histidine. Many of these amines are powerful vasopressor substances the amino acid tryptophan undergoes a series of reactions to form indole and methylindole (skatole).



Indole skatole (methylindole)

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the substances particularly responsible for the odor of feces, the sulfur-containing amino acid cysteine undergoes a series of transformations to form mercaptans such as ethyl and methyl mercaptan as well as H_2S.



methyl mercaptan — methane and hydrogen sulfide

The large intestine is a source of considerable quantites of ammania, presumably as a product of the putrefactive activity of the intestinal bacteria on nitrogenous substrates. This ammonia is absorbed into the portal circulation, but under normal conditions it is rapidly removed from the blood by the liver. In liver disease, this function of the liver may be impaired, in which case the concentration of ammonia in the peripheral blood will rise to toxic levels. It is believed that ammonia intoxication may play a role in the genesis of hepatic coma in some patients. In dogs on whom an Eck fistula has been performed (complete diversion of the portal blood to the vena vaca), the feeding of large quantities of raw meat will induce symptoms of ammonia intoxication (meat intoxication) accomplanied by elevated levels of ammonia in the blood. The oral administration of neomycin has been shown to reduce the quantity of ammonia delivered from the intestine to the blood, owing to the antibacterial action of the drug.

Under normal circumstances, the dietary proteins are almost completely digested to their contituent amino acids, and these end products of procin digestion are then rapidly absorbed from the intestine into the portal blood.

There is a difference in the rate of absorption from the intestine between the 2 isomers of an amino acid. The natural (L) isomer is actively transported across the intestine from the mucosa to the serosa. Vitamin B_6 (pyridoxal phosphate) may be involved in this transfer. The D-isomers, on the other hand, are transported only by free diffusion. This active transport of the L-amino acids is energy dependend, as evidenced by the fact that, in studies of small pieces of segmented intestine, 2, 4-dinitrophenol, the uncupler of oxidative phosphorlylation, inhibits the concentration of L-amino acids. Amino acids are transported through the

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bruch border by a multiplicity of carriers, many having Na^+ - dependent mechanisms similar to the glucose carrier system. It has been postulated that four specific mechanisms exist for amino acid transport across the membrane being basic, acidic, neutral and for iminoacid (proline-hydroxy pyroline)

When groups of amino acids are fed, there is some evidence that one amino acid fed in excess can retard the absorption of another. These observations are similar to those made with respect to reabsorption of amino acids by the renal tubules. A puzzling feature of protein absorption is that in some individuals sensitivity to protein (in the immunologic sense) results when they eat certain proteins. It is known that a protein is antigenic, is, able to stimulate an imminologic response, only if it is in the form of a relatively large molecule. The digestion of a protein even to the polypeptide stage destroys its antigenicity. Those individuals in whom an immunologic response to ingested protein occurs must three fore be able to absorb some unhydrolyzed protein. This is not entirely undocumented, since the antibodies of the colostrum are known to be available to the infant.

There are some disorders resulting from malabsorption. Pancreatic and small intestinal diseases are major causes of protein maldigestion and malabsorption. Yet in some anemia cases; iron, vitamin B_{12} and folate, in osteoporosis cases lactose, in steatorrhea cases (fatty stools); lipids and fat-soluble vitamins and in Hartrup disease; neutral amino acids malabsorbtion are seen. Mucosal diseases may affect protein assimilation by reduction in the number of mucosal cells, increased turnover of intestinal cells, or increased losses of plasma proteins from damaged intestinal surface. Surgical resection of the intestine not only reduces the total intestinal absorbtive surface but may remove a segment of the gut that is specialized for absorbtion of certain nutrients. Another case for reduced protein assimilation is protein-losing enteropathy, in which inflamed or ulcerated mucosa exudes considerable quantities of plasma proteins, which, is not subsequently digested and reabsorbed, represent large fecal losses of nitrogen, these losses will cause hypoproteinemia.

The laboratory plays an important part in detecting or ruling out malabsorption and in elucidating its cause shown the many diagnostic modalities that are avaitable. (Table 32) There are some visualization techniques. These are; angiography, camputed axial tomography (CAT scan), endoscopic retrograde cholangiopancreatography (ERCP), ultrasound scan, and radiologic examination of intestine with contrast media.

TABLE 32: Selected tests used in diagnosis of pancreatic and intestinal diseases

Pancreatitis,

Amylase, serum

Amylase, urine

Isoamylase serum

Amylase/creatinine ratio, urine

Lipase, serum

Immunoreactive trypsin, serum

Miscellaneous tests: calcium, serum glucose, creatinine serum, albumin serum and hematocrit

Visualization techniques

Pancreatic Exocrine Insufficiency

Pancreatic juice or duodenal content after stimulation with secretin, or secretin-CCK for volume, concentration of HCO_3^-, trypsin, chymotrypsin, lipase, and amylase

PABA test after stimulation with Lundh meal

Stool for trypsin or chymotrypsin, with or without Lundh meal

Stool for fat

Malignant Disease

Carcinoembryonic antigen, serum

Pancreatic oncofetal antigen, serum

a_1 - Fetoprotein, serum

Special enzyme tests, pancreatic juice or duodenal contents

Pancreatic exocrine function test, with cytologic examination

Visualization techniques

Malaborption

Hydrogen test, breath

Disaccharide tolerance tests

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Xylose absorbtion test

Fat, stool

Tests for pancreatic exocrine insufficieny

Intestinal biopsy, histochemical study for intestinal enzymes

Iron, folate, vitamin B_{12} in serum

Visualization techniques

Investigation of some amino acids in stool or urine.

Some Malabsorbtion Disorders

Sprue Syndrome

Sprue syndromes are diseases of disturbed small intestine function characterized by impaired absorption, particularly of fats an motor abnormalities. Celiac sprue responds to a gluten-free diet, whereas tropical sprue does not. The polypepide gliadin is the offending substance in gluten. Although an infectious cause has not been conclusively demonstrated, tropical sprue behaves clinically like an infectious disease. It responds to folic acid and broad spectrum antibiotics.

The clinical severity of sprue syndrome varies depending upon the extent of the lesion in the small intestine and the duration of the disease. Severe wasting, gastrointestinal protein loss, multiple vitamin deficiencies, and adrenal and pituitary deficiency may be associated with the severe forms of the disease. A flat intestinal mucosa without vili in the small intestine is noted, and some observers have described degenerative changes in the myenteric nerve plexuses.

Rare secondary varieties of sprue syndrome in which the cause of the small intestine dysfunction is konwn include gastrocolic fistulas, obstruction of intestinal lacteals by lymphoma, whipple's disease, extensive regional enteritis, and parasitic infections such as giardiasis, strongyloidiasis, and coccidiosis. Diagnosis essential to sprue syndrome are;

- Bulky, pale, frothy, foul-smelling, greasy stools with increased fecal fat on chemical analysis of the stool.

- Hypochromic or megaloblastic anemia;

- Deficiency" pattern on small bowel x-ray.

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Hartnup Disease

This is a hereditary metabolic disorder of tryptophan catabolism. It is characterized by a pellagralike skin rash, intermittent cerebellar ataxia, and mental deterioration. The urine of patients with Hartnup disease contains greatly increased amounts of indolacetate and tryptophan.

Protein - Losing Enteropathy

Leakage of plasma proteins into the intestinal lumen is an intestinal disease states, excessive protein loss into the intestinal lumen may be responsible for hypoproteinemia noted in these entities. Excessive loss of plasma protein may be due to increased mucosal permeability to protein, inflammatory exudation, excessive cell desquamation, or direct leakage of lymph from obstructed lacteals. Gastrointestinal diseases associated with protein-losing enteropally include gastric carcinoma, lymphoma, gastric rugal hypertrophy, sprue, allergic gastroenteropathy, regional enteritis, diffuse jejuno-ileitis, and others. Treatment consists of management of the primary disorder.

Disaccharidase Deficiency

The specific disaccharidase deficiencies in the intestinal mucosa have become increasingly important in our understanding of malabsorption. The congenital absence of these enzymes leads to an acidic diarrhea (fecal pH 4.5-6.0). There are large amounts of lactic acid in the stool abnormal cramps bloating distention. The symptoms follow the ingestion of milk. Some individuals suffer from severe diarrhea and malnutrition from the first day of life. There is a severe form disaccharidase deficiency. The infant fails to thrive. The onset of these symptoms in the adult may follow intestinal surgery, regional enteritis, ulcerative colitis, or the sprue syndromes. The diagnosis is confirmed by a flat oral tolerance to the specific disaccharide and the onset of an acidic diarrhea with ingestion of the offending sugar. Secondary diaccharidease deficiencies have been described in both children and adults with giardiasis, celiac disease, ulcerative colitis, short bowel syndrome, cystic fibrosis and after gastrectomy. Removal of the offending sugar from the diet will result in remission.

Intestinal Lipodystrophy (Whipple's Disease)

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Whipple's disease is an uncommon malabsorption disorder of unknown cause with widespread systemic manifestation. Histologic examination of a small bowel mucosal biopsy specimen reveals characteristic large, foamy mononuclear cells filled with cytoplasmic material which gives a positive Periodic Acid-Schiff (PAS) staining reaction. Electron microscopic studies reveal bacterial bodies in the epithelium and lamina propria. The disease occurs primarly in middle-aged men and is of insidious onset: the course, without treatment, is usually downhill. The clinical manifestations include abdominal pain, diarrhea, steatorrhea, gastrointestinal bleeding, fever, lymphadenopathy, polyarthritis, edema, and gray to brown skin pigmentation. Anemia and hypoproteinemia are commen.

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PART II

CARBOHYDRATES

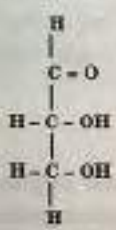
General Description

Carbohydrates are composed of carbon, hydrogen and oxygen. Sometimes they contain other element as a trace quantity. They are probably the most abondant naturally occurring organic compounds. They are found as constituents of both plants and animals. They also represent stored energy.

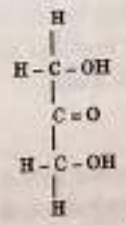
As the name implies, carbohydrates can be represented as hydrates of carbon. But this is not a very meaningful definition. Because many organic compounds which are not carbohydrates, can also be written as hydrates of carbon. For example (1)

$$CH_3COOH = C_2(H_2O)_2 \quad \text{Acetic acid}$$
$$HCHO = C(H_2O) \quad \text{Formaldehyde} \qquad (1)$$

As seen above acetic acid is not carbohydrates but it can be written as hydrates of carbon. The best definition for carbohydrate is "A carbonyl group with poly alcohol". Glyceraldehyde represents one of the simplest compounds of this description. Another simple carbohydrate is dihydroxyacetone. There are two hydroxy groups plus a carbonyl functional group. In glyceraldehyde also there are two hydroxy group and a carbonyl group. Carbohydrates can exist in two distinc forms. Those are aldehyde and ketone group. Those bearing an aldehyde group are referred to as aldoses. Those with a ketone group as ketoses. The suffix "ose" represents the compounds as carbohydrates. These are shown below (2)



Glyceraldehyde

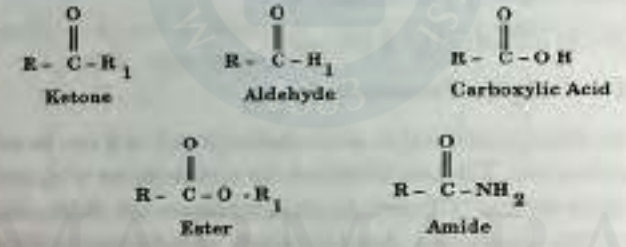


Dihydroxyacetone

(2)

Another type of nomenclature takes into consideration. It is based on the number of carbons which occur in a continuous chain in the molecule. For instance, glyceraldehyde and dihydroxyacetone are called trioses. This does not allow for a distinction between the aldoses and ketoses. By combining the two systems of nomenclature, glyceraldehyde can also be called aldotriose. Because it has three carbons and an aldehyde group, and the suffix ose indicates it is a carbohydrate. Similarly, dihydroxyacetone can be called a ketotriose. Here keto indicates that the carbonyl group is a ketone, the tri portion refers to three carbons, the ose ending signifies a carbohydrate.

It is better to learn what is the carbonyl group clearly. "A carbon and oxygen atom bound together by a covalent double bond' This group is very active because; there is a double bond and oxygen being a more electronegative element than carbon. Some important carbonyl compounds are given as an example are very important in biochemistry. (3)



(3)

We are concerned with the first 2 members of carbonyl groups, aldehydes and ketones because carbohydrate is concerned with these two groups. The functional group of aldehyde and ketone is written like this - CHO and -CO . We never write COH instead of - CHO because it might then be confused with the functional group of an alcohol. Both aldehyde and ketones show similar physical properties. Except of formaldehyde which is a gas, they are all either liquids or solids at room temperature. Because of the polar nature of carbonyl group low formula weight aldehydes and ketones are soluble in water.

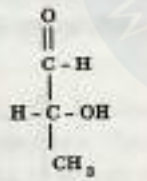
Monosaccharides

Carbohydrate may be divided into 3 groups mono-di and polysaccharides. They are all polyhydroxy aldehydes or ketones. Monosaccarides can be divided into 2 groups depending on whether they have the charac-

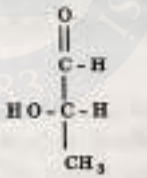
teristic of aldehydes or ketone. Sometimes they are called aldose or ketose sugar. The number of carbon atoms in the molecule can vary. Ketotriose and aldotriose are the simplest monosaccharides.

Isomerism

If a carbon atom is bonded to four different groups it carbon is called asymmetric. Theroretically, there are two isomers for each asymetric carbon atoms. The prefixes D or L in small capital letters designate configuration, not the direction of rotation. The signs (+) and (-) are used to denote dextrorotation (+) and levorotation (-) respectively. For glyceraldehyde, the D isomer is dextrorotatory, thus, the complete name would be D (+) glyceraldehyde. Since the L isomer for glyceraldehyde is levarotatory, the correct name would be L (-) glyceraldehyde. There is no consistent relationship between the structure and rotation the isomers of lactic acid are D (-) lactic acid and L (+) Lactic acid (4)



D (-) lactic acid



L (+) lactic acid

(4)

As is seen D-lactic acid is (-) and L-lactic acid is (+) Glyceraldehyde has two optical isomers. These are D and L isomers and they are accepted as the standard for comparison D,L configuration of other organic molecules. Hexoses and pentoses are the most important monosaccharides in human the isomers are mirror images (5)

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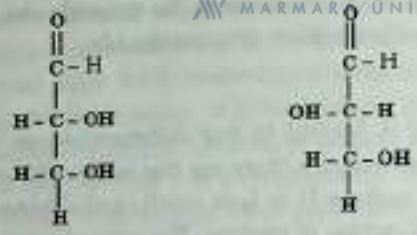
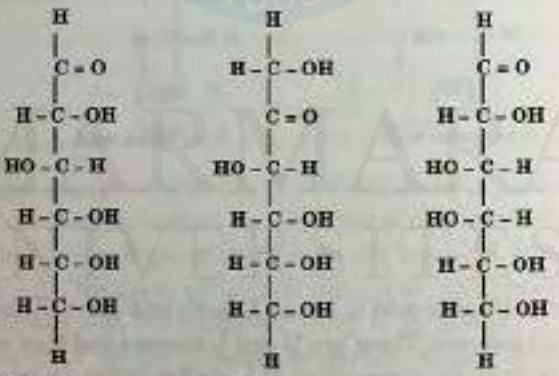


D - Glyceraldehyde L - Glyceraldehyde (5)

In glyceraldehyde, there is one asymmetric C atom, as seen above.

An aldohexose structure is given as an example below (6) represents sixteen possible isomers because it has 4 asymmetric carbon. It gives 8 different (2x4) D isomers and 8 different L isomers total of 16 isomers per glucose molecule. In a similar way ketohexose sugar can be established.



D - Glucose D - Fructose D - Galactose (6)

These molecule given above is a simple linear molecule It is known that the molecule has a ring structure. The orientation of the OH group by ring clasure results in two further isomers called the alpha and beta forms. The alpha and β forms of glucose spontaneously undergo interconversion **mutoratation** in water. A heterocyclic ring form is the structure

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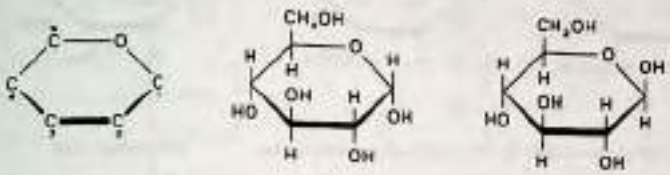
of pyran. Thus, glucose also called α - D or β - D glucopyranose. Two conformations of D glucose and pyranose form are represented in (7).



(7)

numbering for a monosaccharide in the pyranose form

α-D-Glucose

β-D-Glucose

Glucose or dextrose is one of the most important hexose sugars. It is found in sweet fruits, particularly ripe grapes. For this reason it is sometimes called grape sugar. It is very important source of energy for maintaining living processes. It is found in blood stream as well as other tissue fluids.

Disturbances of glucose metabolism resulting from insulin deficiency are called diabetes. There are many other diseases in carbohydrate metabolism due to enzyme deficiency. The glucose tolerance test is often used to diagnosis diabetes condition. In such cases the fasting patient is given a large oral dose of glucose. Blood and urine samples are colected at timed intervals. These samples are later analysed. A graph of blood sugar concentration against time is plotted. The height which blood sugar rises, and the time taken to return to fasting levels can give an indication whether a patient has diabetes or not.

Glucose has also some biologically important derivates glucosamine galactosamine and glucuronic acid (8) are a constituent of mucopolysaccharides and also plays an impoetant metabolic role in removal of toxic materials from the body by forming soluble glucuronides. They are readily excreted in the urine.

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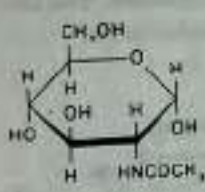
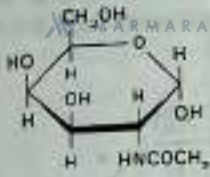
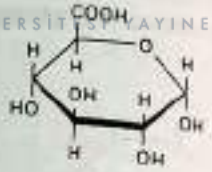


N-Acetyl α-D-glucosamine



N-Acetyl α-D-galactosamine



Glucuronic acid

The other important monosaccharides, in living cell is galactose. It does not occur free in nature. But it is combined with glucose to form disaccharide called lactose (milk sugar) Galactose is found in nervos tissue associated with fats called glycolipids. Because of an enzyme defect sometimes galactose can accumulate in the tissues. These cases are called galactosemia. In the tissue the normal convertion of galactose to glucose can not be occured in this condition. The resulting accumulation of galactose leads to mental retardation, liver damage and cataract formation. The trestment must be started at early stage to avoid these side effects.

The other important monosaccharides is fructose. It is an aldoketos. It is found in honey and fruit. For this reason sometimes it is called fruit sugar. It is also found in sucrose as a bound form with glucose. It is called tea sugar (sucrose)

Ribose and deoxyribose both these aldopentoses are found in nucleic acids.

Hemiacetal Structures

Carbonyl groups are extremely active in aqueous solvents. They readily react with water to yield hydrated forms. In the glucose molecule intramolecular hemiacetal occurs as shown below: An oxygen bridge takes place between carbons 1 and 5. This is the most stable configuration of the molecule. Similar reactions occur with fructose. The oxygen bridge here is shown extending to carbons 2 and 5 (9).

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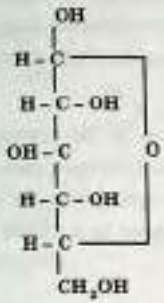


Oxygen bridge
(D-Glucose)



Oxygen bridge
(D-Fructose)

(9)

When hemiacetal form carbonyl group containing carbon becomes asymmetric, thus glucose contains 5 asymmetric carbon instead of 4. Ring form of hemiacetal structure was shown in (7).

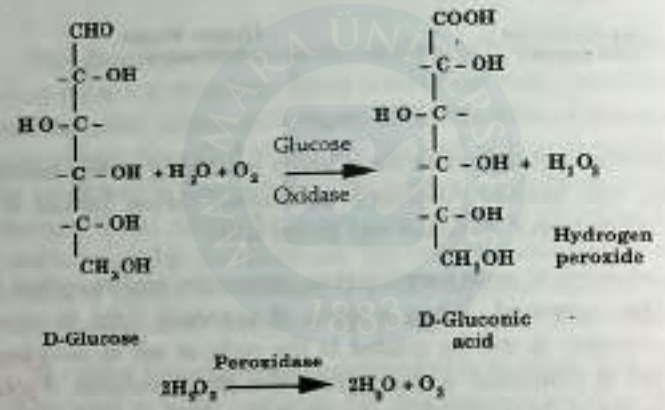
The presence of asymmetric carbon atoms also confers optical activity on the compound. When a beam of polarized light is passed through a solution, it will be rotated to the right or left in accordance with the type of compound. A compound which causes rotation of polarized light to the right is said to be dextrarotatory and a plus (+) sign is used to designate this fact. Rotation of the beam to the left (levorotatory action) is designated by a minus (-) sign. When equal amounts of dextrorotatory and levorotatory isomers are present, the mixture has no optical activity a mixture is said to be a **racemic mixture**. Stereoisomerism and optical isomerism are independent properties. Thus, a compound might be designated D (-) or L (+) indicating structural relationship to D or L glucose but exhibiting the opposite rotatory power. The naturally occurring form of lactic acid, the L (+) isomer, is an example as was mentioned.

Important chemical reactions of monosaccharides

Various reactions dependent upon the presence of aldehyde or ketone groups, are particularly important because they form the bases for most analytical tests for the sugars. The best known tests involve reduction of metallic hydroxides together with oxidation of the sugar. The alkaline metal is kept in solution with sodium potassium tartrate (Feh-

ling's solution) or sodium citrate (Benedict's solution). Varios modifications permit quantitative detection of the copper reduced as a measurement of the sugar content. Other metallic hydroxides may be used which is called, Nylander's test, Tollens' test.

Glucose is oxidized by atmosphenic oxygen in the presence of the enzyme glucose oxidase to give gluconic acid and hydrogen peroxide. Peroxidose catalyses the decomposition of the hydrogen peroxide into water and oxygen. This inturn oxidises the dye substance to a shade of purple (10)



(10)

Osazone formation is a useful means of preparing crystalline derivatives of the sugars. These compounds have characteristic crystal structures, melting points and precipitation times. All these are valuable in the identification of sugars. They are obtained by adding a mixture of phenylhydrazine hydrochloride and sodium acetate to the sugar solution and heating in a boiling water bath. The reaction involves only the carbonyl carbon (altehyde or ketone group) and the next adjacent carbon. For example with an aldose the following reaction occurs (11)

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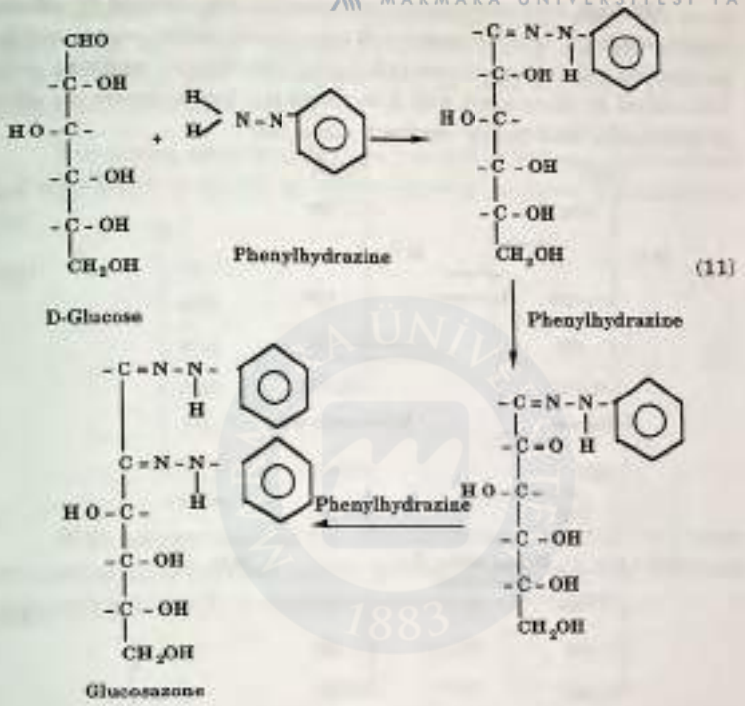


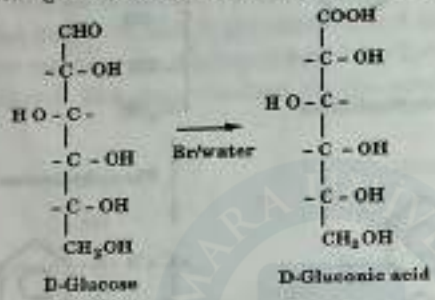
(11)

The reaction with a ketone is similar. Glucose, fructose and mannose from the same osazones, but since the structure of galactose differs in that part of the molecule unaffected in osazone formation, it would form a different osazone.

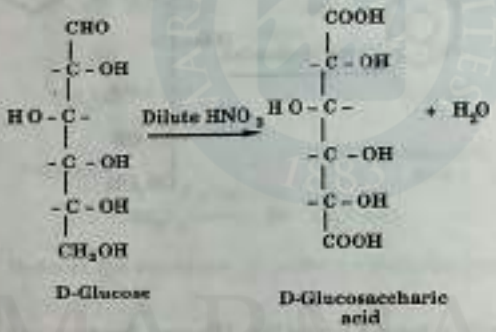
Glucose, fructose and mannose are interconcertible in solutions of weak alkaline such as $Ba(OH)_2$ or $Ca(OH)_2$. The interconversion of these sugars which take place in the body is explained by a similar reaction. But these reactions have need some kind of enzyme.

Oxidation of aldoses may form acids as end products. As shown in below, oxidation of the aldehyde group forms "aldonic acids". If the aldehyde group remains intact and the primary alcohol group at the opposite end of the molecule is oxidized, "uronic acids" are formed instead. Hexuronic acids are important in connection with conjugation reactions and detoxification reactions.

Although both ketose and aldose sugars are oxidised by alkaline copper solutions, bromine water will only oxidise aldose sugars and has no reaction wih ketose sugars. (12) Under the stronger oxidising conditions afford by dilute nitric acid beth aldose and ketose sugars are effected yielding the dicarboxylic saccharic acids. (13)

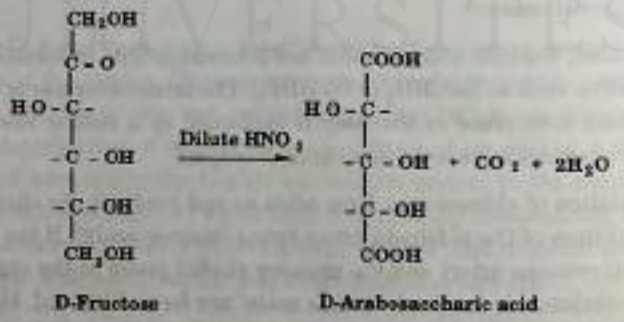


(12)



(13)

D - Fructose yields D - Arabosaccharic acid by dilute HNO_3 (14)



(14)

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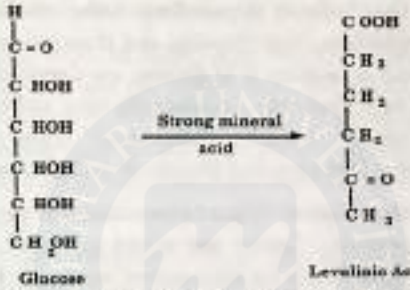
Oxidation of galactose with concentrated HNO_3 yields the dicarboxylic music acid. This compound crystallizes readily, and this is useful as an identifying test. Galacturonic acid is found in natural products. pectins, are one of the examples.

With strong mineral acids there is a shift of hydroxyl group toward and of hydrogen away from the aldehyde end of the chain as shown below (15)



(15)

With sodium amalgam the aldehyde group of an aldose is reduced to a primer alcohol to yield a sugar alcohol. Similarly a ketone functional group of a ketose will be reduced to a secondary alcohol. (16)



(16)

With alkali monosaccharides react in various ways:

1) In dilute alkali the sugar will change to the cyclic alpha and beta structures with an equnilibrium between the 2 isomeric form (mutarotation increases in alkaline medium)

D Glucose may exist in two different isomeric forms differing in specific ratation, alpha D-glucose, has + 112.2 and β D glucose has + 18.7 . Both have been isolated in pure form. Although they do not differ in elementary composition, their physical and chemical properties differ. When the alpha and B isomers of D glucose are dissolved in water, the optical rotation of each gradually changes with time and approaches a final equilibrium value of + 52,7 , This change, called mutorattation. There are one-third = ,D glucose and two-thirds β-D-glucose at 20 C.

2) In concentrated alkali, sugar caramelizes and produces a series of decomposition products. Yellow and brown pigments develop, salts may form, many double bonds between carbon atoms are formed, carbon to carbon bods may rupture.

Monosaccharides are stable to hot dilute mineral acids Concentrated acids, however, cause dehydration of sugar to yield furfurals (aldehyde derivatives of furan ring).

If monosaccharides react with organic phenols some characteristic color occurs. Color test for the various sugar are based on such reactions. For example, D-glucose heated with strong HCl yields 5-hydroxymethyl furfural. Furfural condense with phenols, such as orcinol to give caharacteristic colored products often used for colorimetric analysis of sugars.

Epimer Sugars , Isomers differing as a result of variation in configuration of the - OH and - H on carbon atoms 2,3 and 4 of glucose are known as epimers. Biologically, the most important epimers of glucose are mannose and galactose. Formed by epimerization at carbons 2 and 4 respectively.

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Some important derivatives of monosaccharides Sugar acids: There are three important types of sugar acids

Aldonic acid, The aldoses are oxidized at the aldehydic carbon to yield corresponding carboxylic acids. These are called generally aldonic acids. For example, D-glucose yields D-gluconic acid. This an important intermediate in carbohydrate metabolism.

If a stronger oxidizing agent is employed, e.g. nitric acid, both the aldehydic carbon atom and the carbon atom bearing the primary hydroxyl group are oxqidized to carboxyl groups, yielding saccharic acids. They are also called **aldaric acids**. For example with D-glucose the product is called D-glucaric acid. Aldaric acids are sometimes useful for the identification of sugars, but they are no great biological significance.

However, the third class of sugar acids the **uronic acids**, are biologically very important. In uronic acids, only the carbon atom bearing the primary hydroxyl group is oxidized,, to a carboxyl group. The uronic acid derived from D-glucose is D-glucuronic acid. Other important uronic acids are D-glacturonic acid and D-mannuronic acid. The uronic acids are components of many polysaccharides. They are also useful in detoxification reaction.

The oxidation of carbohydrates can lead to several products. The conversion of gluose to gluconic acid by the oxidation of the aldehyde group to a carboxyl group can be accomplished in the laboratory withaut difficulty. However, under laboratory canditicns oxidation of glucose to glucosiduronic acid is difficult because it involves the oxidation of a primary alcohol group in a molecule which contains an aldehyde group. Further oxidation of either gluconic or glucosiduronic acid leads to the dicarboxylic oxidation product glucaric acid (17) the oxidation of the aldohexoses to the corresponding dicarboxylic acid is readily acoomplished in the laboratory. Similarly, galactose can undergo oxidation to form a dicarboxylic acid, music acid, this reaction is often important in the identification of galactose.

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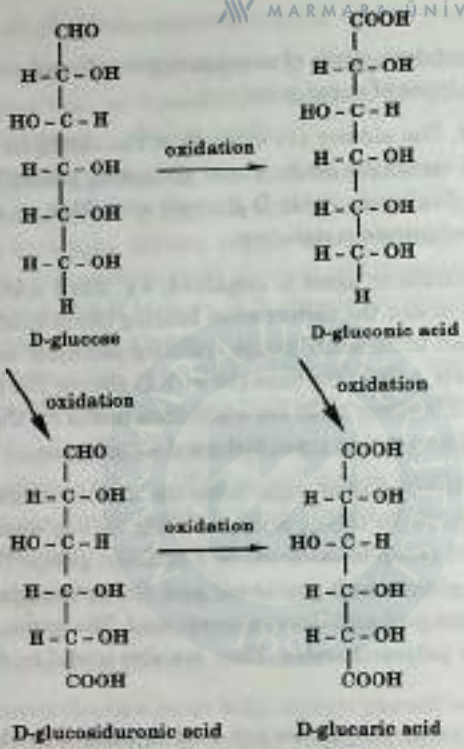

(17)

One of the most important sugar acids is ascorbic acid or vitamin C. Below gamma lactone of a hexonic acid and endiol is shown. This is a very unstable compound and readily undergoes oxidation to dehydroascorbic acid (18). Prolonged lack of ascorbic acid in the diet of human beings results in scurvy. Less severe deficiency of ascorbic acid produces alterations in connective tissue structure and may also cause decreased resistance to some infections. Ascorbic acid is present in large amounts in citrus fruit and tomatoes.

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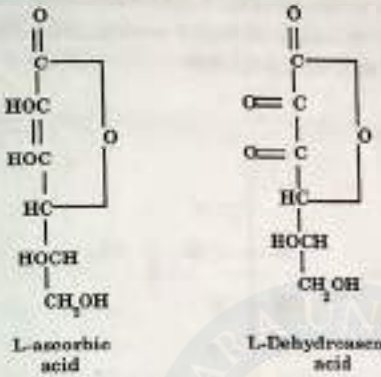
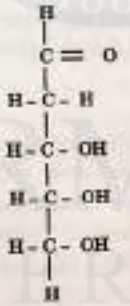


L-ascorbic acid

L-Dehydroascorbic acid

(18)

Deoxy sugars: those containing fewer atoms of oxygen than of carbon are called **deoxy sugars**. Several deoxy sugars are found in nature. The most aboundant is 2-deoxy - D ribose (19) This sugar is component of deoxyribonucleic acid. Some deoxy sugars are important components of some bacterial cell walls.



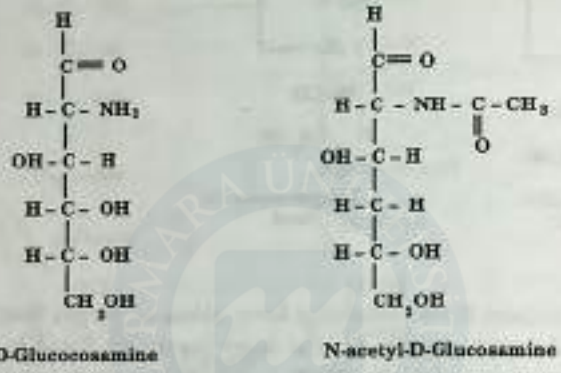
2-deoxy-D-ribose

(19)

Amino sugars:

D-galactosamine and D-glucosamine in which the hydroxyl group at carbon atom 2 is replaced by an amino group are the most wide distribution of amino sugars. Glucosamine occurs in many polysac charides of vertebrate tissues and is also a major component of chitin. D-galactosamine is a component of glycolipids and of the major polysaccharide of cartilage, chondrotin sulphate. Muramic acid and neuraminic acid

are important building blocks of the structural polysaccharides found in the cell walls of bacteria and cell coats of higher animal cells. In these sugars the amino group is usually acetylated (20).



(20)

Sugar Phosphates: These are found in all living cells. Because they are important intermediates in carbohydrate metabolism, such as glucose - 6 - phospate and fructose - 1,6 diphosphate.

Disaccharides

Disaccharides consist of two monosaccharides joined by a glycosidic linkage. The composition of a disaccharide may be establishes by breaking down this chemical combination by enzymatic or acid hydrolysis the liberated monosaccharides may then be identified by chorornatography. Table indicates Disaccharides and their monosaccharides building blocks.

Disaccharide	Monosaccharide building blocks
Maltose	Glucose + Glucose
Lactose	Glucose + Galactose
Sucrose	Glucose + Fructose
Cellobiose	Glucose + Glucose

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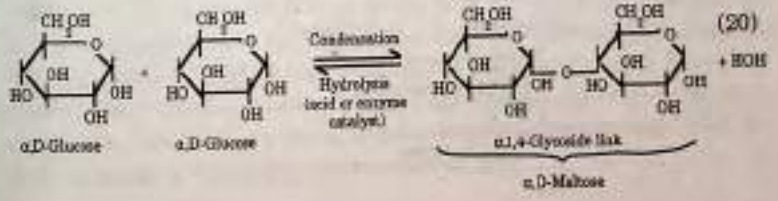
Disaccharides may be divided into two catogories depending upon their ability to reduce alkaline copper solutions. Table in belov represents reducing and non reducing disaccharides.

Reducing disaccharide	Non-reducing disaccharide
Maltose	Sucrose
Lactose	
Cellobiose	

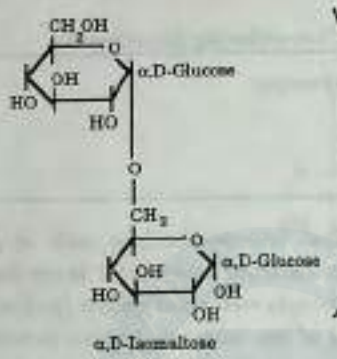
We have already seen that monosaccharides exist as ring structures which is the result of an internal hemiacetal being formed. You may also remember that hemiacetals well react with a further alcohol to form an acetal. The hemiacetal of one monosaccharides reacting of with an alcohol group on another monosaccharide hemiasetals are relatively unstable and easily broken down, but an acetate is quite stable. This stability is shown by the glycoside link which can be hydrolysed but requires the presence of acid or enzyme act as catalyst.

The most common disaccharides are maltose lactose and sucrose. Maltose is formed as an intermediate product of the action of amylases on starch. It contains two D-glucose residues (20). It is a mixed acetal of the anomeric carbon atom 1 of D-glucose and carbon atom 4 of a second D-glucose molecule. The first glucose residue cannot undergo oxidation in maltose molecule but the second residue can. It is called the reucing end. The position of the glycosidic linkage between the two glucose residues is symbolized 1-4.

Because maltose possesses a potential aldehyde group it will exhibit the properties of an aldehyde, will reduce alkaline copper solutions and will form an osazone with phenylhydrazine hydrochloride. It also exhibits mutarotation and therefore exists in both alpha and beta forms.



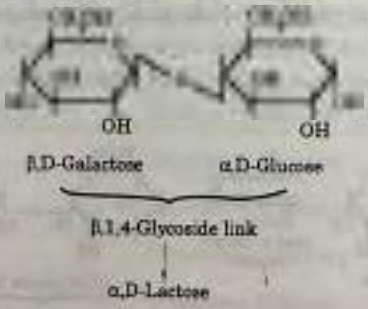
Isomaltose is formed in the enzymatic break down of starch and differs from maltose in that glucose units are linked alpha 1,6 instead of alpha 1,4. (21)



(21)

The other common disaccharides that contain two D-glucose units are cellobiose. The repeating disaccharide unit of cellulose has beta L 4 glycosidic linkage. The other disaccaride is gentiobiose. The glycosidic linkage in that sugar is 1 - 6 beta. Since both these disaccharides have free anomeric carbons are reducing disaccharides.

The dissaccharide lactose β (1 - 4) is found in milk. Otherwise it does not occur in nature. It yields D-galactose and D- glucose with hydrolysis. (22) Since it has a free anomeric carbon on the glucose residue, lactose is a reducing disaccharide. Lactose possesses a potential aldehyde group and will reduce alkaline copper solitions as well as forming an osazone, and exhibiting mutarctation.



(22)

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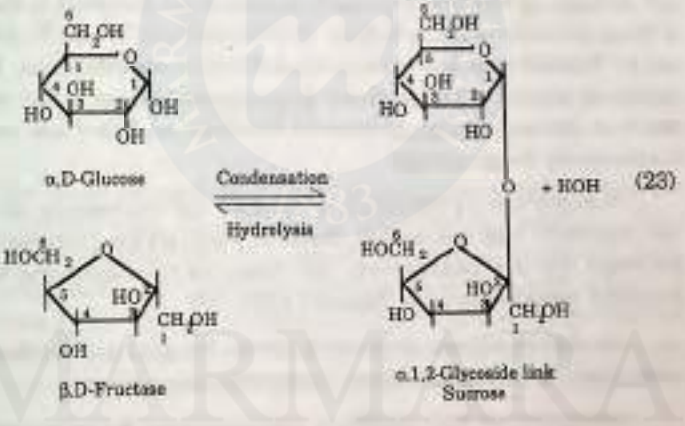
Sucrose or cane sugar, is a disaccharide of glucose and fructose (23). It is extremely aboundant in the plant world it is also table sugar. Unlike most disaccharides and oligosaccharides sucrose contains no free anomeric carbon atom. The anomeric carbon atoms of the two hexoses are linked to each other. For this reason sucrose does not undergo mutarotation, does not react with phenyl-hydrazine to form osazones and does not act as a reducing sugar. It is much more readily hydrolyzed than other disaccharides. The hydrolysis of sucrose to D-glucose and D-Fructose is called inversion. The product is named **invert sugar**. The specific optical rotation of sucrose is 66.5 and is dextrorotory. If the optical rotation of sucrose is measured during the course of hydrolysis it is found to change direction and become levorotory. The hydrolysis of sucrose is catalyzed by enzyme invertase. This can be followed with a polarimeter.



Polysaccharides (Glycans)

Most of the carbohydrates found in nature occur as polysaccharides of high molecular weight. On complete hydrolysis with acid or specific enzymes, these polysaccharides yield monosaccharides and/or simple monosaccharide derivatives. D-glucose is the most prevalent monosaccharide unit in polysaccharides, but polysaccharides of D-mannose D-fructose, D and L-galactose, D-xylose and D-arabinose are also common. Monosaccharide derivatives commonly found as structural units of natural polysaccharides are D-glucosamine, D-galactosamine, D-glucuronic acid, N-acetylmuramic acid, and N-acetylneuraminic acid.

Polysaccharides are also called glycans. They are divided into homopolysaccharides, which contain only one type of monomeric unit, and heteropolysaccharides which contain two or more different monomeric units.

Glycans are large molecules. They have diverse functions; being structural units of bacterial cell walls, the hard chitinous shell of crab, lobster and insect coats, fabric of plant cells is supported by the cellulose. Cellulose is also the storage form of carbohydrate in plant cells (starch), glycogen is mammalian liver and muscle tissue. Polysaccharides serve as a food reserve in various plant types.

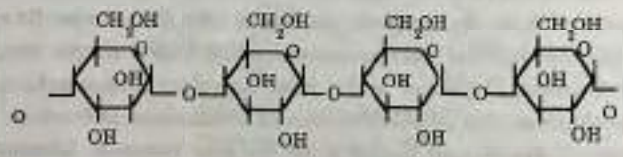
Storage Polysaccharides

These polysaccharides, of which is the most aboundant in plants and glycogen in animals. These polysaccharies are deposited in the form of large granules in the cytoplasm of cells. Glycogen or starch granules can be isolated from cell extracts by differential centrifugation. If it is necessary glucose units are stored by enzymatic linkage to the ends of starch or glycogen chains, in times of metabolic need they are released enzymatically for use as fuel.

Starch occurs in two forms, α- amylose and amylopectin. An amylose consists of long unbranched chains in which all the D-glucose units are bound in α (1-4) linkages (24). The chains are polydispers and vary in molecular weight from a few thousand to 500.000.

Amylose is a linear molecule of 250 to 300 α , 1-4 Linked glucose units, which are called up to form, a spring like arrangement.

Amylose is not truly soluble in water but forms hydrated micelles, which give a blue color with iodine.



(24)

α,1,4-glycoside links (Amylose)

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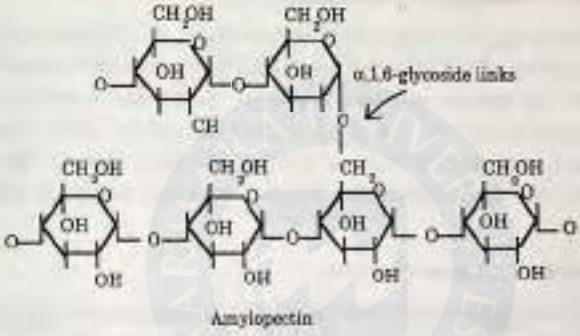
Amylopectin is highly branched, the average length on the branches is from 24 to 30 glucose residues., depending on the species. The backbone glycosidic linkage is α(1-4), but the branch points are a (1-6) linkages (25). Amylopectin yields colloidal or micellar solutions, which give a red-violet color with iodine. Its molecular weight may be as high as 100 milion.



(25)

Amylopectin

The major components of starch can be enzymatically hydrolyzed in two different ways. Amylose can be hydrolyzed by a amylase α (1-4) glucan 4-glucanohydrolase). It is present in saliva and pancreatic juice and participates in the digestion of starch in the gastrointestinal tract. It hydrolyzes α (1-4) linkages at random to yield a mixture of glucose and free maltose. The polysaccharides of intermediate chain length that are formed from starch components by the action of amylases are called dextrins.

Neither α nor β amylases can hydrolyze the α (1-6) linkages at the branch points of amylopectin. A debranching enzyme α (1-6) glucosidase can hydrolyze the α (1-6) linkages at the branch point. The cambined action of amylase and glucosidase can therefore completely degrade amylopectin to maltose and glucose.

Glycogen is the main storage polysaccharide of animal cells, the counterpart of starch in plant cells. Glycogen is especially abundant in the liver. It may attain up to 10 % of the wet weight It is also present to about 1 to 2 % in skelatal muscle. In liver cells the glycogen is found in large granules, highly branched molecules with a molecular weight of

several million. Like starch glycogen is a polysaccharide of D glucose in α (1-4) and α (1-6) linkage. But it is a more highly branched and more compact molecule than starch. The branches in glycogen occur about every 8 to 12 glucose residues. Glycogen can be isolated from animal tissues by digesting them with hot KOH solutions Glycogen gives a red violet color with iodine.

Other storage polysaccharides

Dextrans are also branched polysaccharides of D glucose, but they differ from glycogen and starch in having backbone linkages other than α (1-4). Found as storage polysaccharides in yeasts and bacteria. Their branch points are, 1-2, 1-3, 1-4 or 1-6 in different species. Inulin, found in the artichoke, consists of D-fructose residues in β (2-1) linkage. Mannans are mannose homopolysaccharides found in bacteria, yeasts, molds and higher plants

Structural polysaccharides

Many polysaccharides serve primarily as structural elements in cell walls and coats, intercellular spaces and connective tissue. It gives shape, elasticity or rigiity to plant and animal tissues. It also supports to unicellular organisms. Cell walls and coats are not only important in maintaining the stucture of tussues but also contain specific cell-cell recognition sites important in the mophogenesis of tissues and organs. They also contain other protective elements, such as cell-surface antibodies of vertebrate tissues. For this reason we shall examine the structural polysaccharides in the context of the molecular organization of cell walls and coats and cell membrane. The most abundant cell-wall and structural polysacharide in the plant is cellulose. It is a linear polymer of D glucose in β (1-4) linkage (26). The only chemical difference between starch and cellulose, both homopoly-saccharides of D-glucose, is that starch has α (1-4) linkages and cellulose β (1-4). Cellulose is not attacked by either α or β amylase. Enzymes capable of hydrolyzing the β 1-4 linkages of cellulose are not secreted in the digestive tract of most mammals, and they cannot use cellulose for food. However, the ruminants, e.g. the cow are an exception. They can utilize cellulose as food since bacteria in the lumen form the enzyme **cellulase**, which hydrolyzes cellulose to D-glucose. The minumum molecular weight of cellulose from different sources has been estimated to vary from about 50.000 to 300.000 in different species,

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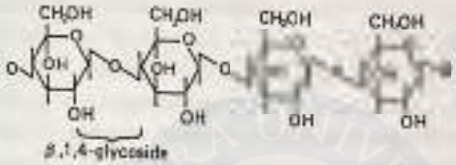
equivalent to 300 to 15.000 glucose residues. X ray diffraction analysis indicates that cellulose molecules are organized in bundles of parallel chains to form fibrils. although cellulose has a high affinity for water, it is completely insoluble in it.



(26)

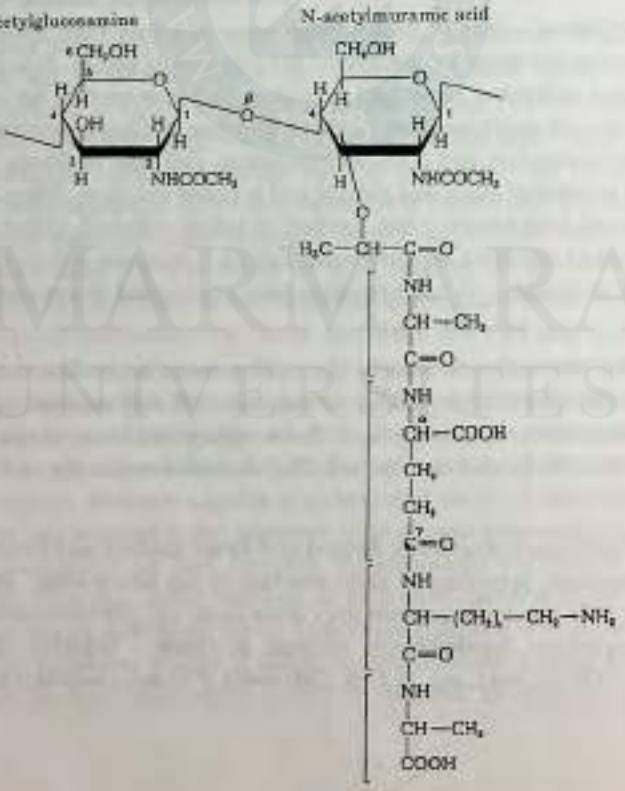
Cellulose

Celluluse is seen in such diverse forms as cotton wool, filter paper, linen and is the main constituent of the cell wall of plants. It is also the basis for a number of important derivates. Cellulose asetate for example is used as a support medium in electrophoresis, Cellulose trinitrate has explosive properties and is called gun cotton. Cellulose dinitrate is soluble in a mixture of ether and alcohol and is called collodion. When collodion is mixed with camphor the, product is called celluloid. Celluloid was important at one time because when heated it becomes soft and pliable and can be moulded. It has nowadays been superceded by synthetic plastics.

The cell walls of bacteria are rigid, porous, its boxlike structures that provide physical protection to the cell. Cell wall prevents swelling and rupture of the cell membrane. The structure and biosynthesis of bacterial cell walls is inhibited by penicillin and other medically useful antibiotics.

Bacteria are classically divided into Gram positive and Gram negative organisms, according to their reaction to the Gram stain. In Gram positive cells the walls contain very little lipid, e.g. Streptococcus albus and Micrococcus lysodeikticus, whereas in Gram - negative cells the walls are rich in lipid, e.g. E. Coli. Cell walls of Gram - negative bacteria

can be isolated by shaking thick suspensions of the cells with very fine glass beads to rupture the walls. The bacterial contents can then be washed out. Cell walls make up about 20 to 30 % of the dry weight of Gram-negative bacteria. Gram positive and gram negative cell walls have one molecular feature in common: in both the rigid structural framework consists of parallel polysaccharide chains covalently cross-linked by peptide chains. This framework makes up 50 % or more of the weight of the cell wall. This structure is called a peptidoglycan or murein. There is recurring muropeptide in bacterial cell wall. This structure were largely elucudated during investigations on the mechanism of action of the antibiotic penicillin by Strominger and his colleagues. The basic recurring unit in the peptidoglycan structure is the muropeptide as seen in (27). It is a disaccharide of N-acetyl-D- glucosamine and N-acetylmuramic acid in β (1-4) linkage. Here D glutamic acid residue is linked to L lysine by its gammacarboxyl group.



(27)

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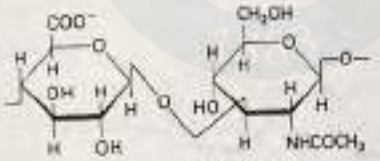
Acid Mucoplysaccharides

The acid mucopolysaccharides are a group of related heteropolysaccharides usually containing two types of alternating monosaccharide units of which at least one has an acidic group. This acidic group either a carboxyl or sulfuric group. When they occur as complexes with specific proteins, they are called mucins or mucoproteins. In this class of glycoproteins the polysaccharide makes up most of the weight. Mucoprotoins are are jellylike, sticky, or silppery substances. Some provide lubrication and some function as a flexible intercellular cement.

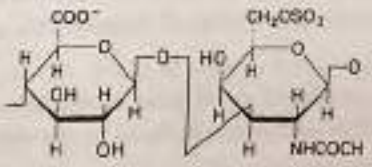
The most abundant acid mucopolysaccharide is hyaluronic acid, (28) present in cell coats and in the extracellular ground substance of the connective tissues of vertebrates. It also occurs in the synovial fluid in joints and in the vitreous humor of the eye. The repeating unit of hyaluronic acid is a disaccharide composed of D-glucuronic acid and N-acetyl D-glucosamine in β (1-3) linkage. Hyaluronic acid is soluble in water, in which it forms highly viscous solutions. The enzyme hyaluronidase catalyzes hydrolysis of the β (1-4) linkages of of hyaluronic acid, this hydrolysis is accompanied by a decrease in viscosity.



(28)

Hyaluronate

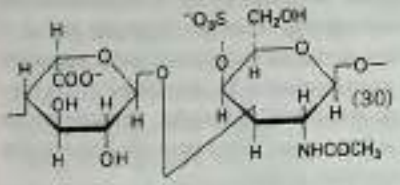
Another acid mucopolysaccharide is chondritin, which is nearly identical in structure to hyaluronic acid. The only difference is that it contains N-acetyl D-galactosamine instead of N-acetyl - D-glucosamine residues. Chondritin itself is only a minor component of extracellular material, but its sulfuric acid derivatives, chondroitin 4 sulfate, Chondroitin A_4 and chondroitin 6-sulfate Chondroitin C. (29) are major structural components of cell coats, cartilage, bone, cornea and other connective tissue structures in vertebrates.



(29)

Chondroitin 6-sulphate

Dermatan sulfate (30) and keratan sulfate (31) are acid mucopolysaccharides found in skin, cornea and bony tissues.



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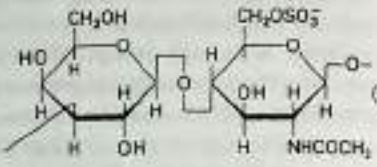
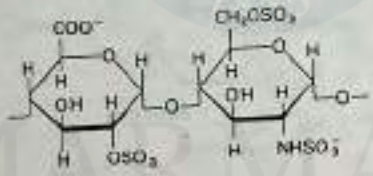


(31)

Dermatan sulphate

Keratan sulphate

Another acid mucopolysaccharide is heparin, which prevents coagulation of blood. (32) It is found in the lungs and in the walls of arteries. Recently it has been discovered that some acid mucopolysaccharides contain the element silicon. This is essential in the nutrition of rats and chicks. The silicon is bound to the polysacharides in covalent form.



(32)

Heparin

Glycoproteins (GP)

Glycoproteins contein carbohydrate groups attached covalently to the polypeptide chain. GP has considerable biological significance and wide distribution. The carbohydrate groups in different glycoproteins may vary frum less than 1 % to as high as 30 % in the mucoproteins. Glycoproteins having a very high content of carbohydrate are called proteoglycans.

Glycoproteins are found in all forms of life. Many different monosaccharides and derivatives have been found in glycoproteins. The linear

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or branched side chains of glycoproteins may contain from two to dozens of monocacharide residues. Often the terminal monosaccharide unit is a negatively charged residue of N-acetylneuraminic acid, a sialic acid. Some glycoproteins, grouped according to biological function. Note the most glycoproteins are found in extracellular. Biological distribution of major types of glycoproteins are shown below:

Blood Plasma

Fibrinogen, Immune globulins, thyroxine-binding protein blood group proteins, coagulation proteins and some others.

Urine:

Urinary glycoprotein

Hormones:

Thyroid-stimulating hormone

Follicle-stimulating hormone

Some gonadotrophin

Enzymes

Ribonuclease B

β- glucuronidase

Pepsin

Serum cholinesterase

Egg white

Ovalbumin, Avidin, Ovomucoid

Mucus secretions

Submaxillary glycoproteins

Gastric glycoproteins

Connective tissue

Collagen

Cell membranes

Basement-membrane glycoproteins

Lens capsule glycoproteins

The oligosaccharide groups of most glycoproteins are covalently attached to the R groups of specific amino acid residues in the polypeptide chain. Three different types of linkages have been found in glycoproteins. In some GP for exopmple ovalbumin and the immunoglobins, the oligo-

saccharide is attached via a glycosylamine linkage between N-acetyl-D-glucosamine of the oligosaccharide to the amide nitrogen of an asparagine residue in the polypeptide chain. In second class of glycoproteins, including the submaxillary mucoprotein there is a glycosidic bond between N-acetyl-D-galactosamine of the side chain oligosaccharide and the hydroxyl group of a serine or threonine residue. The submaxillary mucins have recurring units of about 28 amino acid residues. Each recurring unit contains three oligosaccharide side chains. In the third class of glycoproteins, represented by collagen, the oligosaccharide side chains are attachad to the hydroxyl groups of hydroxylysine residues.

Cell Wall and Membrane

The cell wall contains a rigid framework of polysaccharide chains cross-linked with short peptide chains. Its outer surface is coated with lipopolysaccharide. The cell wall protects bacteria against swelling in hypotonic media. It is porous and allows most small molecules to pass. The cell coat of hepatocytes is flexible and sticky. It is composed of acid mucopolysaccharides, glycolipids, and glycoproteins. The adhesive properties of cell coats are specific and play an important role in cell,cell recognition and thus tssue organization. under the cell coat cell membrane is present. Cell membrane also called plasma membrane. The plasma membrane is about 9 nm thick and contains about equal amounts of lipids and proteins, the lipids are arranged in a bilayer. It contains a greater variety of, lipids than bacterial membranes. The plasma membrane is selectively permeable. It contains active-transport systems for Na+ and K+, glucose, amino acids, and other nutrients, as well as a number of important enzymes. Plasma membranes contain specific receptors for external stimuli. This stimuli may result in different responses as oriented movement of the cell eg. stimulation of membrane bound enzymes (result in release of cAMP, cGMP or electrical or nerves).

Proteins and lipids are the major constituents of the plasma membrane, generally in the ratio of approximately 1:1, but in some instances, eg. myelin, lipid concentration may be higher than of protein. The major membrane lipids are phosphoglycerides, glycolipids, and cholesterol. The proteins are largely glycoprotein The membrane protein divided into two types, peripheral and integral. The former term is used to desighate those glycoproteins are readily extractable from the plasma membrane by non-detergent solevents, whereas the integral glycoproteins are more

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tightly associated with the membrane and can be solublized readily with the use of detergants. A generalized model of the plasma membrane is shown schematically in figure 1.

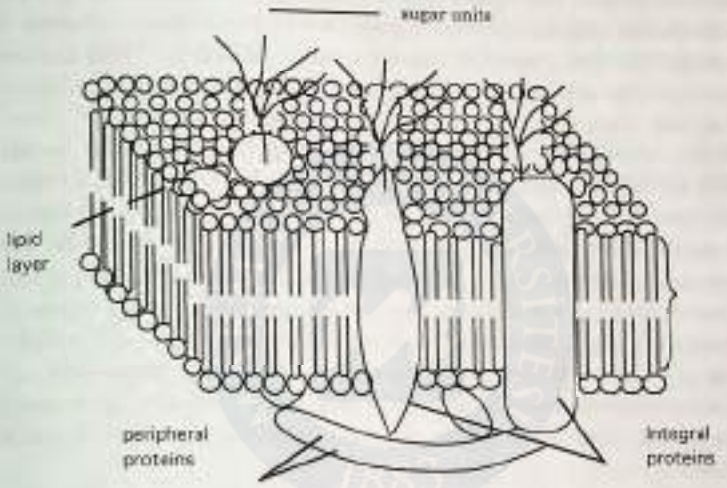

Figure 1: The lipid-globular protein model of plasma membranes. Schematic representation of the fluid mosaic model (From S.J. Singer and G.L. Nicolson)

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CARBOHYDRATE METABOLİSM

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The term metabolism can be defined as all the physical and chemical changes that occur within living systems. The concept is easy to grasp if we consider that substances like glucose are absorbed into the organism and oxidized to CO_2 and water. In this case metabolism refers to the degradation of glucose to its end products with concomitant production of energy. The degradative processes are called catabolism. There are also accumulative aspects for which the term anabolism is used. Changes that take place during growth of plants and animals are anabolic, whereas the wasting away processes are catabolic. In the normal adult animal, both proceses are balanced to a large degree: as a result, one could easily conclude that metabolism is the chemical changes which occur to various compounds during their exposure to living systems. Unfortunately, it is also used to describee the transport and compartmentalization of substances which do not undergo chemical changes. For instance, many investigators speak of the metabolism of sodium. Sodium enters the organism as a sodium ion, spends a variable period of time in the system, and is released in the same form in which it entered. obviously, no chemical change has occurred. Metabolic pathways for food melecules are shown in Figure 2.

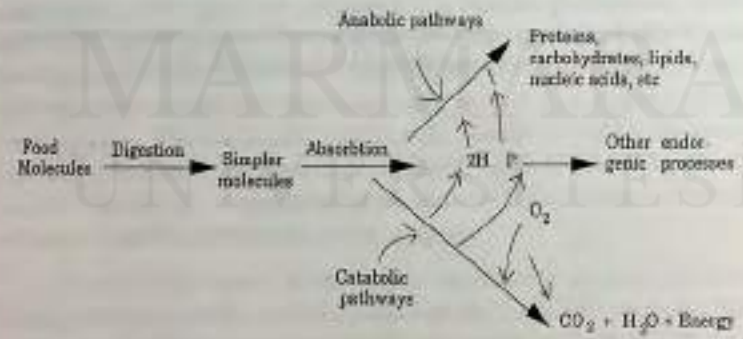

Figure 2: General metabolic pathway for food molecules

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The chief function of carbohydrate in the animal organism is that of a fuel, the degradation of which, to carbon dioxide and water represents major source of energy. Carbohydrate can also be used as a starting material for the biological synthesis of other types of compounds in the body, such as fatty acids and certain amino asids. In addition, it has a role in the structure of certain biologically important compounds, such as glycolipids, glycoproteins, heparin, nucleic acids and other substances.

Digestion and Absorption of Carbohydrates

Ingested diasaccharides (mainly sucrose and milk lactose) and polysaccharides (starch and glycogen) are converted into monosaccharides by the action of enzymes present in saliva (amylase), pancreatic secretion (amylase) and the mucosa of the small intestine (disaccharidases).

The polysaccharides are made up of glucose and molecules combined together by types of linkage, the so-called 1 - 4 and 1 - 6. The former forms straight chains and the latter is the linkage at the point where one straight chain branches from another. In this way highly branched structures are formed. The amylases of saliva and pancreatic juice can attack only the 1 - 4 linkages. In this process molecules of maltose are split off to glucose as well as isomaltose, which represents the original 1 - 6 linkage. Glucose produced in this way, and some from the diet itself, represents the only polysaccharide-derived monosaccharide material presented to the intestinal mucosa for absorption.

The remainder, in fact, the large bulk of carbohydrate is presented to the mucosa in the form of disacharides (sucrose and lactose in the diet and maltose and isomaltose derived from polysaccharide digestion). Enzymes capable of splitting disaccharides into monosaccharides are known as glycosidas or disaccharidases. It is now well recognized that these enzymes occur in the plasma membranes covering the microvili at the bruch borders of the intestinal mucosal cells. There appear to be at least seven such enzymes in human material. Five are glucosidases and two are galactosidases.

As a result of the disaccharidase activities, the disaccharides within the plasma membrane are broken down to the monosaccharides, glucose, fructose and galactose. Other monosacharides presented to the intestinal mucosa are mannose derived from glycoprotein and pentoses derived from digestion of nucleic acids.

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The comparative rates of absorption of the monosaccharides may be indicated as follows: galactose > glucose > fructose > mannose > pentoses. These striking differences in conjunction with the fact that they are not absorbed from the peritoneal space at the same low rate.

Galactose, glucose and fructose are transferred across the intestinal mucosa by some active process., and not entirely by diffusion. Moreover, the rate of absorption of certain sugars is modified by simultaneoous administration of another e.g. that of galactose is diminished when it is given with glucose. Two mechanisms are apparently involved: 1 - simple diffusion, dependent on the sugar concentration gradients between the intestinal lumen, mucosal cells and blood plasma, 2- active transport, independent of concentration gradient, and dependent upon the provision of energy by metabolic process in the intestinal tissue. Although the transport machanisms for different sugars posses certain features in common, they also differ in certain respects.

It appears that the transport processes for monosaccharide absorbtion are also located within the brush border plasma membrane. One active transport process, which is sodium dependent, is shared by glucose and galactose. The mechanism for absorption of fructose is a different one. The mechanism for absorption of fructose is different. It does not seem to show the characteristics of active absorption, but is nevertheless more rapid than simple diffusion.

Active transport of sugars is depressed by agents that inhibit respiration (cyanide, azide, malonate, fluoroacetate) and by dinitrophenol which uncouples oxidation from phosphorylation. Phloridzin, which apparently interacts with the membrane site at which the sugar enters, also inhibits intestinal absorbtion of glucose and galactose.

Absorption of glucose from the intestine is influenced by the general condition of the organism, being interfered with in infections, varios intoxications, prolonged undernutrition and vitamin deficiencies, especially of thiamine, pantothenic acid and pyridoxine. Absorption may be decreased in the presence of abormlities, structural or functional of the mucous membrane, as in inflammation (enteritis), edema (congestive heart failure) and celiac disease. It is retarded in hypothrhyroidism and accelerated in hyperthyroidism. In experimental animals, active absorbtion of glucose is influenced strikingly by alterations in the concentra-

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tions of cations, especially Na^+ and probably K^+ Diminished absorption in adrenal cortical insufficiency is dependent upon the decreased concentration of Na in the body fluids, and probably not upon a specific influence of the adrenal hormones on the absorbtive process. Malabsorption of carbohytrate may also occurs in following conditions:

1- Generalise intestinal disease

2 - Pancreatic disease, causing amylase deficiency

3 - Intestinal disaccaridase deficiency

4- Defect of monosaccharides transport

Fate of Absorbed Carbohydrates

After absorption into the portal blood, carbohydrates must pass through the liver before entering the systemic circulation. Certain hepatic mechanisms contribute to the withdraval of carbohydrates from the blood: This process has considerable physiological significance, these are:

1- Uptake of hexoses such as fructose and galactose for conversion to glucose by the liver cells,

2- Conversion of glucose to glycogen for storage in the liver **(glycogenesis),**

3- Utilization of glucose by oxidation, for energy production,

4 - Utilization of glucose for the synthesis of other compounds, such as fatty acids and certain amino acids

Opposed to these mechanism there are others which lead to the release of glucose by the liver to the blood:

1- Formation of blood sugar from hexoses other than glucose by the liver,

2- Conversion of liver glycogen to blood glucose **(glycogenolysis)**

3- Synthesis of blood glucose by the liver and kidney from noncarbohydrate sources, such as certain amino acids **(gluconeogenesis).**

Once it is in the systemic circulation, blood glucose becomes available for utilization by the extrahepatic tisses. From what has been been said about portal absoption, it is evident that these tissues are presented with carbohydrate which has already been "picked over" by the liver in a selective manner. Overview of carbohydrate metabolism is shown in figure 3.

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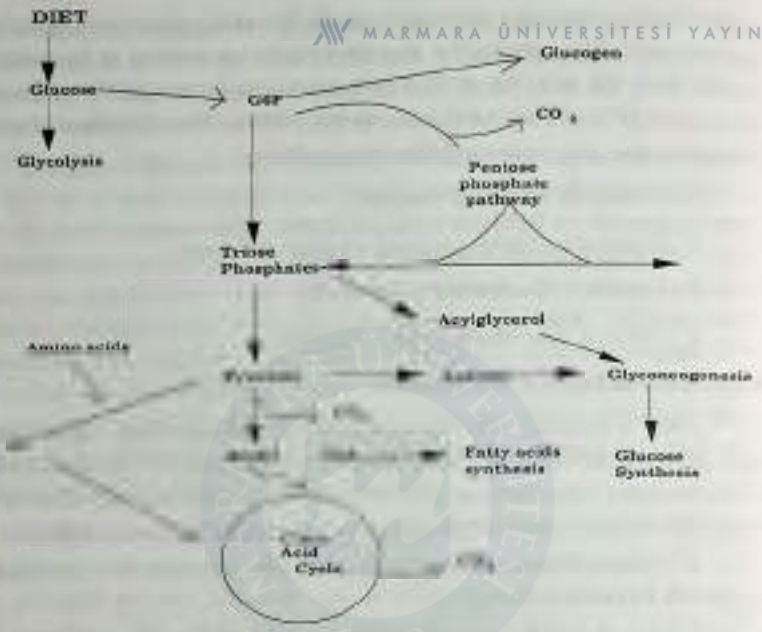

Figure 8 : Overview of carbohydrate metabolism showing the major end products

Endogenous sources of glucose:

The major endogenous carbohydrate source is liver glycogen. The glycogen of muscle is not directly convertible to blood glucose the lactic acid formed during glycolysis of muscle glycogen can be converted to glucose and glycogen in the liver.

Certain noncarbohydrate sources are available to the organism for production of glucose. Although it is generally believed that the fatty acids cannot undergo net conversion to carbohydrate, their oxidation provides the energy for gluconeogenesis. Also, the glycerol moiety of lipids can serve as a source of glucose (from which it can also be derived). However, amino acids are the major raw material for the synthesis of carbohydrate from noncarbohydrate sources (gluconeogenesis). It has been estimated that over half of the average animal protein is potentially capable of conversion to carbohydrate. Thoe amino acids which are carbohydrate formers are called "glucogenic". The major site of gluconeognesis is liver.

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UTILIZATION OF GLUCOSE

Storage:

When a molecule of glucose is introduced into the body several metabolic pathwasy are open to it. In the absence of urgent physiological demands for oxidative energy or conversion to special products, excess glucose may be deposited as glycogen in the liver, muscles and other tissues (glycogenesis). In as much as the amount of glycogen which can be stored in the body is limited, quantities of glucose in excess of this upper limit are converted to fatty acids and stored as triglycerides in the fat depots.

GLYCOGEN STORAGE (Glycogenesis)

The liver is one of the main storage sites for glycogen. Following the ingestion of carbohydrates, the quantities of the various hexoses exceed the immediate need. The excess is converted by the liver to glycogen. Later, when to organism has a requirement for raditional glucose, the glycogen can bu converted to the phosphorylated glucoses and the hydrolysis of glucose 6- P yields glucose which then enters the bloodstream to be distributed to the various body cells.

Another storage site for glycogen is skeletal muscle. The metabolic scheme in muscle is very similar to that of the liver, with one notable exception: skeletal muscles do not have the phohsphatase enzyme for hydrolyzing glucose-6 pohsphate to glucose and inorganic phosphate. Therefore, muscle **cannot supply glucose** to the bood stream and other tissues directly.

Glycogenesis refers glycogen synthesis. In this metabolic pathway first glucose is phosphorylated to glucose-6-Phosphate Glycose-6-Phosphate is then converted to glucoe 1-P in a reaction catalysed by the enzyme phosphoglucomutase. Next, glucose 1 P reacts with uridine triphospate (UTP) to form the active nucleotide uridine diphosphoglucoe (UDPG).

The reaction between glucose 1-P and uridine triphosphate is catalyzed by the enzyme UDPG pyrophosphorylase. By the action of the enzyme glycogen synthase (or glucosyl transferase) the C_1 of the activated glucose of UDPG forms a glycosidic bond with the C_4 of a terminal glucose residue of glycogen, liberating uridine diphosphate (UDP). After

that the "branches" of the glycogen "tree" become elongated as successive 1-4 linkages occur as seen in figure When the chain has been lengthened to between 6 and 11 glucose residues, a second enzyme, the branching enzyme (amylo-1,4-1,6 transglucosidase) acts on the glycogen. This enzyme transfers a part of the 1,4 chain (minimum length 6 glucose residues) to a neighboring chain to form a 1,6 linkage, thus establishing a branch point in the molecule.

The structure of glycogen is shown in Figure. 4 It will be seen to be a branched polysaccharide composed entirely of alfa D-glucose units. These glucose units are connected to one another by glucosidic linkages between the first and fourth carbon atoms except at branch points, where the linkages are between corbon atoms 1 and 6 The molecular weight of glycogen may vary from 1 milion to 4 milion or more.



Figure 4 : The Glycogen Molecule

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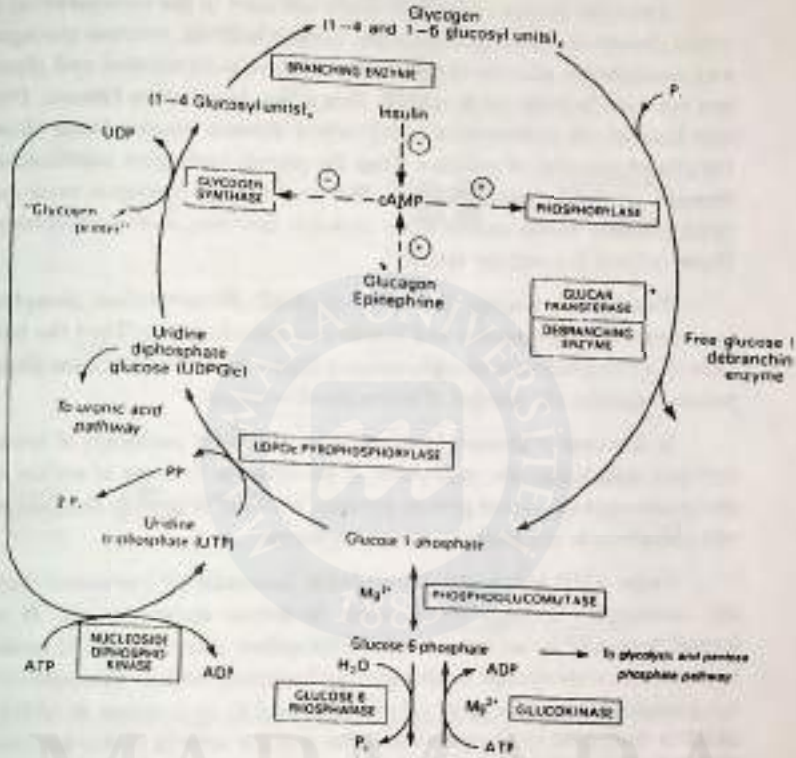


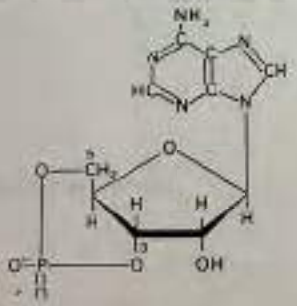
Figure 5 : Glycogenesis and glycogenolysis in the liver (From ref. 26)

Two high energy phosphate bonds are used in the incorporation of 1 mol glucose into glycogen molecule. Insulin inhibits, whereas glucagon and epinephrine activate cAMP. Phosphorylase is stimulated and glycogen synthase is inhibited by cAMP. This molecule activates kinases. Protein kinases are mediators of cAMP action. Protein kinases bring about the phosphorylation of proteins. Thus the protein undergoes modification through a covalent bond addition. This modification activates some enzyme proteins. In the case of other proteins, however, such a phosphorylation reduces the enzyme activity.

Two kinases amplify the action of cAMP. Phosphorylase phosphatase removes the phophates and inactivates phosphorylase. Thus the balance of activity between phosphorylase b kinase and phosphorylase phospatase regulates the amount of active phosphorylase.

In the case of kinases involved in regulation of pathways of intermediany metabolism the phosphate is esterified to hydroxy of serine or threonine residues. Other protein kinases, involved in growth regulation, add phosphate to the phenolic group of tyrosine.

Cydic AMP is the (33) intracellular intermediate compound (second messenger) through which many hormones appear to act. It is formed from ATP by an enzyme adenylate cyclase occurring in cell membranes. Adenylate cyclase is activated by hormones such as epinephrine, norepinephrine and glucagon, all of which lead to an increase in cAMP. cAMP is destroyed by phosphodiesterase, and the activity of this enzyme that maintains the level of cAMP at its normally low level. Insulin has been reported to increase its activity in liver. Thyroid hormones may increase the synthesis of adenylate cyclase, thus potentiating the effects of epinephrine in stumulating the formation of cAMP.



(33)

3', 5' - Adenylic acid (cyclic AMP, cAMP)

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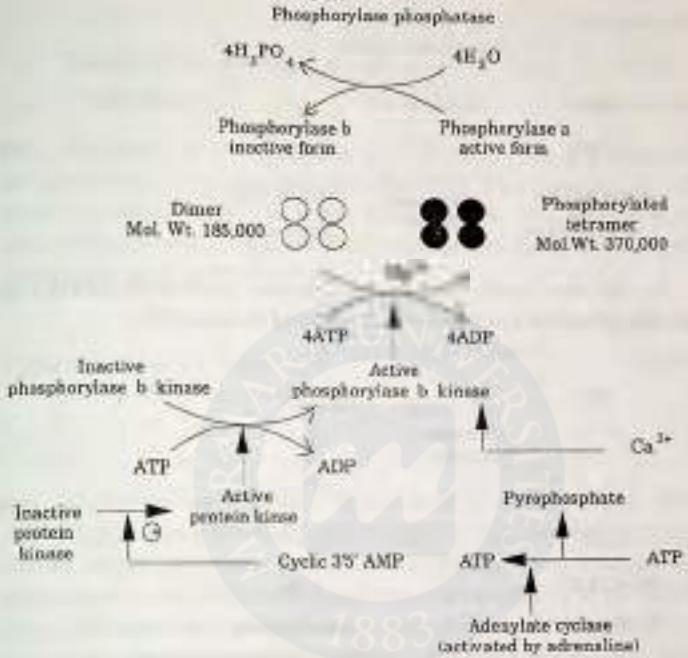

Figure 6 The relationship between cAMP, protein kinase and phosphorylase. (From ref. 6)

In liver the phosphorylase exists in both an active and an inactive form. **Active phosphorylase** (or phosphorilase a) has one of its serine hydroxyl groups phosphorylated in an ester linkage. As it is seen in figure 6 the inactive form of phosphorylase is a dimer termed phosphorylase b, phosphorylase b is activated by the addition of four phosphate groups, donated by ATP, a reaction brought about by phosphorylase b kinase, the addition of the phosphates causes the dimers to associate to form a tetramer.

By the action of a specific phosphatase (phosphorylase phosphatase), the enzyme is inactivated to dephosphophosphorylase in a reaction which involves hydrolytic removal of the phosphate from the serine residue. In the case of kinases involved in regulation of pathways of intermediany metebolism the phosphate is esterified to hydroxyls of serine or threonine residues (34).

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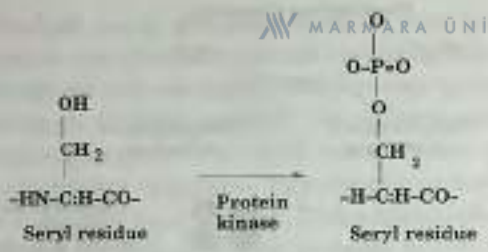
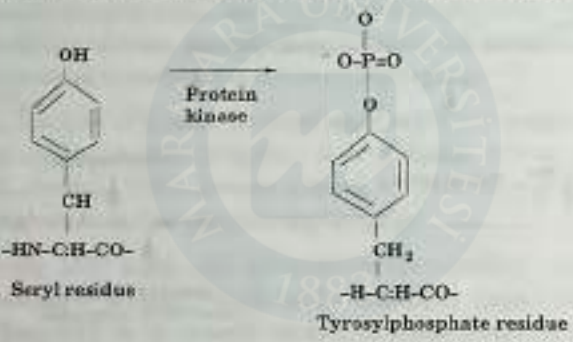


(34)

On the other hand, other protein kinases, involved in growth regulation, add phosphate to the phenolic group of tyrosine (35).



(35)

Studies in liver have shown that α receptars are the major mediators of catecholomine stimulation of glycogenolysis. This involves a cAMP-independent mobilization of Ca^{++} from mitochondria into the cytosol, followed by the stimulation of a Ca^{++} / calmodulin-sensitive phosphorylase kinase. Reactivation requires ATP and a specific enzyme, phosphorylase b kinase or dephosphophosphorylase kinase.

Muscle phosphorylase is immunologically distinct from that of liver. It is present in 2 forms phosphorylase a, which is active in the absence of 5'-AMP, and phosphorylase b, which is active only in the presence of 5'-AMP. Phosphorylase a is the physiologically active form of the enzyme. It is a tetramer containing 4 mol of pyridoxal phosphate. When it is converted to a dimer by phosphorylase phosphatase, which removes phosphate from phosphoserine residues, phosphorylase b formed. This contains 2 mol of pyridoxal phosphate.

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$$\text{Phosphorylase a} + 4H_2O \xrightarrow[\text{Phosphatase}]{\text{Phosphorylasephe}} \text{2 phosphorylase b} + 4\ Pi$$

(Active tetramer) (inactive dimer)

Conversion of phosphorylase b to phosphorylase a is considered to be the mechanism for increasin glycogenolysis. Two dimers of phosphorylase b may recondense to an active phossporylase a tetramer in the presence of phosphorylase b kinase which reposphorylates the serine residues at the expense of ATP. (Fig. 5 a)

$$\text{2 phosphorylase b} + 4\ ATP \xrightarrow[Mg^{++}]{\text{Phosphorylase b kinase}} \text{Phosphorylase a} + 4\ ADP$$

Phosphorylase in muscle is activated by **epinephrine**. However, this occurs not as a direct effect but rather by way the action of epinephrine on adenylate cyclase to form cAMP. Phosborylase b kinase is then activated by a second protein kinase system considered to be identical to the cAMP dependent protein kinase responsible for inactivation of glycogen synthetase. In this way glycogen catabolism is activated while, simultaneously, glycogen synthesis is inhibited. Activation of phosphorylase b kinase is also caused by musclular contraction, an effect due to Ca^{++} rather than cAMP.

The reactions for the inactivation of muscle phosphorylase are in some respects similar to those of the liver enzyme except that no cleavage of the protein molecular structure is involved in the case of liver phosphorylase. Furthermore, skeletal muscle phosphorylase is not affected by glucagon although heart muscle is. Another important difference is that liver synthetase phosphatae is inhibited by the active form of phosphorylase.

Phosphorylase catalyses the removal of 1,4 glucosil residues from the outermost chains of the glycogen molecule until approximately 4 glucose residues remain on side of 1,6 branch. Another enzyme (a 1,4 to a 1,4 glucan transferase) transfers a trisaccharide unit from one side to the other., thus exposing the 1,6 branch points. The hydrolytic splitting of the 1,6 linkages requires the ac-

tion of a specific **debranching enzyme (amylo 1,6 glucosidase).** Combined action of phosphorylase and these other enzymes converts glycogen to glucose 1-phosphate phosphoglucomutase is reversible, so that glucose 6 phosphate can be formed from glucose 1 phosphate. In **liver** and **kidney** (but not in muscle) there is a specific enzyme, **Glucose -6-phosphatase,** that removes phosphate from glucose 6 phosphate, enabling the free glucose to diffuse from the cell into the extracellular spaces, including blood. This is the final step in hepatic glycogenolysis which is reflected by a **rise in the blood glucose.**

GLYCOGEN STORAGE DISEASES

These are a group of inherited disorders characterized by deposition of an abnormal type or quantity of glycogen in the tissues

In type I glycogenenosis **(von Gierke's disease);** both the liver cells and the cells of the renal convuluted tubules are characteritically loaded with glycogen. In these patients hypoglycemia and a lack of glucose release under stimulus by epinephrine or glucagon are seen. Ketosis and hiperlipemia are also present in these patients. In liver, kidney and intestinal tissue, the activity of glucose 6-phosphatase is either extremely low or entirely absent. Thus, in the fasting state glucose formation by liver cannot accur because glycogen breakdown to glucose is blocked and gluconeogenesis is also blocked. Thus, hypoglycemia results. Hormones are secreted response to the hypoglycemia e.g. glucocorticoids which cause excessive mobilization of muscle amino acids, which enter the liver as shown in figure 7.

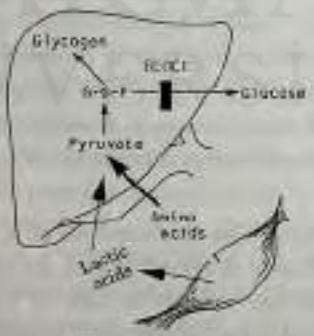

Figure 7: Glucose 6- phosphatase deficiency in type I glycogen storage disease (From ref.6)

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Type II (Pompe's disease). This is characterized by a deficiency of lysosomal α 1,4 glucosidase (acid maltase) whose function is to degrade glycogen, which otherwise accumulates in the lysosomes.

Type III (limit dextrinosis) this is characterized by the absence of debranching enzyme, with the result that a polysaccharide having few branch points accumulates.

Type V (myophosphorylase deficiency or Mc Ardle's disease or syndrome). Patients with this disease exhibit a markedly diminshed tolerance to exercise. Although their skeletal muscles have an abnormally high content of glycogen (2,5 - 4.1 %) little or no lactate is detectable in their blood after exercise. After administration of glucagon or epinephrine, a rise in blood sugar does occur, which indicates that hepatic phosphorylase activity is normal. An absence of muscle phosphorylase is seen in this patients. In some of the repoted cases, myoglobinuria has been an associated finding.

Type VI, VII, and **VIII** have also been reported in literature. Enzyme deficiency in glycogenesis and glycogenolysis pathway is shown below:

Condition		**Enzyme with deficient activity**
Glycogen Storage Disease	I	Glucose -6- Phosphatase
Glycogen Storage Disease	II	α 1-4 glycosidase
Glycogen Storage Disease	III	Amylo 1,6 glucosidase
Glycogen Storage Disease	IV	Amylo (1.4 to 1.6) tranglucosidase
Glycogen Storage Disease	V	Muscle phosphorylase
Glycogen Storage Disease	VI	Liver phosphorylase
Glycogen Storage Disease	VII	Muscle phosphofructo kinase
Glycogen Storage Disease	VII	Liver phosphorylase kinase

OXIDATION OF GLUCOSE

To obtain energly, glucose may be completely oxidized to CO_2 and H_2O. This process occurs in all tissues. at certain times special circumstances in muscle may result in only partial degradation of glucose (Glycolysis). The prodact, lactic acid, is then largely disposed of by other tissues, notably liver. (figure 7)

GLYCOLYSIS

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Glycolysis refers to the breakdown of carbohydrates to lactic acid with the liberation energy. It occurs under anaerobic condition. It was noted that when a muscle contrcts in an anaerobic medium, glycogen disappears and pyruvate and lactate appear as the principal end products. When oxygen is admitted aerobic recovery takes place and glycogen reappears, while pyruvate and lactate disappear. However, if recovery takes place under aerobic conditions, lactate does not accumulate and pyruvate is oxidized further to Co_2 and water. As a result of these observations carbohydrate metabolism is seperated into anaerobic and aerobic phases.

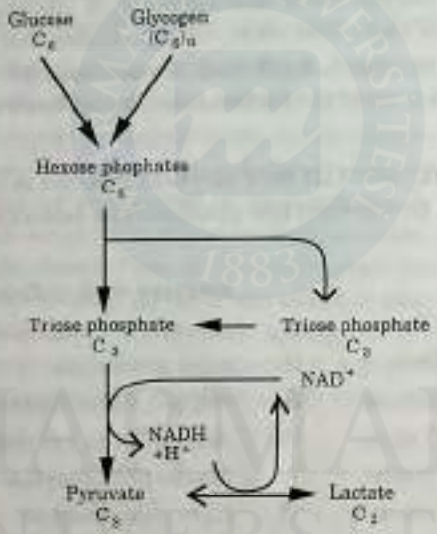

Figure 8: Summary of glycolysis.

Glycolysis can be subdivided into the phases of

a- Initial phosphorylation,

b- Conversion to trioses (cleavage)

c- The oxidative step and

d- Formation of lactic acid. These steps are outlined in figure 8, 9, 10.

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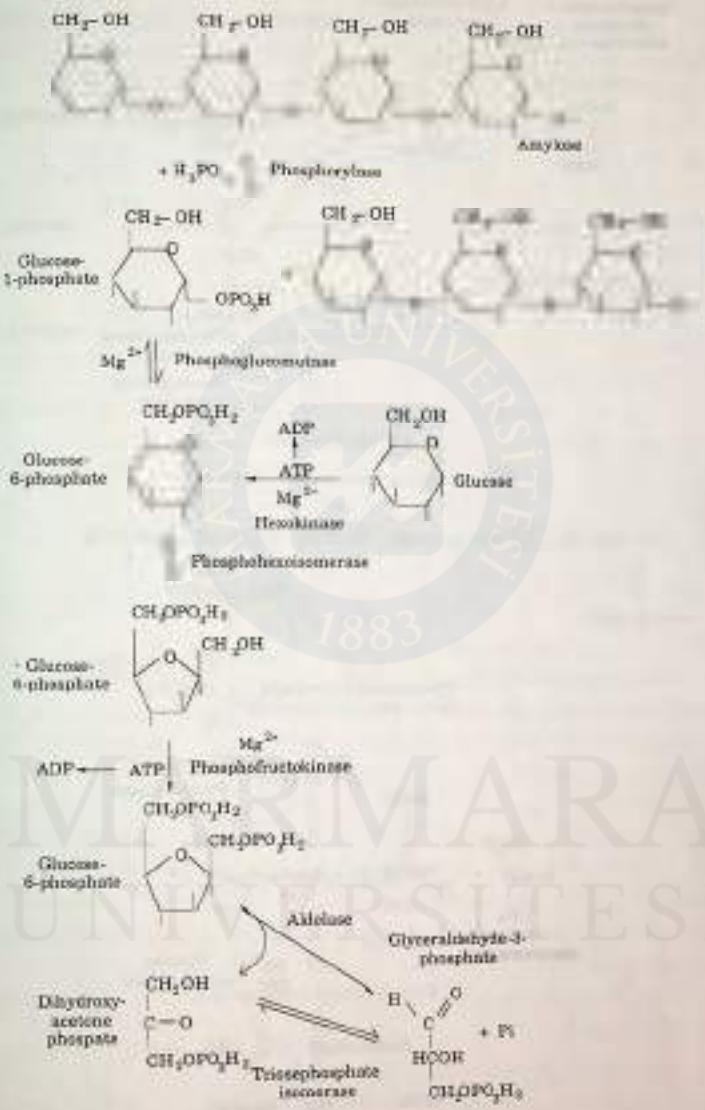

Figure : 9 Glycolytic reactions with molecular levels and formation of ethanol from glucose or pyruvic acid by alcohol dehidrogenase

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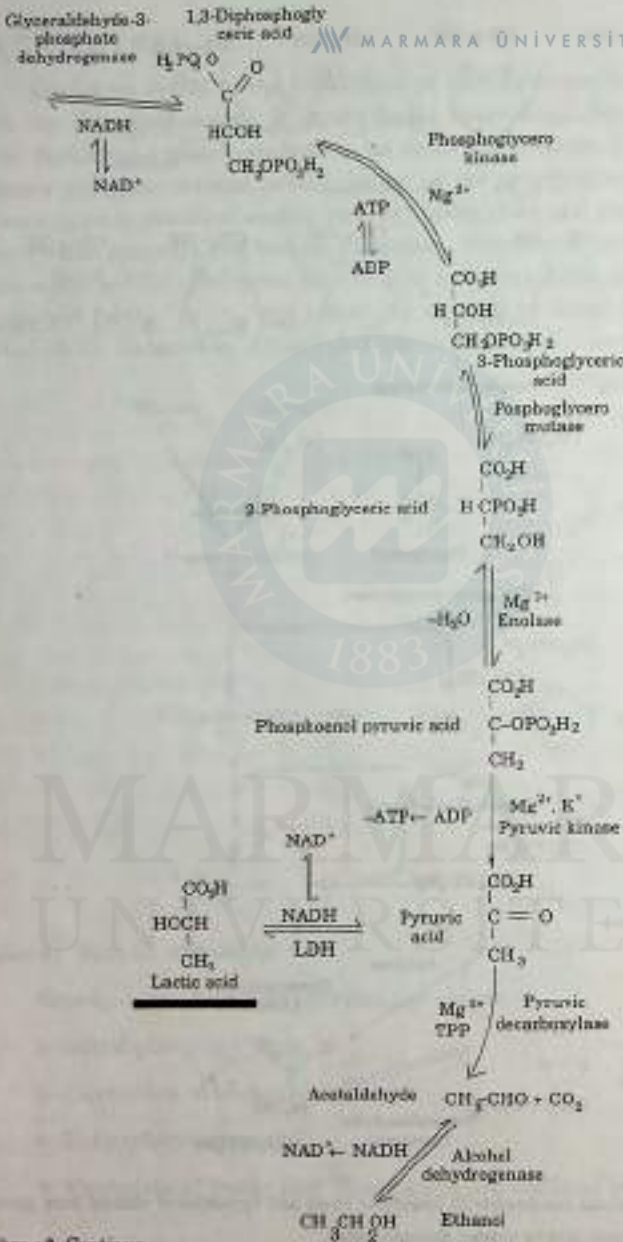

Figure : 9 Continue

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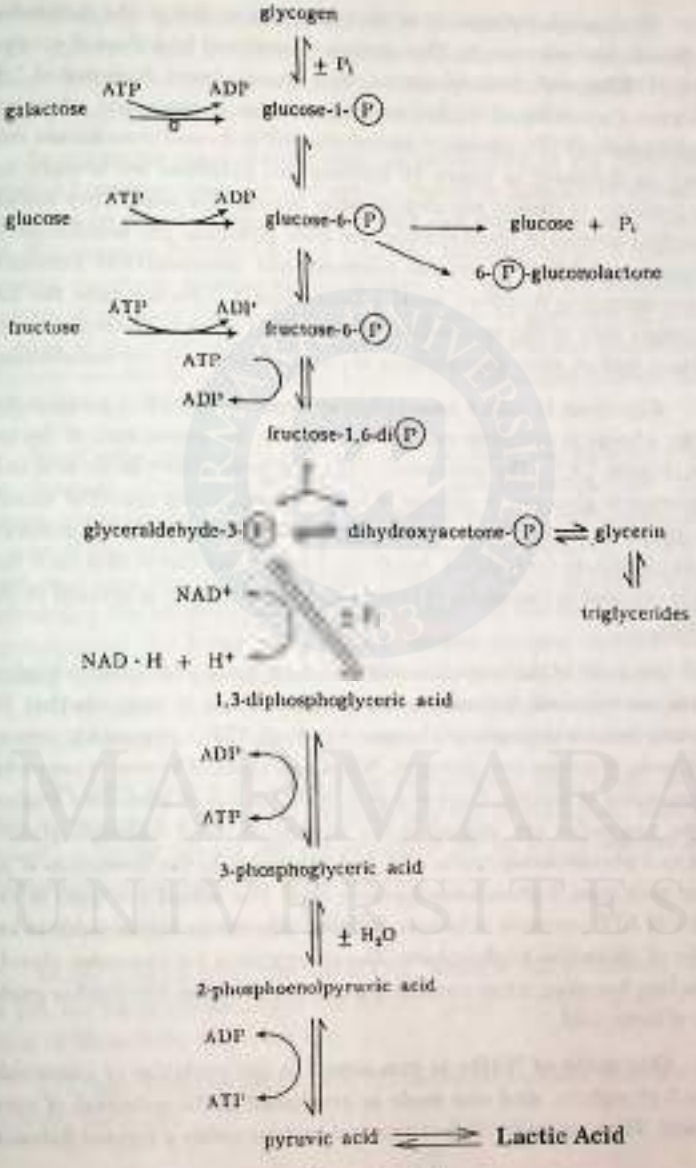

Figure : 10 A simplified presentation of glycolytic pathway

The phosphorylation is an obligatory initial step in the metabolism of glucose and other sugrs. This rection is catalyzed by a class of enzymes termed **"Kinases"** that concerned with glucose being deesignated "glucokinase (hexokinase). In the presence of this enzyme and adensosintriphasphate (ATP), glucose 6 phosphate G6P is formed (hexokinase reaction). As indicated in figure 10 fructose and galactose are brought into the pathway of glucose metabolism at or beyond this point. They are also potential sources of blood glucose and liver glycogen. The hexokinase reaction is inhibited by growth hormone and adrenocortical hormones. These hormones therefore, exert a fundamental influence upon the first oblgatory step in the metabolism of glucose. If there is abnormal supply of these factors some disturbances are seen in carbohydrate metabolism.

Glycolysis is called **anaerobic system.** A simplified presentation of the scheme is complete enough to illustrate the importance of the system (Figüre 7,8,9). The reversibility of the scheme allows lactic acid to be converted to glycogen or glucose or both. An interesting aspect of anaerobic glycolysis is that the components are almost invariably interconverted as phosphate derivatives. Another aspect to consider is that each hexose is oxidized to two moles of lactic acid, but no oxygen is utilized in this system.

An audit of the system reveals that two moles of adenosine triphosphate are required for entry of each hexose. This is true whether the starting point is glycogen or glucose. A mole of ATP is required to convert each mole of hexose into glycogen. When each mole of hexose is converted to two moles of lactate, there is a gain of four moles of adenosine triphosphate. two moles are gained in the conversion of 1,3 diphosphoglyceric acid to 3 phosphoenolpyruvic acid and two moles in the formation of pyruvic acid from 2-phosphoenolpyruvic acid. The actual net gain is two moles of ATP per mole of hexose, because there is an initial debit of two moles of adenosine triphosphate. The energy yield for anaerobic glycolysis is low, however, when compared with that obtained the further oxidation of lactic acid.

One mole of NAD+ is consumed in the oxidation of glyceraldehyde-3 phosphate, **and one mole is produced** in the reduction of pyruvic acid. Thus, an audit of electrons and protons yields a zeronet balance.

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The energy of the mature erythrocyte is supplied primarly by anaerobic glycolysis and the phosphogluconate pathways Because in contrast the other cell erythrocyte lacks mitochondria, hence the cytochrome system is absent and there is no the tricarboxylic acid cycle.

In erythocyte many genetic enzymes deficiencies in the glucolysis pathways have been described and usually **result in hemolytic anemia as well as in other disturbances of red cell structure and function.** For example Pyruvate kinase deficieny, inherited as an autosomal recessive, results in a rather severe anamia. The level of 2,3 diphosphoglycerate is elevated due to diminished formation of pyruvate, in turn, the value of the P for oxygen is markedly elevated, whereas hexokinase deficiency results in a diminished level of 2,3 diphosphoglycerate and hence a decreased value for the P for oxygen.

Many other enzymes in glycolitic pathway have also been reported (For example; Triosphosphate isomerase, phosphofructokinase, 2,3-diphospoglyceromutase deficiencies. In all these enzymic deficiencies, the life time of the erythrocytes is reduced. The reason is unknown yet but it is assumed that the energy supplied by these pathways is essential for maintaining the integrity of the cell membrane. On the other side these enzymes effect the **Bohr effect. That means oxygen equilibrium binding curves of hemoglobin.** This curves is effected by many factors. CO_2 in the medium surrounding the erythrocyte, pH, and some glycolitic enzyme concentration and their product are among these factors.

The Bohr effect is of considerable physiological importance. As arterial blood enters tissues, CO_2 diffuses into the erythrocyees, thus potentially lowering the pH and the affinity of Hb for O_2, is reduced and therefore oxygen is delivered to the medium.

$$H^+ + HbO_2 \longrightarrow O_2 + HHb +$$

In the lung (in turn), the loss of CO_2, which would potentially raise the pH, increases the affinity of the Hb for O_2, thus permitting the saturation of Hb with O_2 at a lower Po_2.

CO_2 also binds to Hb to form carbaminohemoglobin, shown schematically as follows:

$$Hb - NH_2 + CO_2 \rightleftharpoons Hb - NH - \overset{\overset{O}{\|}}{C} - O^- + H^+$$

The reaction is freely reversible, and the P_{CO_2} determines the amount of carbaminohemglobin formed. Only the NH_2 terminal alfa-amino groups bind CO_2. This is called carbamylated Hb.

Influence of 2,3, diphosphoglycerate (DPG): A major fraction of the posphate in erythrocytes is present as DPG. Its intracellular concentration is approximately 4 to 5 mM, about the same as the Hb. DPG binds to tetrameric Hb in a 1:1 molar complex at neutral pH with a binding constant K_{as} of about 10^5 M. K_{as} for HbO_2 and DPG is only one hundredth as large. The major effect of this difference in K for DPG is to decrease the affinity of Hb for O_2 illustrated schematically as follows: (Figure 12)

$$HbO_2 + DPG \rightleftharpoons Hb\,DPG + O_2$$

Thus, at a fixed HbO_2 increasing DPG promotes dissociation of HbO_2.

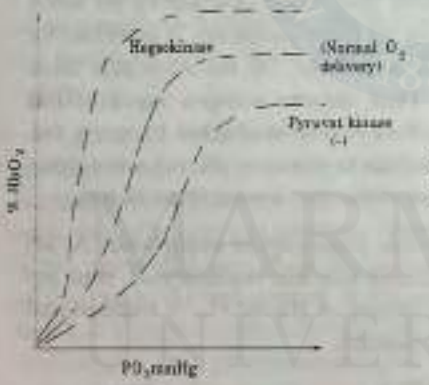

Figure 11: Some changes on the Bohr Curve due to enzyme deficiencies

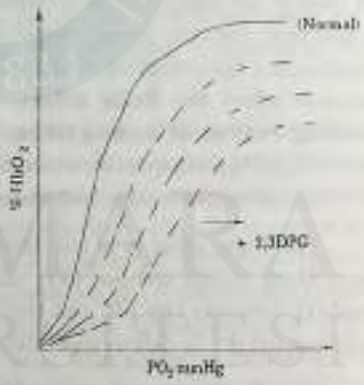

Figure 12: Effect of 2,3 DPG on Bohr Curve.

As it is shown in figure 11,12 if the 2,3 DPG concentration is elevated the curve slides to the right as seen in pruvate kinase deficiency. If the 2,3 DPG concentration is diminished the curve for oxygen slips to the left as seen in hexokinase deficiency.

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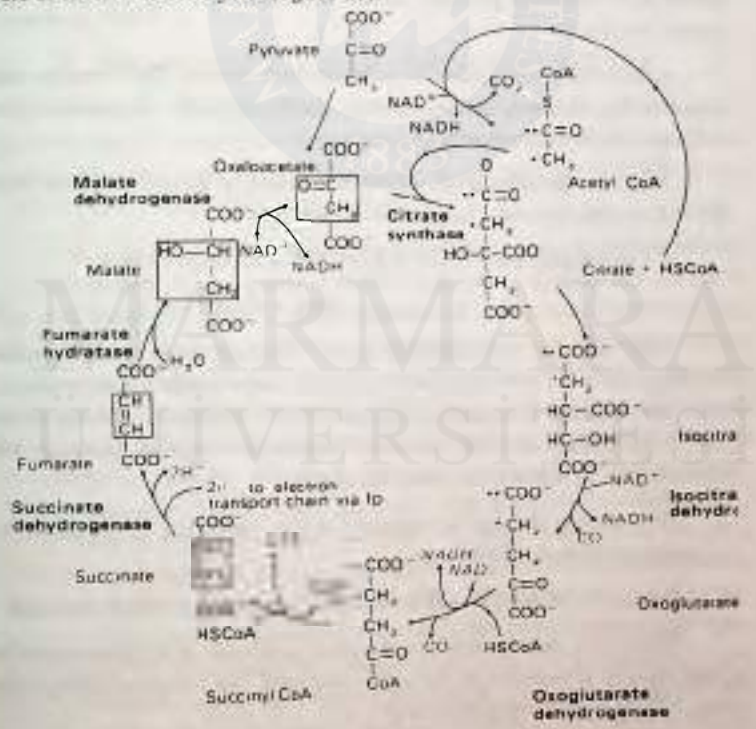
THE CITRIC ACID CYCLE

The citric acid cycle (Krebs cycle, tricarboxylic acid cycle) is a series of reactions in mitochondria. In this system, 1- liberation of hydrogen equivalants, 2- Catabolism of acetyl residues, 3- Most of the free energy of the tissue fuels takes places. The acetyl residues are in the form of acetyl-CoA an ester of coenzyme A. This is an active acetate compound (CH_3–CO–S-CoA). Asetyl -CoA is very important metabolit in carbohydrate, lipid and protein metabolism. Nearly all corbohydrate and fat molecules form acetyl-CoA during oxidative catabolism, as do many of the amino acids resulting from the degradation of proteins. Figure 13, 14, 15. In addition, Acetyl-CoA serves as the source of acetyl units in the synthesis of long chain fatty acids, cholesterol and others teroids and ketone bodies (aceto-acetate, B-hydroxybutyrate and acetone). These metabolic pathways will be the subjects of following chapters. The peresent chapter is concerned solely with the catabolism of acetyl-CoA and the role of the citric acid cycle. (figure 13).



Figüre 13: The Krebs Cycle with molecules (From ref. 6)

The major function of the citric acid cycle is to act as the final common pathway for the oxidation of carbohydrate, lipids and protein. Furthermure, the the citric acid cycle is the mechanism by which much of the free energy liberatted during the oxidation of carbohiydrate, lipids and amino acids is made available. The first reaction in the citric acid cycle is condensation of acetyl CoA and oxolcacetate. The reaction is catalyzed by a condensing enzyme ctrate synthase. Citrate is converted to isocitrate by the enzyme aconitase which contains iron in the Fe^{++} state, the reaction is inhibited by florocetate.

Isocitrate undergoes dehydragenetion in the presence of isocitrate dehidrogeuase to form oxalcsuccinote. Three different isocitrate dehydrogenoses have been described. One which is NAD^+-specific, is found only in mitochondria the other 2 enzymes are $NADP^+$ specific and are found in the mitochondria and the cytorsol, respective respiractory chain-linked oxidation of isocitrate preceeds almost comletely through the NAD^+ dependet enzyme.

Later, decarboxylation to ketoglutarate occurs. This reation also catalyzed by isocitrate dehidrogenate Mn^{2+} (or Mg^{2+}) is an important component of the decarboxylation reaction.

Next, α – ketoglutariate undergoes oxidative decarboxylation. Just like to the oxidative decarboxylation of pyruvate.

$$\alpha - \text{Ketoglutarate} + NAD^+ + \text{COA-SH} \rightarrow \text{Succinyl COA} + CO_2 + NADH + H^+$$

The reaction, catalyzed by an α – Ketoglutarate dehidrogenase complex, also requires identical cofactor to decarboxylation of pymvate. These are; thiamin diphosphate, lipoate, NAD^+, FAD and COA As in the case of pyruvate oxidation, arsenite inhibits the reaction, causing the substrate, α – ketoglutarate, to accumulate.

To continue the cycle, succinyl-COA is converted to succinote by the enzyme succinate thiokinase.

$$\text{Succinyl - COA} + Pi + GDP \rightleftharpoons \text{Succinate} + GTP + \text{COA-SH}$$

This is the only example in the citric acid cycle of the generation of a high-energy phosphate at the substrate level. By means of phosphokinase, ATP may be formed from GTP.

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$$GTP + ADP \rightleftharpoons GDP + ATP$$

Next, succinate is metabolyzed to fumarate

$$Succinate + FAD \rightleftharpoons Fumarate + FADH_2$$

This reaction is catalyzed by succinate dehydrogenase which is bound to the inner surface of the inner mitochondrial membrane. It is the only dehidrogenation reaction in the citric acid cycle that involves the direct transfer of hydrogen from the substrate to a flovoprotein without the participation of NAD^+, the enzyme contains FAD and iron-sulfur (Fes) protein. Fumarate is formed as a result of the dehydrogenation. Addition of malonate and oxalacetate inhibits succinate dehydrogenase competitively, resulting in succinate accumulation

$$Fumarate + H_2O \rightleftharpoons L\text{-} Malate$$

malate is converted to oxaloacetate by malate dehydrogenase, a reaction requiring NAD^+

$$\alpha - Malate + NAD^+ \rightleftharpoons Oxaloacetate + NADH + H$$

Although the equilibrium of this reaction strongly favors malate, the net flux is toward the direction of oxaloacetate because this compound, together with the other product of the reaction (NADH), is removed continuously in further reactions.

As a result of oxidation catalyzed by dehydrogenase enzymes of the citric acid cycle, 3 molecules of NADH and one of $FADH_2$ are produced for each molecule of acetyl - COA catabolized in one revolution of the cycle. These reducing equivalents are transported to the respiratory chain in the inner mitochondriol membrane. In respiratory chain, reducing equivalents from NADH generate 3 ATP. However, $FADH_2$ produces only 2 high-energy phosphate bonds because it transfers its reducing power to Koenzyme Q , thus by passing the first site for oxidative phosphorylation in the respiratory chain. Thus; 12 high energy phosphate bonds (ATP) are generated for each turn of the cycle. All these reactions in the citric acid cycle and respiratory chain are shown in figure 14 During the oxidation of acetyl COA in the cycle, reducing equvalants in the form of hydrogen or of electrons are formed as a result of the activity of specific dehydrogenases. These reducing equivalants then enter the respiratory chain, where large amounts of high energy phosphate are generated in the process of oxidative phosphorylation. As shown in figure 14 Co_2 and reducing

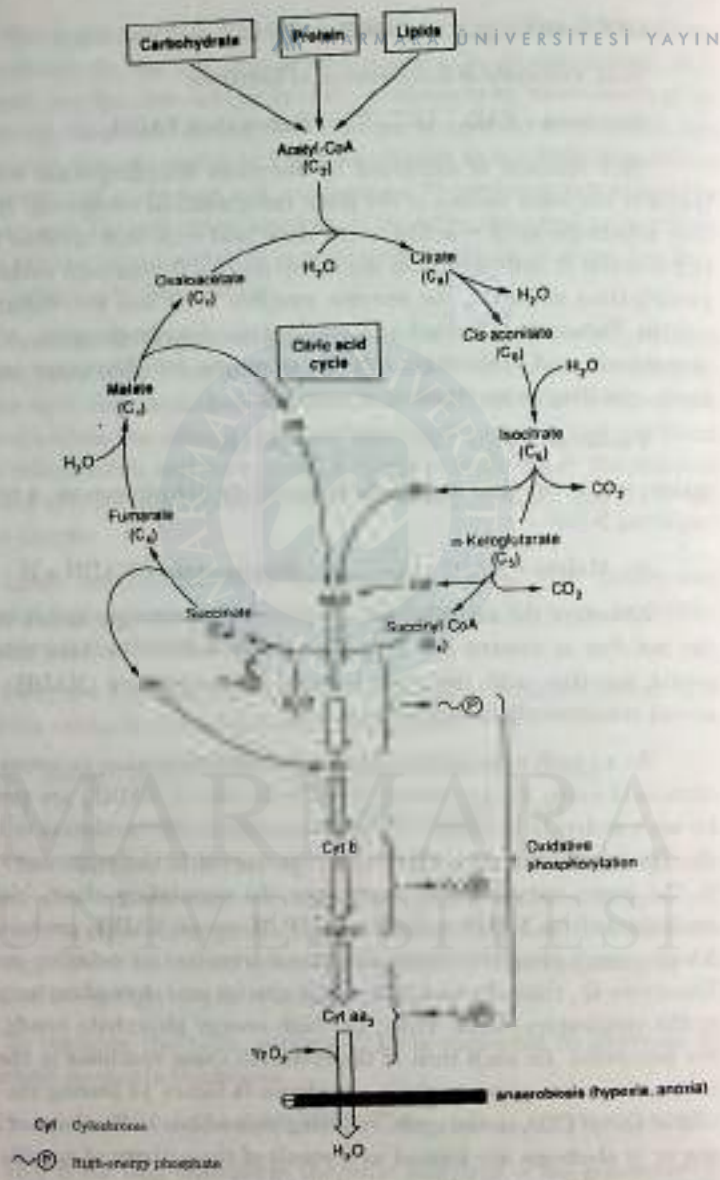

Figure 14 Oxidation of acetyl COA (obtained from carbohydrate, protein and lipids) by citric acid cycle to CO_2 with the release of reducing equivalents and oxidation of reducing equivalents in the respiratory chain. (From ref. 26)

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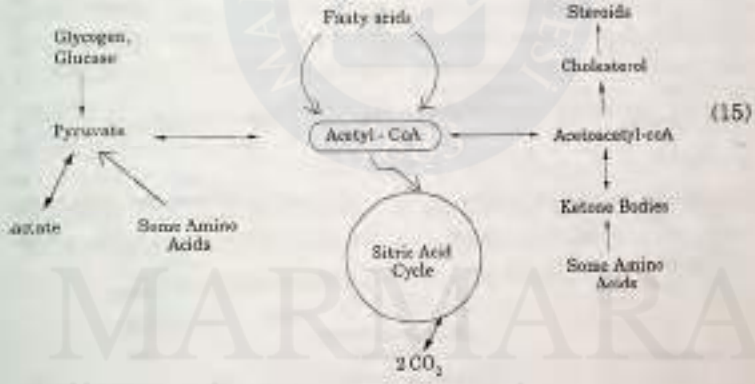
equivalents (2H) release from the citric acid cycle then enter the respiratory chain. Oxidation of 2H in respiratory chain leads to coupled phosphorylation of ADP to ATP. For one turn of the cycle, 11 ATP are generated via oxidative phosphorylation and one arises at substrate level from the conversion of succinyl. COA to succinate, thus 12 ATP generation occurs for one turn of the acetyl-COA in the cycle.

The enzymes of the citric acid cycle are located in the mitochondrial matrix, either free or attached to the inner surface of the inner mitochondrial membrane. Some of this enzyme farilitates the transfer of reducing equivalents to the adjacent enzymes of the respiratory chain, situated in the inner mitochondrial membrane.

It is of further significance that the citric acid cycle has a dual or amphibolic role, as it is a source of molecules for anabolic processes such as fatty acid and amino acid synthesis and gluconeogenesis. These will be discussed in greater detail in subsequent chapters.



Figüre 15: Overview of acetyl CoA metabolism

Acetyl-coA (asetate) only breaks down by the reactions of the citric acid cycle. There remains the basic question of how some organisms (many bacteria) can utilize acetate as the the only carbon source for all the carbon compounds of the cell. How can they give rise to carbohydrates as well as amino acids derivable from the TCA? This challenging problem was dissolved by sequence called glyoxylate cycle. In those organisms acetate converts to carbonhydrate glyoxylate cycle bypasses reactions. The by pass consists of two reactions that convers isocitrate to malic acid, in the process 1 mole of acetyl-coA is assimilated and 1 mole of succinic acid is formed.

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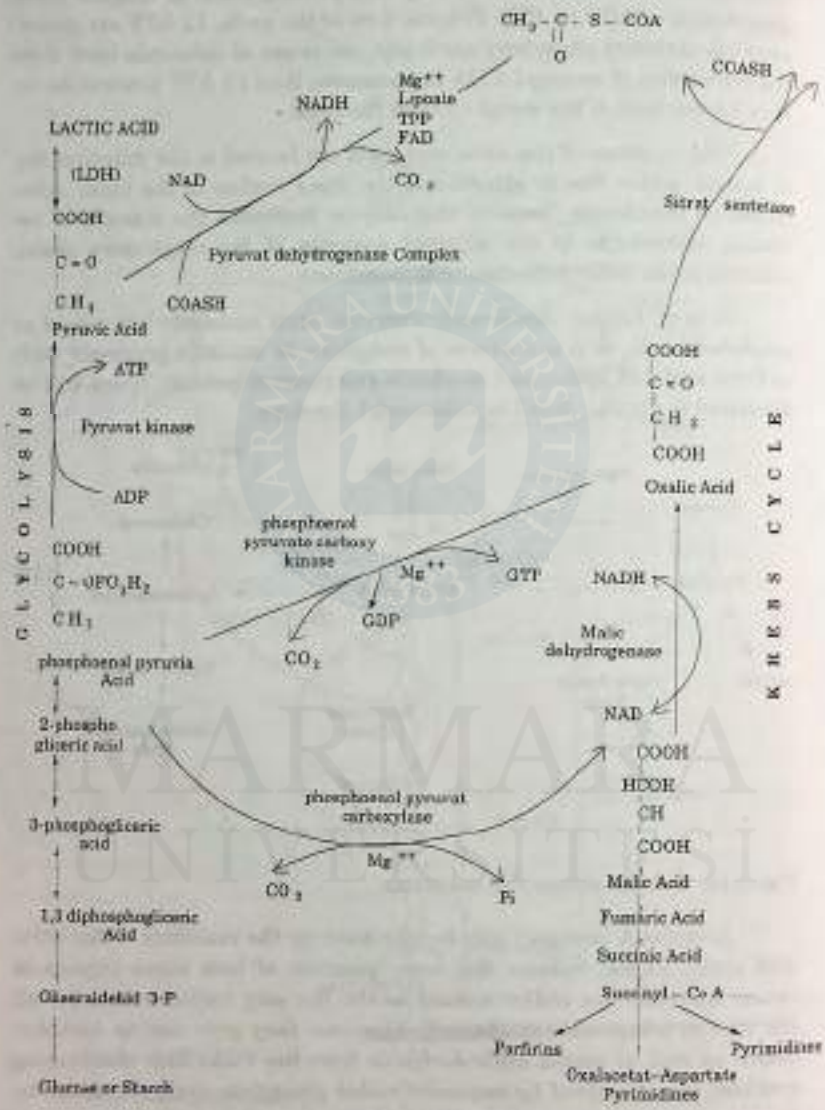

Figure 16: The relationship between glycolysis and krebs cycle.

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Energetics of Carbohydrate Oxidation

When 1 mol of glucose is combusted in a calorimeter to CO_2 and water, approximately 2870 kj are liberated as heat. When oxidation occurs in the tissues, some of this energy is not lost immediately as heat but is "captured" in high energy phosphate bonds. On the order of 38 high energy phosphate bonds are generated per molecule of glucose oxidized to CO_2 and water. Assuming each high energy bond to be equivalent to 36.8 kj, the total energy captured in ATP per mol of glucose oxidized is 1398 kj, or approximately 48.7 % of the energy of combustion. Most of the ATP is formed as a consequence of oxidative phosphorylation resulting from the reoxidation of reduced coenzymes by the respiratory chain. The remainder is generated by phosphorylation at the substrate level Table in below (Table 1) indicates the reactions responsible for the generation of high energy phosphate during oxidation of glucose and the net production under aerobic and anaerobic conditions. As seen in the table not 38 mol ATP produces in, indirect oxidation of glucose. But it is assumed that NADP formed in glycolysis is transperted into mitochondria via the malate shuttle. If the glycerophosphate shuttle is used, only 2 p would be formed per mol of NADH the total net production being 36 instead of 38,.

TABLE 1 Generation of high energy bonds in the catabolism of glucose in the aerobic oxidation

Pathway	Reaction Catalyzed By	Method of production	Number of
Glycolysis	Glyceraldehid 3-P-dehid	Resp.Chain 2 NADH	6 ATP
	Phosphoglycerate kinase	Oxid.at sbs.level	2 ATP
	Pyruvate kinase	" " "	2 ATP
			10 ATP
Allow for consumption of ATP by reactions catalyzed by hexokinase and phosphofructokinase			-2
			8 ATP
	Pyruvate dehydrogenase	Resp. Chain Ox. 2NADH	6 ATP
	Isositrat dehydrogenase	" " " 2NADH	6 ATP
	alfa ketoglutarat "	" " " 2NADH	6 ATP
	Succinate thiokinase	Oxidation at sub. level	2 ATP
	Succinate dehyrogenase	Resp.Chain Ox. $2FADH_2$	4 ATP
	Malate dehydrogenase	" " " 2NADH	6 ATP/30
			38 ATP

HEXOSE MONOPHOSPHATE SHUNT

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Hexose monophosphate shunt is the alternate routes of glucose catabolism. The Embden -Meyerhof scheme is a major route for the anaerobic degradation of hexoses to pyruvate. But we must take pains to stress that this is not the only pathway for the degradation of hexoses. Hexose monophosphate shunt is also known as the pentose phosphate pathway. The chief architect, of the pathway are B. Horecker and E. Racker, among the earlier workers three who should be mentioned are Warburg, Lipmann, and Dickens. The most important enzyme of the pentose phosphate pathway is glucose 6 phosphate dehydrogenase. Reaction 1 in figure 17. Warburg's discovery of this enzyme and its coenzyme NADP is one of the classic episodes of biochemistry. Many disturbances are seen in the cell, especially in the red blood cell, lack of this enzyme.

Significance of the Pentose Phosphate Pathway

Although many of the reactions of the pentose phosphate pathway were initially worked out in yeast and bacteria, the pathway exists in animals as well. There are two important roles of this pathway. The first of this is the production of NADPH. During the oxidation of one molecule glucose -6- phosphate to ribose -5- phosphate, one molecule CO_2 occurs and 2 moles of NADPH for each mole of glucose produces.

In contrast to NADH, NADPH plays a special role in biosynthetic processes within the cell. Whenever a biosynthetic step involves a reduction with a nicotinamide nucleotide, the coenzyme is, with few exceptions, NADPH. For example, NADPH is specifically utilised in th biosynthesis of long chain fatty acids. It is the reducing agent employed in the reduction of glucose to sorbitol, the reduction of dihydrofolic acid to tetrahydrofolic acid, and the reduction of glucuronic acid to L-gulonic acid. In addition, NADPH is used in the reductive carboxylation of pyruvic acid to malic acid by the malic enzyme. In the other way NADPH plays a unique role in hydroxylation reactions involved in the formation of unsaturated fatty acids, the conversion of phenylalanine to tyrosine, and the formation of certain steroids. Further evidance of the role of the pentose phosphate pathway in producing NADPH for biosynthetic purposes is found in the fact that the pathway enzymes are especially prominent in tissues such as adipose tissues, mammary gland or adrenal cortex that carry out these biosynthesis.

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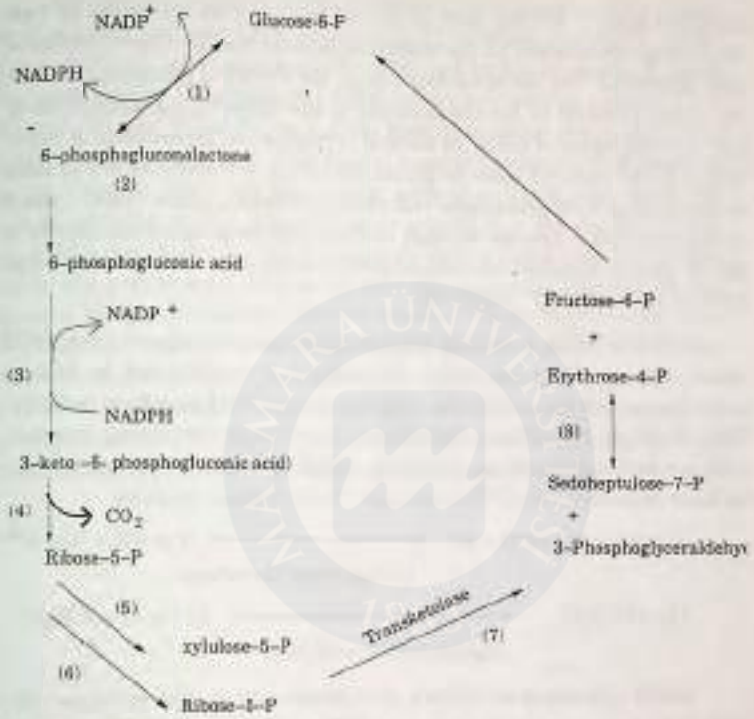

Figure 17: Summary of the pentose phosphate pathway.

Finally, NADPH plays an important role in erythrocytes to prevent oxidative damage within the cell. in fact, this role is made by glutathione.

Hemolytic anemia is due to inadequate energy producution and NADPH production. The erythrocyte can not maintain their normal elliptical shape. They readily destroyed in the spleen. The life span of the erythrocyte is 110 to 120 days. Mature red cells are constantly destroyed and replaced by new cells. The daily turnoer, calculated from the amount of bile pigment exereted represents about 20 ml of erythrocytes, that is, 0.85 % of the red cell mass. In the adult, about 6x109 erythrocytes are

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produced hourly. The life span of the red cell has been measured by various isotope techniques. In the hemolytic anemia the life time of erythrocytes shortened. Ths life span depends on the severe of hemolysis. Hemolsis seems to relate to hypersensitivity to peroxides as a concequence of low concentration of GSH. In normal erythrocytes, glutathione is maintained in the reduced state by glutathisensitive erythrocytes are present in individuals with gluathione reductase deficiency, hence GSSG cannot be reduced as it is formed. In most instance, the primaquine sensitivity is due to glucose 6 phosphate dehydrogenase deficiency, with resultant inability to reduce NADP (Figure 18).

(GSSG), which is present almost entirely as the reduced form, GSH (about 0.2 mmol/100 ml cells). Glutathione is synthesized in human erythrocytes and has a half-life of about 4 days. GSH may help detoxify H_2O_2 through glutathione peroxidase, a selenium-containing enzyme, with concomitant oxidation to GSSG, which is rapidly reduced by glutathione reductase. NADPH is necessary both of these enzymes.

$$\text{G-S-S-G} + \text{NADPH} + \text{H}^+ \xrightarrow[\text{Glutathione Reductase}]{} 2\,\text{G-SH} + \text{NADP}^+$$

$$2\,\text{G-SH} + \text{H}_2\text{O}_2 \xrightarrow[\text{Glutathione Peroxidase}]{} \text{G-S-S-G} + 2\,\text{H}_2\text{O}$$

G6PD (glucose-6-phosphate dehydrogenase) is the principal enzyme in this pathway, if there is not G6PD in the cycle NADPH does not produce and hemolytic anemia is seen in the human being because of these reactions (Figur 18). As many as 100 million people in the world may have genetically determined low levels of G6PD owing to variant forms, the variant forms can be distinguished by electrophoresis and other techniques. Reactions in above is important, since accumulation of H_2O_2 may decrease the life span of the erytrocyte by increasing the rate of oxidation of hemoglobin to methemoglobin. An inverse correlation has been found between the activity of G6PD and the fragility of red cells. A mutation present in some populations causes a deficiency in this enzyme. It results in impairment of the generation of NADPH, when the susceptible individual is subjected to oxidants such as the antimalarial primaquine, aspirine, or sulfonamids, or when the susceptible individuals has eaten fava beans (this conditions is called favism). Many of the enzyme deficiency cause hemolytic anemia as shown in figure 18.

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Anemias are associated with abnormally low numbers of erythrocytes or released with abnormal erythrocyte metabolism, a group of anemias of nutritional origin are those characterized by the presence of unusually large cells in the peripheral blood (mean corpuscular volume, 95 to 160 um^3, normal about 87 um^3). This kind of anemias due to deficiency in vitamin B_{12} or folic acid. This type of anemia associated with impaired gastric function. Although the gastric juice of patients with pernisious anemia contains no HCl and little or no pepsin, it is not the lack of pepsin or HCl that underlies the hemopoietic failure but a defect in production by the gastric mucosa of one or more mucoproteins (Intrinsic factor), essential for the normal absorbtion of dietary vitamin B_{12} from the intestine. Purified intrinsic factor from human gastric juice (MW = 50.000) binds 1 mol of vitamin B_{12} per pol of complex. The protein contains about 13 % carbohydrate including glucose, mannose, fucose, N-acetylglucosamine, N-acetylgalactosamine and a sialic acid. Biological activity is rapidly destroyed by incubation with neuraminidase.

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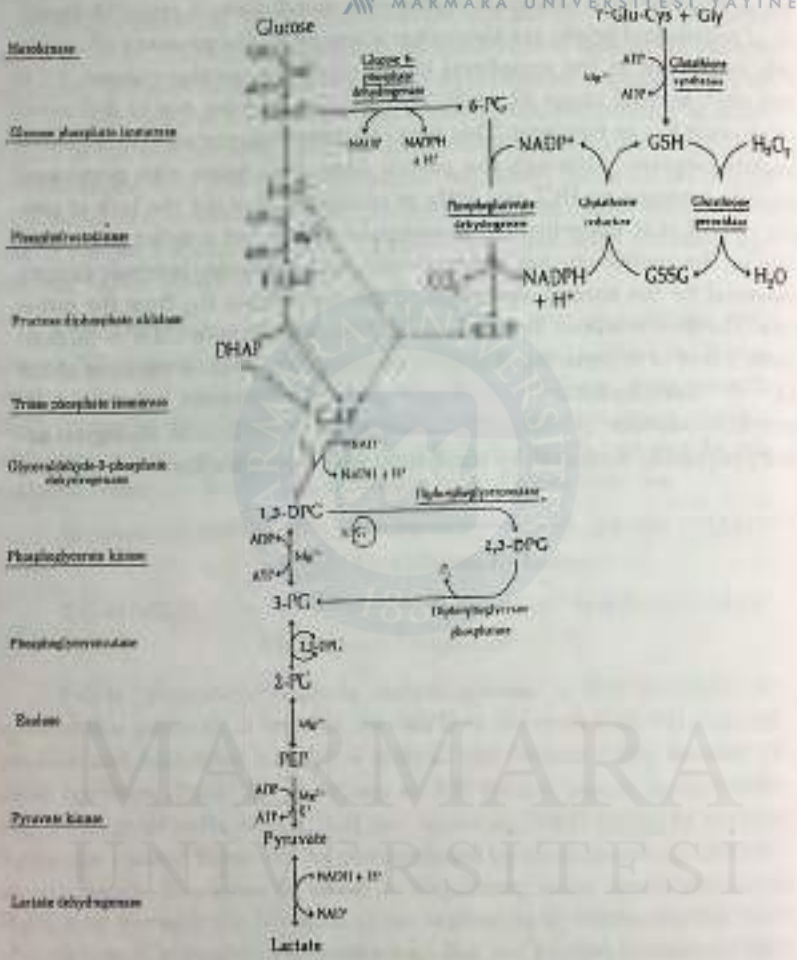

Figure 18: Glycolysis, pentose phosphate and related pathways of glutathione metabolism in the human erythrocyte. Enzymic steps in which the deficiencies are associated with hereditary hemolytic anemia are indicated by lines under the name of the enzymes and put in parenthesis. (From ref. 30)

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Hemolytic anemias may occur in malaria, blackwater fever or other infections due to accelerated erythrocyte destruction. Hemolysis also occurs following mismatched blood transfusion and in erythroblastosis fetalis (hemolytic disease of the newborn). Unusual anemia of paroxymal hemoglobinuria is characterized by hemolysis and hemoglobinuria in certain individuals after exposure to cold. This appears to be due to the presence of so-called "cold" hemolysins in the blood. The blood of person with familial or congenital hemolytic jaundice is characterized by the peresence of spherical erythrocytes of unexplained subnormal diameter (spherocytes), with a markedly increased fragility to hypotonic solutions. The tendency to spherocyte formation is transmitted as a mendelian dominant characteristic.

An unusual disorder associated with hemolytic anemia involves a fifty-to eightyfold increase in erythrocyte adenosine deaminase. This leads to lower than normal levels of other compounds requiring adenosine as a precursor, namely, ATP, NAD, NADP, etc. Presumably the defect is in a regulatory gene.

The other abnormal erythrocyte metabolism is methemoglobinemias which occurs due to lack of NADPH. Erythrocytes are exposed to higher O_2 than most cells and potentially are more susceptible to damage by oxidants than other cells The O_2 consumption of erythrocytes is very low, and is associated primarly with oxidation of Hb to metHb. As much as 0.5 % of the total Hb is converted to metHb each day. A hemecontaining methemoglobin reductase (MW = 185.000) catalyzes reduction of methemoglobin, utilizing NADH as a cofactor. A second system, of unknown nature, utilizes NADPH but oly the presence of an intermediary such as methylene blue.

Elevated amounts of intracellular metHb, methemoglobinemia are found in individuals with HbM or after exposure to a variety of agents, including amyl nitrite, animiline, nitrobenzene, nitrates, nitrites, etc. Methemoglobinemia also has been observed clinically after administration of sulfonamides, acetanilid, phenactin, and salicylates. A third type of methemoglobinemia, familial methemoglobinemia, is a rare hereditary deficiency of methemoglobin reductase in the erythrocytes of afflicted persons. In these circumstances, as much as 25 to 40 % of the total hemoglobin may be present as metHb, with accompanying evidence of cyanosis.

As we know the erythrocyte is the most important cell due to the fact that they transport the oxygen from the lung to the tissue cell and Co_2 from the tissue to the lung. The hemoglobin in the erythrocyte must be found in the reduced form to do above function. These functions are related with ATP and NADPH, content of the red cell. The energy requires also for preservation of its biconcave shape and for maintaining the $Fe^{++}$of hemoglobin in the reduced state. The reduction of methemoglobin Fe^{+++} is provided by the metemoglobin reductase which depends on NADPH. Congenital deficieny of red cell methemoglobin reductase leads to an accumulation of methemoglobin, since this forms spontaneously in the erythrocyte. There is a persistent cyanosis and moderate erythrocytosis. The enzyme activity can be inhibited by a number of drugs.

The energy of the mature erythrocyte is supplied primarly by anaerobic glycolysis and the phosphogluconate pathways. Many genetic deficiencies in the enzymes of these pathways have been described and usually result in hemolytic anemia as well as in other disturbances of red cell structure and function. Several enzymes involved in the glycolytic pathway have been found to be hereditarily deficient. Pyruvate kinase deficiency which results in a rather severe anemia is the most important of these.

The phosphogluconate (pentose phosphate shunt) begins with glucose 6 phosphate, which is oxidized to 6 phosphogluconic acid under the influence of glucose 6 phosphate dehydrogenase in the presence of NADP This is a key reaction in this requence because NADPH and energy produceses in this reaction. A widespread, genetically transmitted disorder, in this pathway is glucose 6 phosphate dehydrogenase deficiency, genetically transmitted disorder. In this disturbance, erythrocyte hemolyze on administration of a variety of agents, including the antimalarial drug primaquine, sulfonamides, fava beans (favism), naphthalene, acetanilid and acetophenetidin, aspirin, fenasetin, Vitamin K. The electrophoretic studies showed that there are two big type of glucose 6 phosphate dehidrogenase enzyme. And there are also many subgroup of this enzymes. The different enzyme type make disease in negro and white people. In some cases not only antioxidan agents fava beans caused the hemolytic anemia. This kind of people must (patients) be protected antioxidan agents and fava beans. As was mention above.

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The second important role of pentose phosphate pathway is the biosynthesis of ribose-5 phosphate, an essential component of nucleotides and RNA. In this case, the ribose-5-phosphate can be formed by the action of phosphoriboisomerase on ribulose-5- phosphate produced by the irreversibl portion of the pathway. Equally possible is the formation of ribose 5 phosphate from fructose 6 phosphate and glyceraldehyde 3 phosphate by reversal of reactions in that order. Thus, the cell possessing the pentose phosphate pathway has the option of producing ribose 5 phosphate by an oxidative route or by a nonoxidative route.

Indivuduals who excrete large amounts of L-xylose in their urine have a hereditary enzyme deficiency called **pentosuria.** It is apparently an innocuous condition. Xylose, a ketopentose, is formed from D-ribulose-5 phosphate, but the enzyme required for further metabolism is absent.

Sequence of pentose phosphate pathway reactions

The sequence of reactions of the pathway may be divided into 2 phases: oxidative and nonoxidative route. In the first, Glucose 6. phosphate undergoes dehydrogenation and decarboxylation to give a pentose, ribulase 5- phosphate. In the second route, ribulase 5. phosphate is converted back to glucose. 6. phosphate by a series of reactions involving mainly 2 enzymes transketolase and transaldolase (Figure 17 and 19)

The enzmes of the pentose phosphate pathway are found in the extramitochendrial soluble portion of the cell, the cytosol. As in glycolysis, oxidation is done by dehydrogenation, but NADP is used as hydrogen acceptor instead of NAD.

The oxidative route:

Dehydrogenation of glucose 6-phosphate to 6-phospho gluconate occurs via the formation of 6-phosphogluconolactone cotalyzed by G-6-P-D. The hydrolysis of 6-phosphogluconolactone is done by the enzyme gluconolactone hydrolase. A second oxidative step is catalyzed by 6-phosphogluconate dehydrogenase, which also requires $NADP^+$ as hydrogen acceptor. Decarboxylation follows with the formation of the ketopentose, ribulose 5-phosphate, the reaction probably takes place in 2 steps through the intermediate 3-keto-6-phosphogluconate.

The nonoxidative route:

Ribulose 5-phosphate now serves as substrate for different enzymes. Ribulose 5-phosphate 3-epimerase alter the configuration about carbon 3, forming the epimer xylusose 5-phosphate, another ketepentose,

Ribose 5-phosphate ketoisomerase converts ribulose 5-phosphate to the coresponding aldo pentose, ribose 5-phosphate.

Transketolase transfer the 2-carbon unit comprising carbon 1 and 2 of a ketose to the aldehyde carbon of an aldose sugar. The reaction requires, B vitamin, thiamin as the coenzyme thiamin diphosphate in addition to Mg ions. Transketolase catalyses the transfer of 2-Carbon unit from xylulose 5-phosphate to ribose 5-phosphate producing the 7 carbon ketose sedoheptulose 7-phosphate and the aldose glyceraldehyde 3-phosphate. These 2 products than enter another reaction known as transaldolation. Measurement of transketolase activity in blood reflects the degree of thiamin deficiency. The only condition in which its activity is raised in pernicious anemia.

A further reaction takes place, again involving transketolase, in which xylulose 5-phosphate serves as a donor of "active glycoaldehyde". In this the erythrose 4-phosphate formed above acts as acceptor and the products of the reaction are fructose 6-phosphate and glyceraldehyde 3-phosphate.

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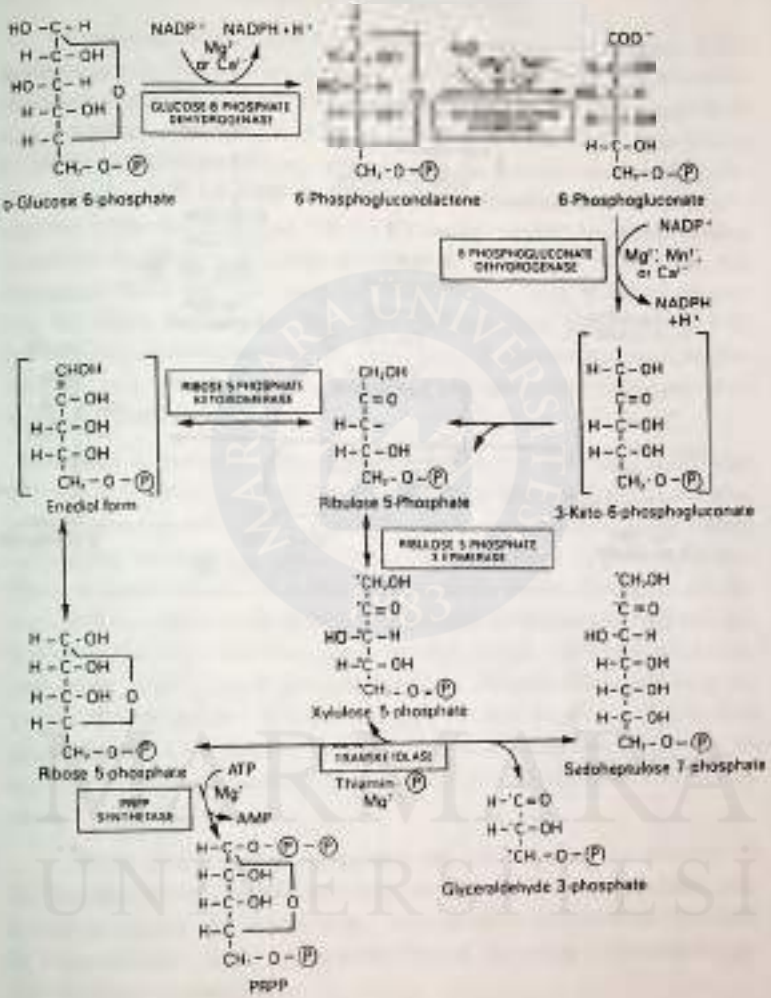

Figure 19 : The pentose phosphate pathway with molecules (From ref. 26)

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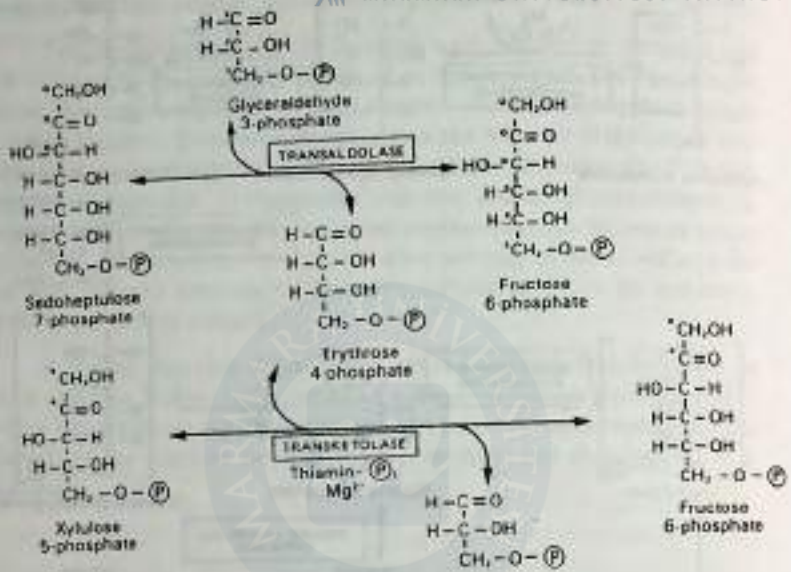

Figure 19: Continue

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GLUCONEOGENESIS

Gluconeogenesis meets the needs of the body glucose when carbohydrate is not available in sufficient amounts from the diet. Glucose is the most important source of energy, especially for the nerovous system and red blood cells. Glucose is also required in adipose tissue as a source of glyceride-glycerol, Glucose is important because of maintaining the level of intermediates of the citric acid cycle in many tissues. In addition, glucose is the only fuel that will supply energy to skeletal muscle under anaerobic conditions. It is the precursor of milk sugar (lactose) in the mammary gland and it is taken up actively by the fetus. It is not surprising, therefore, to find that enzymatic patways have been developed in certain specialized tissues for the conversion of noncarbohydrates to glucose. In fact, gluconeogenesis mean is the conversion of noncarbohydrates to glucose.

Such as glycerol, amino acids and lactate are the major substrates for gluconeogenesis as shown in Fig. 20. Only alanine directly provides after removal of the amino group. The carbon skelotons of other amino acids must undergo a series of metabolic conversions to yield pyruvate. There is some evidence that this may be partly carried out in muscle after which the pyruvate is converted to alanine by transamination and delivered to the liver where is readily yields pyruvate after another transamination step. Lactate provide pyruvate directly after oxidation by lactate dehydrogenase. Glycerol can be converted to glucose if it is first phosphanylated (by glycerol kinase) to glycerol phosphate, which can be oxidized to dihydroxyacetone phosphate by glycerol phosphate dehydrogenase.

These gluconeogenic mechanism are used to clear the products of the metabolism of other tissues from the blood. These are, lactate, produced by muscle and erythrocytes, and glycerol, continuously produced by adipose tissue. Some amino acids are also converted to glucose by gluconeogenitc mechanism.

In mammals, the liver and the kidney are the principal organs responsible for gluconeogenesis. The main pathway for gluconeogenesis is essentially a reversal of glycolysis. In fact, if there is active gluconeogenesis in these organs low glycolytic activity is seen. This research shows that liver and kidney play an important role in the gluconeogenesis.

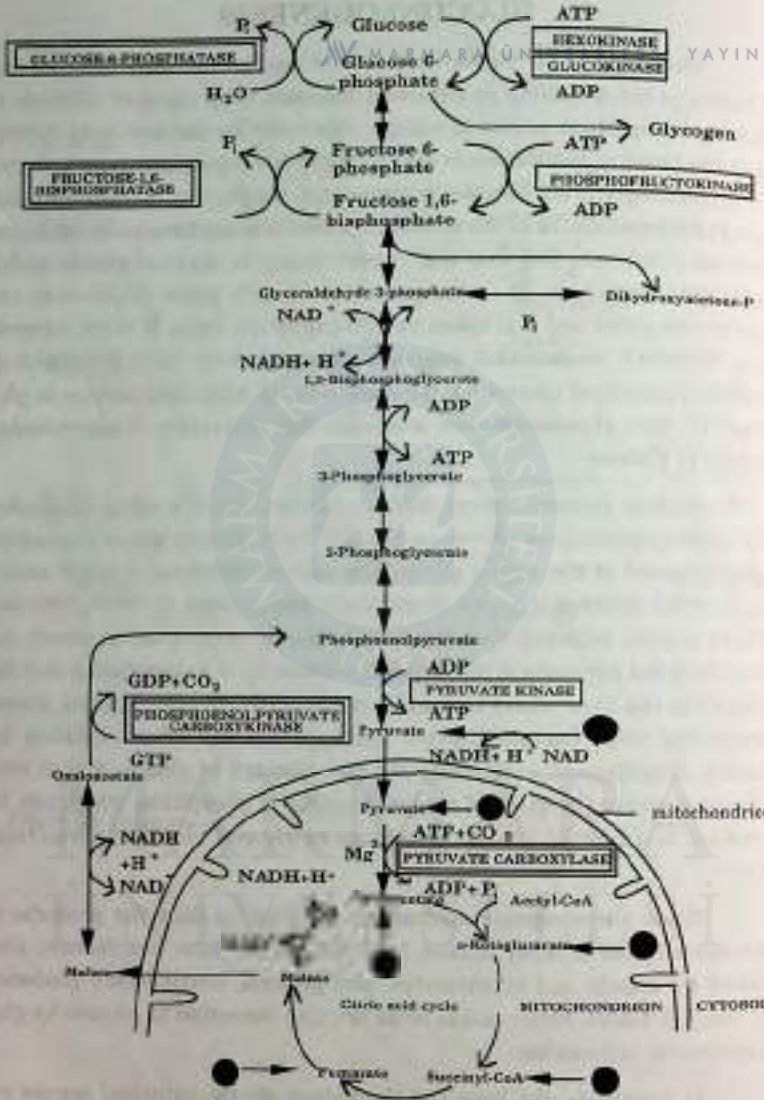

Figure 20: Gluconeogenesis pathway in the liver. Entry points of glucogenic amino acids after transamination are indicated by ●, entry points of lactate are indicated by ●. The key gluconeogenic enzymes are shown by ▭. (From ref. 26)

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Metabolic pathways in the gluconeogenesis:

As seen in figure 20 this pathway is reversal of glycolysis. They are concerned with the conversion of glucogenic amino acids, lactate, glycerol to glucose or glycogen. It has been pointed out by Krebs that energy barriers obstruct a simple reversal of glycolysis. **These barriers are:**

1 - Between pyruvate and phosphoenolpyruvate

2 - Between fructose 1,6 disphosphate and fructose 6 phosphate

3 - Between glucose 6 phosphate and glucose.

These barriers are circumvented by special reactions described below:

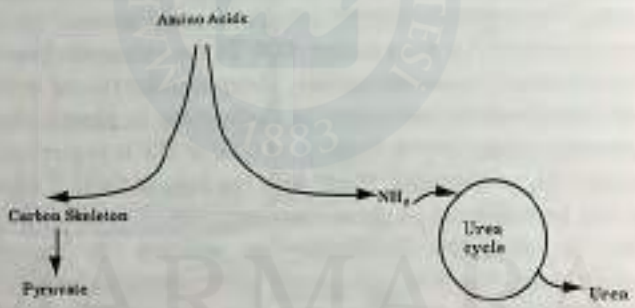
1- Pyruvate carboxylase enzyme takes place in this reactions. This is enzyme is present in the mitochondria, but ATP and biotin must be found the medium to activation of enzyme. CO_2 is also necessary for this reaction. This enzyme converts pyruvate to oxalacetate. The function of biotin is to bind CO_2 from bicarbonate. In the extramitochondiral part of the cell is found a second enzyme, phosphoenolpyruvate carboxykinase, which catalyzes the conversion of oxalacetate to phosphoenolpyruvate. High energy phosphate in the form of GTP or ITP is required in this reaction and CO_2 is liberated. Thus, with the help of these 2 enzymes pyruvate can be converted to phosphoenolpyruvate. Lactate can be also converted to phosphoenolpyruvate in addition to enzyme lactate dehydrogenase. However oxalacatate does not diffuse readily from mitochondria it converts into other compounds that can diffuse from the mitochondria. Such a compound is malate, but conversion via aspartate, a-ketoglutarate, glutamate, and citrate has also been proposed. Their formation rom oxalacetate in the extramitochondrial compartment involve citric acid cycle reactions and transaminations. There are species differences differences with regard to the distribution of phospheenolpyruvate carboxykinase. The extramtochondrial location of this enzyme is true for the rat and mouse, but in the rabbit and chicken the enzyme is located in the mitochondria and in the guinea pig and human its is found in both the mitochondria and cytosol.

2- The conversion of fructose 1,6 diphosphate to fructose 6-phosphate, necessary to achieve a reversal of glycolysis, is catalyzed by a specific enzyme, fructose-1,6 disphosphatase. This is a key enzyme in the

sence that its presence determines whether or not a tissue is capable of resynthesing glycogen from pyruvate and trinsephosphates. It is present in liver and kidney and has been demonstrated in striated muscle.

3- The conversion of glucose 6 phosphate to glucose is catalyzed by another specific phosphatase glucose-6 phosphatase. It is present in intestine, platelets, liver, and kidney where it allows these particular tissues to add glucose to the blood. It is absent from muscle and adipose tissue.

Pyruvate is important intermediate product in the gluconeogenesis because as it is shown below skeleton of amino acid is converted to pyruvate and amino group is converted to urea by the urea cycle, which has increased activity during gluconeogenesis, giving an elevated urinary concentration of urea. That is indicative of the protein breakdown.



The relationships between these key enzymes of gluconeogenesis and the Embden-Meyerhof glycolytic pathway are shown in Figure 21. After transamination or deamination, glucogenic amino acid form either pyruvate or members of the citric acid cycle. Therefore, the reactions described above can account for the conversion of both glucogenic amino acids and lactate to glucose or glycogen. Thus, lactate forms pyruvate and enters the mitochondria before conversion oxalacetate and ultimate conversion to glucose, As mentioned above.

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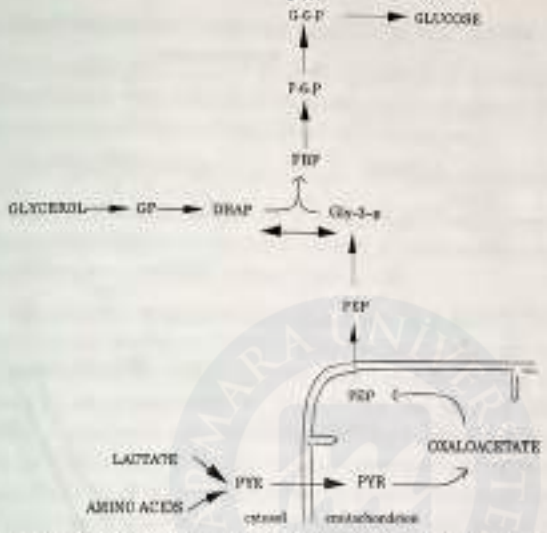

Figure 21 : Summary of the relationships between glycolysis and glyconeogenesis

The Cori Cycle:

The lack of glucose-6-phosphatase in muscle prevents the formation of free glucose in that tissue. However, muscle metabolism can contribute to blood indirectly. As it is shown in Figure 22. Here, lactate formed by the oxidation of glucose in the skeletal muscle and by red cells. It is transported to the liver and kidney where it re-forms glucose. It again becomes available via the circulation for oxidation in the tissues. This process is known as the Cori cyle or lactic acid cycle. It has been noted that of the amino acids transported from muscle to the liver during starvation alanine predominates so, cori cycle also called as glucose-alanin cycle. Glucose alanine cycle has the effect of cycling glucose from liver to muscle and alanine from muscle to liver.

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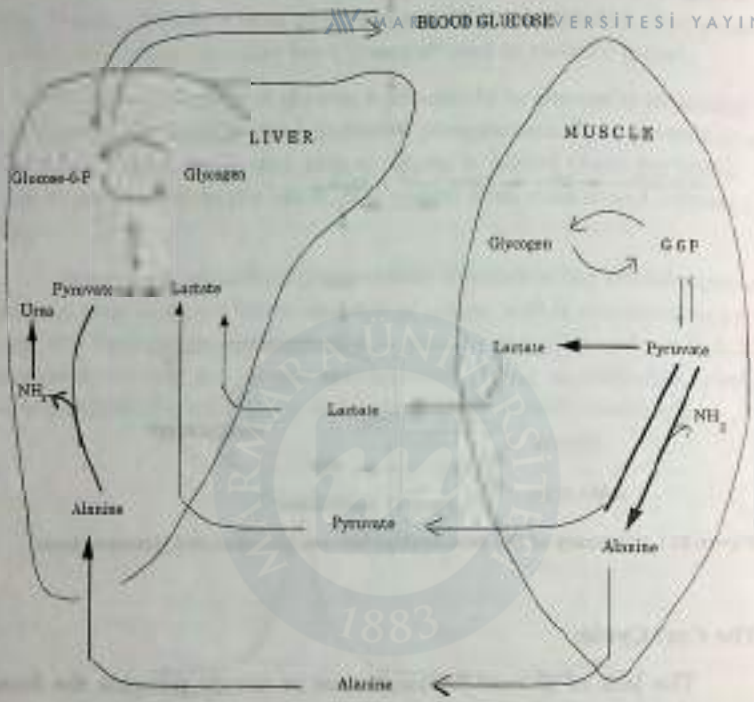

Figure 22 : The lactic acid (Cori cycle) and glucose alanine cycle

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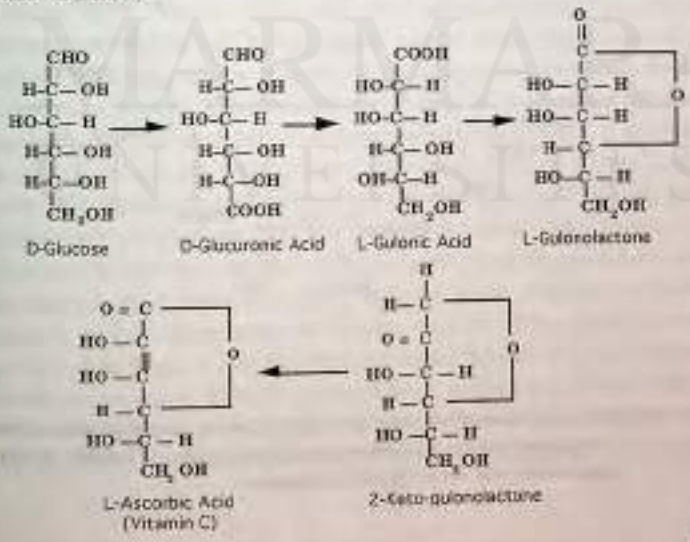
GLUCOSE METABOLISM VIA GLUCURONIC ACID

The other pathways of glucose oxidation is glucuronic acid pathway. Glucuronic acid is formed from glucose by oxidation of UDP-glucose in the presence of an NAD dependent dehydrogenase:

$$\text{UDP-glucose} + 2NAD^+ + H_2O \rightarrow \text{UDP-glucuronide} + 2NADH + 2H^+$$

Glucuronic acid, in the form of the UDP complex, is available for the formation of glucuronides of a variety of compounds, such as bilirubin (physiological) or menthol (nonphysiological).

The further metabolism of glucuronic acid glucuranate involves its conversion to L-gulonic acid, and this compound can serve as a source of the vitamin ascorbic acid, as well as of the ketopentose xylulose. Since xylulse can be metabolized to glucose via the pentose phosphate pathway, these reactions may be written in the form of a cycle. This is illustrated in Figure 23). This figure also notes various sites where the congenital absence of an enzyme leads to inborn errors of metabolism. The most interest as the absence in man, monkey and guinea pig of the enzme that converts L-gulunolactone to 2-keto-L-gulonolactone. These species are therefore vulnerable to ascorbic acid deficiency. The rat, on the other hand, has the enzyme and cannot be made deficient in this vitamin by dietary means. But if there is no ascorbic acid in man, monkey and guinea pig diet they show vitamin C deficiency. Conversion of glucose to vitamine. Molecular changes occuring during the convertion of vitamin C is shown below.



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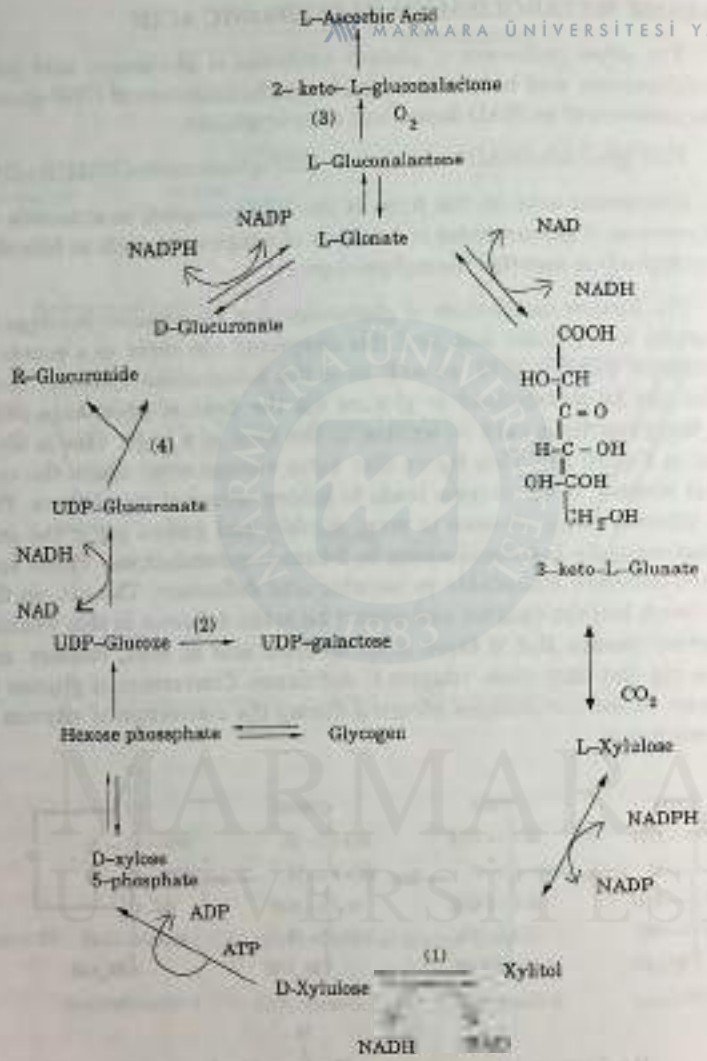

Figure 23: The glucuronate-xylose cycle, showing sites where congenital defects have been known to occur: (1) Pentosuria, (2) Galactosuria., (3 Inability to sythesize ascorbic acid and (4) Hyperbilirubinemia.

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In the rare hereditary disease esential pentosuria - xylulose appear in the urine. It is now believed that this may be explained by the absence in pentoseuric patients of the enzyme necessary to accomplish reduction of L-xylulose to xylitol. Parenteral administration of xylitol may lead to oxalosis involving calcium oxalate deposition in brain and kidneys, this results from the conversion of D-xylulose to oxalate via xylulose-1-phosphate, glycolaldehyde, and glyucolate formation. Various drugs markedly increase the rate at which glucose enters the glucuronic acid pathway. For example, administration of barbital or chlorobutonol to rats result in a significant increase in the conversion of glucose to glucuronate, L-gulonate and ascorbic acid.

METABOLISM OF FRUCTOSE

Fructose may be phosphorylated to form fructose -6 P-hosphate, by hexokinase. Its affinity of the fructose is very low compared with its affinity for glucose. Fructokinase which is present in the liver, kidney and intestine, effects the transfer of phospate from ATP to fructose, forming fructose 1-P. Unlike glucokinase, its activity is not affected by fasting or by insulin. This may explain why fructose disappears from the blood of diabetic patients at normal rate. The Km for fructose of the enzyme in liver is very low, this indicate that enzyme has high affinity for its substrate. Because of this, this is the major route for the phosphorylation of fructose (Figure 24). **Essential fructosuria** results from a lack of hepatic fructokinase. Fructose 1-P is split into D-glyceraldehyde and dihydroxacteone phosphte by aldolase B, found in liver. The enzyme also attacts fructose 1,6- diphosphate. Absence of this enzyme leads to a hereditary fructose intolerance. D-glyceraldehyde may gain entry to the glycolysis sequence. It also converts to ∝-glycero-glycerophosphate by glycerokinase. This reactions is imprtantant for lipid metabolism. Studies have indicate that fructose is metabolized by adipose tissue and it is metabolized independently of glucose. Free fructose is found in seminal plasma. It accumulates in the amniotic and allantoic fruids. Both fructose and sorbitol are found in the human lens. They increase in the diabetes and may be involved in the pathogenesis of diabetic cataract. The sorbitol pathway (not found in liver) is responsible for fructose formation from glucose. It activity increases in diabetic patients such as glucose sorbitol does not diffuse through cell membranes easily and therefore accumulates. Glucose undergoes reduction by NADPH to sorbitol catalyzed by aldose re-

ductase. Then it oxidases to D-fructose in the presence of NAD and sorbitol dehydrogonase. Aldose reductase is found in the placenta and is responsible for the secretion of sorbitol into the fetal blood. When sorbitol is administered intravenously, it is converted to fructose rather than to glucose. When it given by mouth, much escapes absorption from the gut and is fermented in the colon by bacteria to products such as acetate and H_2 abdominal pain may be caused by "sugarfree" sweeteners containing sorbitol. (Sorbitol intolerance)

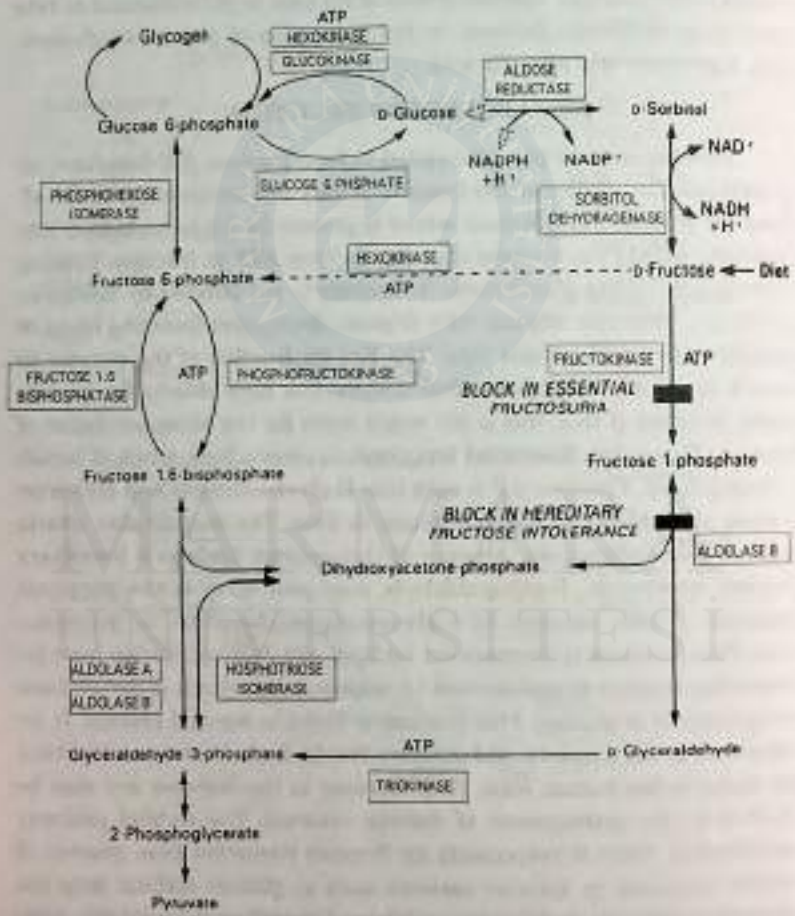

Figure 24: Fructose metabolism (D-sorbitol is converted to D-fructose) (From ref. 26)

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Metabolism of Galactose

Galactose is derived from intestinal hydrolysis of the disaccharide lactose, the sugar of milk. It is readily converted in the liver to glucose. The ability of the liver to accomplish this conversion my be used as test of hepatic functin in the galactose tolerance test, The pathway is shown in Figure 25. As shown in figure, glucose can be converted to galactose,

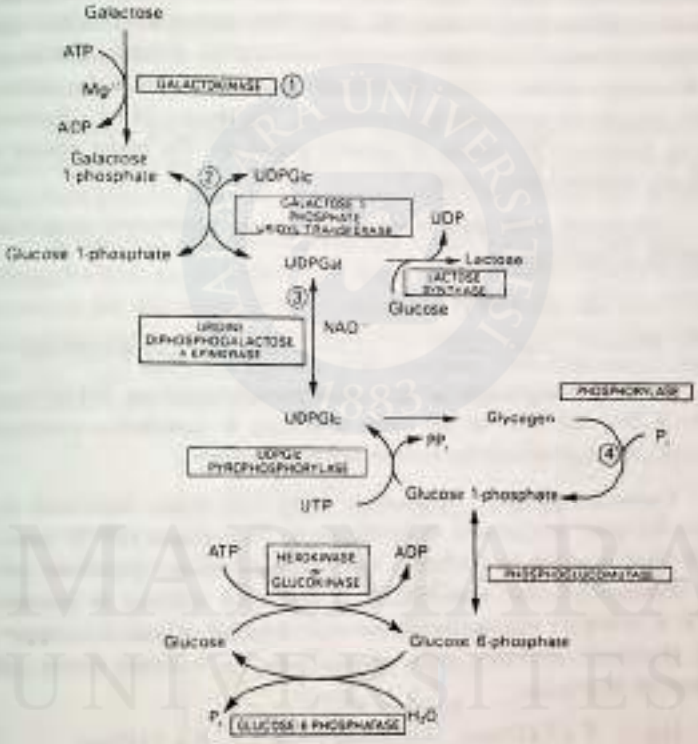

Figure 25: Galactose metabolism (From ref. 26)

so that preformed galactose is not essential in the diet. Galactose is required in the body not only in the formation of lactose, but also as a constituent of glycolipids (cerebrosides), proteoglycans and glycoproteins. In the synthesis of lactose in the mammary gland, glucose is converted to UDP galactose by the enzymes described above.

Marmara Üniversitesi
Kütüphane ve Dokümantasyon
Daire Başkanlığı

An inherited deficiency of certain enzymes can produce benign or profound effects on organisms. Deficiencies of this type are sometimes referred to as "newborn errors of metabolism".

In the condition called galactosemia, the enzyme galactose - 1 - P - uridyl transferase (GPUT) is absent or insufficient. The enzymatic conversion of galactose to glucose is blocked and the concentration of blood galactose elevated. High concentrations of blood galactose are toxic, and infants with hypergalactosemia have diarrehea, gastric distress and irritabilitly. Prolonged hypergalactosemia can result in irreversbil damage to the central nervous system. It is therefore imperative that the activity of this enzyme be measured in all newborns. The treatment for galactosemia is removal of the source of diateary galactose. The major source is milk so a nonmilk diet is used.

Individuals with galactosemia develop an alternative metabolic pathway as they mature.

$$\text{UTP} + \text{Gal-1-P} \xrightleftharpoons{\text{UDGP}} \text{UDP - Gal} + \text{P-Pi (pyrophosphate)}$$

At birth uridinediphosphogalactose pyrophosphrylase (UDGP) activity is low, but later in life there is enough to metabolize ordinary amounts of galactose and the intolerance disappears.

Normally, galactose 1-phosphate uridyl transferase and other enzymes for assay are present in erythrocytes. To evaluate GPUT, hemolyzed blood is added to a solution containing galactose-1-phoshate, uridine diphosphoglucose, and NADP. If enzymatic activity is present, NADP H is formed and can be measured or detected or both flourometrically. Qualitative detection of the enzyme using the reactions shown can be done on filter paper.

$$\text{Gal - 1 - P} + \text{UDP-glu} \rightleftharpoons \text{Glu - 1 - P} + \text{UDP-gal}$$

$$\text{Glu-1-P} \rightleftharpoons \text{Glu-6 - P}$$

$$\text{Glu - 6 P} \xrightarrow[\text{G6P dehydrogenase}]{\text{NADP NADP + H + H}} \text{G-gluconalactone-6-P}$$

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Inability to metabolize galactose occurs in the galactosemias, which may be caused by inherited defects in any of the 3 enzymes marked 1,2,3 in figure 25. Deficieny in the uridyl transferase is the best known enzyme in clinic. If galactose concentration increases in the blood, it is reduced by aldose reductase in the eye to the corresponding polyol (galactitol), which accumulates, causing cataract. If there is uridyl transferase deficieny, since galactose 1 phosphate accumlates and depletes the liver of inorganic phosphate the general condition is severe. Ultimately, liver failure and mental deterioration result.

The term **galactorrhea** is not relaated to galactose metebolism, it means breast discharge in women. Tumors of prolactin (PRL) secreting cells cause galactorrhea and amenorrhea (cessation of menses) in women.

In inherited galactose 1 phosphate uridyl transferase deficieny affecting the liver and red blood cells (reaction 2). Galactose free diets used to control the symptoms of the disease. The epimerase (reaction 3) has been found deficient in erythrocytes but present in liver and elsewhere, and this third condition appears to be symptom, free. Chromatographic seperation of galactose and other sugars is shown in figure 26.

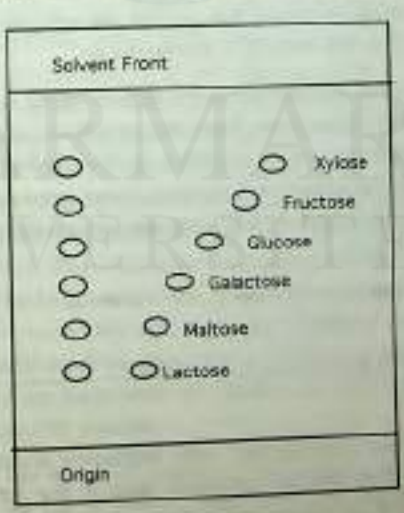


Figure 26:Schema of a typical chromatographic separation of individual sugars.

To identify a urinary carbohydrate other than glucose correctly, paper or thin-layer chromatography is employed. This is necessary when a urine sample gives a positive sugar reaction and the reducing sugar is not glucose, as demonstrated by a negative result with use of glucose oxidase method. Clinically important carbohydrate have been detected in urine and identified by several methods, as shown in figure 26.

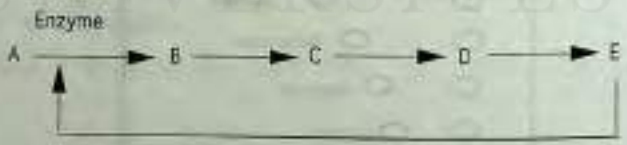
Metabolism of amino sugars

Amino sugars are important components of glycoproteins. The major amino sugars are glucosamine, galactosamine and mannosamine and the 9 carbon compound sialic acid. The principal sialic acid found in human tissues is N-acetylneuraminic acid. Glucosamin is the major amino sugar. It is formed as glucosamine 6 phosphate from fructose 6 phosphate using glucosamine as the donor of the amino group. The amine sugar occur mainly in the N-acetylated form. The acetyl donor is acetyl CoA.

METABOLIC CONTROL

The regulatory mechanism are quite complex and will not be presented in datail but some important aspecs will be mentioned. For example; 13 enzymes and reactions are required for the conversion of hexoses to lactate. The small stepwise changes are advantageous to the organism because they permit control of the processes.

One regulatory mechanism is the **inhibiton of some enzymes by the product of the reaction.** Some enzymes that catalyze one reaction in a series of reactions can be inhibited by the product of another reaction of the series. For exaple in following sheme, product E exerats an inhibitory effect on the enzyme catalyzing the conversion of A to B.



In this manner, an enzyme at the beginning of metabolic sequence can be regulated by a product produced later in the series. This type of regulation is called "negative feedback"a term used in the field of electronnics. When adequate amounts of product accumulate, inhibition is

increased and the system is slowed or stopped. A decrease in product concentration decreases inhibition and the rate of the system increases.

The **availability of cofactors** is another mechanism of regulation system. For example, in anaerobic glycolysis, a limited amount of NAD^+ would limit the rate at whech 3-phosphogly-glyceraldehyde is converted to 1,3-diphosphoglyceric acid. Similar control can be effected by the availability of adenosine diphosphate and inorganic phosphate in glycolysis. **Hormones** also exert great regulatory influence on metabolic processes.

Without control, large quantities of hexose would produce correspondingly large amounts of lactic acid, glycogen and if their rate of formation was solely dependent on the hexose concentration. With regulatory mechanisms, large amounts of glucose generally are converted to glycogen and stored until needed. If energy is required and the oxqygen supply is limited, the glucose is converted to lactic acid with concomitant energy production. May be the best example of a **lack of metabolic regulation is cancer.** Malignant cells apparently have little growth regulation and may continue to accumulate until the host is destroyed, unless they are treated.

Regulation of Gluconeogenesis and Glycolysis

Gluconeogenesis is new glucose synthesis from such precursors as pyruvate, lactate, certain amino acids, and intermediates of the tricarboxylic acid cycle. So the control of the pathway is very important. The first reaction in the uphill pathway from pyruvate to glucose is catalyzed by a regulatory enzyme. This enzyme is pyruvate carboxylase, which exerts primary control. This reaction is promoted by the allosteric modulator acetyl CoA. As a consequence whenever excess mitochondrial acetyl CoA builds up beyond the immediate needs of the cell for fuel, glucose synthesis is promoted. The secondary control point of this pathway is the reaction catalyzed by hexosediphosphatase. This enzyme is stimulated by the glucose precursors citrate and 3 phosphoglycerate and inhibited by AMP. Thus the pathway from pyruvate to glucose is regulated both by the level of respiratory fuels such as acetyl CoA and citrate and by the energy charge of the ATP system.

In contrast, the downhill pathway from glucose to pyruvate is primarily regulated by the allosteric pathway. Like many allosteric enzymes, it has a rather high molecular weight (380 000), contains a num-

ber of subunits, and shows a complex dependence of its reaction velocity on the concentration of its substrates. Phosphofructokinase has multiple allosteric modulators. It is inhibited by high concentrations of ATP, citrate, and long-chain fatty acids but is stimulated by ADP or AMP. Therefore, as I mention before, whenever other fuels such as fatty acids or citrate available, 6 phosphofructokinase is inhibited and turns off glycolysis. Conversely, whenever the ATP concentration is low and AMP and ADP thus predominate or whenever the concentration of other fuels such as citrate or fatty acids is low, 6-phosphofructokinase activity is stimulated. Thus the kinetic behavior of 6 phosphofructokinase, though very complex, is extraordinarily well adapted for the regulation of this important step of glycolysis. This positive and negative allosteric modulators of this enzyme vary from one type of cell to another. The 6 phosphafructokinase reaction is essentially irreversible in the cell, it will be recalled that most regulatory enzymes catalyze irreversible reactions. All these stimulators and inhibitors are shown in figure 27.

A secondary point of regulation of glycolysis is provided by hexokinase. This enzyme is inhibited by glucose 6 phosphate and possibly acetyl CoA and phosphoenolpyruvate.

Another regulator of glycolysis is pyruvate kinase, which is inhibited by ATP, NADH and alanine but stimulated by fructose 1, 6 diphosphate and glucose 6 phosphate.

We therefore see that the important regulatory enzymes of the opposing pathways of glycolysis and gluconeogenesis are located at those points where the two pathways are not identical thus providing independent regulation of each.

If we summarize all these activatory or inhibitory effect whenever the cell has an ample level of ATP, and whenever respiratory fuels such as acetyl Coa, citrate, or NADH are readily available, glycolysis is inhibited and gluconeogenesis promoted. On the contrary, when the energy charge is low or respiratory fuels are not available, glycolysis is accelerated and gluconeogenesis is inhibited. Administration of excess glucose, fructose or glycerol to fasted rats depresses the phosphoenolpyruvate carboxykinase activity of the liver: It will be recalled that this enzyme catalyzes the formation of phosphoenolpyruvate from oxaloacetate. This effect is due to suppression of the biosynthesis of the enzyme, presumably

because it is not needed when the liver is provided with ample supplies of glucose, a more direct precursor of glycogen than phosphoenolpyruvate. On the other hand, feeding excess glycogenic amino acids enhances the biosynthesis of phosphoenolpyruvate carboxykinase because it is required for the conversion of these amino acids into glucose it appears that food can function as a regulatuar system. Figure 28 summarises the control points in the pathways between pyruvate and glucose in animal tissues.

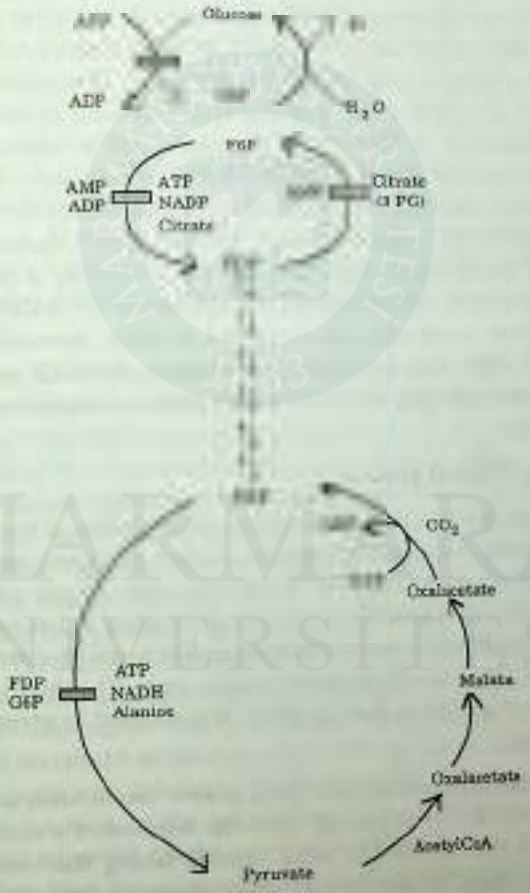


Figure 27. Allosteric regulation of opposing pathways from pyruvate to glucose (From ref. 28)

Regulation of the citric acid cycle

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It is difficult to identify the citric acid cycle enzyme because of the many pathways interacts with this cyle. On the other hand cycle enzymes located in mitochondrion because of this the measurement of enzyme activity and substrate levels is relavitely uncertain. In most tissues, where the primary function of the citric acid cycle is to provide energy, Thus the control of citric acid cycle activity depend on the supply of oxidized dehydrogenase cofactors (eg NAD) In addition to this overall or coarse control, the properties of some of the enzymes of the cycle indicate that control might also be exerted at the level of the cycle itself. In a tissue such as brain, which is largely dependent on carbohydrate to supply acetyl-CoA, control of the citric acid cycle may occur at the pyruvate dehydrogenase step. In the cycle proper control may be exercised by allosteric inhibition of citrate synthase by ATP or long chain fatty acyl-CoA. Allosteric activation of mitochondrial NAD-dependent isocitrate dehydrogenase by ADP is counteracted by ATP and NADH. Succinate dehydrogenase is inhibited by oxalacetate and the availability of oxalacetate controlled by malate dehydrogenase, depends on the the NADH/NAD ratio. Oxalacatate could also play to control of citrate formation. An increased ATP /ADP ratio is considered to raise the GTP/GDP ratio at the succinate thikenase step. May be this is the another control sistem in the citric acid cylo.

Regulation of blood glucose

Sources of blood glucose are from carbohydrates of the diet and from the various glucogenic compounds. Glycerol, glycogenic amino-acids and lactic acids become available via the Cori cycle or lactic acid cyle or gluconeogenetic mechanisms in the liver and kidney. It has been noted that, some amino acids transported from muscle to the liver during starvation. This sistem is called glucose alanine cycle. The third important source of blood glucose is liver glycogen. It is supplied by glycogenolysis from the liver.

In postabsorptive state, the blood glucose concenration in humans varies between 80 and 100 mg/dl. After the ingestion of a carbohydrate meal, it may rise to 120-130 mg/dl. During fasting the level falls to around 60-70 mg/dl. Under normal circumstance the level is controlled within these limits.

The ability of the body to utilize carbohydrates is measured by carbohydrate tolerance test. It is indicated by the nature of the blood glucose curve following the administration of glucose. Diabetes Mellitus (sugar diatebes) is characterized by decreased tolerance to carbohydrate due to decreased secretion of insulin. This is manifested by elevated blood glucose levels (hyperglycemia) and accompanying glycosuria and may be accompanied by changes in fat metabolism. Tolerance to carbohydrate declines not only in diabetes but also in liver damaged, in some infections, in obesitiy and sometimes in atherosclercesis. It would also be expected in the presence of hyperactivity of the pituitary or adrenal cortex. Because these endocrine glands have the antogonis effect to insulin. Injection of insulin lowers the content of the glucose in the blood and increases its utilization and its storage in the liver and muscle as glycogen. An excess of insulin may lower the blood glucose level then hypoglycemia occurs. It results in convulsions and even in death unless glucose is adminisrede promptly. In humans, hypoglycemic convulsion may occur when the blood glucose is lowered acutely to about 20 mg/dl or less. In creased tolerance to carbohydrate is also observed in pituitary or adrenocortical insufficiency.

When the blood glucose rises to relatively high levels, the kidney also exerts a regulatory effect. Glucose is continually filtered by the glomeruli but is ordinarily returned completely to the blood by the reabsorptive system of the renal tubules. The reabsorption of glucose is linked o oxidative phosphorilation and the provision of ATP in the tubular cells. Some process takes place in the intestine while the absorbtion of suguar into the blood. The capacity of the tubular system to reabsorb glucose is limited to a rate of about 350 mg/min. When the blood levels of glucose are elevated, the glomerular filtrate contain more glucose than can be reabsorbed. The excess glucose pases into the urine to produce **glycosuria.** In normal indivuduals, glycosuria occurs when the venous blood sugar exceeds 170-180 mg/dL. This level of the venous blood sugar is termed the **renal threshold** for glucose. Glycosuria may be produced in experimental animals with phlorhizin. This chemical agent inhibits the glucose reabsorptive system in the tubule. This is known as renal glycosuria. **Glycosuria of renal origin** may result from inherited defects in the kidney. Sometimes it may be acquired as a result of disease processes.

The maintenance of stable levels of glucose is provided some homeostatic mechanisms. The liver is the most important homeostatic organ in the blood sugar regulation as mentioned before. But the extrahepatic tissues and several hormones play an important role in this mechanism. Liver cells appear to be freely **permeable** to glucose, whereas cells of extrahepatic tissues are relatively **impermeable.** The concentration of glucose in the blood is an important factor controlling the rate of uptake or output of glucose from liver. Hexokinase is inhibited by glucose 6 phosphate, so some feedback control may be exerted on glucose uptake in extrahepatic tissues dependent on hexokinase for glucose phosphorylation. The liver is not subject to this because glucokinase is not affected by glucose 6 phosphate. Km of the glucokinase higher for glucose than hexokinase. After a carbohydrate meal when glucose uptake into the liver at the higher concentration glucokinase activity increases at the same time.

Insulin plays a central role in the regulation of the blood glucose concentration by enhancing the uptake of glucose into both the liver and peripheral tissues. It is produced by the β cells of the islets of Langerhans in the pancreas and is secreted into blood as a direct response to hyperglycemia.

There are some substances causing release of insulin these are amino acids, free fatty acids, ketone bodies, glucagon, secretin and tolbutamide. On the other hand, epinephrine and norepinephrine block the release of insulin. In invitro probably in invivo, insulin has an immediate effect on tissues such as adipose tissue and muscle of increasing the rate of glucose uptake. It is considered that this action is due to an enhancement of glucose transport through the cell membrane by effects on transporters of glucose. In contrast, there is no effect of insulin on glucose penetration of hepatic cells. However insulin does indirectly enhance uptake of glucose by the liver as a result of its actions on the enzymes controlling glycolysis and glycogenesis. (See page 217 further reading for insulin)

The adrenal medulla secretes epinephrine and norepiehprine. Epineprine stimulates glycogen breakdown in muscle and in liver. In muscle, as a result of the absence of glucose -6-phosphatase glycogenolysis ensues with the lactate formation. Lactate then diffuses into the blood is converted by the gluconeogneetic mechanisms back to glycogen in the liver (Cori cycle). Hypoglycemia causes a neural sympathetic discharge that increases epinephrine secretion. It in turn stimulates glycogenolysis and results in an increase in the blood glucose concentration.

Glucagon is the hormone produced by the α cells of the islets of Langerhans of the pancreas. Its secretion is stimulated by hypoglycemia. When it reaches the liver, it causes glycogenolysis by activating phosphorylase in a manner similar to epinephrine. Unlike epinephrine, glucagon does not have an action on muscle phosphorylase. Glucagon also enhances gluconeogenesis from amino acids and lactate. Both hepatic glycogenolysis and gluconeogenesis contribute to the hyperglycemic effect of glucagon.

Thyroid hormone should also be considered as affecting the blood glucose. There is experimental evidence that thyroxine has a diabetogenic action. Thyroidectomy inhibits the development of diabetes. In humans, the fasting blood glucose is elevated in hyperthyroid patients and decreased in hypothyroid patients. However, hyperthroid patients apparently utulize glucose at a normal or increased rate, whereas hypothyroid patients have a decreased ability to utulize glucose. In addition hypothyroid patients are much less sensitive to insulin than are normal or hyperthyroid individuals. These effects of thyroid hormone on carbohydrate metabolism may be related to rates of destraction of insulin or organ response.

The anterior pituitary gland secretes hormones that tend to elevate the blood glucose and therefore antagonise the action of insulin. These are grojth hormone, ACTH (adrenocorticotropin) and possibly other "diabetogenic" principles. Growth hormone secretion is stimulated by hypoglycemia. Growth hormone decreases glucose uptake in certain tissues, eg, muscle. Some of this effect may not be direct, since it mobilizes free fatty acids from adipose tissue which themselves inhibit glucose utilization. Chronic administration of growth hormone leads to diabetes. By producing hyperglycemia, it stimulates secretion of insulin, eventually causing B cell exhaustion. ACTH could have an indirect effect upon glucose utilization. Because it enhances the release of free acids from adipose tissue. Major effect of ACTH on carbohydrate metabolism is due to its stimulation of the secretion of hormones of the adrenal cortex.

The adrenal cortex secretes a number of steroid hormones of which glucocorticoids are important in carbohydrate metabolism. Administration of glucotorticoids lead to gluconeogenesis. It has antagonistic effect to insulin. Upon administration of glucocorticoids following metabolic changes are seen;

- increased protein catabolism in the tissues
- increased hepatic uptake of amino acids
- increased hepatic activity of transaminases and other enzymes concerned with gluconeogenesis,
- inhibited the utilization of glucose in extrahepatic tissues.

DIABETES MELLITUS AND LABORATORY APPROACHES

Diabetes mellitus, a familial disorder with a prevalence of about 2%, is characterized by a hyperglycemia. Clinically, Diabetes Mellitus may be classified as idiopathic (primary or essential) or as secondary diabetes. Idiopathic diabetes may be caused by factors in the blood which antagonize or inhibit the action of insulin, or by production of an abnormal form of insulin, or by inability of the pancreas to produce sufficient insulin. Idiopathic diabetes is divided into types I and II.

Secondary diabetes occurs as a consequence of other disease, either pancreatic or endocrine, there are also other groups of patients in whom abnormalities of glucose tolerance detected. But they do not fit into either of these catagories.

There is insulin deficiency or resistance to the action of insulin in diabetes mellitus. About 90 % of persons with diabetes have non-insulin dependent (type II) diabetes melliutus (NIDDM). Such patients are usually obese. Diabetes mellitus, as currently undersood is almost certainly more than a single disorder. Of particular importance, yet poorly understood, is the propensity for persons with diabetes mellitus to develop specific complications (retinopathy with blindness, kidney failure with uremia, nerve damage and circulatory problems that predispose to tissue damage and may lead to amputations, heart disease and stroke). Diabetes mellitus is a common disorder, but the actual prevalence of this disease depends on the criteria used for its diagnosis. There are perhaps as many as 10 million persons with diabetes mellitus in the United States, half of them are not aware that they have the dissease. diabetes has been classified into various stages.

The diagnostic bases for classification have been defined somewhat differently by different authors. According to the some authors it is classified latent diabetes, chemical diabetes and overt diabetes.

Latent diabetics demonstrate a normal fasting glucose concentration and a normal hyperglycemic response to an oral glucose load. In some circumtances, usually in response to stress, they demonstrate a greater than normal normal hyperglycemic response to glucose administration.

Chemical diabetics are generally asymptomatic and do not demonstrate abnormal fasting glucose levels. However, they do demonstrate a greater than normal hyperglycemic response to an oral glucose load. This is mild form of diabetes, which is the majority of cases does not require the administration of insulin for management of the disease. Chemical diabetes is also sometimes referred to as maturity-onset diabetes, although onset can occur in childhood. Detection of the presence of this form of diabetes usually arises as the result of a chance finding of glycosuria at the time of a routine urinalysis.

In overt diabetes all of the acute symptoms described above are present. Fasting glucose levels are significantly elevated. Management of this form of diabetes requires the administration of insulin. Overt diabetes is sometimes referred to as growth-onset or juvenile diabetes, although onset can occur at any time from the neonatal period into adulthood. Overt diabetes is characterized by a persistent fasting hyperglycemia. The hyperglycemia is great enough to exceed the capacity for complete reabsorption of glucose by the renal tubules. As a result glucose appears in the urine (glycosuria) Despite the hyperglycemia, a fasting type of metabolic response occurs in that glycogen stress are depleted and fats and proteins are mobilized and catabolized. Ketones from fat breakdown accumulatte in the blood (ketonemia) and urine (ketonuria). Unlike in the normal prolonged fasting state, the brain does not adapt and use the ketones, eventually ketoacidosis can occur followed by coma and death. The causal relationship between ketoacidosis and coma are not clear at present. In addition to these acute symptoms of diabetes, long-term diabetes is associated with pathologic changes in many organ systems, which appear to be related to vascular insufficiency (chronic syndrome). In diabetes the greater than normal conversion of glucose to sorbitol (glucitol) by aldose reductase. As the result of the high glucose concentrations has been suggested as a causstive factor in the diabetic complications of neuropathies, cataract formation and nephropathies. Sorbitol has also been considered as the source of the idiogenic osmoles that can lead to a fatal cerebral edema following the treatment of hyperglycemia in diabetic coma.

All of the acute symptoms of diabetes are consistent with a lack of

insulin and insulin administration is capable of reversing the features of acute diabetes. Thus traditionally diabetes was considered to result solely from a deficiency in circulating insulin. Recent important observations support the view that the acute symptoms of diabetes are not only the result of a lack of insulin but also the result of an accompanying hyperglucagonemia. According to the hormonal theory, both the ∝ and β cells are defective in their normal response to fluctuation in blood glucose. The actions of insulin can be seen in table through several critial pathways in the absence of hormone.

Classification and some properties of hyperglycemic disordes according to some autors are as follows:

Insulin-dependent diabetes mellitus (IDDM, type I). Persons with this type of diabetes mallitus are usually acutely ill at the time of diagnosis with severe hyperglycemia and other metabolic erangements. These individuals produce very little insulin and thus have an absolute deficiency of insulin. Without insulin treatment, these metabolic changes will cause death in a short time.

TABLE 2: Actions of insulin on several pathways

Enzyme	Change in Activity	Possible Mechanism
cAMP metabolism		
Phosphodiesterase (low K_m)	Increase	Phosphorylation
Protein kinase (cAMP-dependent)	Decrease	Association of R and C subunits
Glycogen metabolism		
Glycogen synthase	Increase	Dephosphorylation
Phosphorylase kinase	Decrease	Dephosphorylation
Phosphorylase	Decrease	Dephosphorylation
Glycolysis and gluconeogenesis		
Pyruvate dehydrogenase	Increase	Dephosphorylation
Pyruvate kinase	Increase	Dephosphorylation
6-Phosphofructo-2-kinase	Increase	Dephosphorylation
Fructose 2,6-bisphosphatase	Decrease	Dephosphorylation
Lipid metabolism		
Acetyl-CoA carboxylase	Increase	Phosphorylation
HMG-CoA reductase	Increase	Dephosphorylation
Triacylglycerol lipase	Decrease	Dephosphorylation
Other		
Tyrosine kinase (the insulin receptor)	?	Phosphorylation

With insulin treatment, satisfactory control of blood glucose can be obtained, yet many of these individuals will develop significant microvascular changes with time. Diagnosis of this type of diabetes is fairly straightforward when the patient presents in the acute stages. Type I, insulin dependent diabetes mellitus, makes up about 10% of all patients with diabetes mellitus. IDDM appears to be due to autoimmune destruction of β cells. Several studies have shown that circulating antibodies to pancreatic islet cells are present in patients for months or years prior to the development of overt diabetes. Even when glucose and insulin responses to an oral glucose tolerance test are normal, these "prediabetics" have impaired early insulin release after intravenous glucose.

In a few centers, clinical trials are underway using cyclosporine, an immunosuppressive drug, in patients with new-onset IDDM with hopes of preventing or minimizing β cell destruction. Preliminary data are encouraging.

Non-insulin-dependent diabetes mellitus (NIDDM, type II)

NIDDM is the most common form of diabetes mellitus (> 90%). Most individuals with NIDDM have plasma insulin concentrations that are higher than normal but not sufficient to maintain a normal blood glucose concentration in the face of insulin resistance.

Thus, most persons with the form of diabetes mellitus have a relative deficiency of insulin activity. Obesity is commonly associated with this form of diabetes, and a reduction in body weight usually brings about an improvement in hyperglycemia and, often, normal glucose concentrations. Patients with NIDDM are subdivided into: 1) those patients who are not obese and 3) those who require insulin for control of hyperglycemia and b) those who do not require insulin. Early diagnosis of this type of patient is difficult, since the disease is usually slow to develop and progress, and precise separation of all persons with NIDDM from those who are normal may not be possible. Although insulin dependent diabetes is most commonly seen in young persons and non-insulin-dependent diabetes usually develops in middle age, these disorders are not age specific.

Impaired glucose tolerance (IGT): This category identifies individuals who are not clearly normal at the time of testing but are not sufficiently abnormal to be certain that they have diabetes mellitus. They have a greater than usual risk for the development of diabetes mellitus at a later age and there is a slow rate of progression to overt diabetes (1-5 % a year). In this group there is also a greater than normal risk for the

development of cardiovascular disease is quite rare in this group, even in patients in whom definite hyperglycemia appears with time. This category is useful for identifying persons whose glucose tolerance is not clearly normal, but it avoids the inappropriate label of diabetes mellitus for persons in this group.

Gestational diabetes mellitus (GDM)

Gestational diabetes is diagnosed when an abnormal glucose concentration is discovered for the first time during pregnancy, this is not the same as a known diabetic who becomes pregnant. Early diagnosis of gestational diabetes is important so that the patient can be monitored and treatment can be instituted when necessary. There is a high probability (30-60 %) that a woman with gestational diabetes will develop diabetes mellitus later in life, but carbohydrate tolerance often reverts to normal after delivery and may remain normal.

Previous abnormality of glucose tolerance (Prev. AGT):

This category includes persons known to have had an abnormality of glucose tolerance at one time but who have been shown to have normal results on repeat testing. For example, this would include someone who had type II diabetes mellitus, was obese, was not treated with insulin, and after weight reduction had a normal glucose tolerance test.

Potential abnormality of glucose tolerance (Pot.AGT): This includes perons at greater than the average theoretical risk for the later development of diabetes mellitus, for example, the identical twin of a patient with type II diabetes or the child of parents who each had type II diabetes.

Glucose intolerance associated with certain conditions and syndromes.: This category includes the uncommon patient in whom hyperglycemia is due to a specific underlying disorder (such as chronic pancreatitis with endocrine and exocrine insufficieny, Cushing's disease, acromegaly, or glucagon-producing islet-cell tumor of the pancreas) to other diseases, or to the use of certain drugs that are known to cause hyperglycemia.

The previously used terms prediabetes, latent diabetes, chemical diabetes, subclinical diabetes, borderline diabetes, and asymptomatic diabetes do not appear in this classification but these conditions are covered in the categories of Pot.AGT, Prev.AGT and IGT. Juvenile-onset or ketosis-prone, diabetes is now classified as Type I diabetes, and maturity-onset, or ketosis resistant, diabetes as Type II diabetes.

LABORATORY APPROACEES IN DIABETES MELLITUS

The demonstration of significant hyperglycemia is the key to the diagnosis of diabetes mellitus. For type I diabetes, the diagnosis is usually simple, since hyperglycemia appears abruptly, is severe, and is accompanied by serious metabolic derangements (e.g metabolic ketoacidosis). But in Type II diabetes mellitus (NIDDM early diagnosis becomes troublesome. The risk for the later development of microvascular disease makes it important to identify patients with type II diabetes. Although it seems that bringing glucose levels to normal or near normal will help prevent or minimize microvascular disease, the evidence is inconclusive.

Diagnostic Strategies

Fasting plasma glucose: An elevation of fasting plasma glucose values is highly specific for diabetes mellitus. Values > 120 mg/dl on two or more occasions would be diagnostic. Fasting hyperglycemia however, occurs relatively late in Type II iabetes mellitus, so that waiting for fasting hyperglycemia to appear delays the time of diagnosis. It must be done oral glucose tolerance test.

Postprandial plasma glucose: 2 hours or more after a mixed meal is considered diagnostic for diabetes mellitus. The postprandial plasma glucose results either will be normal or will quickly establish the diagnosis where it clear cut. Only a small number of individuals with borderline values who need to be studied further.

A person with a clear elevation of plasma glucose needs no further diagnostic testing. A person with a value < 140 mg/dl is unlikely to have diabetes mellitus.

Post-challenge plasma glucose : Administration of a standard oral glucose load (75 g recommended, 50 and 100 g loads have been used). After 2 hours later glucose determination is performed. This is more objective conditions than the post prandial method

Collection and handling of specimens: When blood is drawn and permitted to clot and to stand uncentrifuged at room temperature, the average decrease in serum glucose is about – 7% in 1 hour. This decrease is the result of glycolysis. Plasma removed from cells after moderate centrifugation contains leukocytes that also metabolize glucose. In separated unhemolyzed serum, the glucose concentration is generally stable up to 8 h at 25 C, if kept free of bacterial constamination and up to 72 h t 4 C.

Glycolysis can be prevented and glucose in whole blood stabilized for up to 24 h at room temperature by adding sodum fluoride to the specimen. Fluoride ions prevent glycolysis by inhibiting enolase. Fluoride ions also inhibit coagulation by binding calcium. But clotting may occur after several hours. It is therefore advisable to use a combined fluoride-oxalate mixture, such as 2 mg of potassium oxalate and 2,5 mg of sodium fluoride per ml. of blood, to prevent late clotting. Fluoride ions in high concentration inhibit the activity of urease and certain other enzymes, consequently, specimens are unsuitable for determination of urea in some procedures that require urease and for direct assay of some serum enzymes.

Cerebrospinal fluid frequently contains bacteria or other cellular constituents and should be analyzed for glucose without delay. Glucose may be preserved in 24 h collections of urine by adding 5 ml of glacial acetic acid to the container before starting the collection. The final pH of the urine is usually between 4 and 5, and bacterial activity is inhibited at this level of acidity. The use of 5 g sodium benzoate per 24 h speciman is also effective in preserving the urine.

Factors affecting determination of reference ranges

Glucose is uniformly distributed between plasma and erythrocayte water. Plasma contains about 15 % more water than whole blood, thus plasma or serum glucose values are higher than whole blood values. Multiplication of whole blood values by 1.15 to convert them to serum or plasma values has been utilized in order to unify interpretation of the glucose levels obtained in oral glucose tolerance tests. Deproteinization of serum or plasma samples results in a concentration of the solutes. As a result, the glucose values in deproteinized plasma or serum are approximately 5% higher than in untreated samples.

The reference range is also affected by the procedure used for glucose measurements. The o-toluidine procedure yields slight higher values (about 4% than the glucose oxidase procedure and hexokinase procedures. Somogyi Nelson, ferricyanide methods yield values 10-15 % higher than the enzymatic methods. Thus in fasting adults the reference range for the oxidation reduction methods is about 70-120 mg/dl, while the reference range for the o-toluidine procedure is essentially the same as for the enzymatic methods 60-110 mg/dl.

In fasting individuals venous, capillary or arterial blood glucose concentration are essentialy identical. The concentrations are higher in capillary than in venous blood after the ingestion of glucose. This is an important consideration in the interpretation of postprandial glucose measurements or glucose tolerance tests when capillary blood is used as the sample.

GLUCOSE TOLERANCE TESTS

Both fasting blood glucose levels and glucose levels obtained 2 hours after ingestion of a meal (or a standard glucose load of 50 or 100 g) are used in diagnosting diabetes. after food intake the glucose level tends to return within 2 hours to the individual fasting level. Some diabetics show decisively elevated fasting and postprandial plasma glucose levels. In these cases a glucose tolerance test is not necerrary to establish the diagnosis and in fact may be dangerous because of ketoacidosis occurs. However, in cases of mild diabetes neither the fasting nor the 2 hour postprandial glucose levels may be clearly elevated. The oral glucose tolerance test is considered a useful procedure for detecting doubtful cases of diabetes.

Oral glucose tolerance test (GTT): Serial measurement of plasma glucose before and after glucose is given orally should provide a standard method to evaluate individuals and establish values for normal and disease states. There are a number of factors that may affect glucose tolerance. So it should be controlled or eliminated before such a test in performed. other condition altering the glucose response need to be controlled during the test. Otherwise, abnormal values may occur in the absence of diabetes mellitus. When an oral glucose tolerance test is ordered, the following conditions should be met: omit medications known to affect glucose tolerance, perform the test in the morning after 3 day of unrestricted diet and activity, and perform the test after a 10-16 hours fast. Pasma glucose should be measured fasting, then every 30 min for 2 hour after an oral glucose load. For adults, the recommended load is 75 g and for children, 1.75 g/kg up to 75 g maximum. There is still some question as to what constitutes the ideal glucose load. In adults, 50 g is suboptimal, whereas 100 g frequently causes nausea and vomiting: 75 g is the consensus but may not be a maximal stimulus.

According to the National Diabetes Data Group, no GTT is necessary if fasting plasma glucose is > 140 mg/dl or the 2 hour postprandial value is > 200 mg/dl. Diabetes is confirmed if the 2 hour plasma glucose and at least one other glucose value in o oral glucose tolerance test are > 200 mg/dl. An oral glucose tolerance test is not always necessary for the diagnosis of diabetes mellitus, particularly in a person with classic symptoms of diabetes mellitus (thirst, frequent urination, unexplained weight loss) The oral glucose tolerance test is indicated in the following situations:

1- Dignosis of gestational diabetes melliutus

2- Further evaluation of an individual with a borderline elevation of fasting or postprandial plasma glucose.

3- Risk counseling in an individual with a previously abnormal glucose tolerance test under suboptimal conditions (repart results will often be normal)

4- Risk counseling in persons at high risk for the development of diabetes mellitus but with normal fasting and postprandial plasma glucose values (a normal result, however, does not mean) than diabetes mellitus will not develop in the future).

5- Evaluation of a patient with unexplained nephropathy, neuropathy, or retinopathy (abnormal results in this setting do not necessarily indicate a cause and effect relationship and further studies will beneeded to exclude conclusively other diseases)

6- Population studies for epidemiologic deta.

Factors that affect glucose tolerance:

Factors during the test that affect glucose Tolerance:

Posture	Anxiety	Cigarettes	Activity
Nausea	Coffee	Time of day	Amount of glucose ingested

Factors before the test that affect glucose Tolerance:

Carbohydrate intake	Stress
Time of previous food intake	Propranolol
GI surgery and malabsorption	Corticosteroids
Thiazides	Age
Estrogens	Inactivity
Diphenylhydantoin	Weight

When an oral glucose tolerance test is performed for diagnosis of gestational diabetes, the conditions and criteria for diagnosis are different from those when the test is done for the diagnosis of idiopathic diabetes mellitus. 3 hours glucose tolerance test using a 100 g glucose load is used for the diagnosis of gestational diabetes.

Carbohydrate restriction results in a decreased tolerance for glucose and can result in the observence of a diabetic curve in a nondibetic individual. Thus a minimum carbohydrate intake of 150 g (preferably 300 g) for each of the 3 days preceding the glucose tolerance test has been recommended. Illness and trauma can decrease glucose tolerance and the test should not be conducted until at least week after recovery. Pregnancy can also decrease glucose tolerance. Any medication that alters blood glucose levels should also be discontinued prior to testing according to the length of the duration of action of the drift. After the evening meal the patient should be permitted only water. After

administration of the glucose blood and urine specimens are taken exactly 0.5, 1.0, 1.5, 2.0 and 3.0 hours. In testing for carbohydrate reactive hypoglycemia or chemical diabetes in children the test is extended and additional samples are obtained after 4.0 and 5.0 hours. The urine samples are analyzed semiquantitatively and the blood samples are analyzed quantitatively for glucose. If a preservative is not used in collecting the blood samples the plasma or serum must be separated from the cells within 30 minutes after collection of each sample. With the nondiabetic individual all urine specimens should show a negative reaction. Usually unless the blood glucose exceeds 180 mg/dl **(the renal threshold)** glucose does not appear in the urine. Below this level the glucose in the glomerular filtrate is totally reabsorbed in the tubules. Some individuals demonstrate a lower renal threshold for glucose, and even thouht the peak glucose cocenration does not exceed 180 mg and the glucose tolerance curve is normal, glucose is excreted in the urine. In young infants, obese children and elderly adults the peak occurs at 60 minutes, while in other groups of normal children and adults the maximum is achieved in 30 minutes. The mild diabetic may show a peak level within 30-60 minutes, but with more severe diabetes a peak level will be reached later.

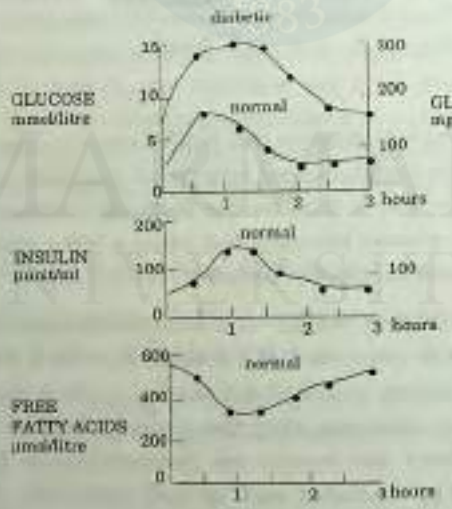

Figure 28: The glucose tolerans test (after 75 g glucose orally). Glucose, insulin and fatty acids concentration in the blood (From ref. 6).

Other Tolerance Tests

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Intravenous glucose tolerance test: Poor absorption of oral glucos may result in a "flat" tolerance curve. Some patients are unable to tolerate a large carbohydrate load orally (vomiting) or may have altered gastric physiology (e.g. gastric resection). In these cases an intravenous glucose tolerance test may be performed. Plasma insulin assays may also be requested in conjunction with the test.

The dose of glucose is 0.5 g/kg of Body weight, given as a 25 g/dl solution administered intravenously wethen 2-4 min. Blood is collected every 10 min for 1 h. if insulin assays are performed a specimen is also obtained 5 min after the start of the enjection.

Epinephrine tolerance test: This test is used to evaluate one form of glycogen storage disease (Type I, von Gierke's disease) This is a deficiency of absence of the enzyme G-6-Phosphatase in the liver. As I said before this enzyme is the catalyst for the final step in the formation of blood glucose from hepatic glycogen, which is normally stimulated by epinephrine. Individuals with von Gierke's disease have low blood glucose, increased liver glycogen, after intravenous administration of epinephrine there is no increase in blood glucose in von Gierke disease.

Lactose tolerance test: A lactose tolerance test can be used to evaluate deficiency of small intestine mucosal lactase. This may be associated with intolerance to lactose and manifested by diarrhea and other symptoms following ingestion of milk.

Tolbutamide test: Tolbutamide (ormase) stimulates the normal pancreas to produce insulin. The test is useful in evaluating hypoglycemic states caused by insulinomas, when a marked decrease in blood glucose to values in the range of 20-30 mg/dl and hypoglycemia persist up to 3 hour. After intravenous injection of this drug, the normal response is a decrease in blood glucose to 50% of the fasting level by 30 min. followed by a return to normal. Patients must be watched carefully for hypoglycemic reactions, in which case the test is terminated by intravenous administration glucose.

DETERMINATION OF GLUCOSE IN BODY FLUIDS

Many analytical procedures have been developed to measure blood glucose. In the past, analyses were often performed with relatively non-specific methods, which resulted in falsely elevated values. Procedures in common use can be classified as **enzymatic** and **colorimetric.**

The enzymatic procedure in commen use are: glucose oxidase hexokinase, and glucose dehydrogenase. The principle of these methods are principle of as follows:

Glucose Oxidase Methods

The enzyme glucose oxidase catalyses the oxidation of glucose to gluconolactone and hydrogen peroxide:

$$\text{Glucose} + O_2 \xrightarrow[\text{Glucose oxidase}]{} \text{D-Gluconolactone} + H_2O_2$$

Addition of the enzyme peroxidase and a chromogenic oxygen acceptor, such as o-dianisidine, results in formation of color that can be measured:

$$\text{o-Dianisidine} + H_2O_2 \xrightarrow[\text{Peroxidase}]{} \underset{\text{(colored)}}{\text{Oxidized-o-dianisidine}} + H_2O$$

Glucose oxidase is highly specific for β-D-glucose. Glucose in solution exists as 36 % ∝ and ~ 64% β form. Complete reaction of glucose, therefore, requires mutarotation of the ∝ to β form. Some commercial preparations of glucose oxidase contain an enzyme mutarotase, that accelerates this conversion. Otherwise, extended incubation time allows for spontaneous conversion.

The second step, involving peroxidase, is much less specific than the glucose oxidase reaction. Various substances such as uric acid, ascorbic acid, bilirubin and glutathione inhibit the reaction, which results in lower values. Standards and knowns should be analyzed simultaneously under conditions such that the rate of oxidation is proportional to glucose concentration. Glucose oxidase are not directly applicable to urine specimens, because uric acid is the interfering substance in urine. But it is suitable for measuring glucose in blood serum and cerebrospinal fluid. Some instruments employ a polarographic oxygen electrode, This electrode measures the rate of oxygen consumption after addition of the sam-

ple to a solution containing glucose oxidase. Normal range for fasting adult plasma or serum glucose is 70-110 mg/dl. For whole blood the range is 60 - 95 mg/dl. There is no sex difference and the ranges given are applicable to children after the first few weeks of life. In the premature newborn, plasma levels may range from 25 to 80 mg/dl. In the full-term infant, expected values are 30-90 mg/dl. Fasting plasma glucose values increase about 2 mg per decade in the adult years. Postprandial values increase at the rate of 4 mg/dl per decade.

Hexokinase Methods

Hexokinase is an enzyme that catalyzes phosphorylation of glucose by adenosine triphosphate (ATP) to form glucose 6 phosphate and ADP. To follow the reaction a second enzyme, G-6-P dehydrogenase (G6PD is used to catalyze oxidation of G-6-P by NADP to form NADPH in proportion to the glucose originally present. The increase in absorbance at 340 nm due to NADPH provides a measure of glucose content.

$$\text{Glucose} + \text{ATP} \xrightarrow{\text{Hexokinase}} \text{D-G-P} + \text{ADP}$$

$$\text{Glucose-6-P} + \text{ADP}$$

$$\text{G-6-P} + \text{NADP} \xrightarrow{\text{G6PD}} \text{6-Phosphogluconate} + \text{NADPH} + \text{H}^+$$

This is a generally accepted reference method based on this principle has been developed and validated. In this method serum or plasma is deproteinized by adding solutions of barium hydroxide and zinc sulfate. The clear supernatant is mixed with a reagent containing ATP, NAD^+, hexokinase, and G-6-PD. The mixture is incubated at 25 C until the reaction is complete and the absorbance related to the production of NADH is measured at 340 nm. Standards and blanks are carried through the entire procedure, including the deproteinization step.

O-Toluiding Methods

Various aromatic amines react with glucose in hot acetic acid solution to produce colored derivatives. Among those used are aniline, benzidine, and o-toluidine Sines the others are suspected carcinogens, only o-toluidine finds wide use.

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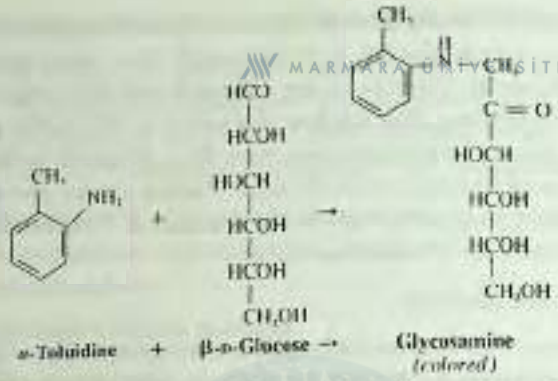

Moderate hemolysis of the specimen does not interfere significantly with the toluidine method for measuring glucose. Bilirubin contributes some increase in absorbance because it is partially converted to the green pigment biliverdin. This limitation of the method must be considered when measuring glucose in serum of infants with elevated bilirubin levels. EDTA in concentration > 1 g/ml and sodium fluoride at levels 5 mg/ml in the specimen will cause some increase in color. Thymol preservative should be avoided since it inhibits color formation. Reference ranges for the o-toluidine is the same as glucose oxidase method and other enzymatic methods, since all three methods give similar results. In patients wih uremia, somewhat higher values are obtained with the o-toluidine method.

Oxidation-Reduction Methods for Glucose Analysis

Most older established methods for measurement of serum glucose were based on the ability of glucose to directly reduce cupric ions (Cu^{++}) to monovalent couprous ions (Cu^{+}). In the presence of heat, the reduced cuprous ions can form cuprous oxide (Cu_2O) which can be detected by a variety of methods. The most popular methods related with reduction are;

Somogyi-Nelson: These autors introduced the use of arsenomlybdate to determine the Cu^{+} formed in the oxidation of glucose. Arsenomolybdate yields a more stable and intensely colorde complex than that obtained with phosphomolybdate. Principle of this method is as follow:

If it is used phosphomolybdate instead of arsenomolibdate it is called Folin-wu method. In both copper reduction is done.

Copper reduction **Principle**

phosphomolbdate (Folin-Wu)

$$Cu^{++} + \text{Glucose} \xrightarrow[OH^-]{\text{heat}} Cu_2O \text{ (colored)}$$

Arsenomolybdate (Somogyi-Nelson)

$$Cu^{+} + \text{Molybdate } (Mo^{++}) \longrightarrow \text{Blue molybdenum complexes}$$

These methods are quantitative. These give large positive because of chemical interference by other sugars creatine, ascorbic acid and other compounds.

Alkaline ferric reaction

In hot alkaline solution yellow ferricyanide ($Fe(CN)_6^{-3}$) oxidizes glucose, and clorless ferrocyanide ($Fe(Cn)_6^{-4}$) is formed.

This reaction gained use as a tool for measuring glucose because ferrocyanide is less susceptible to reoxidation by air than is Cu+. Hagedorn-jensen introduced the technic in which glucose is oxidized with a known excess of ferricyanide, and the unchanged ferricyanide is reacted with potassium iodiate to form iodine.

Later it is titrated with thiosulfate. The difference obtained in titration of the blank and sample is proportional to the glucose. Spectrophotometric measurement of the ferricyanide was introduced.

Reactions:

$$\text{Glucose} + Fe(CN)_6^{-3} \xrightarrow[\text{heat}]{OH^-} Fe(CN)_6^{-4} + \text{Sugar acids}$$

$$2Fe(CN)_6^{-3} \text{ (excess)} + 2HI \longrightarrow 2Fe(CN)_6^{-4} + I_2$$

$$2S_2O_3^{-2} + I_2 \longrightarrow 2I^- + S_4O_6^{-2}$$

Measurement of ferrocyanide

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$$\text{Glucose} + \underset{\text{(yellow)}}{FeCN)_6^{-3}} \xrightarrow[\text{heat}]{OH^-} \underset{\text{(colorless)}}{Fe(CN)_6^{-4}} + \text{Sugar acids}$$

or

$$Fe(CN)_6^{-4} \text{ formed} + \text{Reactant (e.g. } Fe^{+3} \longrightarrow \underset{\text{(Blue)}}{\text{Colored complex}}$$

Condensation Methods

- Aromatic amine reaction

-o-Toluidine (see page 82)

- Phenol reaction (e.g. Anthrone)

$$\text{Glucose} \xrightarrow[\text{Heat}]{H_2SO_4} 3H_2O + HOCH_2-\underset{O}{\text{(furan ring)}}-\text{CHO (HMF)}$$

hydroxymethylfurfural (HMF) is formed from glucose in hot strongly acidic solutions. The aldehyde group of this roduct condenses with a phenol to yield a colored compound that can be quantitated spectrophotometrically.

$$\underset{\text{+}}{\underset{\text{Antrone}}{\text{Hydroxymetyl furfunal}}} \longrightarrow \underset{\text{product}}{\underset{\text{colored}}{\text{Green}}}$$

Other phenols that have been utilized include resorcinol, thymol α-napthol, m-aminophenol and phloroglucinol.

Home Monitoring of Blood Glucose

Home monitoring of blood glucose is useful for all patients with diabetes, but is especially recommended for the followig:

- diabetic women during pregnancy,

- users of insulin infusion pumps,

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Patients with renal disease and patients with unstable, insulin dependent diabetes. Two methods are described briefly; both depend on the glucose oxidase-peroxidase chromogenic reaction. In both methods, a small surface area of the test strips is impregnated with combined reagents in dry form.

In these methods a drop of blood is placed on the reagent pad, let stand exactly 60 second, then washed off for 2 second with water from a wash bottle. The strip is read on a reflectance colorimeter (several models are available). The meter must becalibrated and controlled daily prior to use. To obtain valid results, the test must be performed exactly as directed. Readings must be made immediately, since the color is unstable, hence, the strip may not be kept for later validation.

Testing urine for glucose is a simple but unreliable guide since the renal threshold must be exceeded before glucose appears in the urine. Renal thresholds very considerably tend to be higher in diabetic individuals, especially in elderly person who have decreased glomerular filtration rates associated with atheriosclerosis and glomerulosclerosis. Insulin pumps, which now replace daily injections for many patients, should not be used without frequent monitoring of blood glucose.

KETONE BODIES

The metabolism of fatty acids results in the formation of a small amount of acetoaceate, which is subsequently metabolized in peripheral tissues. When there is carbohydrate deprivation (e.g. starvation) or decreased utilization of carbohydrates eg. diabetes mellitus, there is increased production of acetoacetate, which may exceed the capacity of peripheral tissues to metabolize this compound. Thus, acetoacetate accumulates in blood. A small part is converted to acetone by spontaneous decarboxylation and the greater part is converted to β-hydroxybutyrate in accordance with the following reactions:

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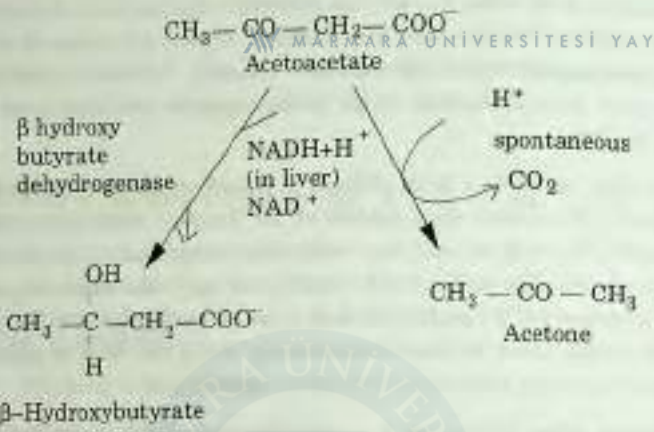


Blood concentrations of these chemicals are present vary: average figures are 78% β hydroxybutyrate, 20% acetoacetata and 2% acetone. None of the most commonly used methods for the detection and determination of ketone bodies in serum or urine react with all three ketone bodies. Tests employing nitroprusside are 15-20 times more sensitive for acetoacetate than for acetone and give no reaction at all with β-hydroxybutyrate. Thus, the tests to be described essentially detect or measure acetoacetate only. Test for β hydroxybutiryrate are indirect. They require brief boiling of the urine to remove acetone and acetoacetate by evaporation (acetoacetate first breaks down spontaneously to acetone), followed by gentle oxidation of β hydroybutyrate to acetoacetate and acetone with peroxide, ferric ions, or dichromate.

Excessive formation of ketone bodies results in increased blood concentrations (**ketonemia**) and increased excretion in the urine (**ketonuria**). This process is observed in conditions associated with a decreased intake of carbohydrate, such as starvation, digestive disturbances, dietary imbalance, and frequent vomiting. **The most frequent** cause of excessive ketone formation is decreased utilization of carbohydrates, as in diabetes mellitus.

Diabetic ketosis can be thought of as an extreme of the of metabolism seen during fasting. In diabetic ketosis, large amount of oxoloacetate are continually being formed in the liver, due to the exaggerated glu-

coneogenic response that occurs in the absence of insulin, at the same time larger amounts of acetyl CoA are being formed due to the exaggerated ketogenic response that is also occurring As it is shown in Figure 29. However, these do not condense to form citrate to be oxidized by the citric acid cycle. In the absence of insulin there is no regulator to keep the action of hormones such as glucocorticoids, glucogon, ACTH, and growth hormone within.

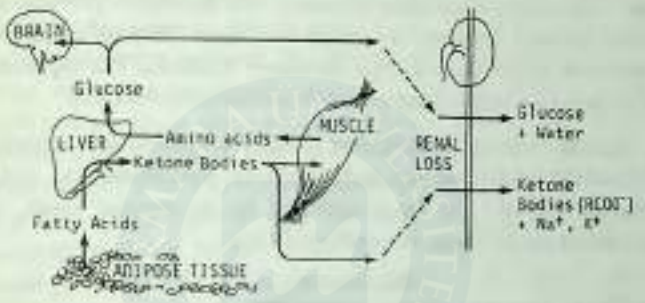
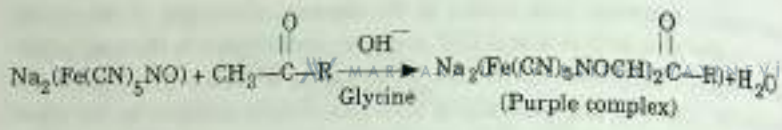


Figure 29: Diabetic ketosis with loss of cations, water and glucose. (From ref. 6)

Semiquantitative determination of ketone bodies in plasma or urine is a helpful guide in the treatment of diabetic ketoacidosis. Excess production of ketone bodies in the diabetic leads to ketoacidosis with a decrease in plasma pH and bicarbonate. Treatment of such patients must be directed to the metabolic abnormalities and to the associated acid-base and electrolyte problems.

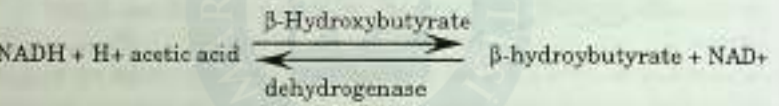
Determination of Ketone Bodies in Serum and Urine

The reaction between ketones and nitroprosside (sodium nitroferricyanide) under alkaline conditions is a widely used procedure. Acetoacetic acid and acetone both form a purple color in this reaction. It is most frequently used as a semiquantitative measure of ketones in serum and urine.



This assay is found in the form of a paper impegrated with nitroprusside, glycine, and sodium phosphate (Ketostix). The phosphate provides a suitable buffered pH, enabling the reaction to occur. This technique is also incorporated into multitest urine chemistry dipsticks. The dipsticks are most accurate for urines with a specific gravity of 1.010-1.020.

Several techniques have been recently developed that measure the individual ketone bodies with high specificity. Acetoacetic acid and beta-hydroxybutyric acid can be quantitated by an enzyme assay, which makes use of the following reversibl reactions



Well-centrifuged urine, serum or plasma can be used for the semi-quantitative nitroprusside test. Serum or plasma can be used for the enzymatic assays as well. Reference range for serum acetoacetate of 5 to 30 mg/L, in enzymatic procedure it is 3 to 23 mg/L. There does not appear to be any age-related differences in reference ranges for ketone bodies. At these levels, serum and urines will be negative by the nitroprusside semi-quantitative secreening tests.

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Interpretation of ketone bodies:

Acetoacetic acid, acetone, and beta-hydroxybutyric acid accumulate in large amounts when the liver's capacity to metabolize the final oxidative catabolite of fatty acids, acetyl coenzyme A This occurs in states of starvation, impaired carbohydrate metabolism (such as diabetics) or acute ethanol abuse. In these states, the oxidative capacity of liver cells is diminished and the ratio of NAD/NADH is greatly decreased, resulting in an inability to oxidize or transfer acetyl CoA or to allow metabolism by the Embden-Meyerhof pathway. The type and amount of the ketone bodies formed depend on the oxidative state of the cells. In early, severe diabetic ketoacidosis or in acute alcoholic ketoacidosis, the cells are in a more reduced state (that is, decreased NAD/NADH ratio) and beta hydroxybutyric acid is usually the predominant ketone body form. If these states are associated with a concomitant lacticacidemia, there can be an even greater tendency toward production of beta hydroxybutyric acid from acetoacetic acid. As the biochemical status of the diabetic ketoacidotic patient improves and the NAD/NADH ratio increases, the betahydroxybutyric acid levels decrease while the levels of acetoacetic acid increase. Generally, the reported proportions of total serum ketone bodies are 2% acetone, 20% acetoacetic acid and 78% beta-hydroxybutyric acid.

Serum pyruvate and lactate levels will change in response to the oxidative-reductive state of peripheral tissues. They do not to change dramatically because of the biochemical abnormalities associated with diabetic ketoacidosis. If the cell's NAD/NADH ratio decreases, because of tissue anoxia, the **lactate pyruvate** ratio will increase.

Clinical Significance of Lactate and Pyruvate

Lactic acid, present in blood entirely as lactate ion. It is an intermediary product of carbohydrate metabolism and is derived mainly from muscle cells and erythrocytes. It is normally metabolized by the liver. The blood lactate concentration is affected by its rate of production as well as its rate of metabolism. During exercise, lactate levels may increase significantly from an average normal concentration of 0.9 mmol/L to 12 mmol/L.In normal conditions ratio of lactate to pyruvate remains approximatiely 6.1 or 7.1.

Severe oxygen deprivation of tissues results in a blokage of aerobic oxidation of pyruvic acid in the TCA and subsequent glycolytic reduction of pyruvate to lactate. This leads to a severe acidosis, called latic acidosis, which is associated with an increase in the lactate-pyruvate ratio in blood and lactate levels to as high as 25 mmol/L (225 mg/dl) or more. Such extreme findings are associated with marked hyperpnea, weakness, fatique, stupor, and finally coma. Clinical examples are irreversible stage of shock, diabetic coma without ketosis, and a variety of Ilnesses in the terminal stage. Hypoxia seen in shock, cardiac decompensation, hematological disorders and pulmonary insufficiency are associated with less extreme increases in blood lactate. The liver can normally metabolize more lactate than is produced. In the case of decreased perfusion of the liver, however, removal of lactate by the liver may be significantly reduced. The amount of lactate in cerebrospinal fluid normally parallels blood levels. In cases of biochemical alterations in the central nervous system, however, CSF lactate values change independently of blood values. Increased CSF levels are detected in cerebrovascular accidents, interacranial hemorrhage, bacterial meningitis, epilepsy, and other CNS disorders.

Determination of Lactate in Whole Blood:

Lactate in the peresence of NAD^+ and lactate dehydrogenase (LDH) is oxidized to pyruvate, the NADH formed is measured spectrophoto metrically at 340 nm and serves as a measure of the lactate concentration.

$$\text{L-Lactate} + \text{NAD} \xrightleftharpoons{\text{LDH (pH 9-9.6)}} \text{Pyruvate} + \text{NADH} + \text{H}^+$$

Fasting venous blood has a lactate concentration of 0.5-1.3 mmol/L (5-12 mg/dl). Arterial blood contains 0.36-0.75 mmol/L (3-7 mg/dL) Normal 24 h urine output of lactate is 5.5-22 mmol/day

Determination of Pyruvate in Whole Blood:

This is the reverse reaction which used in the lactate procedure.

$$\text{Pyruvate} + \text{NADH} + \text{H} \xrightleftharpoons{\text{LDH (pH 7.5)}} \text{Lactate} + \text{NAD}^+$$

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Fasting venous lood has a pyruvate concentration of 0.03-0.10 mmol/L (0.3-0.9 mg/dl). Arteril blood contains 0.2-0.7 mg/dl. Normal 24 hour urine output of pyruvate is 0,1 mmol/day o less.

SOME PROPERTIES OF INSULIN

Insulin is stored as an inactive precursor which is called proinsulin. It has 9000 mol. weight. It contains a single polypeptide which has 3000 daltons. It is known as C-peptide. Proteolytic cleavage of the proinsulin polypeptide releases one molecule of insulin and one molecule of the C-peptide. Molecular weight of active insulin is 6000 daltons and it contains 51 amino acid. It is composed of two chains (A and B), They are joined by disulfide bridges. The insulin molecules of humans, horses, cattle, sheep, pigs and rabbits are biologically and immunologically similar.



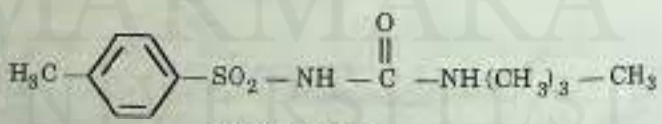
Figure 30 : Structure of human proinsulin and C-peptide (From ref. 10).

The human pancreas secretes 40-50 units of insulin daily, which represents about 15-20 % of the hormone stored in the gland. Insulin secretion is an energy requiring process that involves the microtubuler microfilament system in the cells of the islets. A number of mediators have been implicated in insulin release. **These are;**

Glucose: an increase in plasma glucose concentration is the most important physiologic regulator of insulin secretion. Insulin secretion in response to glucose is biphasic. There is an immediate response that begins within 1 minute and lasts for 5-10 minutes. This is followed by a more gradual, prolonged second phase Glucose is much more effective in releasing insulin when given orally than intravenously. Various gastrointestinal hormones including secretin, cholecystokinin, glucagon and gastrin have been implicated in insulin release.

Hormonal Factors: Various hormones affect insulin release, adrenergic agonists stimulate insulin release, probably by increasing intracellular cAMP. Chronic exposure to excessive levels of growth hormone, cortisol, placental lactogen, estrogens and progestins also increases insulin secretion. It is therefore not surprising that insulin secretion increasese markedly during the later stages of pregnancy.

Pharmacologic Agents: Many drugs stimulate insulin secretion. But the sulfonylurea compounds are used most frequently for therapy in humans. In the treatment of Type II (NIDDM) tolbutamide is widespread used.



Tolbutamide

Physiologic effects of insulin

In spite of adequate caloric intake, polyuria, polydipsia and weight loss are the major symptoms of insulin deficiency. Because insulin effects different metabolic pathways. There are summarised as follows:

A-Effects on membrane transport: D-Glucose and other sugar with a similar configuration at the C_1-C_3 positions (galactose, D-xylose and L-arabinose) enter cells by carrier mediated facilitated diffusion,

This process is enhanced in many cells by insulin. Insulin does not promote the facilitated diffusion of glucose into hepatocytes, but it indirectly enhances action of glucokinase.

Insulin also promotes the entry of amino acids into cells, particularly, on muscle, and enhances the movement of K^+, Ca^+, nucleosides and inorganic phosphate. These effects are independent of the action of insulin on glucose entry.

B.Effects on Glucose utilization: Insulin influences the intracellular utilization of glucose in number of ways, such as glycolysis, converted to fat, converted to glygogen.

C-Effects on glucose production (Gluconeogenesis)

D-Effects on Lipid Metabolism: Insulin also is a potent inhibitor of lipolysis in liver and adipose tissue and thus has an indirect anabolic effect. This is due to the ability of insulin to decrease tissue cAMP levels. Insulin also inhibits hormone sensitive lipase activity. Insulin therefore decreases circulating free fatty acids. In patients wth insulin deficiency lipase activity increases, resulting in enhanced lipolysis and increased concentration of free fatty acids in plasma and liver. In poorly controlled diabetics level of choesterol and consequently VLDL and LDL are often elevated. Accelerated atherosclerosis, a serious problem in many diabetics, is attributed to this metabolic defects.

E: Effects on Protein Metabolism: Insulin has an anabolic effect on protein metabolism. It stimulates the uptake of neutral amino acids into muscle. The effects of insulin on general protein metabolism in skeletal and cardiac muscle and in liver are thought to be exerted at the level of mRNA translation.

F: Effects on cell replication: Insulin stimulates the proliferation of a number of cells in culture. It may be involved in the regulation of growth in vivo.

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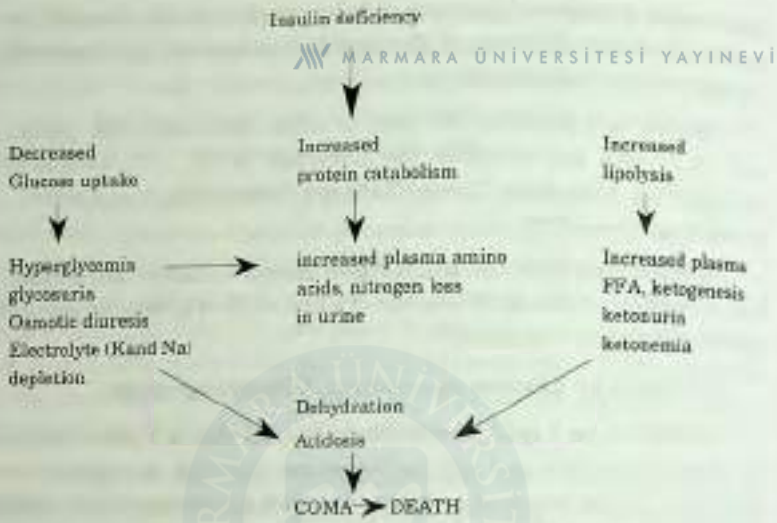

Table 31 : The pathogenesis of diabetic ketoacidosis (From ref. 37)

Insulin deficiency results in hyperglycemia. This is the central biochemical feature of the disease. It is due to impaired transport of glucose into muscle and adipose tissue and to the release of glucose by the liver. Above a blood level of about 160 mg per 100 ml, the renal tubules are unable to absorb all the glucose filtered by the glomeruli. The renal excretion of water and thus produces an osmotic diuresis. Loss of water causes an increase in the serum osmolelity, which stimulates the thirst center in the hypothalemus. The three "polys" of diabetes mellitus (polyuria, polydipsia, and polyphagia) are thus explicable by the body's loss of large quantities of glucose and water with a compensatory increase in hunger and thirst.

More severe insulin deficiency causes, hyperglycemia, glycosuria and ketonemia because of marked increase in lipolysis and serum fatty acids. Both acetoacetic acid and beta-hydroxybutyric acid dissociate to yield hydrogen ions, depressing the plasma pH. at apH level of 7.2, the respiratory center is stimulated and the patient's breathing becomes deep and rapid this hyperventitation is a defensive effort o prevent a fur-

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ther decline in plasma pH. It will be recalled from the Handerson. Hasselbalch equation that pH is determined by the ratio of plasma bicarbonate to

carbonic acid $\frac{HCO_3^-}{H_2CO_3}$ which normally is about 20 to 1. With loss of bicarbonate (utilized to buffer hydrogen ions derived from ketone bodies), this ratio decreases, as does the pH. the increased loss of Co_2 from the lungs reduces plasma carbonic acid and tends to restore the ratio to normal. A further decline in the pH is associated with increasing depression of cerebral function, exentually culminating in coma and death. The major components in the pathogenesis of ketoacidosis are represented schematically in Fig. 30. at several points the consequences of ketonemia and hyperglycemia act synergistically with one another to accelerate the course of events. For example, the cations sodium and potassium are lost into the urine secondary to the osmotic diuresis and in association with the acidic ions. Acetoacetate and beta-hydroxybutyrate. Hyperosmolatlity, dehydration, hypotension, and acidosis effect one another in causing deterioration of cerebral function.

Insulin deficiency prompts protein catobolism which results in the release of nitrogen and potassium into the circulation. With the administration of insulin, potassium re-enters cells along with glucose. Fluid therapy causes exponsion of plasma volume and a return of normal renal function. In the past, patients often did well initially only to develop profound muscle weakness and cardiac dysfunction without apparent cause. With te advent of frequent monitoring of plasma electrolytes, hypokalemia was soon identified as the cause of these complications. They are now readily prevented by the administration of potassium. Although initially ketonemia is the only cause of acidosis, the subsequent occurrence of hypoension, and tissue hypoxia leads to lactate accumulation and a further lowering of the plasma pH. Lactate accumulation sufficient to produce acidosis can also occur in nondiabetic patient severely ill from a variety of causes in which tissue hypoxia is found.

Hyperlipidemia in insulin deficiency

Ketoacidosis and hyperosmolar nonketotic coma are the most dramatic life threatening manifestations of acute insulin deficiency. Infrequently, the uncontrolled diabetic will present with evidence of hyperlipidemia as the major manifestation of disease. There may be widespread

skin eruption. These lesions, which are yellow orange in color, papular and about 2 to 4 mm. in diameter, are eruptive xanthomas. on funduscopic examination, the color of the venules is startling, instead of being deep red, they are white, gray, or link pink. This finding, lipemia retinalis, is directly related to the character of the patient's blood, which is pink or rote when aspirated by venipuncture. after clotting, the appearance of the supernatant serum is indistinguishable from creans. Chemical analysis reveals a marked increase in triglycerides to several thousand mg per 100 ml. Analysis by paper electrophoresis or ultracentrifugation reveals that the increased triglycerides consists mostly of dietary cylomicrons, although endogenous triglycerides are usually present as well. Treatment with insulin promptly increases the activity of enzyme lipoprotein lipase, which is inactive in insulin deficiency, the plasma triplyceride level falls to normal in a short time, and skin and retinal changes disappear.

Hypoglycemia

The common causes of hypoglycemia are;

1) Addison's disease due to impaired gluconeogenesis

2) Liver disease due to impaired gluconeogenesis and glycogen storage.

3) Fasting + alcohol due to impaired glyconeogenesis and depleted glycogen.

4) Insulin dependent diabetes, due to excessive insulin

5) Islet cell adenoma due to excessive unregulated insulin release.

Since hepatic production of glucose sustains the blood level during periods of fast, impaired glycogen storage and glyconeogenesis will lead to fasting hypoglycemia, which typically occurs in the early morning after 8 or 9 hours of fasting. This pattern also seen with hypoglycemia due to insulin-secreting islet cell adenomas. Hypoglycemia, minimized during the weaking hours by constant eating, becomes apparent during the hours of sleep.

The most frequent cause of clinically significant hypoglycemia is insulin self-administration by the diabetic patient. There are many other reasons for hypoglycemia as mentioned above. Regardless of its underlying cause the manifestations of hypoglycemia tend to evolve in a charac-

teristic pattern. With mild hypoglycemia the patient experiences hunger, tremor, perspiration, weakness, blurred vision, and impaired mentation. Mental confussion is often followed by bizarre behavior. The patient may become bellicose and resistant to help. Neuromuscular function becomes impaired. Finally the patient becomes impaired If severe hypoglycemia remains untreated, permanent brain damage or death results. The sequence of event just described is typical, but the exact pattern varies among patients and even in the same patient from one episode to another. For example, on one accasion the patient may wander about in confused state for 2 to 3 hours at approximately the same level of function. another time, he might pass from an alert and rational state to coma in a half hour or less.

Insulin Receptor in The Cell Membrane

Insulin action begins when the hormone binds to a specific glycoprotein receptor on the surface of the target cell. The actions of hormone can occur within seconds or minutes or after a few hours (protein and DNA synthesis and cell growth occurs after few hours; but transport protein phosphorylation, enzyme activation or inhibiton occur within seconds or minutes.) The insulin receptor has been studied in greet detail using biochemical and recombinant DNA techniques. It is a heterodimer consisting of 2 subunits, linked by disulfide bonds. (Fig. 31) Both subunits are extensively glycosylated and removal of sialic acid and galactose decreases insulin binding and insulin action. The δ subunit has 135.000 MW is entirely extracellular and it binds insulin. The β subunit 95.000 MW is a transmembrane protein and performs the second major function of a receptor e.g. signal transduction. The cytoplasmic portion of the subunit has an autophosphorylation site.

The insulin receptor is consistantly being synthesized and degraded and its half life is 7-12 hours. The receptor is synthesized as a single chain peptide in the rough endoplasmic reticulum and is rapidly glycosylated in the Golgi region. The precursor of the human insulin receptor has 1382 amino acids, has a molecular weight of 190.000 and it is cleaved to form the mature α and β subunits. The human insulin receptor gene is located on chromosome 19. Insulin reseptors are found on most mammalila cells in concentration of up to 20.000 per cell. **When insulin binds to the receptor several events occur:** 1) There is a conformational change of the receptor, 2) the receptors cross link and form micro-

aggregates, patches, or caps, 3) the receptor is internalized and 4) some signal is generated. In conditions in which plasma insulin levels are high (obesity or acromegaly) the number of insulin receptors is decreased and target cells become less sensitive to insulin.

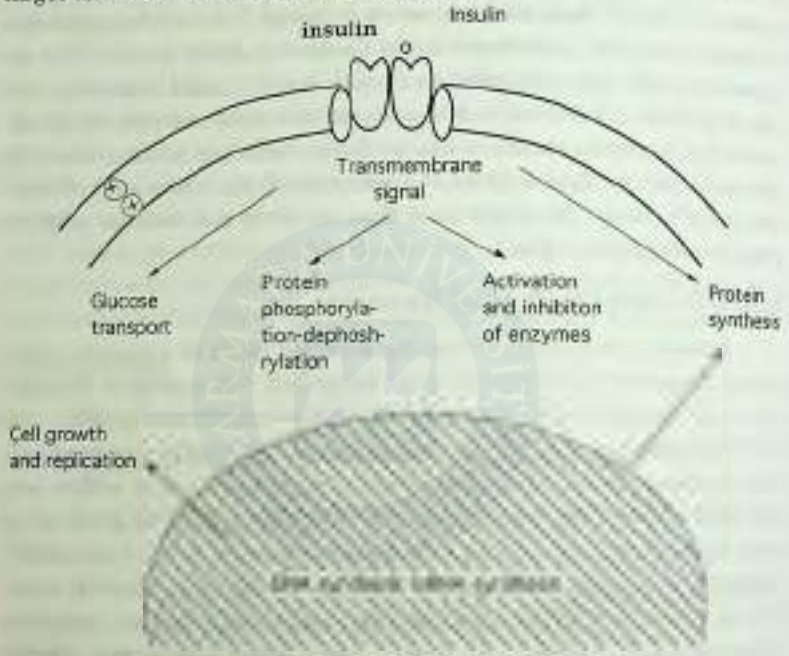

Figure 32 : Relationship of the insulin receptor to insulin action (From ref. 26)

Biosynthesis of Insulin:

Insulin is synthesized as a preprohormone (MW about 11.500). The sequence of subcellular location of insulin is different. It is found as linear form in the polysome during protein synthesis. Later, in the endoplasmic reticulum folded proinsulin occurs by proper disulfide bridges. As shown in Figure 32 The proinsulin molecule undergoes a series of site-specific peptide cleavages that result in the formation of equimolar amounts of mature insulin and C peptide. These enzymatic cleavages, start with a protease with trypsinlike activity that cleaves at the carbox-

yterminal. A second enzyme, with carboxypeptidase B like activity then removes the C terminal basic amino acids from what will become insulin and the C peptide. In the secretion granule Insulin and C peptide are found in equimolar concentration.



Figure 33: The formation of secretion granule from proinsulin.

In the secretion granule insulin and C peptide are found in equimolor concentration. C peptide has no known biologic activity. It is a distinct molecule from an antigenic standpoint. Therefore, C peptide immunoassays can distinguish insulin secreted endogenously from insulin administered exogenously and can quantitate the former when antiinsulin antibodies preclude the direct measuremeat of insulin.

Methods for Insulin Analysis

The analysis of insulin is very important in clinical chemistry. This substances is first measured by RIA methods. It is used widely but there is others. Other assays include insulin radioreceptor methods using cultured leukocytes, bioassays that measure the stimulation of glucose oxidation, and most recently enzyme immunoassay (EIA).

In RIA, radioactive iodine (^{125}I)-labeled insulin competes with sample insulin for binding sites on anti-insulin antibodies. Separation of bound from free ligand can be accomplished by double - antibody precipitation, solid phase and so on. Many persons who have received diabetic therapy have circulating endogeneous antibodies to insulin. These antibodies may increase or decrease the apparent insulin concentration because of an effect on the binding antibody or on the separation step. The endogenous antibodies can be measured and can be removed by precipitation. So, RIA can be affected by endogenous antiinsulin antibodies. For assay purposes, antibodies to insulin are usually raised in guinea pigs, using purified porcine insulin. Because human and rabbit insulin are very similar, antisera developed in rabbits generally do not have a high affinity for human insulin. Insulin is sufficiently immunogenic to initiate an antibody response without being covalently linked to a carrier. Insulin is readily labeled with radioactive iodine using the chloramine-T procedure. Separation of bound and free labeled insulin has been carried out by use of most available techniques. Such as dextran coated charcoal, double-antibody precipitation.

Enzyme immunoassays for insulin show good correlation with RIA procedures but are not so easily performed. This techniques, are heterogeneous assays employing the sandwich technique. Here, antibodies to insulin are adsorbed onto a solid phase (such as disks or beads). Insulin from the sample remains bound to the solid phase antibodies after a washing step.

Assays for C-peptide are used for detecting self-induced hyperinsulinism, since pharmaceutical preparations of insulin do not contain C,peptide. C peptide RIA development is difficult, since the molecule does not contain tyrosine, and synthetic or tyrosylalated molecules must be used.

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For routine insulin assay, RIA is the approach of choice. The selected method should not employ an antibody that cross-reacts more than 1% with C peptide or growth hormone. With dextran-coated charcoal used to separate bound from free insulin, endogenous antibodies bind tracer, falsely lowering the values. Double-antibody separation methods may give falsely increased values in the presence of endogenous antibodies. Therefore an appropriate method for removal of endogenous antibodies should be available. A method for the detection of insulin antibodies is sometimes required. For this purpose, the method of Gerbitz and Kemmler is recommended.

Insulin concentrations are identical in serum and plasma. The hormone is stable for at least 12 hours at room temperature, for a week at 4 C., and for a month at - 10 C.

Insulin values in fasting individuals are usually less than 25 µU/ml (1042 pg/ml). During a glucose tolerance test the insulin value usually peaks between 50-100 µU/ml (2083 to 4167 pg/ml). It follows the glucose curve by about 15 minutes. In hypoglycemic attacks in which glucose level goes below 50 mg/dl the insulin should be suppressed below 20 µU/ml during hypoglycemia, the glucose/insulin ratio should be greater than 25. Otherwise, an insulin-secreting tumor should be suspected.

Interpretation:

Serum insulin levels can be increased in type 2 diabetes, hyperthyroidism, type IV hyperlipoproteinemia, renal failure, liver cirrhosis, and in generalized acute illnesses. Decreased serum insulin levels are found in type I diabetes and can be associated with pheochromocytoma, malnutrition and cystic fibrosis. However, the findings of increased or decreased insulin levels in these pathological states are incidental findings and rarely of diagnostic use. In type 2 diabetes mellitus have down-regulated insulin receptor may be due to elevated plasma insulin levels. Some individuals produce antibodies directed against their insulin receptors. These antibodies prevent insulin binding to the receptor, so such persons develop insulin resistance. Because there is specificity and selectivity of hormone receptors. Many different molecules circulate in the extracellular fluid, but only a few are recognized by hormone receptors. Receptors must select these molecules from high concentrations of the other molecules. There can be one class of receptor on each cell, or a given cell can have receptors for several hormones.

The primary reason for the measurment of serum insulin is to evaluate fasting hypoglycemia. Neurological and behavioral symptoms are frequently seen in patients with insulin-induced hypoglycemia. The neurological symptoms (excessive sweeting, palpitation, tachycardia, tremor and weakness) are the result of increased epinephrine levels. Another frequently seen cause of insulin-induced hypoglycemia is factitious hypoglycemia, that is, a self-induced hypoglycemia. In this situation, the patients are either purposefully or accidentally overtreating themselves with pharmaceutical preparations of insulin. In this hypoglycemia, the most useful test is a C-peptide measurement. Since pharmaceutical insulin preparations do not contain C-peptide, an elevated serum insulin level accompanied by a normal C-peptide level should raise the suspicion of self-induced hypoglycemia.

The Importance of Glycosylated Hemoglobins for the Diagnosis of Diabetes

In adults, circulating hemoglobin (Hb) typically consists of Hb A (97% of the total), HbA_2 (2.5%) and Hb F (0.5%). Chromatographic analysis of Hb A has shown that it contains a number of minor hemoglobins, identified as Hb A_{1a}, Hb A_{1b}, Hb A_{1c}. These are referred to as Hb A_1, "Fast hemoglobins" glycosylated hemoglobins, glycated hemoglobins, or simply glycohemoglobins. Hemoglobin A consists of four polypeptide chains, two ∝-chains and two β chains. Hemoglobin A is formed by the condensation between glucose and the N-terminal valine amino acid of each β-chain to form an unstable Schiff base (aldimine), which then undergoes an Amadori rearrangement to form a stable ketoamine. (Figure 33) HbA_{1c} is the major fraction of the fast hemoglobins.

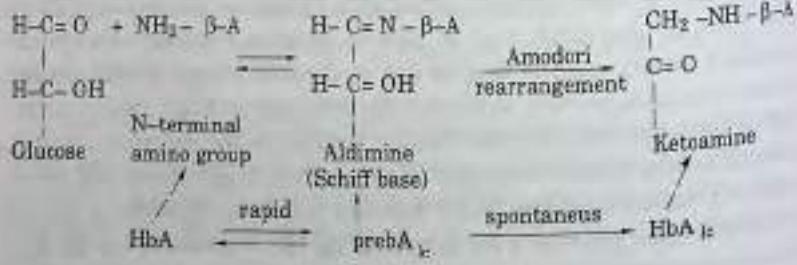

Figure 34: AFormation of HbA_{1c}

The measurement of glycosylated proteins has significantly improved the objective assessment of diabetic control. The measurement of HbA_{1c} has been found to correlate with the degree of diabetic abnormality. No correlation has been observed when the patients have been under optimal glucose control. So nowadays glycosylated blood protein is measured instead of fasting blood glucose.

The formation of the glycohemoglobins is nonenzymatic and occurs over the life span of the erythrocyte (average 120 days). The amount of total Hb A_1 or HbA_{1c} depends on the time-averaged glucose concentration over the two or three months prior to the measurement. This value provides a single objective means of reflecting control of diabetes, unrelated to short term fluctuations in plasma glucose levels. The interpretation of HbA_{1c} values is based on the assumption of a normal erythrocyte life span. Patients with hemolytic disease, or other conditions with shortened erythrocyte survival, have a significant reduction in HbA_{1c}.

Glycohemoglobins (hemoglobins with covalently bound sugars) form nonenzymatically in the red cell in amounts proportional to the cellular glucose level. HbA_1 the major most commonly assayed glycohemoglobin fraction, constitutes 6% to 8% of the total Hb of normal persons but can reach levels of 15% to 18 % in diabetics in poor glucose control. HbA_1, is further separated into subfractions HbA_{1c} is the predominant and best characterized component. HbA_{1c} differs from its parent hemoglobin in that it has a glucose residue covelently linked to the almino group of the N terminal amino acid of each beta-chain. As it is shown above. Only HbA_{1c} reference ranges is 3.0 to 6.0 % mg of total blood Hb vary depending on the method.

Other of Glycosylated Proteins in Diabetics

Nonenzymatic attachment of sugars to amino groups of other proteins also occurs. A stable glycosylated form of serum albumin has been found to be elevated in humans with diabetics. Since the circulating half-life of human serum albumin is 19 days, this suggests that monitoring of glycosylated albumin would provide a more recent time-averaging of blood glucose than would determination of HbA_{1c}.

After glycosylation of hemoglobin was first described a number of tissue proteins were also found to undergo nonenzymatic glycosylation. Because incubation of any protein with physiologic concentration of glu-

cose eventually leads to the formation of glycosylated proteins without the aid of enzymes. This reaction has prompted researchers to investigate various tissue proteins with respect to glycosylation.

TABLE 3 : Alterations in protein function following glycosylation

— Inactivation of enzymes

— Inhibition of binding of regulatory molecules

— Decreased proteolysis

— Recognition and endocytosis of glucose modification of protein by macrophages

— Increased Immunogenicity

— Increased tissue cytotoxicity

Excessive glycosylation causes, undesirable changes in the proteins. After glycosylation proteins known to result in functional defects, for example, hemoglobin acquires an increased affinity for oxygen. Glycosylation of animal lens crystallins has been reported as being an important genesis of cataract in diabetes. Glycosylation has been thought to be the cause of microangiopathy and other complication in diabetes. Table 3 shows some alterations in protein function which occur due to nonenzymatic glycosylation. Some glycosylated proteins and consequent impaired physiological functions can be seen in table 4.

TABLE 4 : Some glycosylated proteins and consequent impaired physiological function

Glycosylated proteins	Refecences		Physiological Function
Hemeglobin	Allen et al.	(1958)	Oxygen Exchange (Hypoxia)
Collagen	Robins and Bailey	(1972)	Structural and hemostasis
Glom.Basement Membrane	Kossing and Cerami	(1975)	Renal filtration Barrier (Nephropathy)
Lens Crystallins	Mannier and Cerami	(1982)	Transmission of light to retina (Cataract)

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Collagen	Schneider et al	(1981)	Structural (Enhanced catabolism)
Platelet membrane protein	Emekli, N.	(1981)	Platelet function (Hyperaggregation)
Red cell membrane protein	Wieland, H.	(1981)	Red cell Stability (Deformation)
Collagen (gingiva)	Yavat and Emekli	(1986)	Structural and hemostasis (Defective agg)
Collagen	Bai et al	(1993)	Structural and hemostase (Alters fibril organisation)

According to our findings the platelet aggregation induced by glycosylated collagen was found to have different lag period. This is shown in figure 35.

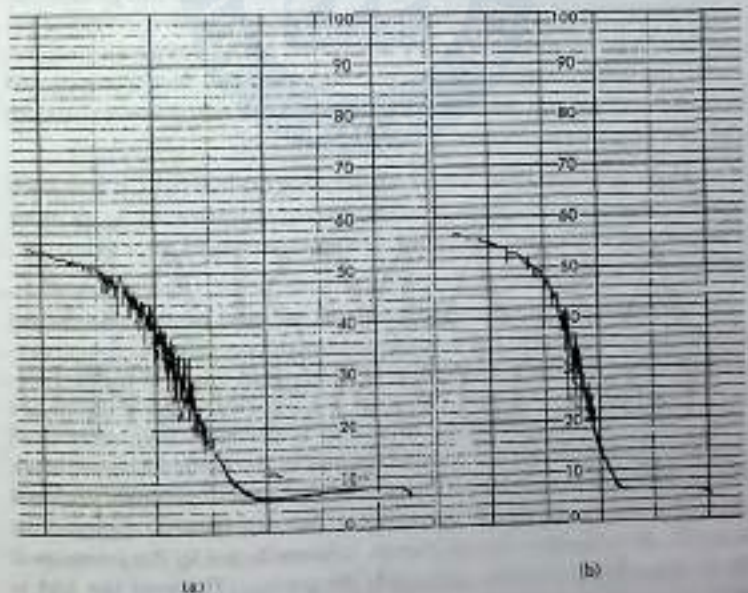

Figure 35: Aggregation of normal platelets by control collagen (a) and collagen from diabetic rats (b) (From ref. 89)

Determination of Glycosylated Proteins

Currently available methods for the determination of glycohemoglobins include ion-exchange chromatography, high performance liquid chromatography, colorimetry, radioimmunoassay, electrophoresis, isoelectric focusing and affinity chromatography.

Ion-exchange chromatography: Short columns are used that contain weakly acidic cation-exchange or carboxymethyl cellulose resin. Glycosylated hemoglobins at a selected ionic strengh and pH of the eluent buffer are less positively charged than Hb A and elute from the column first. A second buffer may be used to elute HbA_{1c}, and the absorbance of the two eluates is used to calculate per cent of total glycocylated hemoglobin. Alternatively, only the Hb A_1 is eluted, and a separate dilution of the original hemolysate is made against which the Hb A_1 is compared. A number of commercial modifications of the procedure are available.

The labile fractions elute with the stable forms and will produce elevated results unless pretreatment of the erythrocytes is included to remove the labile form Hb F typically elutes with HbA_1 and produces falsely elevated results. On the other hand, HbS HbC and their glycosylated derivatives, if present, do not elute. Hence, misleading low values for HbA_1 are obtained in the presence of Hb S and HbC.

High performance liquid chromatograph (HPLC): has been introduced as a method for separating and quantitating HbA_{1c} as other hemoglobin fractions and has since been recommended as a reference method.

Colorimetry: A colorimetric method is based on the observation that HbA_{1c}, when subjected to mild acid hydrolysis, releases 5-hydroxymethylfurfural (5-HMF). Oxalic acid is added to an alqiot of the hemolysate, the mixture is heated at 100 C for 5 h and cooled and proteins are precipitated with trichloroacetic acid. This is the most frequently used nonchromatographic method for the analysis of glycosylated hemoglobin. The supernatant is reacted with thiobarbituric acid to produce a colored solution which absorbance is measured at 443 nm. Because the reaction is specific for ketosamine linked glucose, it is unaffected by the presence of Hb F, hemoglobin variants, and labile Hb pre-A_{1c}. However the test is difficult to standardize because the yield of 5-HMF from Hb A_{1c} is only about 30%. An evaluation of the colorimetric assay indicates that, under controlled conditions, the assay provides reliable result. The reaction is shown below.

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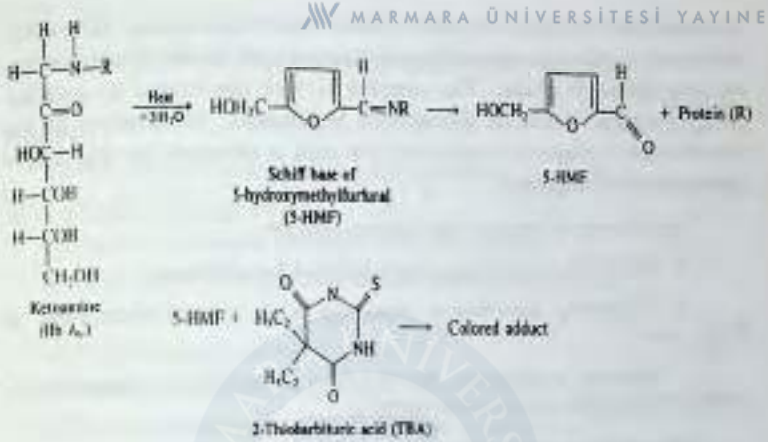

Radioimmunoassay: Sheep antiserum against HbA_{1c} has been prepared and used as the basis for a RIA. Partial cross - reactivity wih HbA_{1c} was noted. At present, the antiserum is not commercially available, and the method has not been fully evaluated.

Electrophoresis: Agar gel electrophoresis on whole blood hemolysas at pH 6.3 has been shown to provide good resolution of Hb A and Hb A_1. In adition, Hb S and Hb C and their glycosylated components are resolved and do not interfere. Hb F migrates to the same region as Hb A_1 and would cause a falsely elevated HbA_1 value. This method includes the labile aldimine as part of the total Hb A_1 fraction.

Isoelectric focusing: Hemolysates are prepared from washed erythrocytes and diluted with a phosphate-glycerol reagent. Ampholines in the pH range of 6-8 are used to establish the gradient in 1 mm thick acrylamide gel slabs. On completion of isoelectric focusing, the gels are fixed in trichloracetic acid and then scanned on a high resolution, integrating microdensitometer. Hemoglobin A_{1c} is adaquately resolved from hemoglobins A_{1a}, A_{1b}, S, and F.

Affinity chromatography: Affinity gel columns are used to separate glycosylated hemoglobin from the nonglycosylated fraction. The gel contains immobilized m-aminophenylboronic acid and crosslinked, beaded agarose. Boronic acid reacts with the cis-diol groups of glucose bound

to hemoglobin to form a reversible 5-membered ring complex, thus selectively holding the glycosylated hemoglobin on the column. Nonglycosylated hemoglobin is eluted. The complex is next dissociated by sorbitol, which permits elution of glycosylated hemoglobin. Absorbances of the two fractions, measured at 415 nm, are used to calculate the per cent of glycosylated hemoglobin.

Advantages of affinity chromatography are

1- No interference from nonglycosylated hemoglobins,

2- Negligible interference from the labile intermediate form of HbA_{1c} and

3- Minimal dependence on variations in ambient temperature in contrast to ionexchange chromatography.

Removal of labile glycohemoglobin from erythrocytes:

The concentration of the labile form of HbA fluctuates rapidly in response to changes in plasma glucose levels. To reflect accurately long-term blood glucose regulation, especially in poorly controlled diabetics, the labile form should be removed prior to assay. This may be accomplished by incubating erythrocytes in saline or buffer solutions at pH 5-6.

Incubation with saline: Collect blood with EDTA anticoagulant, centrifuge and remove the plasma and buffy coat. To 0.5 ml of packed cells add 5 ml of saline (9 g NaCl/L) mix, and centrifuge. Aspirate and discard the supernatant. Add 5 ml of saline mix, and incubate in a 37 C water bath for 5 h. The mixture may be incubated overnight at room temperature, if this is more convenient. Centrifuge and discard the supernatant, add 0.5 ml of saline to the cells, mix, and assay the mixture for glycohemoglobin as if it were whole blood.

Incubation at pH 5: Collect blood with EDTA anticoagulant, centrifuge and remove the plasma and buffy coat. To 0.5 ml of packed cells add 5 ml of citrate buffer (0.1 mol/L, pH 5.) mix, and incubate in a 37 C water bath for 15 min. Centrifuge and discard the supernatant. Add 0.5 ml of saline to the cells, mix and assay the mixture for glycohemoglobin as if it were whole blood.

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PART III

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AMINO ACIDS AND PROTEINS

General Description

Amino acids, as the name implies, are acids carrying amino groups. Amino acids which found in protein are L and D position. The majority of the naturally occurring amino acids are L configuration. They are classified into 7 groups in accordance with the structure of the side chain.

1- Aliphatic side chains amino acids; glycine, alanine, valine, leucine, isoleucine.

2- Hydroxylic (OH) groups side chains; serine, threonine, tyrosine, tyrosine

3- Sulfur atoms side chains; cyteine, cystine, methionine

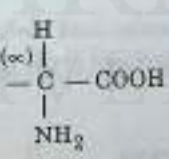
4- Acidic groups or their amides side chains; aspartic acid, asparagine, glutamic acid, glutamine.

5- Basic groups containing side chains; arginine, lysine, histidine,

6- Aromatic rings containing side chains; histidine, phenylalanine, tyrosine, tryptophan,

7- Imino Acids; proline-and hydroxyproline

The general formula for the amino acids is; (1)



(1)

Amino acid have both an amino and carboxylic acid function attached to the same (α) carbon atom. Amino acids have both trivial names (e.g glycine, alanin, valin) and systematic chemical names.

The degradation of proteins to the constituent amino acid monomers yields 20 different amino acids. Glycine is the simplest amino acid (2).

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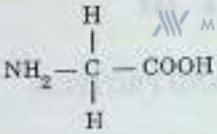
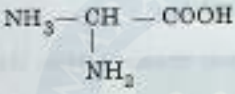


(2)

α – aminoacetic acid (Glycine)

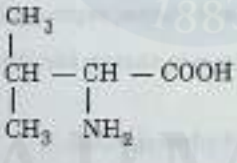
Another monocarboxylic aliphatic amino acid is alanine(3).



(3)

α – aminopropionic acid (alanine)

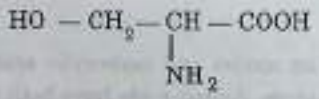
There are also branched-chain aliphatic amino acids, such as valine(4).



(4)

α – aminoisovaleric acid (Valine)

Serine represents an aliphatic amino acid having a hydroxy group. Serine has been encountered as a constituent of the phospholipids(5).



(5)

α – amino – β hydroxypropionic acid (serine)

Amino acids also occur with ∝ thiol group, the sulfur analogue of serine is called cysteine(6).

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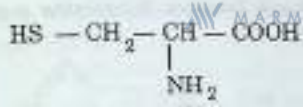
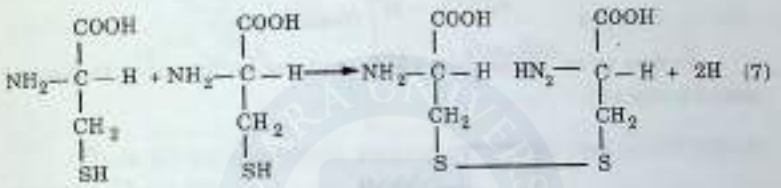


(6)

cysteine

The disulfide amino acid, cystine, is formed by the oxidation of cysteine(7).



(7)

2 molecule cysteine

Cystine

Two amino acids are dicarboxylic: Aspartic and Glutamic acids(8).

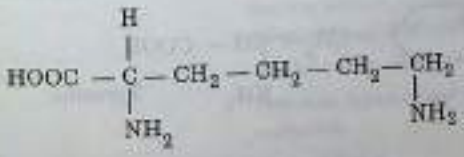


(8)

Aspartic Acid

Glutamic Acid

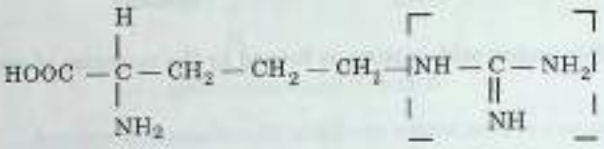
As if to counterbalance this, there are diamino groups such as lysine(9).



(9)

Lysine

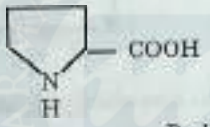
Arginine, is extremely basic because it contains a guanido group (10).



(10)

Arginine Guanido group

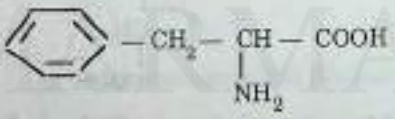
Proline is an amino acid which contains imino group instead of amino group(11).



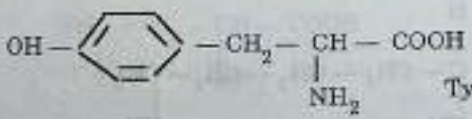
(11)

Proline

Phenylalanine and tyrosine represent two of the aromatic amino acids (12, 13).

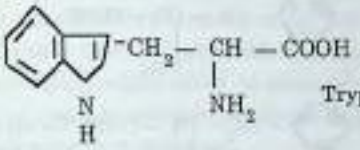


Phenylalanine (12)

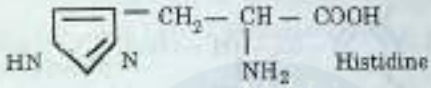


Tyrosine (13)

There are also heterocyclic amino acids such as tryptophan and histidine(14 and 15)



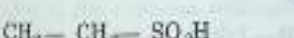
Tryptophan (14)



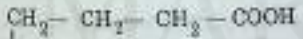
Histidine (15)

There are some naturally occuring amino acids that do not occur in proteins. These are,

βAlanin (3-aminopropanoic acid) — Found in coenzyme A and pantotheine vitamine



Taurine (2 amino ethylsulfonic acid) — Part of bile acid



(GABA - (γ aminobutyric acid) — GABA is a neurotransmitter formed from glutamate in brain tissue

β-aminoisobutyric acid	End product in pyrimidine metabolism; found in urine of patients with an inherited metabolic disease.
Homocysteine	Methionine biosynthesis
Homoserine	Threonine, aspartate and methionine metabolism
Cysteinesulfinic acid	Rat brain tissue
Ornithine	Urea cycle intermediate
Citrulline	Urea cycle intermediate cycle
5-Hydroxytryptophan	Precursor of serotonin

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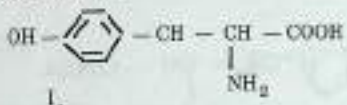
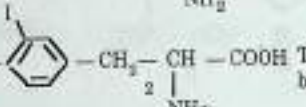
Dopa (3,4-dihydroxyphenylalanine) Precursor of melanin

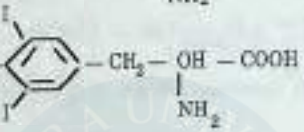


3-monoiodotyrosine



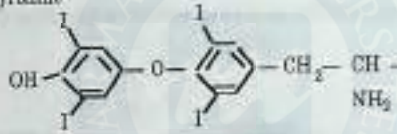
Thyroid tissue and blood serum

3,5 diiodotyrosine



In association with thyroid globulin

3,5,3' Triodothyronine



Thyroid tissue

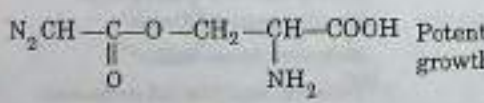
THyroxine
3,5,3',5', tetraiodothyronine



In association with thyroid globulin

Azaserine

This is not a naturally occuring amino acid, but listed here because of its importance as an inhibitor at various steps of purine biosynthesis



Potent inhibitor of tumor growth

Proteins may be broken down to their monomer units by acid, base, or enzyme catalyzed hydrolysis. Complete hydrolysis of most proteins produces approximately 20 different α –amino acids. With a single exception (glycine), Each amino acid has at least one asymmetric carbon

atom and hence is optically active. It can rotate the plane of planepolarized light. Although some of the amino acids found in proteins are dextrorotatory and some are found in lavatory at pH 7.0 All have absolute configuration comparable that of L-glycealdehyde and hence are L – α – amino acids. Although D-amino acids do occur in cells and even in polypeptdies (eg. in polypeptide antibiotics elaborated by certain microorganisms) they are not present in proteins.

Some amino asids such as, threonine, isoleucine, 4-hydroyproline and hydroxylysine each have 2 asymmetric carbon atoms and therefore exist in 4 isomeric forms.

Various other amino acids in free o combined states fulfill important roles in metabolic process other than as consitutents of proteins. Many additional amino acids occur in plants or in antibiotics. Over 20 D-amino acids occur naturally. These include the D-alanine and D-glutamic acid of certain bacterial cell walls and a variety of D-amino acids in antibiotics.

Chemical reactions of amino acids

There are a number of interesting reactions involving amino acids. One is the reaction with acids and bases. There is a basic group and acidic group in amino acid molecule. The intramolecular reaction occurs in solution so that amino acids always exist in an ionic form. Therefore, the previous structures for amino acids are actually incorrect. They should be shown as zwitterions or as specific salts. For instance, if an amino acid is isolated from a HCL solution it would be a hydrochloride salt(16).

$$\overset{-}{C}l\,\overset{+}{N}H_3—\underset{\displaystyle R}{\underset{|}{CH}}—COOH \qquad \text{Amino acid hydrochloride} \quad (16)$$

Simlarly, if it is isolated from a NaOH solution it would be a sodium salt(17).

$$NH_2—\underset{\displaystyle R}{\underset{|}{CH}}—\overset{-}{C}OO\ \overset{+}{N}a \qquad (17)$$

In general, amino acids are not shown in the correct ionic or salt form. This improper representation is due to the fact that it is more convenient to represent the structures as nonionized molecules. This is particularly true in the writing of reactions, since accounting for the various ionic species is thereby eliminated. This type of incorrect representation is common among biochemists. Compounds such as amino acids that form intramolecular salts are called zwitterions, which from the German means "bastard ion". When a zwitterion is treated with a stronger acid the carboxyl group will accept a proton, neutralizng its negative charge, and a net positively charged amino acid results. "bastrad ion" When a zwitterion is treated with a stronger acid the carboxyl group will accept a proton, neutralizing its negative charge, and a net positively charged amino acid results(18).



(18)

Similarly, reaction of the zwitterion with a base will result in the removal of the amino group proton, forming a negatively charged ion(19).



(19)

Thus, the form and molecular charge on the amino acid salt is regulated by the hydrogen ion concentration or pH of the solution. The carboxyl groups of amino acids can be esterified(20).



(20)

Furthermore, the amino group can be acylated, here with acetlyl chloride, to yield the N-acetyl derivative(21).

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In general, then the amino and carboxyl groups of amino acids can form derivatives the same as simple carboxylic acids and amines.

Amino acids react with nitrous acid to yield the unstable diazonium compound, which then undergoes spontanious decomposition to give the corresponding alcohol.

This reaction is of considerable importance in biochemistry because it can be used to quantitative amino acids. The amount of amino acid can be determined by measuring how much nitrogen is released, as there is one mole of nitrogen released for each mole of amino nitrogen. It is nonspecific technique and measures the total number of amino groups present and does not distinguish one amino group from another.

The carboxy and amino group of amino acids exhibit all the expected reactions of these functions. A variety of color reactions specific for particular functional groups in amino acids are known. These are useful in both the qualitative and quantitative identification of particular amino acids combined in peptide or proteins, As shown in below;

Color Reactions for specific amino acids

Arginine	Sakaguchi reaction	α-Naphthol and sodium hypochlorite	Red
Cysteine	Nitroprusside reaction	Sodium nitroprusside in dilute NH_4OH	Red
Cysteine	Salivan reac.	Sodium 1,2 naphthoquinone-4-sulfonate and sodium hydrosulfite	Red
Histidine,	Pauly reaction	Diazonized sulfanilic acid in alkaline solution	Red
Tryptophan	Glyoxylic acid re. (Hopkins-Cole reac)	Glyoxylic acid in 36N H_2SO_4	Purple

Tryptophan	Ehrlich reaction	p-dimetilaminobenzaldehid in 12 N HCl	Blue
Tyrosine	Milon reaction	$HgNO_3$ in HNO_2, heat	Red
Tyrosine	Folin Ciocalteu reaction	Pposphonolibrotuhgstic acid	Red
Tyrosine Tyrptophan Phenylalanine	Xantoprotein reaction	Boiling concentrated HNO_3	Yellow

Ninhydrin gives general reaction with amino acids. It is a powerful oxidizing agent, causes oxidative decarboxylation of α-amino acids, producing CO_2, NH_3 and an aldehye with carbon atoms of amino acids. The reduced ninhydrin then reacts with the liberated ammonia, forming a blue complex which maximally absorbs light of wavelength 570 nm. The intensity of the blue color produced under standard conditions is the basee of an extremely useful quantitative test for α-amino acids. Amines other than α-amino acids also react with ninhydrin, forming a blue color but without evolving CO_2. The evolution of CO_2 is thus indicative of an α-amino acid. NH_3 and peptides also react with ninhidrin but it is more slowly than do α-amino acids. Ninhidrin reaction of amino acids is shown in below proline and 4-hydroxyproline produce a yellow rather than a purple color with ninhydrin.

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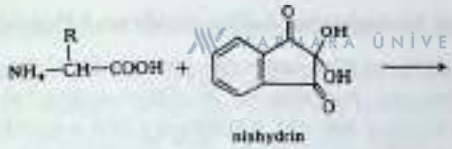
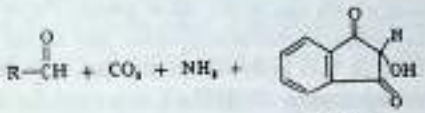
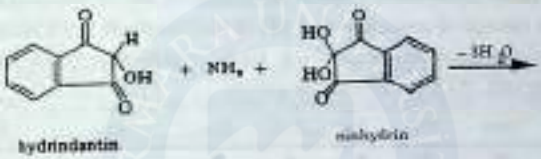


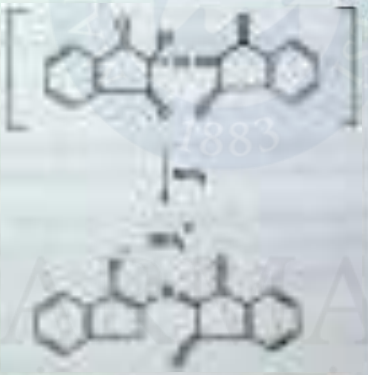
ninhydrin



hydrindantin



hydrindantin ninhydrin



(purple)

A very useful reaction of amino acids is that with ninhydrin. As seen in above the initial reaction causes an oxidative decarboxylation of the amino acid to yield the corresponding altehyde, CO_2 and ammonia. The ninhydrin is reduced to hydrindantin. This first reaction can be used to quantitate amino acids by measuring the CO_2 produced by the reaction, since there is one mole of CO_2 produced for each carboxyl group of the amino acid. Usually, however, the reaction is allowed to proceed. Hydrindantin, ammonia and another mole of ninhydrin react to form a condensation product. In an alkaline medium the product forms a salt which has an intense purple color. This is a very sensitive reaction and is used extensively in amino acid chromatography.

Seperatory Technics for Amino Acids and Peptides

There are different chromographic systems for the seperation different amino acids and peptides. In all chromatographic seperations, molecules are partitioned between a stationary and a mobile phase. Separation depends on the relative tendencies of molecules in a mixture to associate more strongly with one or the other phase. Paper chromatography, automated ion exchange chromatography, gas liquid chromatography, gel filtration technic, thin layer chromatograpy, reversed phase high pressure liquid chromatography (RPHPLC), are used for the separation of amino acids.

The other separation system for amino acids is electrophoresis. In this system charge of molecule and pH of the medium is important. Various electrophretic system is used e.g. high voltage elecrophoresis (HVE) on inert support, HVE on moleculer sieves.



Identification of amino acids present in proteins, after descending paper chromatography in butarol acetic acid (spots were visualyzed with ninhydrin)

The aromatic amino acids tryptophan, tyrosine, histidine and phenylalanine absorb ultraviolet light. As shown in Figure 2 most of the ultraviolet absorption of proteins is due to their tryptophan content.

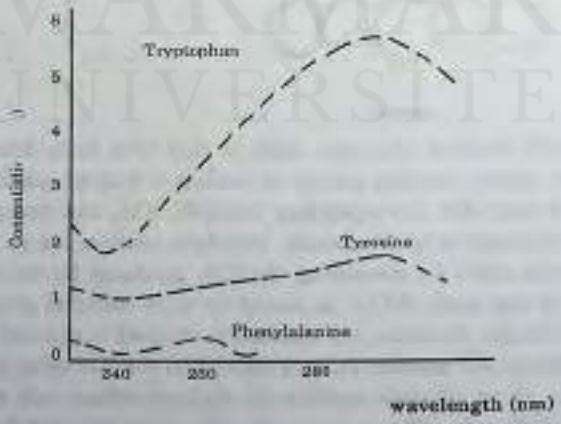


Figure 1: The ultraviolet absoprtion spectra of tryptophan tyrosine, and phenylalanine

Determination of the Primary Structure of Peptides

The linear sequence of amino acid residues in a polypeptide is referred to as its primary structure. The determination of primary structure employs chemical methods whereas the higher orders of protein structure require physical technics such as x-ray crystallography. When the number, kind and linear order of the amino acids are known, an accurate primary structure of a peptide or a protein may be obtained.

The N-terminal (amino terminal) amino acid is always shown at the left and the C - terminal (carboxyl terminal) amino acids at the right of the polypeptide chain. Determination of the number and kinds of amino acids present:

The peptide bonds linking the amino acids are first broken by hydrolysis. The free amino acids are then separeted from one another and identified by chromatography and electrophoresis.

The quantity of each amino acid present is then determined by quantitavive chemical and physical technics (ninhydrin specific color reactions, spectral properties of aromatic residues). Since peptide bonds are stable in water at pH 7.0 to accomplish hydrolytic cleavage of these conds catalysis by acid, alkali, or enzmes must be employed. Each has advantages and disadvantages.

Most proteins are completely hydrolyzed to their constituent amino acids by heating at 110 C for 20-70 hours in 6N HCl. Hydrolysis is carried out in a sealed, evacuated tube to exclude oxygen and prevent oxidative side reactions.

Electrophoresis

The migration of charged particles in an electric field is called electrophoresis. The charged particles move to the cathode or the anode in an electrophoresis system, depending on the net electric charge on the particles. The particles gain positive charge in a more aside solution than its isoelectric point and migrate toward the cathode (negatively charged electrode). In the reverse stiuation the particle is in the anionic form and migrates toward the anode (positive electrode). The particle migrate neither anode nor cathode when its net electric charge is zero. The pH of supporting medium is known as isoelectric point of the particle.

The main factors affecting the rate of migration are as follows.

a) Net electric charge on the particle

b) Size and shape of the particle

c) Electric field strenght

d) Properties of the supporting medium

e) The temperature of the operation

Electrophoretic mobility is directly propotional to the net charge and inversely proportion to the size of the particle and viscosity of the elecrtrohoresis medium.

The charged particles of interest in biochemistry are mainly macromolecules such as proteins. Electrophoresis is useful as an analytical method. Its advantage is that proteins can be visualized as well as sepereted, permitting a reseacher to estimate quickly the number of proteins in a mixture or the degree of purity of a particular protein preparation. Also, electrophoresis allows determination of curical properties of a protein such as its isoelectric point and approximate molecular weight.

There are charge on protein and amino acids. These molecule can be regulated by this charge. The charge of protein allows the seperation of certain proteins from each other. And also another types of compounds.

As mentionad above electrophoresis is a technic for the separation of charged molecules, wihch is dependent on differences in the migration of various molecular species in an electrical field are the result of inequalities in their ionic charges at a given pH. Although electrophoretic fractionations may be performed in a liquid buffered medium (i.e. free Tiselius, or moving boundary electrophoresis), it is usually advantageous to minimize thediffusion of the separated molecules by conducting the electrophoretic migration in an inert stabilizing medium that is saturated with buffer solution. Examples of the stabilizing media used for electrophoresis include filter paper, cellulose acetate, sucrose gradient starch gel acrylamide gel, agarose, dextran gel, and glass blads. A number of synonyms for electrophoresis have been used such as ionophoresis, ionography and electrochromatograpy. The commen applications of electrophoresis in clinical laboratories include fractionations of proteins, lipoproteins glycoproteins, isoenzymes, hemoglobins amino acids and catecholamin metabolities.

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Filter paper electrophoresis

In the electrophoresis apparatus, positively charged protein molecules are introduced at the starting point and a potential difference is applied across the electrodes. A simple schematic representation of an filter paper electrophoresis is seen in figure 2.

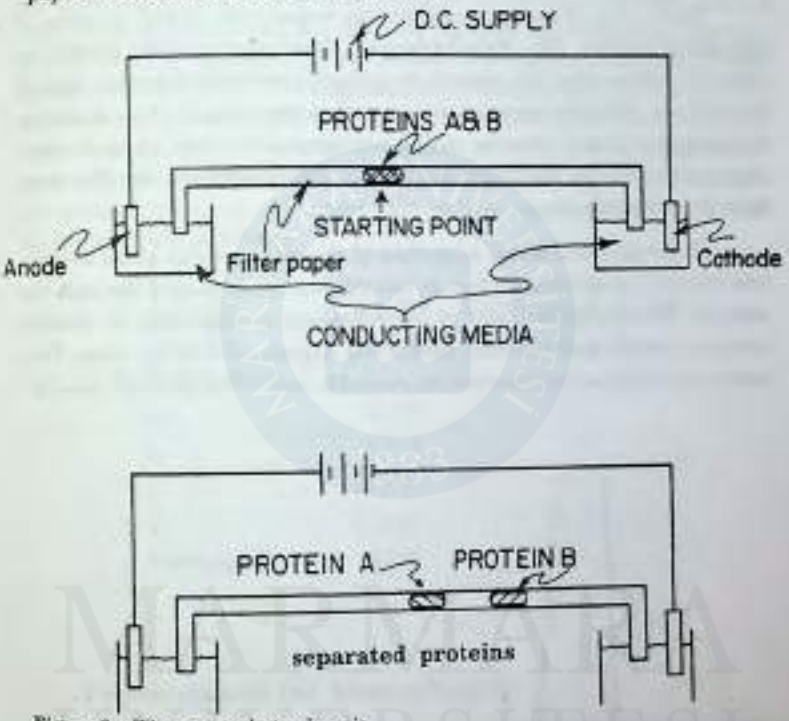

Figure 2 : Filter paper electrophoresis

The protein which conteins positively charge will migrate into the area of the cathode. Thus, electrophoresis can be used to separete positively charged molecules form other molecules. However, it is not ordinarly used for this purpose. Usually electrophoresis is used to characterize proteins by their rate of migration or mobility.

Electrophoretic separations can be accomplished in many types of media besides solutions. Filter paper and cellulose acetate saturated with conducting media have found extensive applications. Various gels on solid supports have been used, including starch, agar-agar, and polyacrylamide.

Electrophoretic techniques are used extensively for serum protein separations. Although serum contains a large number of different proteins, the proteins can usually be separated into five disting groups on the basis of their mobility in an electric field at pH 8.6 As seen in figure 3.

The α_1 , α_2 , β and gamma globulins respectively, follow in terms of decreasing mobility. The globulin fraction, depending upon the particular situation, either does not migrate or perhanps actually migrates toward the cathode. After the proteins are separated according to their mobility, the quantitiy of each fraction can be measured in various ways. Electrophoresis provides a means for separating and quantitang the five fractions of serum proteins.

Electrophoresis is the migration of charged particles in an electrolyte solution which occurs when an electric current is passed through the solution. The isoelectric point of the substance is important in electrophoesis because their isoelecric points will migrate at varying rates. The samples for analysis is dissolved in a suitable buffer (for plasma, usually

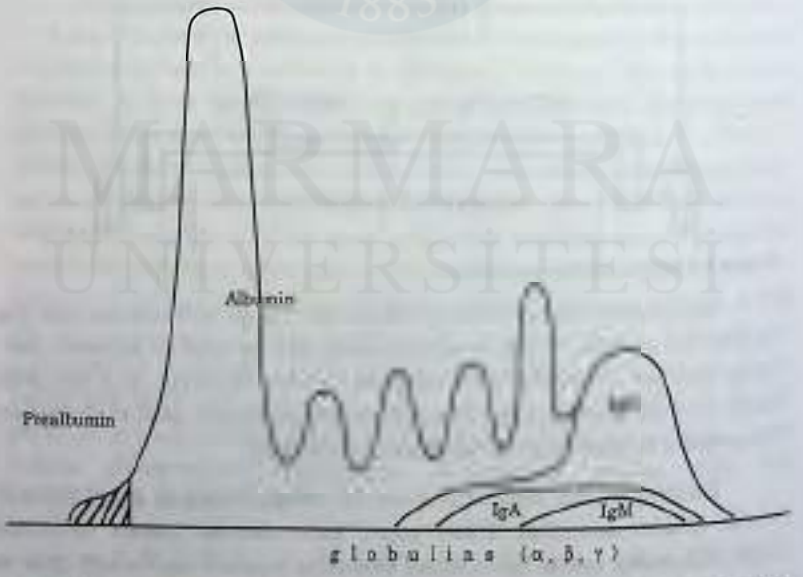

Figure 3 : Fractionated serum proteins obtained by filter paper electrophoresis (From Ref. 29).

0.1 N sodium diethylbarbiturate at pH 8.6). This mixture is then placed in the electrophoretic medium. The positive and negative electrodes are connected to each limb of the cell (electrophores aparatus). When the current is applied, migration of the plasma protein components begins. The albumin molecules, which are smaller and more highly charged, exhibit the most rapid rate of migration follwoed by various globulins. After a time, boundaries between the separate fractions can be detected because of differences in concentrations of protein. A photographic record of these variations constitutes what is termed an electrophoretic pattern. As illustrated above. In normal human plasma 5 or 6 distinct moving boundaries have been identified. These are designated in order of decreasing mobility as albumin, alpha$_1$ and alpha$_2$ globulins, beta globulin, fibrinogen and gamma globulins. The persentage of these fractions to of total plasma proteins are as follows.

Albumin	55.2 %	of total plasma protein
Globulin	44.8 %	"
Globulin fractions		
a_1	5.3 %	"
a_2	8.6 %	"
β	13.4 %	"
γ	11.0 %	"
Fibrinogen	6.5 %	"

Polyacrylamide Gel Electrophoresis

The porous supporting mediums used for the migration of charged macromolecules are usually cellulose paper, vellulose acetate sheets, agarose and polyacrylamide gels. As mentioned above electroporesis of proteins is generally carried out in gels made up of cross-linked polymer polyacrylamide. Polyacrylamide gel acts as a molecular sieve, showing the migration of proyeins approximately in proportion to their mass or molecular weight. The detergents sodium dodecyl sulphate (SDS) is commonly used for estimation of purity and molecular weight of proteins. SDS binds to proteins and contributes a large net negative charge rendering the intrinsic charge of protein insignificant. Native conformation of a protein is altered and proteins assume a similar shape an a similar ratio of charge to mass. Thus smaller polypeptides migrate more rapidly.

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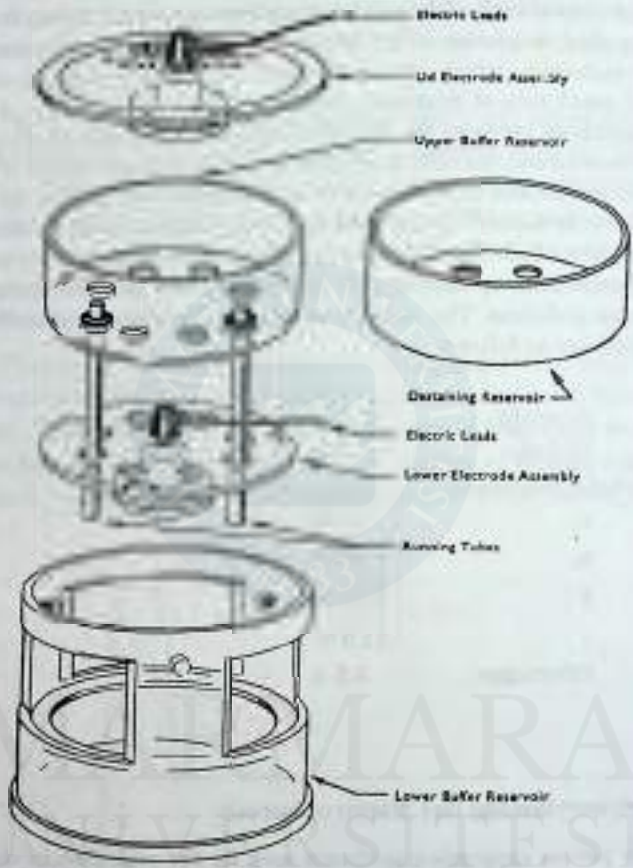

Figure 4: Shematic representation of a polyacrylamide disc gel electrophoresis unit.

Polyacrylamide gel electrophoresis is performed either in cylindrical glass tubes (disc gel electrophoresis) or in flat beds (slab gel electrophoresis). After electrophoresis, the protein are visualised by adding a dye such as commasie blue which binds to proteins but not to the gel itself (Figure 5, 6).

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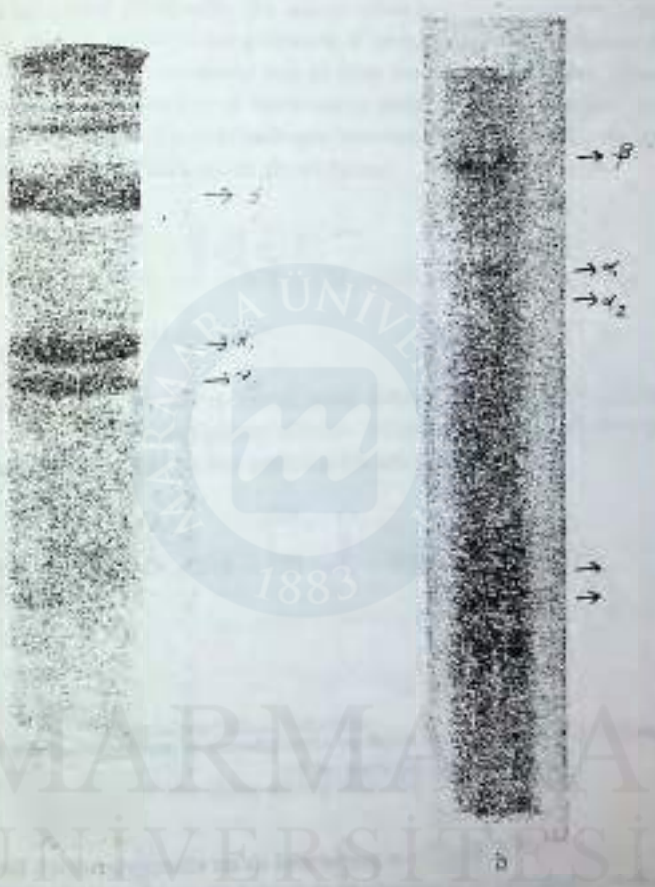

Figure 6 : SDS polyacrylamide disc gel electrophoretic pattern of collagen from tail tendon with commasi brillant blue staining (a) and collagen from gingival tissue with PAS staining (b). (From ref. 60)

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Figure 6 : a) SDS polyacrylamide slab gel electrophoresis of protrombin complex obtained from human plasma (From ref. 61)

If the electrophoresis is performed in an electolyte which has a pH gradient, molecules will migrate to the point where the pH of the solution is the same as their isoelectric pH and remain there. This is the basis of isoelectric focusing techniques. It is used to determined the isoelectric point of a protein. Combining these two electrophoretic methods in two dimension gel permits the resolution of complex mixture of proteins. Two dimensional electrophoresis separates proteins of identical molecular weight that differ in isoelectric point or proteins with similar isoelectric point values but different molecular weight.

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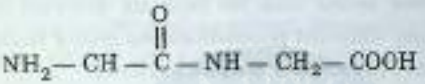
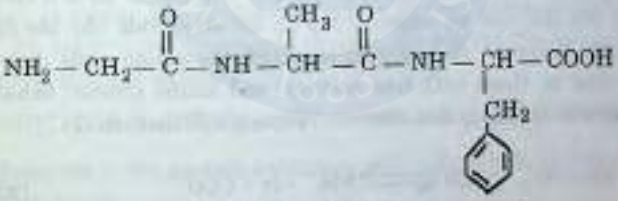
As indicated previously, the amino acids are the monomeric units of protein that is, proteins are polymers of amino acids. The amino acids are linked together by an amine link to form the protein polymers. These amide linkages between two or more amino acids are called peptide linkages or peptide bonds. Peptide linkages between the two molecules of glycine result in a compound called glycylglycine



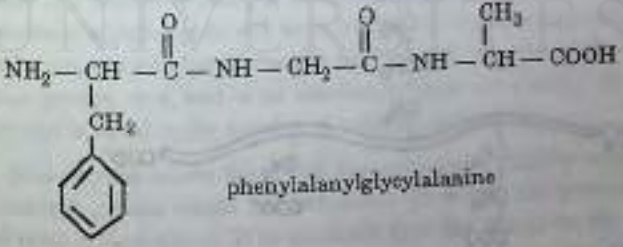
Glycylglycine

As shown below, three amino acids connected together by peptide links produce glycylalanylphenylalanine. Obviously, the structures become complex when only a few amino acids are involved.



Glycylalanylphenylalanine

The sequence of the three amino acids can vary as below,



phenylalanylglycylalanine

Perhaps the importance of the amino acid sequence can be better understood if the possible arrangements of three amino acids are outlined as follows:

A - B - C

B - C - C

C - A - B

B - A - C

C - B - A

A - C - B . As seen here, each arrangement yields a tripeptide, thus with three amino acids we have six different compounds. Now consider how many different possibilities we would have if we had 54 amino acids for one of the smaller simpler proteins.

The nomenclature used to describe polypeptides can often be confusing. As I mention before, glycylglycine is known as a dipeptides, but in reality it has only one peptide linkage. Similarly in a tripeptides there are only two peptide bonds. The prefix tri, di or whatever, is not determined by the number of peptide bonds but rather by the number of amino acids present in the molecule. The di and tripeptides have a carboxyl group on one end and an amino group on the other side like the simple amino acids Figure 7. As might be expected, the amino acids in a polypeptide (that is, those with free carboxyl and amino groups) behave as amino acids in that they demonstrate zwitterion formation(22).

$$NH_2 - Pr - COOH \rightleftharpoons NH_3^+ - Pr - COO^- \qquad (22)$$

the Pr, represents the remainder of the peptide molecule.

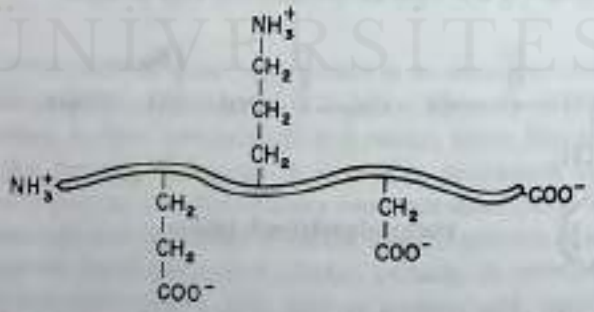


Figure 7 : A polypeptide with amino and carboxyl groups.

In reality, proteins are not as simple as pictured, because it will be recalled that some amino acids have more than one amino group (such as

lysine) and some have more than one carboxyl group (such as aspartic acid). This is depicted in figure above. In the Figure polypeptide chain shows additional amino and carboxyl groups.When the amino and carboxyl groups are considered in addition to the N and C terminals the protein and polypeptide can be represented as;

$$(NH_3^+)_x - Pr - (COO^-)y$$

The terms N and C terminals refer to the ends of the molecule which bear a free amino and a carboxyl group respectively. N terminal amino acid is on the left and C terminal is drawn on the right.

A protein may have the same number of amino groups as carboxyl groups, in which case x=y, or they may be different. A protein may have more free amino groups than carboxyl groups. Then it is spoken of as a basic protein and when dissolved in water yields an alkaline pH. By contrast, other proteins contain more carboxyl groups than amino groups, these are called acidic proteins and demonstrate an acid reaction when dissolved in water. The zwitterion form of protein behaves similarly to that of the amino acids. In an acid medium the polypeptide reacts to give a positively charged molecule.

$$(NH_3^+)_x - Pr - (COO^-)y + yH^+ \longrightarrow (NH_3^+)_x - Pr - (COOH)y$$

Reaction of the protein zwitterion with alkali leads to a negatively charged molecule.

$$(NH_3^+)_x - Pr - (COO^-)y + xOH^- \longrightarrow (NH_2)x - \quad -(COO^-)y$$

A polypeptide in a strongly acid medium is fully protonated and therefore is a positively charged molecule. Conversely, in a strongly alkaline medium a polypepide will be a negatively charged molecule. In an acid solution, the number of charges per molecule is equal to the number of amino groups, or x, and in an alkaline medium the number of molecular charges is equal to the number of carboxyl groups or y.

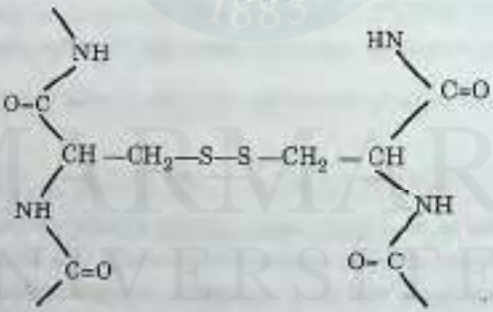
In a solution where the proton concentration is limited, only part of the carboxyl groups would be protonated. However, the amino groups would remain protonated. It is apparent that the charge on the protein molecule is regulated by the hydrogen ion concentration or the pH of the solution. By proper regulation of the pH, a hydrogen ion concentration can be obtained where the number of negative charges and the number of

positive charges are equal, so the overall net charge is zero. That means the sum of the negative and positive charges are equal and opposite, thus the net charge on the molecule is zero. The pH at which the number of negative charges and the number of positive charges are equal is known as the **isoelectric point or the isoelectric pH.** The term isoelectric point is not restricted to proteins and polypeptides but also applies to amino acids.

STRUCTURE OF PROTEINS

The structure of proteins is much more complex than might be expected. There are four structure for proteins and these are not independent from each other. These are; primary, secondary, tertiary and quaternary. Technics used to study protein structure include electron microscopy, x-ray diffraction, streaming birefringence and optical rotatory dispersion. There are some bonds responsible for protein structure these are;

Peptide bonds: long chains of amino acis linked by the peptide bond are called polypeptides. The number and order of the amino acids in the polypeptide chains is made by peptide bonds.



(23)

Peptide chains united by a disulfide bond

Disulfide bonds: This bond may interconnect 2 parallel chains through cysteine residues within each polypeptide. This is stable and thus is not readily broken under the usual conditions of denaturation some acid treatment oxidizes the S-S bonds(23).

Hydrogen Bonds: In addition to S-S bonds one other major force is involved in the pereservation of the structure of a protein molecule, this is the hydrogen bond. This bond is produced by the sharing of hydro-

gen atoms between the nitrogen and the carbonyl oxygen of the same or of diffeent peptide chains. Individual hydrogen bonds are very weak, but the reinforcing action of a large number of suh bonds in the protein molecule produces a stable structure(24).



(24)

Hydrogen bonds

Hydrophobic Interactions The nonpolar side chains of neutral amino acids tend to be closely associated with one another in proteins The relationship is nonstoichiometric. These interactions play a significant role in maintaining protein structure.

Electrostatic Bonds are also responsible for protein structure. These are salt bonds formed between oppositely charged groups in the side chains of amino acids. The epsilon amino group of lysine bears a net charge of + 1 at physiologic pH and the non a carboxyl of aspartate and glutamate a net charge of-1. These may therefore interact electrostatically.

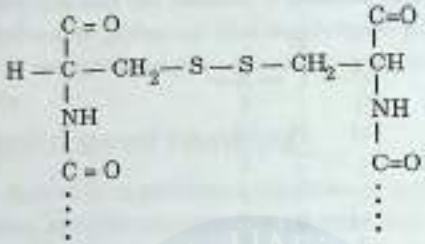
Van der Waals forces is one of the important forces in tertiary structure of protein. It is a weak interatomic forces as hydrogen bonds.

Orders of Protein Structure

Primary

The primary structure is determined by covalent intrapeptide chain bonds. These bonds include the peptide bonds between individual amino acids and disulfide bridges between cyteinyl residues. Cysteine can be oxidized to form cystine, which is two molecules of cysteine connected together by a disulfide bridge. This same event can take place

when cysteine is present in protein molecules forming a disulfide bridge within a single or between two peptide chains(25).



(25)

One type of configuration conferred by intrapeptide chain di sulfide bonds can be represanted schematically Figure 8. The chains are connected by S-S linkages. Oxidation with spesific acid breaks these linkages.

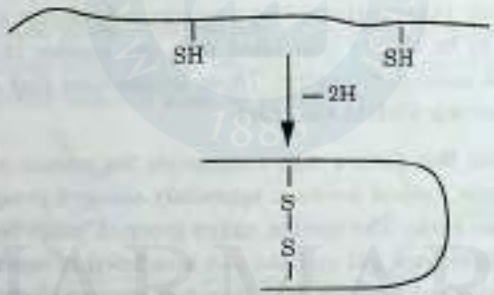


Figure 8: Formation of an intrapeptide disulfide bond.

Configuration that results from formation of an intrapeptide disulfide bond. Some protein molecules contain several disulfide bridges and can assume various shapes.

The covalent bonds of the primary structure are flexible and free to rotate. If proteins had only primary structural bonding, the molecules would assume random coil configuration. A schematic representation of this is seen below (Figure 9).

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Figure 9 : Random coil configuration of a protein molecule

Secondary Structure

Usually, proteins in biologic systems do not have random configurations, instead they possess very ordered and sometimes complex structures. The major part of this ordered structure is due to hydrogen bonding between carbonyl oxygen and amide hydrogen called the secondary structure.We know that some atoms have a greater attraction for electron a than others. Oxygen is a rather aggregssive molecule and likes to associate with more than its share of protons. Since the peptide bonds recur at regular intervals, hydrogen bonding of the secondary structure can also occur with regularity. This regularity frequently allows the protein to assume a helical configuration. The peptide chain is maintained in a coil shape by the hydrogen bonding and helical configuration occur, as seen in figure 10.

As a conclusion, the folding of the polypeptide chains into coiled or pleated structure held together by hydrogen bonds or folding stabilized by disulfide bonds is referred to as the secondary structure of the protein.

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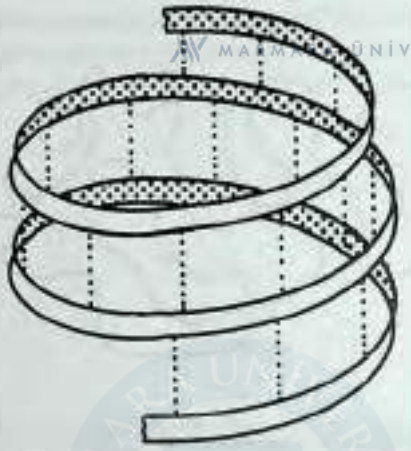
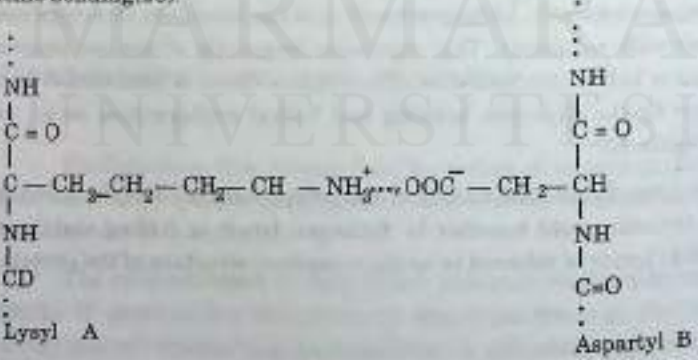


Figure 10 : Helical configuration of a protein molecule due to hydrogen bonding.

Tertiary Structure

All other types of interaction leading to additional folding or intra-peptide bonding is reprsented in the tertiary structure of a protein. This structure depends in part upon hydrogen bonding other than that of the amide (peptide) groups.

Other interactions between amino acid residues in peptide chains also contribute to tertiary fine structure. One type of interaction is called ionic bonding(26).



(26)

Peptide chain A contains a positively charged lysyl residue and peptide B contains a negatively charged aspartyl group. There is the expected attraction between these two oppositely charged groups.

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Quaternary Structure

The term quaternary structure describes all types of bonds and interaction between different peptide chains. A schematic example is seen for bovine insulin in figure 11. There is an intrapeptide chain disulfide bridge (primary structure for alfa chain) and two interpeptide chain disulfide bridge connecting alfa and beta chains, which are part of the quaternary structure of the molecule.

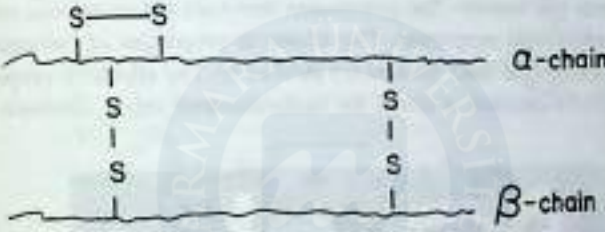

Figure 11 : Representation of bovine insulin showing intrapeptide disulfide bond. Primary structure and interpeptide disulfide bonds of quaternary structure

If a protein consist of 2 or more polypeptide chains united by forces other than covalent bonds (ie, not peptide or disulfide) it shows quaternary structure (Figure 12, 13).



Figure 12 : Representation of quaternary structure of a protein.

The forces that stabilize these aggregates are hydrogen bonds and electrostatic or salt bonds. These bonds formed between residues on the surfaces of the polypepdide chains. Such proteins are termed oligomers

and the individual polypeptide chains of which they are composed are variously termed protomers, monomer, or subunits. The most commonly encountered oligomeric proteins contain 2 or 4 protomers and are termed dimers and tetramers respectively. Oligomers containing more than 4 protomers are also common. Oligomeric proteins play special roles in intracellular regulation because the protomers can assume different spatial orientations. The best studied example is hemoglobin. Hb shows a variety of conformations depending on the degree of oxygenation.

Hemoglobin transports oxygen, carbon dioxide and protons between lungs and tissues. The quaternary structure of hemoglobin confers striking additional properties. The allosteric properties of hemoglobins are consequences of their quaternary structures. The allosteric properties of hemoglobin provide, a model for understanding other allosteric proteins.



Figure 15 : A protein dimer unit illustrating the quaternary structure of a complex globular protein. Peptides stabilized by noncovalent bonds in such proteins

Myoglobin molecule looks like a hemoglobin. But Hb molecule consists of 4 protein chains or subunits. 2 designated alpha and 2 designated beta. It is represented as $(\alpha)_2$ $(\beta)_2$. The alpha chains contain 141 amino acids, the beta chains 146 amino acids. Each has a distinct but similar amino acid sequence. The myoglobin and β subunits of hemoglobin share identical secondary and tertiary structures.

DENATURATION

Proteins can undergo profound changes in physical properties without an apparent change in chemical composition. An example of this is a cooked egg. In this example the characteristic properties of the egg are greatly changed. If part of the egg is analyzed before cooking and com-

pared with the chemical analysis obtained after cooking they will indeed be found the same. This sort of change is called denaturation of proteins. Literally, denatured means "not native". That is, not in the form in which it existed in the biologic system. In some cases denaturation is a reversible process. Physically, denaturation may be viewed as randomizing the conformation of a polypeptide chain without affecting its primary structure as seen in figure 14. After denaturation on oligomeric protein becames monomer units, with or without changes in monomer conformation.

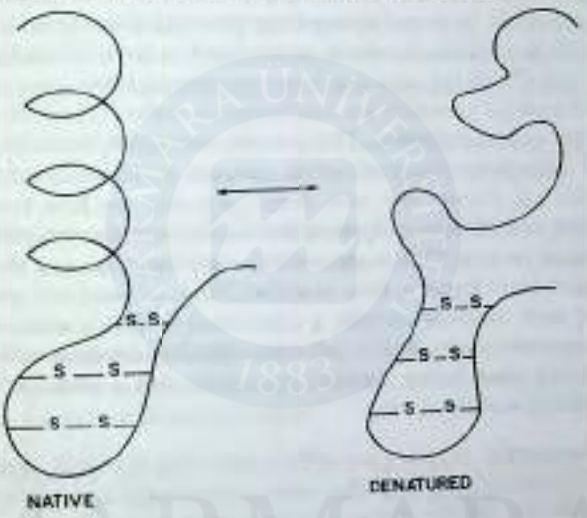

Figure 14: Denaturation of a protein with loss of hydrogen bonding

As seen in figure 14 the native protein has a helical portion which is due to hydrogen bonding and a lower "wrap-around" portion due to disulfide bridges. After denaturation there is disruption of secondary and tertiary structures, the helical configuration is gone, and the upper portion of the molecule is represented as a random coil. Denaturation has importance other than helping to understand what happens when you cook breakfast eggs. It is very important in biologic system, because enzymes, which are proteins, may lose their enzymatic activity when they are denatured. Antibodies no longer function as antibodies when denatured. Hemoglobin no longer transports oxygen when denatured. There are many other examples, but these are sufficient to indicate the importance of denaturation.

These examples suggest that denaturation is a phenomenon that is quite easy to detect. Some proteins are very resistant to denaturation, while others are very sensitive. In addition to the changes in configuration that occur with denaturation, there are obviously changes in physical properties, such as viscosity and solubility. Denatured proteins are generally less soluble than native proteins. If the treatment of proteins is severe enough (exposure to strong acids, organic solvent, heat, mechanical agitation), not only are the proteins denatured but they also coagulate and form a very insoluble precipitate. Because the biologic activity of most proteins is destroyed by exposure to strong mineral acids or bases, heat, ionic detergents, chaotropic agents (such as urea, guanidine), heavy metals (Ag, Pb, Hg) or organic solvents at or above room temperature, denatured proteins generally are less soluble in water. They often precipitate from aqueous solution. This property is used to advantage in the clinical laboratory. Blood or serum samples to be analyzed for small molecules (eg, glucose uric acid, drugs) generally are first treated with acids such as trichloroacetic, phosphotungstic acid to precipitate most of the protein present. This is removed by centrifugation, and the protein-free supernatant liquid is then analyzed. The heat, acid and protease lability of most enzymes provides a simple test to decide whether a reaction is enzyme catalyzed. If a cell extract having catalytic activity loses this activity when boiled, acidified or treated with a protease, the catalyst probably was an enzyme.

Frequently, enzymes are either more or less readily denatured if their substrate is present. Either effect is attributed to a conformational change in the enzyme structure occuring when substrate is bound. The conformation may be either more or less stable than before.

Intermediate between denaturation and coagulation is a process called flocculation. If a denatured protein is in a solution at its isoelectric point it usually will precipitate, all proteins are least soluble at their isoelectric points and denatured proteins are even less soluble. Flocculation, is the precipitation of denatured protein at or near the isoelectric point, without coagulation.

Flocculation usually occurs under mild conditions, whereas coagulation occurs under more severe conditions. Flocculated protein can often be redissolved by adjusting the pH away from the isoelectric point, but coagulated protein is generally insoluble in very strong acid or base.

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Single Proteins: These contain only L-alpha-amino acids or their derivatives and include the following groups:

a) Albumins: Soluble in water, coagulated by heat, precipitated by saturated salt solutions. Examples: lactalbumin, serum albumin.

b) Globulins: Soluble in dilute salt solutions of the strong acids and bases, insoluble in pure water or in moderately concentrated salt solutions, coagulated by heat. Examples: serum globulin, ovoglobulin.

c) Gluteins: Soluble in dilute acids and alkaline insoluble in neutral solvents: coagulated by heat Example: Glutenin from wheat.

d) Prolamines: Soluble in 70-80% alcohol, insolable in absolute alcohol, water and other neutral solvents. Examples: Zein (Corn) and gliadin (wheat)

e) Albuminoids (Scleroproteins): Insoluble in all neutral solvents and in dilute acids and alkalies. These are the proteins of supportive tissue. Examples: keratin, collagen.

f) Histones: Soluble in water and very dilute acids, insoluble in very dilute NH_4OH, not coagulated by heat. Basic amino acids predominate. Example: nucleohistones of nuclei.

g) Protamines: Basic polypeptides, soluble in water or in NH_4OH not coagulated by heat, basic amino acids predominate in their structure, precipitate other proteins. Found principally in egg cells. Examples: salmine and sturine

Conjugated Proteins These type of proteins contain nooprotein substance (the prosthetic group) linked by forces other than ionic attractions. These are:

a) Nucleoproteins: Compounds of one or of several molecules of proteins with nucleic acid. Examples: Nuclein, nucleohistone from nuclei-rich material (eg. glandular tissues)

b) Glycoproteins: Compounds with carbohydrate prosthetic groups which on hydrolysis yield amino sugars (hexosamines) Examples the protein, mucin, and proteins in the plasma which migrate electrophoretically with the alpha1 and alpha2 fractions.

c) Lipoproteins: Lipoproteins facilitate lipid transport between tissues and plasma. After digestion lipids are recombined in the intestinal cells with protein and first secreted into the lymphatic system then into the circulation as a lipoprotein. This is known chylomicnon. Unlike glucose and amino acids, lipopropteins (chylemicron, triacylglycerol) is not taken up by the liver. They are metabolised by extrahepatic tissues possessing the enzyme lipoprotein lipase. Seperation of plasma lipoproteins by electrophoresis is shown in figure 15.

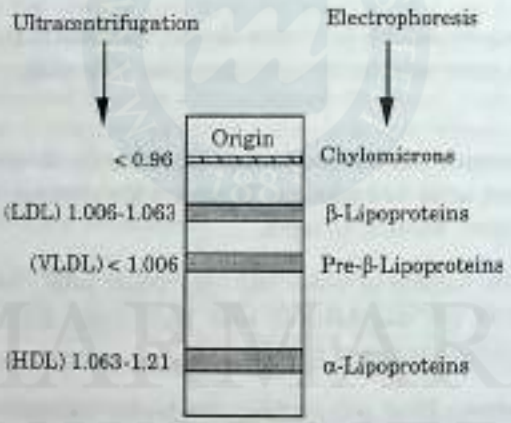

Figure 15 : Seperation of plasma lipoproteins by electrophoresis and ultracentrifugation

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The amino acids from dietary proteins are absorbed and enter the amino acid pool in the cell. Amino acid from this pool are used in the synthesis of proteins and nonprotein constituents, can be oxidized to produce CO_2, water, ammonia and energy. When proteins are degraded the amino acids reenter the pool. Some of the amino acid are ketogenic and some of them glycogenic after specific amino acid catabolism. Some of the amino acids can be synthesized from carbohydrate metabolites. Amino acids metabolism which occurs in mammalian tissues is outlined below Figure 16.

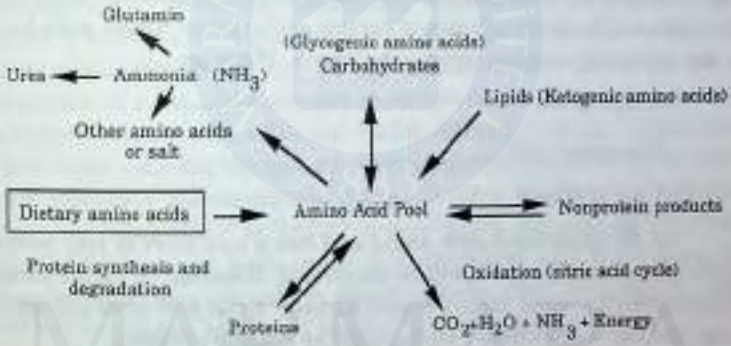

Figure 16: Overview of amino acid metabolism

Some amino acids are **nutritionally essential**, these are; **Valin, leucine, isoleucine, lysine, methionin, threonine, phenylalanin and trptophan**. Others are nutritionally nonessential these are; alanin, serine, proline, hydroxyproline, asparagine, aspartate, glutamate, glutamine, cysteine, glycne tyrosine and histidine.

Nutritionally nonessential amino acids are synthesized by some reactions:

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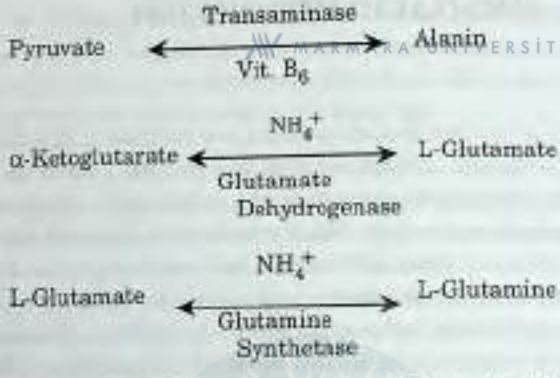

As it is seen above transaminases, glutamate dehydrogenase and glutamin synthetase are essential in amino acid biosynthesis. Their combined effect is to catalyze transformation of inorganic ammonium ion into the organic alfa amino nitrogen of various amino acids. Amino acids have a few metabolic reactions which are common. These are;

- Deamination
- Transamination
- Urea synthesis

On the other hand each amino acid has it own more or less unique metabolic pathway later it will be considered. However, in carbohydrate metabolism, the usual dietary sugars all enter a common metabolic pathway, and the fatty acids are all degraded by a single proceses.

Oxidative Deamination: This reactions is catalyzed by amino acid dehydrogenase (amino acid oxidase). Figure 17 Amino acids are oxidized by the removal of two protons and two electrons to yield the corresponding imino acid. The imino acid is then spontaneously hydrolyzed to the corresponding alfa keto acid and ammonia. Oxidative conversion of many amino acids to their corresponding α-keto acids occurs in mammalian liver and kidney. Amino acid oxidases are auto-oxidizable flavoproteins, ie, the reduced FMN or FAD is reoxidized directly by molecular oxygen, forming hydrogen peroxide (H_2O_2) without participation of cytochromes or other electron carriers. The toxic product hydrogen peroxide is then split to 0_2 and H_2O by catalase, which occurs widely in tissues, especially liver.

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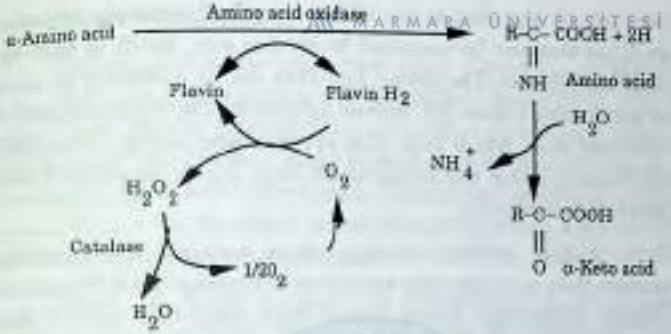

Figure 17: Oxidative deamination of α amino acids

The amino groups of most amino acids ultimately are transfered to α-ketoglutarate by forming L-glutamate reaction of this nitrogen as ammonia is catalyzed by L-glutamate dehydrogenase, an enzyme of high activity widely distributed in mammalian tissues. Liver glutamate dehydrogenase is a regulated enzyme whose activity is affected by allosteric modifiers such as ATP, GTP, and NADH, which inhibit the enzyme, and ADP, which activates the enzyme. Certain hormones appear also to influence glutamate dehydrogenase activity in vitro. Glutamate dehydrogenase uses either NAD^+ or $NADP^+$ as cosubstrate. The reaction is reversible and functions in both amino acid catabolism and biosynthesis. It therefore functions not only to funnel nitrogen from glutamate to urea but also to catalyze amination of α-ketoglutarate by free aminomin.

Deaminasyon and transamination reactions are releated with nitrogen group of amino acid. The other catabolitic pathway for the amino acids are catabolism of the carbon skeletons. At the end of these catabolitic processes amino acids being substrate for carbohydrate and lipid biosynthesis, and some important biological amines, e.g. histamin, triptamin, serotonin are formed.

For man amino acids, the initial reaction is removal of the α-amino nitrogen. After nitrogen removed α-ketoglutarate forms by transamination reaction. Glutamate, proline, orginine, Histidine yield α-ketoglutarate after a series complex reaction. If there is enzyme deficiency in these pathway different metabolic disease can be seen such as hyperprolinemia, histidinemia to generalize about the catabolism of amino

acids, they can be degraded in one of three ways. The glucogenic amino acids are those that can be converted to pyruvic acid, which in turn can be converted to glucose. The amino acid that can be converted to acetic acid and the ketone bodies are termed ketogenic. The third category of amino acids are those which yield both pyruvate and acetate. Phenylalanine and tyrosine, which will be discussed here, can be converted to acetic and pyruvic respectively.

Glisin, alanin, cysteine, cystine, serine, treonine hydroxyproline form pyruvate then pyruvate may be converted to acetyl COA. Phenylalanine, tyrosine, tryptophan, lysine, leucine form acetyl COA without first forming pyruvate.

Metabolic Disorders for some Amino Acids

There are many enzyme defect in specific amino acid metabolic pathway. But some of them are well-characterised.

If catabolic pathway can not be occured for glisin, glysinuria which is a rare familial disorder is seen. Excess Urinary excretion of glysine tendency to form oxalate renal stones. Since plasma glycine levels are normal while Urinary excretion of glycine ranges from 600 to 1000 mg/day, glicinuria is attributed to a defect in renal tubular reabsorbtion of glycine. Excess glycine may be result in primary hyperoxaluria. This is characterised by continuous high Urinary excretion of oxalate unreleted to dietary intake of oxalate urolithiasis, nephrocalcinosis and recurrent infection of the Urinary tract are followed by death in childhood or early adult life from renal failure or hypertension. After deamination glycine is converted to glyoxylate which is a precursor of oxalate.

If there is metabolic disorder in cyteine catabolism, cystinuria and cystinosis are seen, cystinuria is inherited metabolic disorder, urinary excretion of cysteine is 20-30 times excess compare to normal. Excretion of lysine, arginine, and ornithine is also markedly increased, suggesting a defect in the renal reabsorptive mechanisms for these 4 amino acids. Cystnuria is therefore a misnomer. Cystine calculi forms in cystinuric patients, it may be precipitate in the kidney tubules.

In cystinosis, which is also inherited disorder, cysteine crystals are deposited in many tissues and organs particularly the reticuloendothelial system. Cystinosis is usually accompanied by a generalized aminoaciduria. Other renal function are also seriously impaired.

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The aromatic amino acids have many interesting metabolic reactions. Phenylalanine, tyrosine and tryptophan metabolism will be presented here. Several intermediates of tyrosine metabolism were discovered during studies of the human genetic disease alkaptonuria as will be discussed patients with alkaptonuria excrete homogentisate in the urine and much useful information was obtained by feeding suspected precursors of homogentisate to these patients.

Several metabolic disorders are characterised by tyrosinemia, tyrosinuria, phenylaciduria there are two types of tyrosinemia (Tyrosinosis) which are decribed. In tyrosinosis, accumulation of metabolites adversely affects the activities of several enzymes and transport system. Here, the proposed metabolic defect is in fumarylacetoacetate hydrolase. Both acute and chronic forms of tyrosinosis are known. In acute tyrosinesis, infants exhibit diarrhea, vomitting and failure to thrive. Death from liver failure in untreated acute tyrosinosis occurs within 6-8 months. In chronic tyrosinemia, similar but milder symptoms lead to death by the age of 10 years. Plasma tyrosine levels are elevated up to 6-12 mg/dl. Treatment involves a diet low in tyrosine and phenylalanin.

In type II tyrosinemia, probably metabolic defect is in hepatic tyrosine transaminase. Clinical findings includes elevated plasma tyrosine levels, eye and skin lesions, and moderate mental retardation. Urinary metabolites are p-hydroxyphenylacetate, p-hydroxyphenyllactate, N-acetyl-tyrosine and tyramine.

As it is shown in Figure 18, 19. in individuals who lack phenylalanin hydroxylase activity (1) the normal conversion of phenylalanin to tyrosine is prevented. Instead, phenylalanin, and phenylpyrivat, phenyllactic and phenylacetic acids acumulate in elevated concentrations in blood and urine. This condition is called phenylketonuria. These substances are toxic to the developing central nervous system and without proper treatment irreversible nerve system damage will result. For this reason, it is now a standard procedure to analyze the serum and urine or both of all newborns to detect phenylketonuria. Effective treatment is the maintenance of phenylalanine at nontoxic levels. There is no endogenous source for this essential amino acid, but most proteins contains phenylalanine.

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Figure 18: Schematic representation of phenylalanine and tyrosine catabolism. Circled numerals mark the sites of the metabolic defects due to enzyme deficiency.

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Figure 19: Catabolism of phenylalanin and tyrosine. Circled numerals mark the sites of the metabolic defects due to enzyme deficiency.

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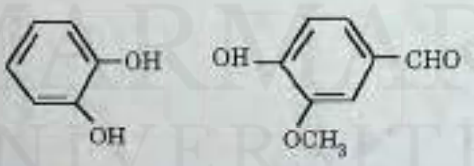
Daire Başkanlığı

Number 2 which is shown in Figure 18 and 19 lack of tyrosinase activity. In normal individuals with phenylalanin hydroxylase activity tyrosine is formed and can be further metabolized to melanins or skin pigments. The lack of tyrosinase activity produces a condition called albinism. Several defects in melanin biosynthesis can cause albinism. There are heritable defects in the pigment cells (melanocytes) of eye and skin in individuals which are called albinos. Several models of albinism are known respect of biochemical, ultrostructural and genetic characteristics.

As shown in Figure 18, 19 tyrosine is a precursor of epinephrine (adrenaline) and norepinephrine (noradrenaline). They are produced in certain parts of the nervous system and by the adrenal medulla. Methylation of norepinephrine yields the 3-methoxy derivativel normetanephrine). Similarly, the methylation of epinephrine produces the 3-methoxy compound, (metanephrine). Both of these compounds are further metabolized to yield 3-methoxy-4-hydroxymandelic acid. These four compounds; epinephrine, norepinephrine, metanephrine, and normetarephrine, are collectively called catecholamines, because all these compounds derived from catechol named benzene ring (27). Further corruption of nomenclature is encountered in the case of 3-methoxy-4-hydroxylmandelic acid. This compound is frequently referred to as VMA (vanilylmandelic acid) because. VMA is somowhat similar to that vanilin.



(27)

catechol vanilin

The analysis of catecholamins is usually done to detect pheochromocytomas and neuroblastomas. These are tumors of the sympathetic nervous system which occur in the adrenal medulla or other sites where there are chromaffin cells. These tumors secrete excessive amounts of epinephrine and norepinephrine, which in turn give rise to elevated quantities of the various metabolites.

Dopa is an intermediate in the formation of epinephrins in neuronal cells, and melanin in melanocytes, but different enzymes carry out the tyrosine hydroxylation reactions in different cell types.

Other well known enzyme deficiency in tyrosine catabolism is seen Figure 18, 19 as number 3 occur due to lack of homogentisate oxidase. This condition is called alkoptonuria. Homogentisic acid is excreted with the urine, which becomes dark open standing or following the addition of alkali. The dark material is formed when homogentisic acid is oxidized to the quinone and then polymerizes. In untreated patients generalized pigmentation of connective tissues and a form of arthritis occur.

The very important other amino acid (thyroxine) is also produced from tyrosine through of synthetic steps of number 4 in figure 18 this iodinated amino acid is a hormon produced in the thyroid glands. The thyroid gland produces 2 iodoamino acid hormones, 3,5,3' triiodothyronine (T_3) and 3,5,3',5'-tetraiodothyronine (T_4, thyroxine). These hormones, whose structures are illustrated in Figure regulate the metabolic rate in mammals. Disease of the thyroid are among the most common afflictions involving the endocrine system. Diagnosis and therapy are firmly based on the principles of thyroid hormone physiology and biochemistry. The using of radioisotopes of iodine has greatly aided in the elucidation of these principles. Radioactive iodine, (because it localizes in the gland) is widely used in the diagnosis and treatment of thyroid disorders. Excessive exposure to radioiodine is dangerous especially to infants and adolescents, whose thyroid cells are still actively dividing.

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TRYPTOPHAN METABOLISM

Tryptophan can be metabolized to serotonin. After hydroxylation of tryptoplan decarboxylation occurs and 5-hydroxytryptophan forms this is called 5-hydroxytryptamin (serotonin). Seretonin is a potent vasoconstrictor and stimulator of smooth muscle contraction (Figure 20).

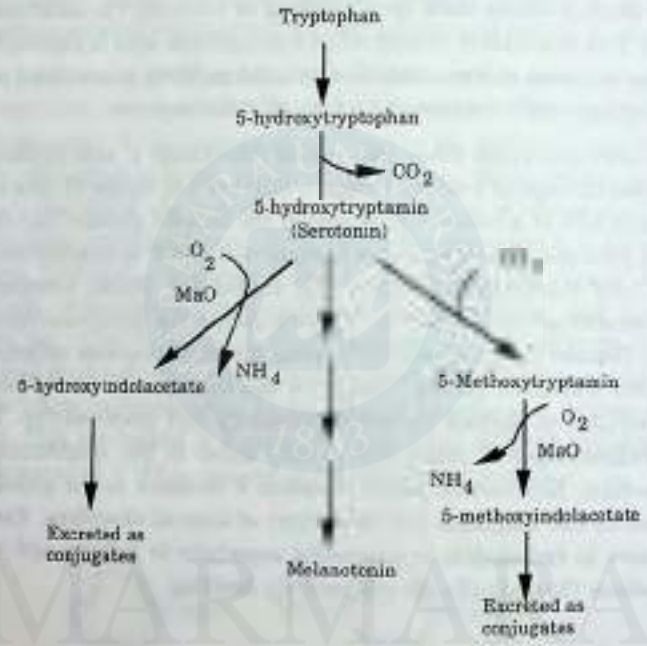

Figure 20: Tryptophan metabolism

Liver phenylalanin hydroxylases also catalyze hydroxylation of tryptophan. Most of the seratonin hydroxylase also catalyzes hydroxylation of tryptophan.

Most of the seratonin is metabolized to 5-hydroxyindolacetate by monoamin oxidase. In normal Urine, 2-8 mg of 5- hydroxyindolacetate is excreted per day. Some drugs inhibit monoamin oxidase. Increased serot-

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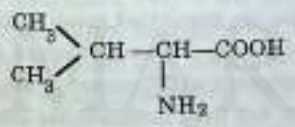
onin production occurs in malignant carcinoid. This is tumors of the argentaffin cells. So this condition is called argentaffinoma and characterized by wide spread serotonin-producing tumor cells in the argentaffin tissue of the abdominal cavity. In normal condition one percent of tryptophan is normally converted to serotonin, but in the carcinoid patient as much as 60 % serotonin occurs in the cell.

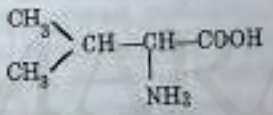
Tryptophan also forms melanotonin. This molecule is derived from serotonin by N-acetylation followed by methylation of the 5-hydroxy group.

Melanins have complex biochemical structure. Melanins form from DOPA and they are also polimers of tryptophan catabolites. Melanins are synthesized in melanosomes-membrane bound particles within melanocytes. Several defects in melanin biosynthesis can cause Albinism.

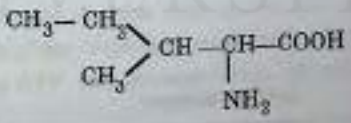
There are also several other abnormalities of amino acid metabolism due to congenital enzyme deficiencies in addition to those presented here. Most of these conditions have including mental retardation and death. One of these is maple syrup disease, so named because the urine of the patients has the aroma of maple syrup. The defect in the metabolism of the branched-chain amino acid,, leucine, isoleucine and valine (28).



valine



leucine (28)



isoleucine

AMMONIA

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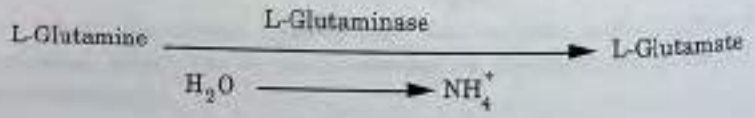
Amonia is present at physiologic pH almost as ammonium ion (NH_4^+) It is produced from the catabolism of amino acids which is toxic to mammals. In addition to ammonia formed in the tissues, a considerable quantity is produced by intestinal bacteria from dietary protein and from urea present in fluids secreted into the gastrointestinal tract. This ammonia is absorbed from the intestine into the portal venous blood, which characteristically contains higher levels of ammonia than does systemic blood. Under normal circumstances, the liver promptly removes the ammonia from the portal blood, so that blood leaving the liver is virtually ammonia free. This is essential since even minute quantities of ammonia are toxic to the central nervous system. The symptoms of ammonia intoxication include a pecular flapping tremor, slurring of speech, blurring of vision, and in severe cases, coma and death. These symptoms resemble those of the syndrome of hepatic coma which occurs when blood and presumably, brain ammonia levels are elevated. Ammonia intoxication is assumed to be a factor in the etiology of hepatic coma. Therefore, treatment includes measures designed to reduce blood ammonia levels.

The ammonia content of the blood in renal veins exceeds that in renal arteries. It indicating that the kidneys produce ammonia and add it to the blood. However, the excretion into the urine of the ammonia produced by renal tubular cells constitues a more significant aspect of renal ammonia metabolism. Ammonia production, an important renal tubular mechanism for regulation of acid-base balance and conservation of cations, is markedly increased in metabolic acidosis and depressed in alkalosis. This ammonia is derived not from urea, but from intracellular amino acids, particularly glutamin ammonia release is catalyzed by renal glutaminase.



Ammonia, constantly produced in the tissues but present only in traces in peripheral blood (10-20 µg/dl). It is rapidly removed from the circulation by the liver and converted to glutamate, to glutamine, or to urea. Formation of glutamine is catalyzed by glutamin synthetase as mentioned above. It is a mitochondrial enzyme present in highest quantities in renal tissue. Liberation of nitrogen of glutamine as ammonia occurs by hydrolytic removal of ammonia catalyzed by glutamine as ammonia occurs by hydrolytic removal of ammonia catalyzed by glutaminase. Glutamine synthetase and glutaminase catalyze interconversion of free ammonium ion and glutamine. Just like of the interconversion of glucose and glucose 6-phosphate by glucokinase and glucose-6-phosphatase. Asparaginase and glutaminase have both been investigated as antitumor agents, since certain tumors exhibit abnormally high requirements for glutamine and asparagine.

In the brain the major mechanism for removal of ammonia is glutamine formation. In the liver the most important pathway is urea formation. Brain tissue can form urea, but this does not play a significant role in ammonia removal.

UREA SYNTHESİS

Urea synthesis is common metabolic reactions of amino acids. Ammonia produced from the catabolism of amino acids is toxic to mammals as mentioned. One of the important mechenism for ammonia removal is the formation of urea. Urea synthesis takes place in the liver, released into the blood and cleared by the kidney. After ingestion 100 g of protein daily must excrete about 16.5 g of nitrogen.

As it is shown in figure 21 first reaction in the urea cycle is formation of carbamoyl phosphate by the enzyme carbamoyl phosphate synthetase(1). This molecule combine with ornitin and citrulline forms(2). Condensation of citrulline with aspartate forms argininosuccinate(3). The reaction requires ATP, and the equilibrium strongly favors argininosuccinate synthesis.

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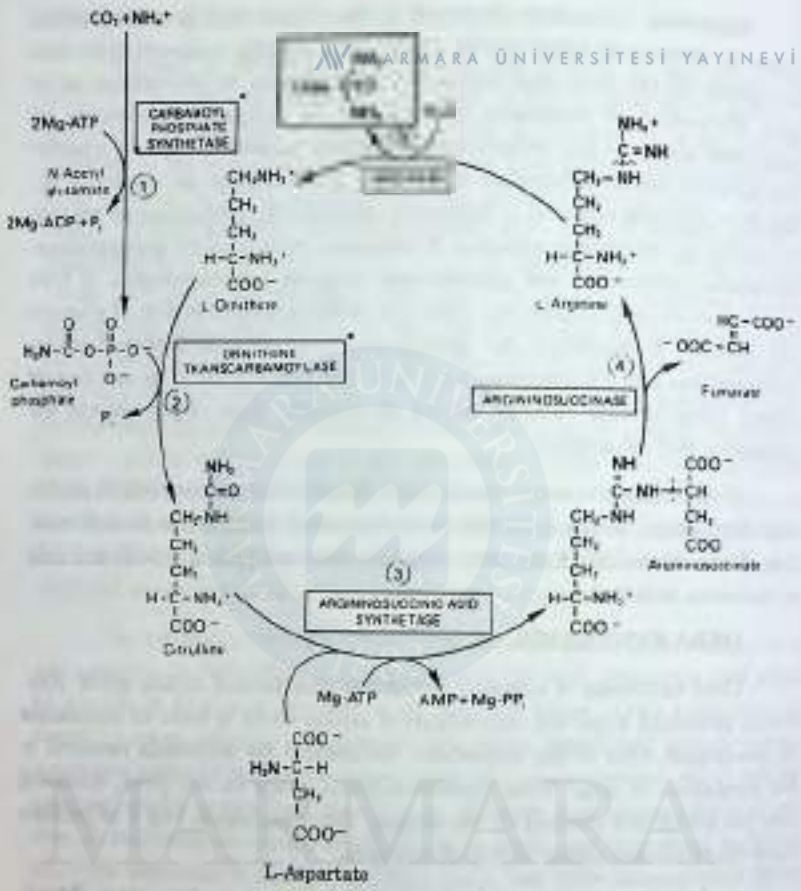

Figure 21: Reactions of urea biosynthesis

Cleavage of argininosuccinate by the enzyme argininosuccinase forms arginine and fumarate, later arginase breaks down the arginine urea and ornitine occur. (4, 5)

Since the urea cycle converts toxic ammonia to nontoxic urea, all disorders urea synthesis cause ammonia intoxication. Hyperammonemia, citrillinemia, hyperargininemia are known disorder releated to urea cycle. Clinical symptoms common to all urea cycle disorders include vomiting in infancy, avoidance of high-protein foods, intermittent ataxia, irritability, lethargy, and mental retardation. In patients food intake should be in frequent small meals to avoid sudden increases in blood ammonia levels.

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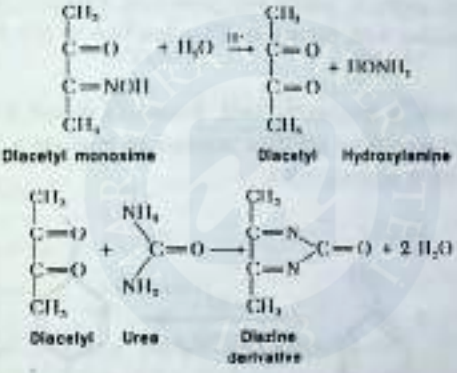
Analysis of urea

There are 2 approaches to the measurement of serum urea: the direct reaction and 2-step reactions.

Direct reaction

In this technic urea reacts directly with diacetyl monoxime to produce a yellow color. The intensity of the color is enhanced by arsenic and thiosemicarbazide. The solvent base is usually sulfuric acid (see below).



The color of this reaction is unstable, and the reagents are foul smelling, deadly poisonous, and highly corrosive. Several manual technics have employed this reaction quite successfully. This reaction is called Fearon reaction and it is highly specific, and only a few minor products of the urea cycle will interfere.

Three other direct methods using hypobromide modified Ehrlich's reagent.

Two-step reaction

All 2-step reactions begin with an initial step that cleaves the urea to produce ammonia; then various methods are employed to quantitate the ammonia.

The first step utilizes the urease reaction. Urease is an enzyme that acts very specifically with urea to yield ammonium and bicarbonate (29).

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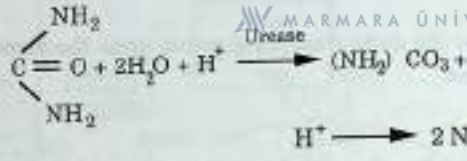
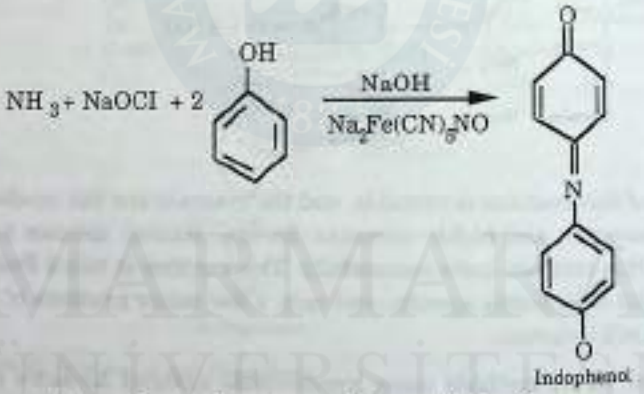


(29)

There are several approaches to the measurement of ammonia.

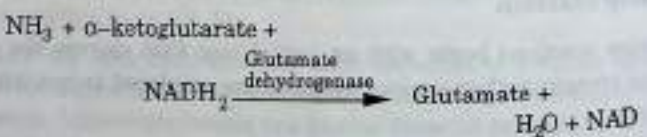
Nessler's reagent of mercuric iodide and potassium iodide in alkali produces a colored complex with ammonia. This reaction is rarely used today.

The Berthelot reaction is frequently used. Ammonia reacts with phenol and sodium hypochlorite in an alkaline medium to produce a blue color. Sodium nitroprusside is a catalyst(30).



(30)

Ammonia may be measured by monitoring its consumption in the reaction



TRANSAMINATION

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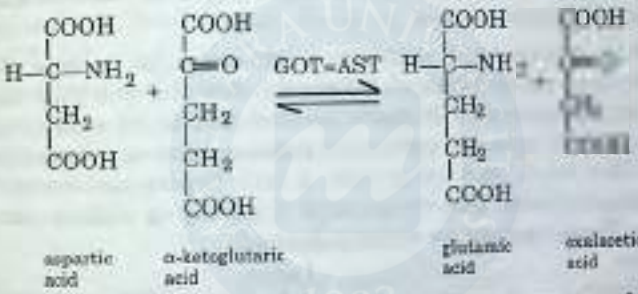
Transamination is another common metabolic reactions for most amino acids. only **lysine, threonine, proline** and **hydroxyproline** are not **substrates for transamination**. Transmanitaion reactions are catalyzed by transaminases or aminotransferases. These enzymes interconvert a pair of amino acids and a pair of keto acids. These generally are α-amino and α-keto acids.

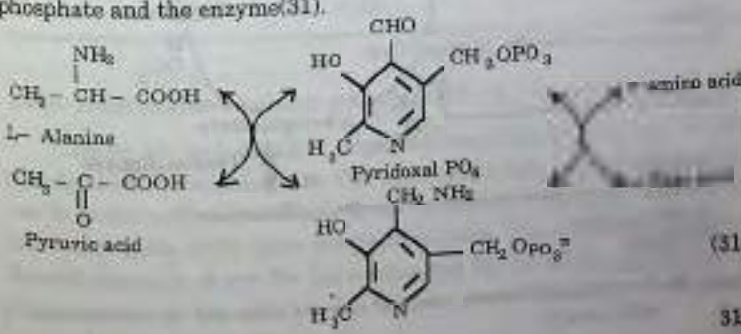
Two transaminases are of clinical interest. One is glutamic oxaloacetic transaminase (GOT) catalyzes the transfer of the amino group of aspartic acid to α-ketoglutaric acid, forming glutamic and oxalic acids.



Other glutamic pruvic acid transaminase (GPT) catalyses the transfer of the amino group of alanin to α-ketoglutanic acid forming glutamic and pyrivic acid.

Vitamin B_6 is essential for transamination reaction. The ability of pyridoxal phosphate is important for its function as a coenzyme in transamination and decarboxylation reactions.

The coenzyme pyridoxal phosphate binds to its apoenzyme via a Schiff base between its 4-aldehyde group and an ε-amino group of a lysine residue in the enzyme and via an ionic bond (salt bridge) between its phosphate and the enzyme(31).



(31)

Transamination reactions is also very important for citric acid cycle. Because some metabolic pathways end in this pathway, while other pathways originate from the cycle. These pathways concern the processes of transamination, deamination, gluconeogenesis and fatty acid synthesis. Although the cycle was discussed in greater detail in carbohydrate chapter, amino acids relationship with citric acid cycle are summarized below.

Transaminase reactions produce pyruvate from alanine, oxaloacetate from aspartate and alfa-ketoglutarate from glutamate. Because these reaction are reversible, the cycle also serves as a source of carbon skeletons for the synthesis of nonessential amino acids. Such as alanin, aspartate, glutamate.

Most of amino acids contribute to gluconeogenesis because all or part of their carbon skeletons are fed into the citric acid cycle after deamination or transamination. It should be noted that amino acids forming pyruvate if follow pyruvate carboxylase pathway converts to oxaloacetat. On the other hand, other amino acids in cycle forming oxaloacetate by transaminase or other pathway, converts to glucose via gluconeogenesis pathway. These reactions are shown in Figure 22.

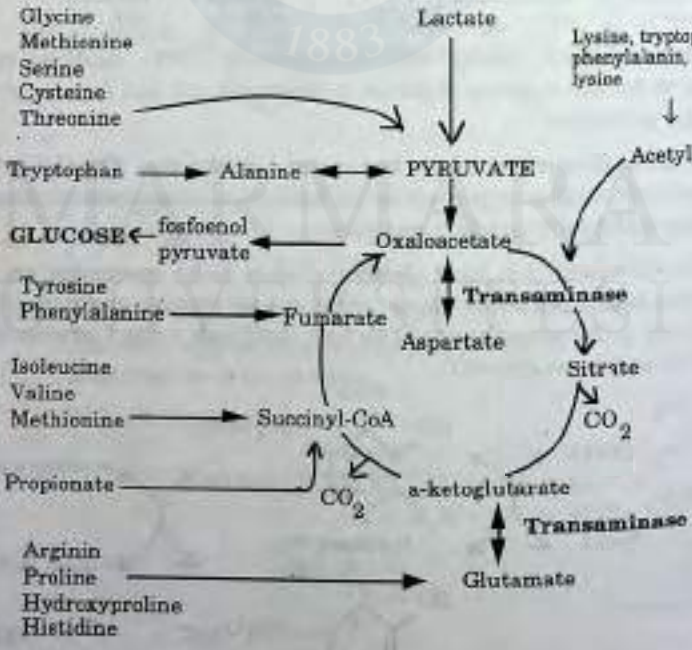


Figure 22: The relationship between citric acid cycle and amino acid via transamination or other reactions.

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Clinical Applications of Transaminases

Transaminases or aminotransferases have a wide tissue distribution and are normally present also in the blood serum in low concentrations. Activity is up to 12 U/l (Reitman, S. and S. Frankel) at 37 C Increased serum aminotransferase activity occurs in certain diseases involving tissues rich in these enzymes, notably the liver and myacardium. **This is due to the liberation of abnormally large amounts from the damaged tissues**. However, there is no consistent parallelism between the extent of increase in the serum and the amount of enzyme in the affected tissue, suggesting that other factors may be involved. Higher values are obtained normally during the first few months of life (GOT < 60 U/l, GPT < 45 U/l). These enzymes are found in high concentration some tissue as shown in table below. It is well known that damage to these tissues by toxins infections, or hypoxia will result in marked incerase in aminotransferase activity.

	Ux10^{-3} g of wet tissue homogenate		Ratio of activity in tissue to that in serum	
Tissue	GPT(ALT)	GOT (AST)	GPT	GOT
Heart	7.1	156	444	7800
Liver	44.0	142	2750	7100
Skeletal muscle	4.8	99	300	4950
Kidney	19.0	91	1188	4550
Pancreas	2.0	28	125	1400
Spleen	0.7	10	44	500
Serum	0.016	0.02	1	1

Table 1: Serum transaminase activity in different tissues (From ref. 41)

There are two forms of aspartate aminotransferase one is mitochondrial others soluble form. The isoenzymes have significant similarities in amino acid sequence and in active site position. Each enzyme comprises two units of the same polypeptide chain. The molecular weights of these dimers are 93.000 for the soluble and 90.000 for the mitochondrial

enzyme. In contrast to aspartate transaminase most of the alanine aminotransferase activity of liver tissue appears to exist as a soluble cytoplasmic enzyme. ALT is a dimer with each subunit weights about 50.000 daltons, the intact molecule is thus 101.000 daltons.

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Aminotransferase Activities in Liver and Biliary Tract Disease

Measurement of plasma AST may be of value when investigating patients with acute liver damage, in inflammatory, degenerative and neoplastic lesions of the liver. In General, the extent of rise reflects the severity and extent of hepatocellular damage. The highest values have been obtained in acute hepatic necrosis, e.g. carbon tetrachloride poisoning (< 15.000 U/l), striking increases occur also in viral hepatitis (<2500 U/l). Lower values (<250 U/l) are usually obtained in uncomplicated portal cirrhosis, biliary cirrhosis, and extrahepatic biliary obstruction, in most types of toxic hepatitis (Chlorpromazine, salicylates, iproniazid, etc) and in hepatic malignancy, primary or metastatic.

Very early acute viral hepatitis and drug induced parenchymal damage may result in elevated aminotransferase activities several weeks before symptoms are felt by the patient. In severe acute hepatitis, the enzyme levels may reach hundreds of times the upper limit of normal. On the other hand obstructive liver disorders are usually associated with minimal aminotransferase alterations. However, when the obstruction is severe or prolonged or when infection spreads from the bile canaliculi into the liver, cellular damage may be produced and aminotransferase levels will rise.

In hepatitis A infection, jaundice occurs 14-35 days after exposure. Course of acute type A hepatitis with recovery is shown in figure 23. AST (SGOT) elevation precede the development of jaundice usually remain abnormal after the serum biluribin returns to normal values. Viremia and fecal excretion of virus usually occur during the preichteric stage of the disease. the appearance and disappearance of serologic markers are depicted by dotted lines in figure 23.

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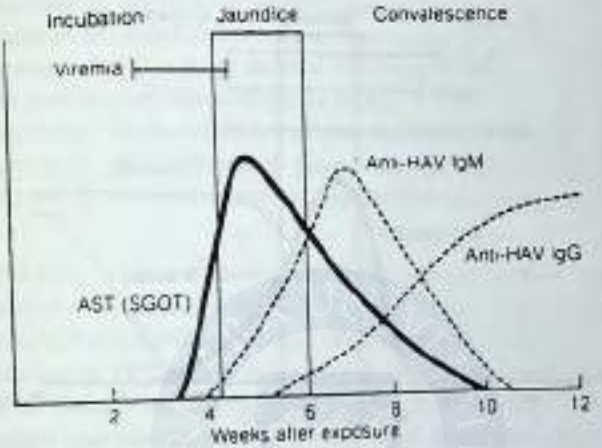

Figure 23: Laboratory indicator in acute hepatitis A infection (From ref. 46)

Course of acute type B hepatitis with recovery is shown in Figure 24. As seen in Figure, onset of hepatitis with jaundice four weeks after exposure. Detection of hepatitis B surface antigen (HB_s Ag) about 2-8 weeks after exposure, followed by apparance of its antibody (Anti-HB_s) about 2-4 weeks after HB_s Ag is no longer detectable. Detection of hepatites Be antigen (HBe Ag) shortly after appearance of HB_s Ag and its disappearance shortly after HB_s Ag disappears, this is usually followed by the appearance of antibody to HBe Ag, (Anti-HBe) that persists. Detection of hepatitis B core antibody (Anti-HBc) at the time of onset of disease 2 to 3 months after exposure. Anti HBc IgM will be detectable in high levels for about 5 months.

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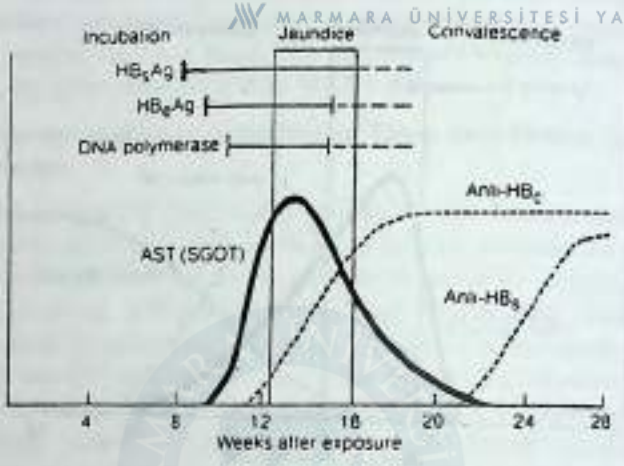

Figure 24: Laboratory indicator in Acute hepatitis B infection (From ref. 46)

Chronic active hepatitis B is illustrated in Figure 25. Ilustration shows prolonged period of abnormal serum alanine amino transferase (ALT) levels, persistance of HB_sAg and HBeAg. High level of anti-HBc IgM for about five months, and no detectable anti-HBe or Anti-HB_s.

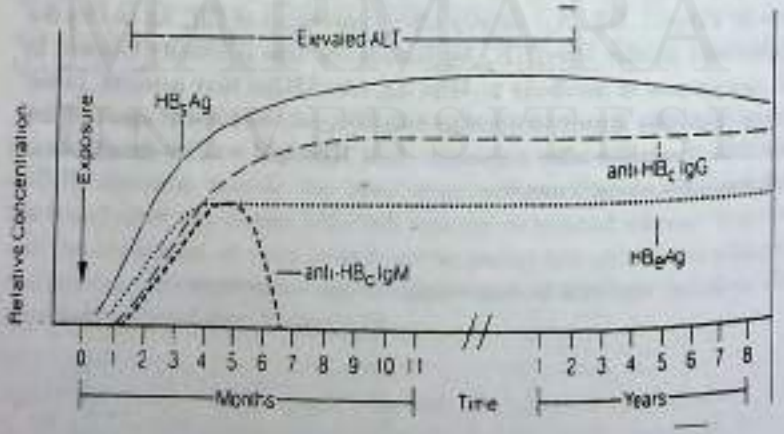

Figure 25: Laboratory indicator in chronic active hepatites (From ref. 47)

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Aminotransferase Activities in Heart Disease:

Increase in serum GOT activity may occur, but inconsistently, in acute right or left-sided heart failure. The values are usually below 100 U/l, but occasionally as high as 1500 U/l. Liver damage due to hypoxia may be a contributory factor. Slight to moderate increaseas may be a contributory factor. Slight to moderate increaeses may occur after intracardiac operations. With occasional exceptions, essentially normal values are obtained in rheumatic carditis, pericarditis and pulmonary infarction.

The form of heart disease in which this determination is of greatest value is acute myocardial infarction. This is characteristically accompanied by significant increase in serum GOT activity. Usually there is little or no increase in GPT except in the case of very large infarctions or associated liver damage (congestive heart failure, hypoxia). The rise in serum GOT begins typically six to twelve hours after occlusion of the coronary artery, reaching a maximum at twenty-four to forty-eight hours and returning to normal at about the third to the fifth day, except in the event of extension or fresh infarction. The GPT level may remain relatively unaffected (Figure 26).

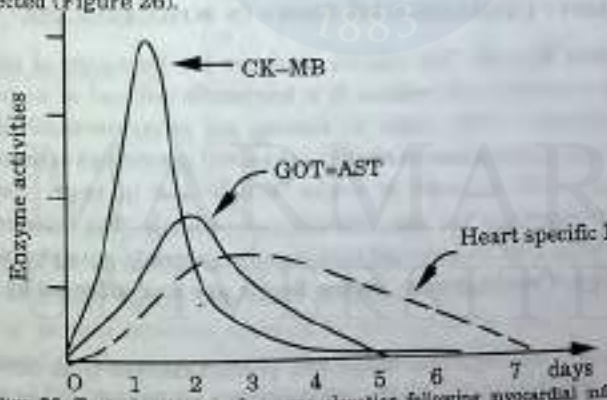

Figure 26: Typical patterns of enzyme elevation following myocardial infarction. (From ref. 1)

The extent and duration of the incerase in GOT and may be GPT are. 250 U/L therefore, of prognostic significance. The peak of rise is usually below values, above 150 U/l suggest a poor prognosis, but lower values, reflecting a smaller lesion, may also be serious, depending upon its location. The smaller increases are found most commonly.

Clinical Applications of Aminotransferases in Other Tissues:

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Increase in serum GOT may occur as a result of muscle trauma (accidental or surgical) and disease (muscular dystrophies, myositis, gangrene). An increase occurs occasionally in patiens with acute pancreatitis, leukemia, and toxemia of pregnancy, liver damage may be a contributory factor in such cases. Increases in serum GOT also occur in patients with acute hemolytic anemia, in acute renal necrosis, after severe burns, as a result of cardiac catheterization and in patients with recent brain injury. In normal individuals, an increase can result from prolonged severe exercise. Little or no incerase occurs, as a rule, in muscular diseases of nervous origin. A number of drugs can cause inceases in serum GOT.

Since ALT (GPT) is widely distributed, increased plasma activity may be observed in many other conditions accociated with tissue injury. For instance, plasma ALT activity sometimes rises in acute pancreatitis, acute renal disease, muscle disease, and disseminated carcinoma, but these findings are not specific enough to be much help in diagnosis.

OTHERS PLASMA ENZYME TESTS IN SOME DISEASE

Creatine Kinase: This enzyme catalyzes the formation of phosphocreatine from ATP and creatine. It is frequently referred to as creatine phosphokinase (CPK), since all kinases are phosphotransferases. The enzyme is found in greatest amount in skeletal muscle but is present in quite appreciable amounts in cardiac muscle and in brain. Small amounts are present in the lung, thyroid and adrenals. The enzyme is apparently not present in liver and kidney and is certainly not detectable in erythrocytes. **Consequently, serum levels are not affected by hemolysis.**

Creatine is an important nonprotein product derived from certain amino acids. The initial step in the synthesis is a reaction between glycine and arginine. The amide group from arginine is transferred to glycine to produce guanidoacetic acid. This type of reaction is called transamidination-not to be confused with transamination(32).

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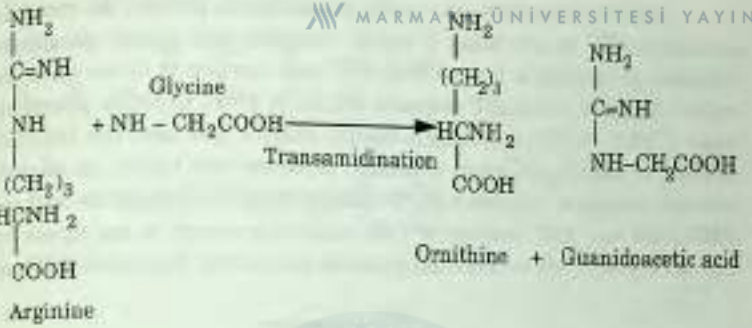
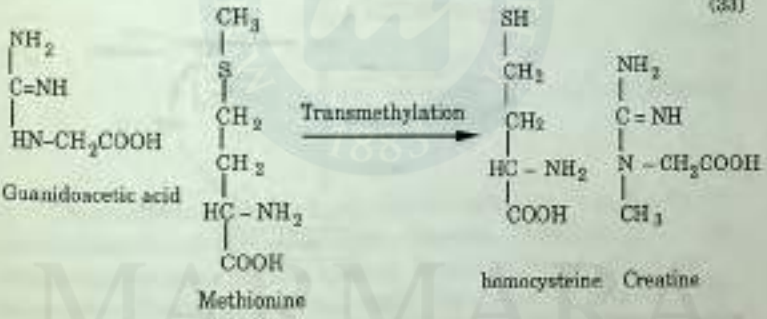


(32)

Later, guanidoacetic acid reacts with methionine in a transmethylation reaction to yield homocysteine and creatine(33).



(33)

Creatine is very important to energy storage in muscles, because it can react with ATP and phosphocreatine or creatine phosphate occurs. This is high energy phosphate compound. It can be stored in muscles for long periods without appreciable decomposition, since the ATP is required for the energy source the contraction-relaxation cycle of the muscle. ATP also can be generated by glycolysis, oxidative phosphorylation, or two ADP molecules by the enzyme myoadenylate kinase as shown in (figure 27) The ATP stores in skeletal muscle are short-lived during contraction, providing energy probably for less than 1 second of contraction, providing energy probably for less than 1 second of contraction. In slow skeletal muscle, which has abundant O_2 stores in myoglobin, oxidative phosphorylation is the major source of ATP regeneration. But in fast skeletal muscles regenerate ATP from glycolysis, mainly.

Creatine phosphate, also called phosphagens, prevent the rapid depletion of ATP by providing a readily available high-energy phosphate. Creatine phosphate is formed from ATP and creatine at times when the muscle is relaxed and ATP demands are not so great. Creatine phosphokinase (CPK), therefore, a muscle-specific enzyme it is used the detection of acute or chronic disorders of muscle. Myoadenylate kinase, an enzyme present in muscle, catalyzes the formation of one ATP molecule and one AMP from two ADP molecules. This reaction is shown in the figure below. ATP hydrolysis occurs during muscle contraction by myosin ATPase.

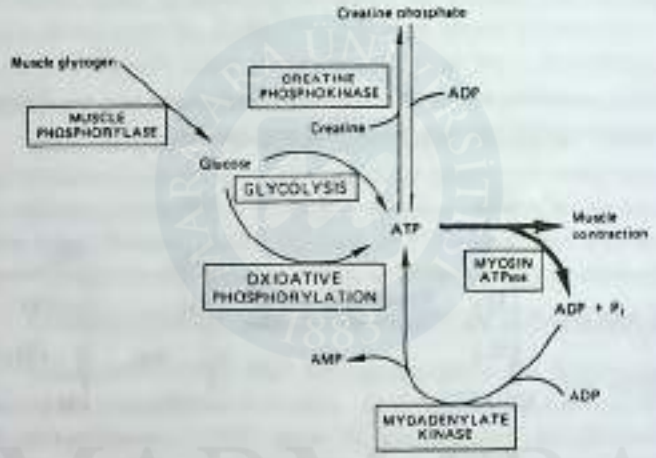


Figure 27: ATP sources in muscle from different pathways and enzymes. (From ref. 15)

Creatine kinase or creatine phosphokinase is a cytoplasmic and mitochondrial enzyme with wide tissue distribution. At neutral pH, the formation of ATP is favored, a pH of 9.0 is optimal for the formation of creatine phosphate, another high - energy compound.

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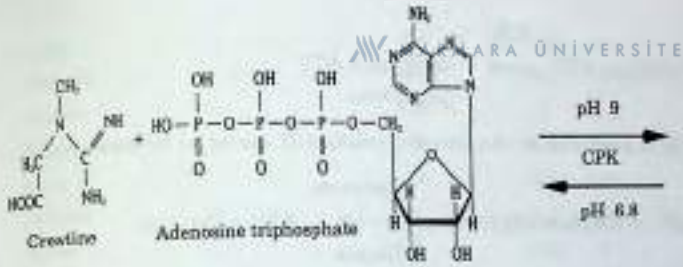

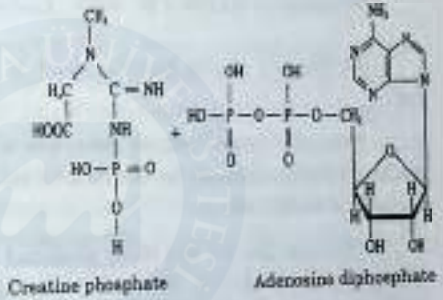

Creatine kinase combines with ATP or ADP and a cation to form a metal nucleotide complex. Magnesium, Mangan, Cobalt, Stronsium and Barium are activators for this reaction. Some cations and anions inhibit this reaction. These are, Berilium, Nikel, Chromium, Calcium, Zinc, Silver, Mercury and Cupper. Anionic inhibitors are, malonate, iodoacetate, citrate, acetate, iodine, clorür, flour sulfate, nitrate, bromium and sulfur.

There are different analytical methods for measuring creatine kinase activity. The analytic methods for serum creatine kinase, either the products formed in the forward or reverse reaction are measured. For exampe, ADP produced is measured by kinetic methods. The reaction is as follows:

$$\text{Creatine} + \text{ATP} \xrightleftharpoons{\text{CK}} \text{Creatine phosphate} + \text{ATP}$$

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ADP is produced in the forward reaction It reacts as follows:

$$\text{ADP} + \text{Phosphoenolpyruvate} \xrightleftharpoons{\text{Pyruvate Kinase}} \text{ATP} + \text{Pyruvate}$$

$$\text{Pyruvate} + \text{NADH} + \text{H} \xrightarrow{\text{LDH}} \text{Lactate} + \text{NAD}^+$$

At the end of this reaction there is decrease in absorbance at 340 nm.

Pyruvate produced in the above reactions reacts with 2,4-dinitrophenylhydrazine to form 2,4-dinitrophenylhydrazone, which is measured at 440 nm.

Creatine kinase has three principal isoenzymes, each composed of two polypeptide chains, denoted B and M. These give dimers with the composition BB, MB and MM. Skeletal muscle has a very high total CK content, over % 98 of this normally being CK-MM, the remaining 0-2 % is CK-MB. Cardiac muscle also has a high CK content, In most other tissues contains in varying amounts in diffeent tissue. A summary of enzyme activity distribution is given in table 2.

Table 2: Creatine kinase and isoenzymes activities in differeat tisues (From ref. 41)

Tissue	CK activity (U/g of wet tissue)	CK-MM %	CK-MB%	CK-BB%
Skeletal muscle (gastrocnemius)	3280	99	<1	0
Skeletal muscle (intercostal)	1884	99	<1	<1
Myocardium (papillary muscle)	620	87	13	0
Myocardium (right atrium)	402	76-78	22	0-2
Placenta	240	19	1	80
Brain	200	0	0	100
Ileum	161	3	1	96
Urinary bladder	160	2	6	92

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Colon	137	3-4	<1	98
Stomach	122	2-4	1-4	94-95
Uterus	89	2	1-3	95-97
Kidney	18	8-13	0	88-92
Lung	11	16-35	0-1	64-84
Prostate	9	3-4	2-4	93-94
Spleen	7	74	0	26
Salivary gland	6	44	0	56

Both total CK activity and CK-MB activity measurements are performed most often in the diagnosis of acute myocardial infarction. Whereas increases in plasma CK-MB activity are relatively specific for myocardial damage, total CK activity is raised whenever there is damage to skeletal muscle, whether this be due to ischaemia, metabolic causes, or inflammation. Very large increases in total CK activity occur in children with Duchenne muscular dystrophy and in patients with rhabdomyolysis. Increases are more variable, but may sometimes be large, following accidental trauma or surgical operations, after intramscular injections, diabetic ketoacidosis, acute renal failure and hypothyroidism. Increased plasma total CK activity is also found after prolonged muscular exercise, especially in untrained individuals. The CK tends to peak 16 to 24 hours after exercise and remains elevated for approximately 72 hours.

In pregnancy, serum CK has been reported to decrease in the second trimester but increase in late pregnancy. after labor, elevated serum CK activities are common.

Raised plasma CK-BB activity is unusual. It may occur in patients with carcinoma, especially of the prostate, and after acute brain damage. The measurement has not been found to be of much value.

Release of CK from skeletal muscle may occur after pharmacological doses of certain drugs, whether injected intramuscularly or ingested orally. Some patients receiving the drugs listed below; had significant increase in the serum CK.

Drug-induced changes at pharmacoligical doses	Relative increase
Danazol	Slight
Halofenate	Slight
Pindolol	Slight
Stanozolol	Slight
Succinylcholine	Slight
Aminocaproic acid	Moderate
Amphotericin B	Moderate
Carbonoloxone	Moderate
Clofibrate	Moderate
Haloperidol	Moderate
Labetalol	Moderate
Lidocaine	Moderate
D-Penicillamine	Moderate
Perphenazine	Moderate
Prochlorperazine	Moderate
Quinidine	Moderate
Cyclopropane	Pronounced
Diethylether	Pronounced
Halothane	Pronounced

Decreased serum values have been observed in small, sedentary persons and in patients with cancer cachexia. Persons with neurogenic muscular atrophy usually have very low serum CK activities.

There is no known enzyme that catalyzes the conversion of creatine to creatinine. The reaction occurs spontaneously in the body. Creatinine is formed by the condensation of creatine into a ring structure with the concomitant loss of a water molecule as shown in above. The amount of creatinine present in a person reflects muscle mass. Therefore women have lower serum creatinine levels than men. Creatinin once formed is essentially exclusively removed by renal excretion, therefore it is used to follow this process. The amount of creatinine excreted in the urine per day for a given individual on a controlled diet is quite constant, and this

amount is directly proportional to the muscle mass of the individual. Adult males secrete from 1.2 to 2.0 g of creatinine per day, while females secrete from 1.0 to 1.8 g per day. Except in pregnancy and certain muscle diseases, **creatine** is normally found in only small concentrations in the urine of adults. However, in the urine of children creatine is normally found in greater concentrations than creatinine until puberty.

Analysis of creatinine in urine can often be used to determine whether a urine specimen is a 24 hour collection. For instance, suppose a 24 hour collection from an adult male is found to contain 0.5 g of creatinine, this would be reason to suspect that the sample did not represent a true 24 hour collection.

Creatinine measurements are primarily performed to estimate renal function. In general, the plasma or serum creatinine level is the most widely used test to monitor renal function. These values, along with urinary excretion rates, can be used to estimate the glomerular filtration rate (GFR). Creatinine clearance is used to estimate the GFR. **Creatinine is chosen because it is freely filtered at the glomerulus and is not reabsorbed by the tubules**. However, a small amount of the creatinine (about 5 %) in the final urine of healthy persons is derived from tubular secretion to do the test, one needs a precisely timed urine collection and a blood sample taken during the collection period. Best results are obtained from a 24,hour urine collection. The test is initiated by having patients empty their bladder at the beginning of the timed period. Urine is collected throughout the period, the bladder is again emptied at the end of the time period, and a blood sample is obtained.

Creatinine determinations are performed on both samples (urine and blood) The creatinine clearance is calculated from the following formula:

$$\text{Creatinine clearance (ml/min)} = \frac{U\,V}{P} \times \frac{1.73}{S}$$

where U is urinary creatinine (mg/l)

V is volume of urine (ml/min)

P is plasma creatinine (mg/l)

S is the calculated surface area of the patient, and 1.73 is the surface area (m^2) of a standard 70 kg person.

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The range of creatinine clearance in healthy persons corrected to a surface area of 1.73 m^2 is 90 to 120 ml/min. At low filtration rates, the creatinine clearance does not parallel true glomerular filtration rate because a relatively large portion of the urine creatinine is secreted rather than filtered.

Serum creatininine is also important following the renal transplantation. For renal transplant patients, an increase in serum creatinine of 2 mg/l has been used as a criterion of establishing rejection. In a healthy person with a serum creatinine of 10 mg/l this change in creatinine of 2 mg/l would represent a 20 % loss in renal function.

The term "**Creatinine coefficient**" is used to indicate the number of miligrams of creatinine (plus creatine) nitrogen excreted per kilogram of body weight in 24-hours, normal values range from 7.5 to 10 (20 to 26 mg creatinine) for men and 5 to 8 (14 to 22 mg creatinine) for women. The creatine coefficient may be assumed to be an index of the amount of active muscle tissue in the body.

Creatine Tolerance: When 1.32 or 2.64 g of creatine hydrate (equivalent to 1 and 2 g respectively, of creatine) are ingested by a normal adult, about 80 per cent is retained by normal men and about 70 percent by normal, nonpregnant women: about 20 and 30 per cent, respectively are excreted in the urine during the subsequent 24 hours. Diminished tolerance is present in conditions accompanied by spontaneous creatinuria, mentioned above, and in mild cases of these disorders in the absence of creatinuria under ordinary dietary conditions.

Creatine tolerance is decreased in the majority of patients with hyperthyroidism and tends to be increased in hypothyroidism. This procedure may be of some value in the diagnosis of hyperthyroidism or hypothyroidism in children. Correction must be made for normal creatinuria, which averages 4.2 mg daily per kilogram of body weight on a meat-free diet, providing adequate calories and 2 g. of protein per kilogram.

Creatine and Creatinine in Urine

Excessive creatinuria may occur in starvation, febrile and wasting diseases, diabetes mellitus, in postencephalitic and parkinsonian rigidi-

ty, and rather consistently, but not invariably, in certain myopathies (myotonia congenita, myasthenia gravis, vaious forms of myositis, congenital muscle dystrophies, congenital muscular hypertrophy, and secondary muscle atrophy). Testosterone diminishes creatinuria in eunichoids and castrates, apparently by increasing the storage, but not the synthesis, of creatine. On the other hand, administration of methyltestosterone, after a latent period of four to sixteen days is followed by increased excretion of creatine and creatinine, due probably to increased synthesis of creatine. Increased creatine excretion follows intravaneous administration of amino acid digests.

Of particular interest in this connection is the influence of glycine upon the clinical and metabolic course of certain of these myopathies, particularly myasthenia gravis. The administration of large doses of glycine results in certain of these cases, in a 100 to 1000 per cent increase in creatine excretion. A similar increase in creatinuria also occurs in majority normal subjects after the ingestion of glycine. After a period of several weeks the creatinura begins to decrease in most instances, eventually falling to almost control levels despite the continued adminisitration of glycine. The observation has been made that cases of primary myopathy in which the average creatinuria increased more than 50 per cent above the control level after the administration of glycine apparently showed both subjective and objective improvement, provided this increased creatinuria disappeared within a few weeks. Little or no improvement was observed in ceses in which the increase in creatinuria was less than 50 per cent.

Excessive creatinuria has also been observed in hyperthyroidism, following the administration of thyroid extract, during the menstrual period and in pregnancy, and when carbohydrate is excluded from the diet. It has been observed after fractures (to 500 mg daily). It disappears gradually during the period of healing of the fracture and after the resumption of activity.

The output of creatinine is relatively constant under normal conditions and is essentially independent of diet and muscular activity. The elimination of creatinine is increased in conditions associated with increased tissue catabolism, as in fevers, and is decreased in the presence of marked muscular atrophy. It has been found that in the primary myopathies the diminished creatinuria which occurs as a secondary phenom-

enon following the administration of glycine is accompanied by an increased excretion of creatinine.

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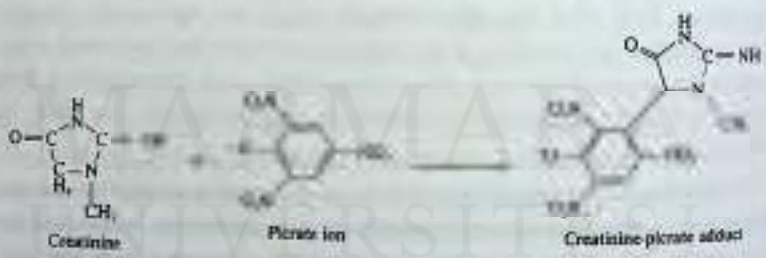
Current Methods for Analysing Creatinine

The jaffé method for creatinine analysis is the oldest method but it still in common use. This assay is based on the reaction of creatinine with an alkaline solution of sodium picrate to form a red Janovski complex. The absorbance of complex is measured between 510 and 520 nm. The major disadvantages of the original Jaffé reaction are related to lack of specificity. Ascorbic acid, pyruvate, acetone, acetoacetic acid, levilase, glucose, uric acid, protein and cephalosporin antibiotics give positive reaction by this method. Because of these Fuller's earth (Floridin) has been used to increase the specificity of the Jaffé reaction by adsorbing the creatinine present in the protein-free filtrate. Thus creatinine is isolated from potential interferents. Other materials with comparable properties are Lloyd's reagent and bentonite. All of these materials are porous aluminum-magnesium silicate clays that form colloidal suspensions with high natural adsorptive power. A small amount of the adsorbent is added to a protein-free filtrate at room temperature, adsorption is esentially complete after 1 minute. The reaction with picrate is as follows:



HPLC (high-performance liquid chromatography) is also used for chromatographic separation of creatinine from other compounds. There is also enzymatic analysis but not widely used. In this process creatinine hydrolysed to creatine, indicator reactions monitored spectrophoto metrically at 340 nm as shown below:

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$$\text{Creatinine} + H_2O \xrightarrow[\text{Amidohydrolase}]{\text{Creatinine}} \text{Creatine}$$

$$\text{Creatine} + ATP \xrightarrow{CPK} \text{Creatine phosphate} + ADP$$

$$ADP + \text{phosphoendpyruvate} \xrightarrow[\text{Kinase}]{\text{Pyruvate}} ATP + \text{Pyruvate}$$

$$\text{Pyruvate} + NADH + H^+ \xrightarrow{LDH} NAD^+ + \text{Lactate}$$

Alkalıne Phospatase

Alkaline phosphatase (ALP) and acid phosphatase (ACP) are enzymes capable of hydrolyzing phosphoric esters with the liberation of inorganic phosphate. Enzymes numbers are 3.1.3.1 and 3.1.3.2 respectively. Three types of phosphomonoesterases of clinical significance may be differentiated on the basis of their activity in different pH ranges:

1) Alkaline phosphatase with optimum activity at about pH 9.3, present in blood plasma or serum, bone, liver, kidney, intestine, mammary gland, spleen, lung, leukocytes, adrnal cortex and seminifirous tubules;,

2) A type acid phosphotase with optimal activity at pH 6, occuring in mammalian erythrocytes and,

3) A type acid phosphatase with optimum actvity at pH 5, occuring in prostatic epithelium, spleen, kidney, blood plasma, liver and pancreas.

Alkaline phosphatase is present in practically all tissues of the body. Different tissues possessing one, or occasionally more alkalin phosphatase activitis. **Liver, bone, placenta and intestine are clinically important sources** of plasma alkaline phosphatase activity. The precise biochemical role of alkaline phosphatase is not known. In many tissues it is attached to cell membranes, suggesting an association between alkaline phosphatase activity and membrane transport. In the liver, for example, activity is localized on the cell membrane that adjoins the biliary canaliculus and on the sinusoidal border of the parenchymal cell.

Alkaline phosphatase can act on a veriety of physiologic and non-physiologic substrate, but at present the in vivo substrates of these enzymes are unknown. The major source of the serum alkaline phosphatase isoenzymes are intestine, liver, bone, placenta, and certain tumor tissues. It is possible, by variety of technics, to differentiate the tissue source of a raised alkaline phosphatase, These include the inhibition of intestinal alkaline phosphatase with L-phenylalanine, the inhibition of of liver and bone alkaline phosphatase with L-homoarginine and differential heat sensitivity are currently the most often used methods of analysis. Heat denaturation remains the most widely used technic for alkaline phosphatase isoenzyme analysis.

Alkaline phosphatases prepared from different human tissues are not identical in all properties. But they are similar in many respects. It can be easily demonstrated that different tissues contain alkaline phosphatases, which migrate at different rates. The liver isoenzyme travels fastest toward the anode and occupies the same position as the fast α_2-globulin. By the electrophoretic separation bone phosphatase gives a more diffuse zone that the liver isoenzyme, Intestinal phosphatase give a more diffuse zone that the liver isoenzyme, Intestinal phosphatase also migrates diffusely but more slowly than the bone enzyme. Kidney phosphatase occurs very rarely in serum, migrates even more slowly. The placental isoenzymes have mobilities of the same order as those of liver and bone, depending on the phenotype.

Recently, two methods have been reported to greatly improve the elerrophoretic separation between bone and liver alkaline phospatases and allow quantitative estimations to be made by densitometric scanning. Both methods exploit differences in the carbohydrate portions of the two forms of alkaline phosphatase. In one method, electrophoretic separation is carried out in the presence of wheat-germ lectin, which retards bone alkaline phosphatase to a greater extent than the liver enzymes An alternative method is to treat the serum briefly, with neuraminidase. Brief treatment with neurominidase removes sialic acid residues of bone phosphatase more readily than those of liver phosphatase, so that the mobility of bone phosphatase is reduced more that of liver phosphatase. treatment with 1 Unit of neuraminidase per 10 µl of serum for 10 min at 37 C gives patterns in which the bone and liver alkaline phosphatase zones are sufficiently well separated to allow densitometric scan of the pattern.

Starch, agar, and polyacrylamide gels, as well as cellulose acetate, have been used for the separation of alkaline phosphatase isoenzymes. The choice of a particular system depends on the resources of individual laboratories, but it is important that the chosen method be used regularly and consistently to ensure repeatable and reliable results. Cerful attention to cooling (e.g. by carrying out the procedure in a cold room) is needed to minimize enzyme inactivation. After electrophoresis, alkaline phosphatase zones are made visible by incubating the gel in a solution of buffered substrate to which a chromogenic system is added. A suitable staining mixture consists of 1-naphthyl phosphate (50 mg) and Fast Blue salt (70 mg) in either Tris-borate-$MgCl_2$ buffer (70 ml) at pH 9.5 or carbonate-bicarbonate buffer at pH 10

Heat inactivation and chemical inhibition of ALP: The most remarkable property of placental alkaline phosphatase is its pronounced resistance to heat. Incubation of the enzyme at a temperature as high as 65 C for 30 min therefore provides a convenient test for the presence of this isoenzyme. Pronounced heat stability is also shown by the "Regan" isoenzyme, This is a placental-like form of alkaline phosphatase that occurs in 5-15 % of specimens from patients with cancers of various types. Thus, heating the sample at 65 C can also be used to detect this abnormal isoenzyme. The similarity between these two enzyme forms is evidenced by the fact that the Regan isoenzyme will react with antibodies raised against the placental isoenzyme. Other isoenzymes of alkaline phosphatase can also be differentiated on the basis of their stability at temperatures lower than 65 C. At 56 C for example liver ALP is more stable than the bone isoenzyme. Thus, if 20% of the serum alkaline phosphatase activity ramains after 10 min. at 56 C, the enzyme present is probably largely of bone type, residual activities of between 25 and 55 % support electrophoretic evidence that liver phosphatase is the predominant isoenzyme. For this test to be meaningful, very careful control of the experimental conditions is essential because the temperature coefficients for inactivation of enzymes by heat are high. At the end of the heating period, specimens must be immediatly immersed in ice water.

Like as L-phenylalanine, urea at high concentrations inhibits the enzyme. Inhibition is irreversible the varies with the tissue of origin of the ALP. This urea inhibition provides an alternative to heat inactivation in isoenzyme analysis. The bone enzyme is most readily inactivated, liver

phosphatase has intermediate resistance, and placental phosphatase is most resistant to urea. Levamisole preferentially inhibits liver and bone alkaline phosphatases and is effective at much lower concentrations.

Some divalent ions such as Mg (II), Co (II) and Mn (II) are activators of the ALP, and Zn (II) is a constituent metal ion. the correct ration of Mg (II)/Zn (II) ions is necessary to obtain optimal activity. Phosphate, borate, oxalate and cyanide ions are inhibitors of all forms of the enzyme. The individual multiple forms are inhibited to different degrees by L phenylalanine, ure, excess Zn (II) or ASO_4^{3-}. Variations in Mg (II) and substrate concentrations change the PH optimum. The type of buffer present may affect the rate of enzyme activity. Buffers can be classified as inert (carbonate, barbital), inhibiting (glycine, propylamine), or activating (2-methyl-2-aminopropanol-1, AMP, hydroxymethyl aminomethane and diethanolamine.

Physiological Changes in ALP Activity

The ALP forms present in the sera if normal adults originate in the liver or the biliary tract and up to half the total activity comes from the skeleton. The relative contributions of bone and liver isoenzymes to the total activity are markedly age-dependent. There is also a significant difference between the sexes in serum alkaline phosphatase activity at some ages, although this is less important for interpretation of values than the dependence on age. The variation in mean plasma alkaline phosphatase activity with age is shown below. A small amount of intestinal alkaline phosphatase may also be present, particularly in the sera of individuals of blood groups B or O who are secretors of blood group substances. The enzyme found in urine is probably derived from renal tissues.

In infancy and childhood, the upper reference value is as much as three or four times the adult value as shown in figure 28. This increase is a direct result of the intensive osteoblastic activity associated with normal bone growth, e.g. at puberty, since osteoblasts secrete alkaline phosphatase.

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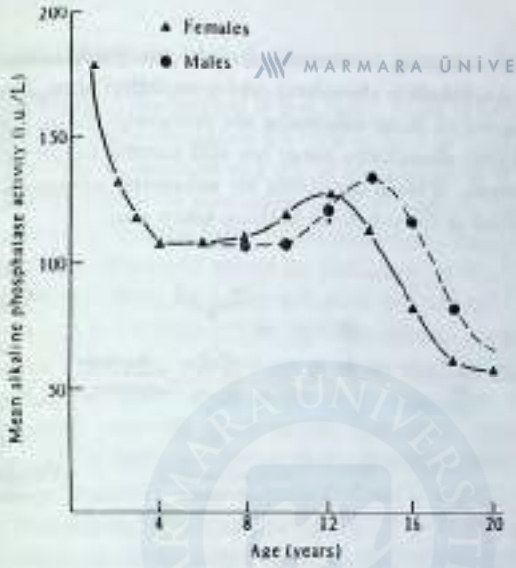


Figure 28: Variation in mean plasma alkaline phosphatase activity with age (From ref. 3)

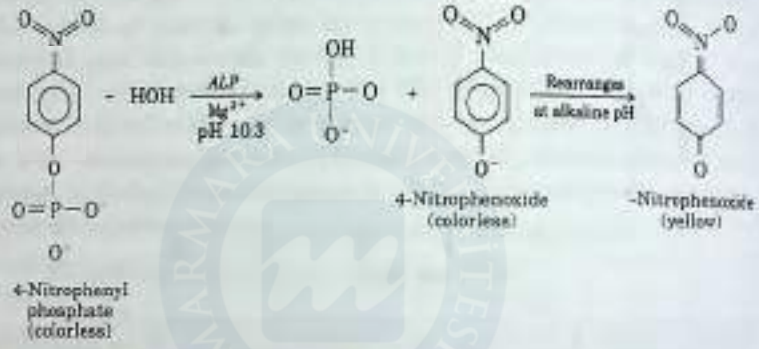
In normal pregnancy, release of alkaline phosphatase from the placenta may cause its activity in plasma to rise in the second and third trimesters to about twice the normal adult levels.

After a meal containing fat, release of intestinal alkaline phosphatase may cause a transient small increase in plasma enzyme activity. Blood for measurement of plasma alkaline phosphatase activity should preferably be collected from fasting subjects.

Methods for the Determination of ALP Activity

Different electrophoretic tecniques have been used for the seperation of alkaline phosphatase isoenzymes was mentioned above. For assay of alkaline phosphatase the most widely used substrate is 4-nitrophenyl phosphate (usually abbreviated to 4-NPP or PNPP from older name, para-nitrophenyl phosphate). This ester is colorless, but 4-nitrophenol (4-NP), the product of enzyme action, is colored at the pH of the reaction. Thus the enzyme reaction can be followed continuously by observing the rate of formation of yellow color. This reaction forms the basis of current recommended and standard methods of alkaline phosphatase assay.

Other self-indicating substrates include phenolphthalein monophosphate, thymolphthalein phosphate, and α-naphthyl phosphate. However, methods based on these substrates are relatively little. Fixed time methods for alkaline phosphates assay are still carried out in considerable numbers because of their suitability for automatic analyzers. 4-NPP is increasingly used as the substrate in these assay also.



The best separation of ALP isoenzymes is obtained using polyacrylamide gel, whereas the poorest separation is obtained using cellulose acetate. Both electrophoretic and heat-inactivation tecniques are popular and relatively simple to perform and yield clinically useful results under most circumstances. Chemical inhibition methods, though not so widely utilized. The ranges given below are based on the L-phenylalanine and urea inhibiton method. But are similar to the results observed by polyacrylamide electrophoresis and heat inactivation methods.

Table: 3 Tissue distribution of ALP

	ALP isoenzyme		
Age group	Bone	Liver	Other (Intestinal and so on)
Child and adolesent	85 %	5 %	10 %
Adult	30 %	60 %	10 %
Elderly	30 %	60 %	10 %

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Reference Range

According to the method of Bodansky, a unit of phosphatase activity (equivalent to approximately 5.5 U) is defined as "equivalent to the actual or calculated liberation of 1 mg. of phosphorus as the phosphate ion during the first hour of incubation at 37 C. and pH 8.6, with the substrate containing sodium beta-glaycerophosphate. Employing this procedure, the range of normal values for plasma or serum alkaline phosphatase activity in adults is 1.5 to 4.0 units per 100 ml (8.0-22 U/l). For children it is 5 to 14 units (27-86 U/l) The alkaline phosphatase activity of plasma is within the adult range at birth, and rises rapidly to a maximum during the first month of life (4-14 units)., the range remaining fairly constant until the third month. The upper limit now falls very slowly until it is about two-thirds of the previous maximum, at about 10 years of age. With the onset of puberty there is a great increase in bone growth. Consequently, within the ages of 11 to 16 years, the maximum may again rise to 14 Bodansky units. In other words, there is a peak value and then the range falls to that of the adult at at about 16 years of age, depending on the age of onset of puberty.

Clinical applications of ALP

Increased activity of plasma alkaline phospatase is found in many conditions two principal categories being bone and liver disease.

Although the precise metabolic function of the enzyme is not yet understood but the enzyme is closely associated with the calcification process in bone. Plasma alkaline phoshhatase activity is increased in patients with diseases in which there is increased osteoblastic activity such as Paget's disease, hyperparathyroidism, rickets and osteomalacia, and carcinoma with osteoblastic metastases.

Alkaline phospatase activity is routinely measured for possile abnormalities of calcium metabolism, these are;

Bone Diseases: The clinical significance of serum alkaline phosphatase activity in skeletal disorders may be indicated by the following generalizations:

1- The bone enzyme originates mainly in functioning osteoblasts.

2- An increase in serum reflects osteoblastic proliferation or increased activity.

3- The usual stimulus to osteoblastic proliferation is bone stress, weakness, or injury.

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4- An increase in bone alkaline phosphatase activity usually reflects; inadequate mineralization, or injury, or attempts at active repair which implies adequate bone matrix.

Abnormalities my occur in both phases of bone construction in skeletal diseases, in the formation of the osteoid matrix and in the subsequent processes of mineralization and demineralization. Accurate diagnosis and proper therapy require that these two clases of skeletal disorders be clearly differentiated.

Osteoporosis is characterized by loss of bone mass. There is not necessarily any great change in the anatomical volume of the bones, but the amount of actual calcified bone tissue in this volume is diminished. The bones are therefore much more porous (less dense) and contain more narrow space. The weight-bearing bones are considerably affected and there is a marked liability to fracture. One or more of the spinal vertebrae may collapse. It is only a matter of degree that distinguishes the condition from the normal loss of bone mass, which commences around the age of 35 years. Such bone tissue as is present seems to differ in no way from that which characterizes normal bone. Calcium deficiency can cause osteoporosis. The condition is relatively common in a number of situations in which intake or absorbtion of calcium is lowered, as in malnutrition, alcoholism, cirrhosis and in some patients with intestinal lactase deficiency, who voluntarily restrict their intake of milk and its products. In spite of this, no constant biochemical defect has been found and the serum levels of calcium, inorganic phosphate and alkaline phosphatase are usually within normal limits. Osteoporosis may be primary or associated with other disorders. Primary osteoporosis can usually be seen postmenopausal situation.

Osteomalacia is characterized by a failure to calcify the osteoid matrix. This results in the preence of an excessive amount of osteoid tissue in relation to the amount of bone mineral. Osteomalacia results from deficiency of or resistance to vitamin D. It can also result from any prolonged systemic acidemia, e.g., ureterocolic anastomosis or renal tubular acidosis, but the mechanism of production is not undersood. Osteomalacia may also be found neurofibromatosis. Serum calcium levels can be very low, but as a rule they are either slightly lowered or normal. The

level of plasma inorganic phosphate in osteomalacia is usually lowered and alkaline phosphatase increased. There is usually a diminition of urinary calcium. Aminoaciduria is occasionally present. There may be some degree of renal tubular acidosis with diminution of plasma bicarbonate.

Hypophosphatasia, the skeletal lesions of which resemble those of vitamin D deficiency, cannot be placed in either category on the basis of the biochemical manifestations. The characteristic feature is deficiency or absence of alkaline phosphatase in various tissues, including the bones and blood, frequently with hypercalcemia in severe cases, and normal or occasionally increased serum phosphate concentration.

Osteosclerosis is a rare form of metabolic bone disease in which there is excessive mineralization of bone. It can be general or local. It sometimes occurs as a result of excessive intake of flouride (fluorosis).

Osteitis fibrosa is usually due to hyperparathyroidism. There is excessive resorption of previously mineralized bone because of increased osteoclastic activity.

Serum alkaline phosphatase activity is consistently considerably increased in active rickets. When bone reconstruction is complete the serum phosphatase is within normal limits. It has been found that the serum phosphatase activity may remain above normal even after healing is apparently complete roenterographically. There is usually a reciprocal relationship between serum phosphatase activity and inorganic phosphate concentration in rickets. Osteitis Deformans (Paget's Disease) increased serum alkaline phosphatase activity occurs rather consistently in patients with osteitis deformans involving several bones. Higher values have been obtained in this condition than in any other clinical disorder. Spontaneous healing by sclerosis was associated with relatively low values for serum phosphatase activity. Age appears to be a factor the oldest patients showing the lowest values. The finding of moderately elevated serum phosphatase activity, together with normal values for serum calcium and inorganic phosphorus, is of diagnostic significance in this condition. A moderate incerase in serum phosphatase activity can occur in clinical and experimental hyperparathyroidism but is not invariably present. Values as high as 65 units have been reported. But the values usually range between 20 and 40 units. A gradual fall to normal levels has been observed following parathyroidectomy in such cases, associated with increasing mineralization of the affected bones.

Slightly elevated values for serum alkamine phosphatase activity can occur in patients with various forms of osteomalacia. Metastatic carcinoma involving bone, osteogenic sarcoma, Gaucher's disease with bone resorption, and osteosclerosis fragilis generalisate (marble bones). In the case of bone tumors, either primary or metastatic, increases occur principally in those that are primarily osteoblastic in nature, e.g. certain types of osteogenic and prostatic carcinoma.

Normal findings are obtained in most cases of chronic arthritis, various forms of osteoporosis, osteomyelitis, bone cysts, tumors not involving bone slight increases have been reported also in occasional cases of renal rickets and multiple myeloma. Decreased serum alkaline phosphatase activity has been reported in hypothyroid children and scurvy. Elevated values have been reported during periods of calcification of hemorrhages in scurvy, with subsequent return to normal as the calcified areas are absorbed.

Liver disease: Cholestasis has a dual effect since it causes increased synthesis of hepatic alkaline phosphatase and solubilization followed by regurgitation of the hepatic enzyme into plasma. In the absence of cholestasis, hepatocelluar damaga causes relatively little release of alkaline phosphatase, presumably because the enzyme is firmly bound to the membrane of the liver cell and therefore does not leak from damaged cells as readily as do soluble cytoplasmic enzymes.

Common causes of increased liver alkaline phosphatase include cirrhosis, metastatic disease of the liver, and a variety of lesions in the liver. Placental ALP is noted during pregnancy. The tumor (Regan) isoenzyme can be seen in malignancy but is relatively rare. The intestinal ALP isoenzyme is seldom encountered in significant quantities. The Nagao isoenzyme, an isoenzyme with a phenotype closely related to the Regan isoenzyme, has been found in patients with pleural carcinoma, metastatic carcinoma of the tail of the pancreas and metastatic adenocarcinoma of the bile duct. This isoenzyme is identical to placental isoenzymes biochemically and immunologically. Unlike placental isoenzymes, however, it is inhibited by L-leucine, L-isoleucine, L-valine and EDTA.

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Acid Phosphatase

The prostate contains high concentrations of acid phosphatase. The range of pH 5-6 is optimum pH for acid phosphatase. Other acid phosphatase isoenzymes are present in small concentration in liver, spleen, red cells and platelets. The prostatic and non-prostatic isoenzymes may be distinguished from one another by using a substrate that is preferentially hydrolysed by one of the isoenzymes, or by measuring enzymic activity in the presence of a selective inhibitor. Prostate gland contributes about one-third to one-half of the enzyme present in sera from healthy males. The source of the remainder of the acid phosphatase in sera from healthy males and females is not known, but there is evidence that it derives from the osteoclasts of bone.

The optimal pH for the individual acid phosphatases varies depending on the tissues from which they originate and the substrate on which the enzyme acts, the more acidic the substrate, the lower the pH at which maximum activity is obtained. The enzymes can hydrolyze a variety of phosphate esters, and indeed every substrate utilized in measuring alkaline phosphatase activity in serum has also been used to determine acid phosphatase activity.

Acid phosphatase measurements are used mainly for the diagnosis and monitoring of metastatic or invasive prostatic carcinoma. While the carcinoma remains confined within the prostate, plasma prostatic acid phosphatase activity is raised in only about 20 % of the patients. However, local or distant spread of the neoplasm causes increased activity, often to high levels, in up to 80% of patients although, when the carcinoma is first diagnosed, activity is raised in only about 50% of patients. Enzyme activity falls rapidly after successful oestrogen therapy, but quickly rises again if relapse occurs.

Increased plasma acid phosphatase activity occurs in several other disorders. The prostatic isoenzyme released when there is prostatic inflammation, trauma or necrosis. A tartratestable (non-prostatic) isoenzyme is often increased in patients with bone disease, especially Paget's disease, but also in patients with hyperparathyroidism and metastatic breast carcinoma. Plasma total acid phosphatase may be increased in liver disease, Gaucher's disease, and thrombocytopenia (when this is due to an increased rate of platelet lysis).

Slight or moderate elevations in total acid phosphatase activity often occur in hyperparathyroidism with skeletal involvement, and in the presence of malignant invasion of the bones by cancers such as female breast cancer. The enzyme in these cases typically is not inhibited by tartrate and thought to come from osteoclasts. In some cases of metastatic breast cancer, however, elevations of the tartrate-inhibited acid phosphatase has been observed. Increased concentration of nonprostatic acid phosphatase have also been observed in patients with Gaucher's and Niemann-Pick diseses, in myelocytic leukemia and in some other hematological disorders. Acid phosphatase is present in very high concentration in semen.

Methods for acid phosphatase activity measurements are essentially adaptations of those developed for alkaline phosphatase. Phenyl phosphate and 4-nitrophenyl phosphate have frequently been used in an acid pH medium for acid phosphatase estimation.

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BIOCHEMICAL ASPECTS OF HAEMOSTASIS

Together with carbohydrates and proteins, lipids constitute the major structural material of living cells. In platelets the most important class of functional lipids are phospholipids, which are esters of fatty acids and phosphoric acid, with glycerol or sphingosine. Platelets also contain glycolipids and others. Lipids rarely exist in cells or tissues as free entities. They usually occur as a macromolecular array with protein or carbohydrate, as lipoproteins or lipopolysaccharides. In addition to their structural importance, they exert a functional role as well.

Eicosanoids and Platelets

As it is shown in figure 29 eicosanoids which is found in diet are converted to their biologically active product by different enzymes.

Following cell stimulation (figure 30) there is also a transient release of free arachidonic acid, which is enzymatically converted to biologically active prostoglandins thromboxanes, prostacylin and hydroxy acids. As will be discussed later, some of these intermediates and products may participate in cell-cell interactions.

Identification and seperation of individual lipid classes from a tissue or blood cell such as platelets developed in the early 1900_s. Gas-liquid chromotography (GLC) provided identification techniques for fatty acids and aldehydes in a given lipid class. Moreover, GLC could be carried out on small samples, making it uniquely suitable for platelets. Paper chromotography was soon superseded by thin-layer chromatography (TLC), which permitted accurate qualitative and quantitative analytical studies of cell and tissue lipids. In addition, seperated lipids could be eluted from TLC plates and further studied. High performance liquid chromatography (HPLC) is the latest available technique, which has been the basis for biochemical studies of hydroxy acids, including leukotrienes. Finally, mass spectroscopy crowned the advances of the analytical phase.

By using these tecgniques platelet were found to contain a complete fatty acid-synthesizing system. Arachidonic acid (20:4) is one of the major fatty acid in human platelets. It is present in both intracellular granules and membranes, mainly esterified to the phospholipid fraction. Platelet arachidonic acid is the precursor of thromboxane and hydroxy fatty acids. Arachidonic acid is not oxygenated unless it is hydrolyzed from phospholipids.

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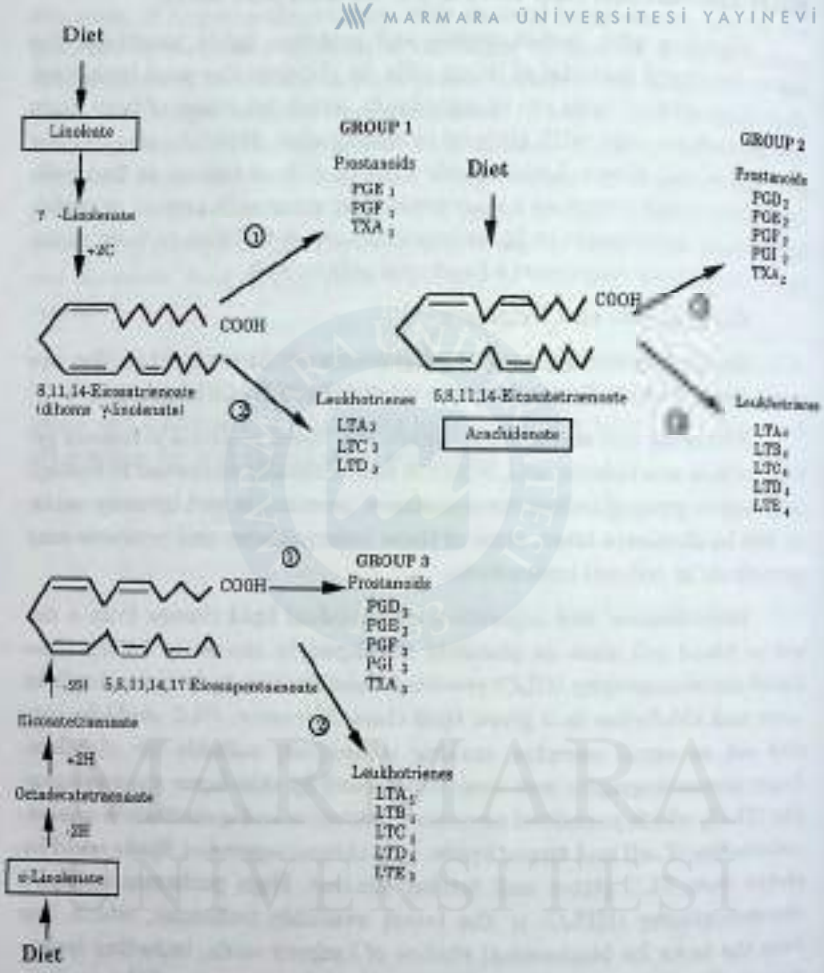

Figure 29: The three groups of eicosanoids and their biosynthetic origins. 1- cyclooxygenase pathway; 2- lipoxygenase pathway.

Platelet cyclooxygenase catalyzes oxygen incorporation at carbons 11 and 15 of arachidonic acid. Cyclization of 20:4 also occurs, and these reactions culminate in formation of endoperoxide prostaglandin G_2 (PGG_2). The endoperoxides are transient intermediates and are the pivotal substances in formation of thromboxane in platelets (TXA_2) and prostacyclin (PGI_2) in endothelial cells. Almost all cyclooxygenase preparations possess peroxidase activity which quickly transforms PGG_2 to the endoperaxide prostaglandin H_2 (PGH_2), its 15-hydroxy derivative. Platelet cyclooxygenase acts on arachidonic acid with much greater efficiency than it does on other 20 carbon fatty acids. Stimulation of platelets by collagen, thrombin or arachidonic acid will result in a transient period (up to 1 min) of oxygen incorporation, which can be recorded concomitantly with the initition of platelet aggregation, the duration of the platelet oxygen burst varies with the stimulus but is much shorter than that of the stimulated leukocyte, which persists for up to 20 minutes. Furthermore, the leukocyte oxygen burst results mainly in production of superoxide and hydrogen peroxide and accompanies the process phagocytosis, platelets are not phagocytic cells and therefore would not be expected to possess an oxygen burst identical to that of the leukocyte. the cyclooxygenase in platelets and other tissues is irreversibly inactivated by the acetyl group of acetylsalicylic asid (Aspirin). Since the platelet is unable to synthesize protein, this acetylation persists for the life span of the platelet (7 to 9 days)

Eicosanoids have important effect on platelet aggregation, thromboxane A_2 which was isolated from platelets is the active aggregating agent thromboxane B_2 is its inactive, stable end product. Thromboxane A_2 has been identified in other tissues and is the major component of the rabbit aorta contracting substance, and also it is powerfull aggregating agent.

Participation of eicosanoids in platelet aggregating may be summarized as follows: Stimulation of platelets with agents such as thrombin, collagen e.g. result in hydrolyse of arachidonic acid by phospholipase. Oxygenation and cyclization of arachidonate leads to PGH_2 formation and subsequent conversion to thromboxane A_2 by platelet thromboxane synthetase. Thromboxane A_2 induces mobilization of calcium from intracellular storage sites. Mobilized calcium in the platelet cytoplasm has at least two effects:

- One is stimulation of release of ADP, serotonin and other secretory products from intracellular grenules.

- The other is inhibition of platelet adenylate cyclase resulting in a fall in platelet cAMP.

There is lipoxygenase pathway in platelets but most research has focused on the cyclooxgenase pathway because this can be directly related to platelet function.

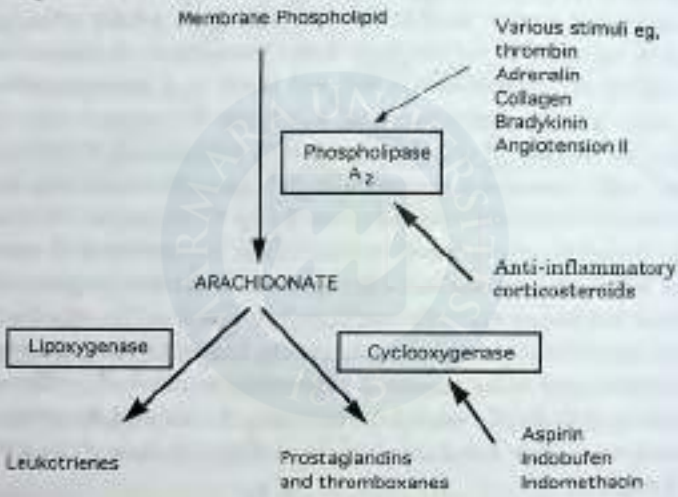

Figure 30: Eicosanoids after cell stimulation

Platelet research is now focusing on the metabolism of arachidonic acid which is an polyunsaturated fatty acid. A polyunsaturated fatty acid synthesized by mammals from dietary linoleic acid. Arachidonic linoleic and linolenic acids are known as the essential fatty acids (EFA_s) and cannot be synthesized by higher mammals; instead, they must be obtained from dietary sources. In the absence of intake of EFA, a metabolic disorder known as essential fatty acid deficiency will develop. The pathways for unsaturated fatty acid biosynthesis are oxygen dependent, as is eicosanoid synthesis. The discovery of eicosanoids and the development of the concept that they originate from arachidonic acid constitute a milestone in biomedical research. Structures of the compounds are shown in figure 29. Eicosapentaenoic acid (20:5), found in fish oils, may also serve as a source of eicosanoids (TXA_3, PGI_3). These omega-3-derived eicosanoids may function as protective factors in vascular disease.

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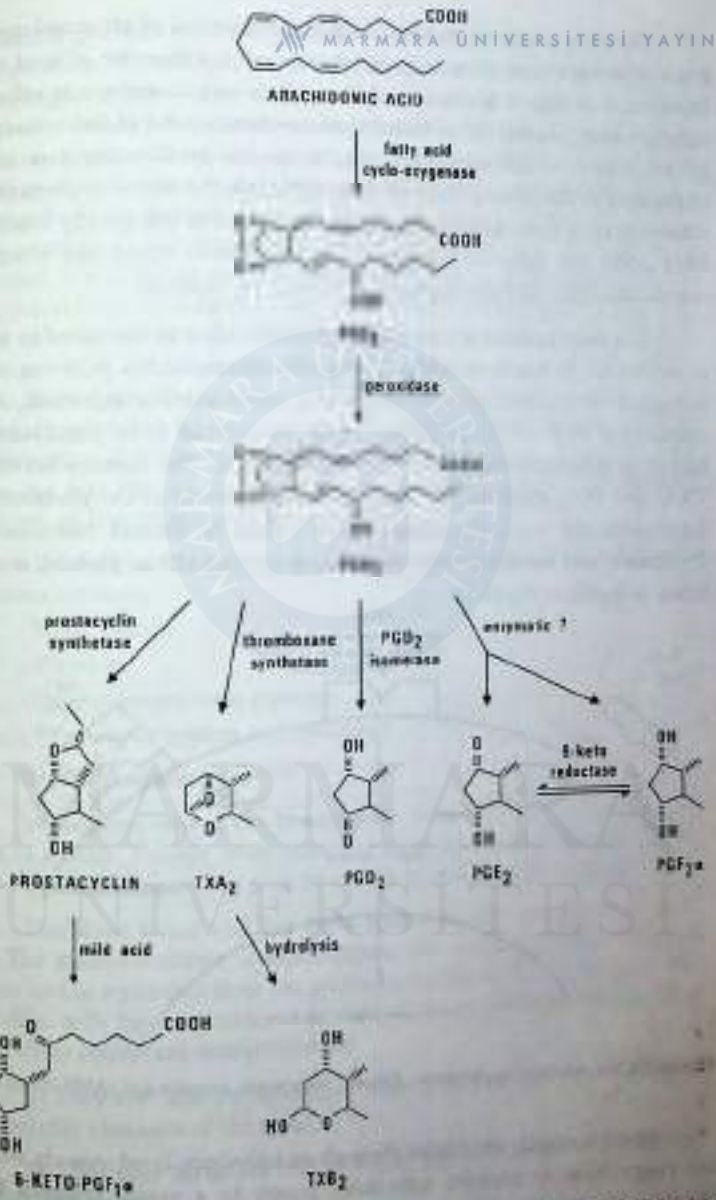


Figure 31: Biosynthetic pathways of prostacyclin, thromboxane A_2 and other product which originate from arachidonic acid. (From ref. 123)

Platelets might be involved in the pathogenesis of atherosclerosis. Since atherosclerosis is considered by some to be a disorder of lipid metabolism, it is logical to consider that platelets may contribute to atherosclerosis and possibly to occlusive vascular disease, the platelet-derived growth factor, which stimulates smooth muscle proliferation has been implicated in the pathogenesis of atherosclerosis. Since it is important in atherosclerosis and vascular disease incorporation of isotopically labelled fatty acids and fatty acid precurcors in to platelet lipids and platelet membrane lipids and glycoproteins was reported in detail.

The total amount of membrane phospholipids was increased in atherosclerotics. It was also observed that the prostaglandin pathway was enhanced in platelet of atherosclerotics. After release induction, the amounts of PGF, PGE, TXB_2 and MDA were found to be significantly higher in atherosclerotics than in normal control. The balance between TXA_2 and PGI_2 shifts to the TXA_2 side. cAMP levels in the platelets of atherosclerotics were significantly lower than in normal individuals. Prostanoic acid receptors and interactions with cAMP in pletelet membrane is shown in figure 32.

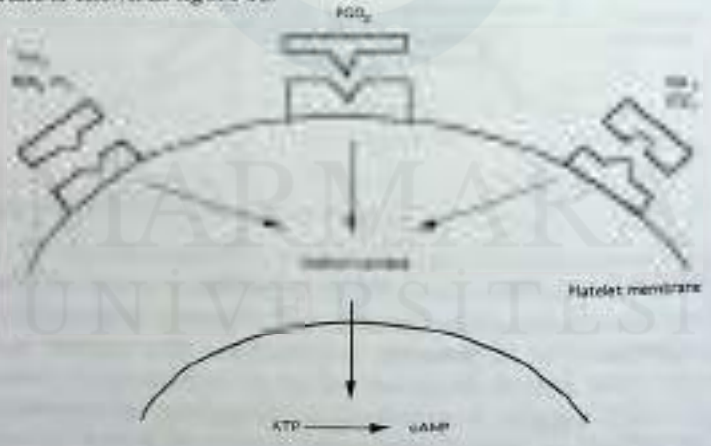


Figure 32: The relationship between Pletelet membrane receptor and cAMP (From ref. 74)

Blood normally circulates through endothelium-lined vessels without coagulation or platelet activation. Injury to a vessel triggers the heamostatic process. After injury adherence of platelets begin to damaged endothelium or exposed subendothelium. At the same time, plasma

coagulation proteins react with the subendothelium and "contact phase" of coagulation. Thrombopastin, which is present in exposed tissues or macrophages, triggers the "extrinsic phase" of blood coagulation. Platelets are stimulated, for instance, by subendothelial collagen, to expose membrane glycoproteins and support platelet aggregation. Secretion mediated by thrombaxane A_2 synthesis and intracellular calcium translocation results in the release of proteins from α-granules. After a number of series reaction Factor X and prothrombin activation occur. The result is thrombin formation the major substrate of thrombin is fibrinogen, which after initial hydrolysis forms fibrin monomers that then undergo spontaneous polymerization to form the fibrin clot. Covalent crosslinking by the thrombin-activated enzyme Factor XIII_a increases the resistance of the clot to fibrinolysis. Plasminogen is converted to plasmin by plasminogen activators produced by endothelial cell, a later fibrinolysis occurs with the formation of fibrin degradation products. Dissolution of the clot prepares the way for the formation of fibrous tissue and wound healing, these are all hemostatic system processes.

- Normal haemostasis depends on a balance and complex interareactions between;

- Blood vessels
- Platelets
- Plasma coagulation proteins
- Fibrinolytic system and inhibitors.

Blood Vessels:

In the first step of the haemostasis vasoconstriction occurs by the help of vessel. Vessels with muscular coats contract following injury, thus assisting haemostatic plug formation by reducing the blood flow.

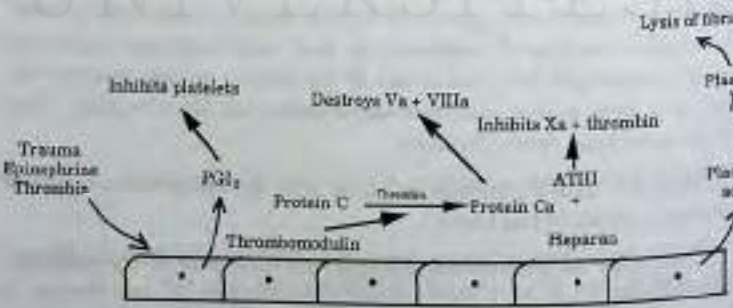
The blood vessel wall has three layers: intima, media and adventitia. The intima consists of endothelium and subendothelial connective tissue and is separated from the media by the elastic lamina interna. Endothelial cells form a continous monolayer lining all blood vessels. They have three important characteristics;

1) They are "non-thrombogenic" that is do not react with plasma or the cellular elements of the blood,

2) They play an active role in supplying nutrients to the subendothelial structures,

3) They act as a barrier to macromolecules and particulate matter circulating in the blood stream.

The luminal surface of the endothelial cell is covered by the glycocalyx, a proteoglycan coat. It contains heparin sulphate and other glycosaminoglycans which are capable of activating antithrombin III, an important inhibitor of coagulation enzymes. Endothelial cells synthesize and release plasminogen activator. Arachidonic acid produces prostoglandin I_2 (PGT_2) from the membrane phospholipids of endotheliol cells by the action of cyclooxypenose enzyme. PGI_2 is a strong antiaggregating substance and a vasodilator. In platelets, it causes an increase in cAMP levels by activating adenylate cyclase. At the same time, it blocks calcium mobilization. Not only anachidonic acid is converted to PGI_2, but also PGH_2 is also converted to PGI_2 in the intima of the endothelial cell. PGH_2 is produced from the PGI_2 by the enzyme PGI_2 synthetase. Some drugs increased the release of PGI_2 from the endothelial cell. A decreased production of PGI_2 in vessel walls has been demonstrated in experimental as well as clinical atheroselenosis with thromboembolism. While decreases in PGI_2 in atherosclerosis, platelets TXA_2 level increase. In animals, during experimental atherosclerosis, PGI_2 formation is increased in the early phases, but it is decreased at later stages. Decreased PGI_2 level is also observed in diabetics. Cigarette smoking decreases PGI_2 production. It has also been shown that some lymphocyte factors increase PGI_2 production. Thrombomodulin, is also found on endothelial cell membranes. It plays a role in protein C activation. Von Willebrand Factor (vWF) is necessary for the adhesion of platelets to the subendothelium. The production of vWF in endothelial cells and megakaryocyte cultures is well known. In addition to these sabstances basal membrane collagens can be synthesized in endothelial cell cultures. Normal vascular endothelium has thromboresistance effect, as is shown in figure 33.



Figür 33: Thromboresistant properties of endothelium (From ref., 123)

The subendothelium consists of conective tissues composed of

- Collagen
- Elastic tissues
- Proteoglycans and
- Non-collagenous glycoproteins such as fibronectin and vWF

After endothelial damage has taken place, components of the subendothelium are exposed and platelets adhere to various elements including collagen of the basement membrane and microfibrils. The adhesion of platelets is regulated by the spesific properties of the platelet membranes and the biochemical characteristics of the subendothelial structures. Plasma level of vWF and characteristics of the blood flow are also affect platelet adhesion.

Platelets

Platelets do not adhere to normal vascular endothelial cells, when the vessel wall is damaged, the subendotheliol structures, including basement membrane, collagen and microfibrils, are exposed. Circulating platelets react with exposed collagen fibres, and their adherence to the damaged surface is mediated by high molecular weight multimers of vWF, and possibly fibronectin. There is exidence that the tertiary structure of collepen fibrils is an essential component of their capacity to "activate" adherent blood platelets. Once activated, platelets immediately change shape from a disc to a tiny sphere with numerous projecting psev dopods; as shown in Figure 34. After adhesion of a single layer of platelets to the exposed subendothlium, platelets stick to one another to form aggregates. Fibrinogen and fibronectin are essential at this stage to increase the cell to cell contact and facilitate aggregation. Certain substances (agonists) react with specific pletelet membrane receptors and initiate platelet aggregetion and further activation.

The agonists include;

- Exposed collagen fibres
- ADP
- Adrenalin
- Serotonin

- Certain Arachidonic
- Acid metabolites
- Including thromboxane
- A_2 (TXA_2).

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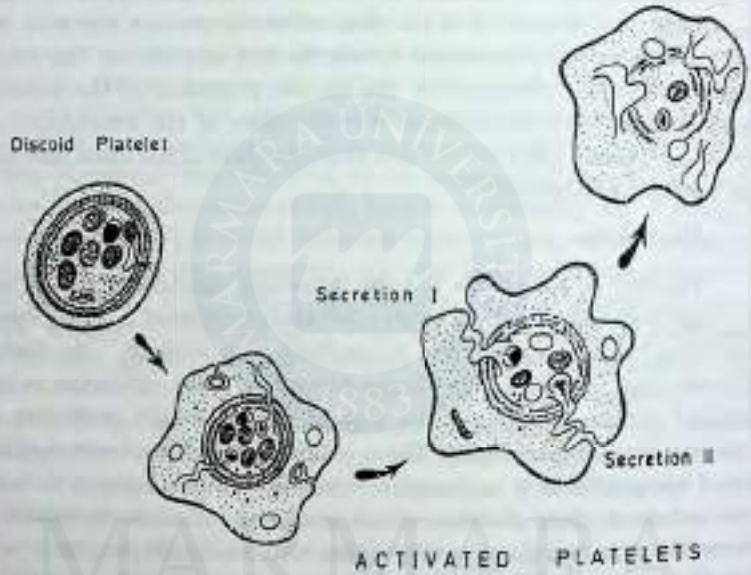

Figure 34: Schematic representing of activated platelets. (From ref. 67)

Many processes observed within activated platelets are known to be calcium dependent, including phosphorylation of myosin light chains by a specific kinase enzyme, and liberation of arachidonic acid from membrane phospholipids by the enzyme phospholipase A. There are at least four separate pathways of platelet secretion by extracellular agonists operating via receptors on the platelet membrane.

Activation of phospholipase A_2 results in the liberation of free arachidonic acid, which is then converted by cyclooxygenase to prosta-

glandin endoperoxides and ultimately forms the potent platelet aggregator thromboxane A_2 (TXA_2), as well as stable prostaglandins such as PGD_2, which inhibits platelet activation. The latter may serve in a regulatory role to modulate platelet activities. TXA_2 has ionophoric activity, enabling it to transport calcium across intracellular membranes, resulting in a redistribution of calcium from intracellular stores to cytoplasm.

A second secretory mechanism involving elevation of cytoplasmic calcium is attributed to the action of ADP. A possible third parallel pathway, involving elevated cytoplasmic calcium levels is via the generation of PAF (platelet activating factor or 1-0-alkyl-2-0-acetylglyceryl phosphoryl choline) This is a lipid product of phospholipid metabolism produced by leukocyte and platelets in some species. PAF is a potent platelet aggregating agent in vitro, but the relevance of this substance to human platelet activity in vivo is in dispute.

A fourth pathway responding to platelet stimulation involves the activation of phospholipase A, with the subsequent production from phosphatidylinositol diphosphate of diacyl glycerol and inositol triphosphate. These substances are known to activate protein kinase C, which phosphorylates a 40000 dalton substrate protein the function of which is not established. Inositol triphosphate raises cytoplasmic calcium levels. Activation of both phospholipase C and protein kinase C requires a low level of calcium (less than 100 nM).

Platelets contain a variety of granules as it is shown in figure some of content of these granules are as fallows;

- Dense bodies; contain serotonin, ATP, ADP, pyrophosphate and calcium.

- α-grenules contain; fibrinogen, vWF, Factor V, high molecular-weight kininggen, fibronectin, α_1-antitrypsin, β-thromboglobulin, platelet factor 4, and platelet-derived growth factor.

- lysosomes contain acid hydrolases.

Centralization of these granules following stimulation of platelets results from activation of the platelet contractile apparotus. In the presence of elevated cytoplasmic calcium this leads to fusion of the granular envelope with the lining membranes of intracellular canaliculi and to external secretion of the granule contents.

Table 4 : The major platelet membrane glycoproteins (From ref. 123)

Glycoprotein	Molecular Weight (Unreduced)	Subunits	Molecular Weight (Reduced)
Ib	170.000	α	128.000-141.000
		β	22.000
IIa	138.000		148.000
IIb	142.000	α	132.000
		β	23.000
IIIa	95.000		114.000
IIIb (IV)	97.000		97.000
V	82.000		
IX	17.000-22.000		17.000-22.000

There are specific glycoproteins in the platelet membrane act as receptors either plasma proteins or haemostotic system releated proteins. As mentioned above, in primary haemostasis, platelets adhere to the subendothelium of damaged blood vessels, becomes stimulated by agonists, and aggregate to arrest bleeding. These processes are mediated by the binding of either plasma proteins or platelet-secreted proteins to specific receptor sites on the pletelet cell surface. The receptor sites are necessary for adhesion and aggregation function of platelets.

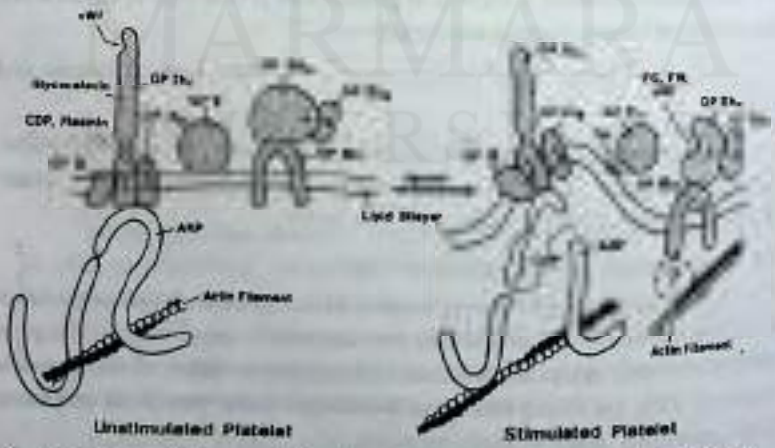

Figure 35: Schematic diagram of the major platelet membrane glycoproteins in the lipid bilayer (From ref. 123)

Platelet membrane glycoproteins are important elements in primary haemostasis. Considerable progress has been made in describing platelet membrane glycoproteins, but the mechanism how platelet-bound ligands mediate adhesion and aggregation remains unclear. Orientation of the major platelet membrane glycoproteins in the lipid bilayer is shown in figure 34.

In secondary haemostasis, coagulation factors interact with the platelet cell surface glycoprateins to generate thrombin for the conversion of fibrinogen to fibrin. although more than 30 individual surface proteins have been detected by one or two dimension. SDS-polyacrylamide gel electrophoresis under reducing conditions with surface labelling techniques. Only seven of these glycoproteins are predominate. Some of these glycoproteins are characterized by intrachain disulfide bonds, and two of them (GPIb and GPIIb) consist of two disulfide linked subunits.

Monoclonal antibodies have been produced against GP_{Ib}, GP_{IIa} - GP_{IIIa} complex, and GP_{IX}. These antibodies have been great value in analyzing the structural and functional properties of these glycoproteins. GP_{Ib} appears to be primarily involved in the adhesion as the receptor for von Willebrand factor, GP_{IIb} and GP_{IIIa}, a Ca^{2+}-dependent heterodimer complex (termed the GP_{IIb} - GP_{IIIa} complex) and function as a receptor for several ligands that are necessary for aggregation GP_V is a thrombin substrate and a possible thrombin receptor. Three glycoproteins, GP_{Ib}, GP_{IIb} and GP_{IIIa}, interact with the platelet cytoskeleton This interaction appears to be important in the maintenance of platelet shape and in clot retraction after aggregation of simulated platelets.

Two rare inherited bleeding disorders, Glanzmann's thrombasthenia and Bernard-Soulier syndrome, involve deficiencies or abnormalities in the GP_{IIb} - GP_{IIIa} complex and GP_{Ib}, respectively. Patient with these disorders have abnormal haemostasis and a lack of membrane receptors for ligands required for platelet adhesion and aggregation. In contrast, amounts of GP_I and GP_{IIb} increased in atherosclerosis (Figure. 36, table 5) this might explain, in part, why there is platelet hyperaggregation in atherosclerotic patients. These glycoproteins are the receptor sites in the membrane of platelets.

The ^{14}C galactose transport into platelets was found to be normal in atherosclerosis and galactose incorporation into protein increased significantly (Figure 37) Thus, the glycoproteins synthesis was normal in the circulating platelets and increased in atherosclerosis. Morover ^{14}C galactose incorporetion is inhibited by aspirin and indobufen. This inhibition was highly significant. Therefore the altered membrane glycoproteins could be changed by some drugs as seen in fig ure 38

Table 5: Borohydrite labelled normal and atherosclerotic platelet membrane glycoproteins in PAGE sections (cpm) (From ref. 92)

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	GP_I (M.W. 160.000)	GP_{IIb} (M.W. 134.000)
Normal Platelets (Mean ± SD)	661 ± 96	298 ± 21
Atherosclerotic Platelets (Mean ± SD)	1214 ± 278	557 ± 61
Increase	p < 0.001 83 %	p < 0.001 92 %

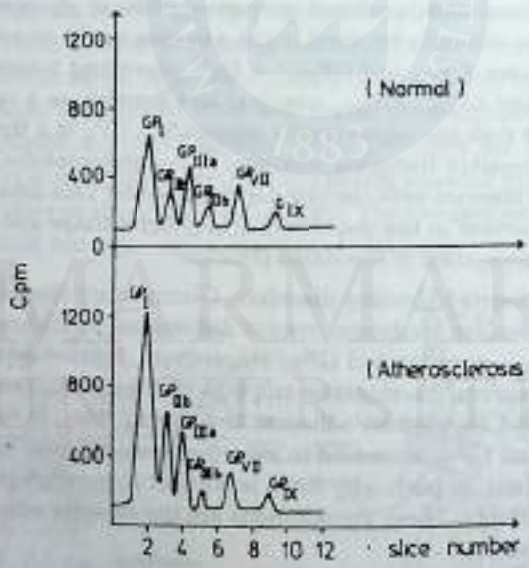

Figure 36: Membrane glycoprotein distribution in a normal control and in a patient with atherosclerosis. (From ref. 80)

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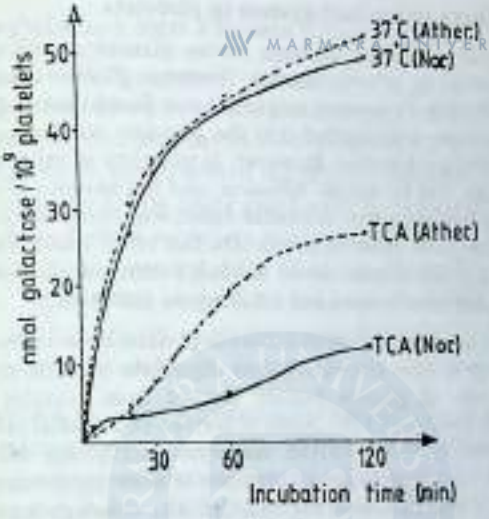

Figure 37: ^{14}C-galactose transport and incorporation into the glycoproteins of platelets from normal controls and atherosclerotics. (From ref. 92)

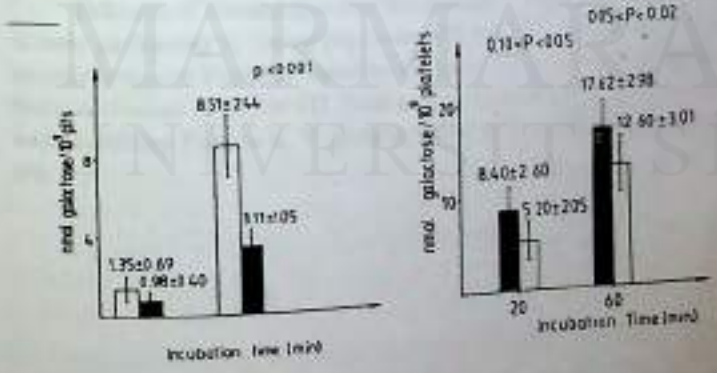

Figure 38: Inhibition of the ^{14}C-galactose incorporation into platelet glycoprotein before and after the administration of aspirin left and indobufen right. (From ref. 93)

Some others important system in platelets

Amino acid transport systems across platelet membranes were found to be normal in atherocelenosis. However, glucose transport was shown to be defective. In normal subjects it was found that at 37 C, ^{14}C-glucose was actively transported into the platelets with concentration-dependent saturation kinetics. However, in platelets of atherosclerotics, glucose transport was by simple diffusion, and the absence of a glucose-binding protein was observed. A similar defect was found in young membrers of families with atherosclerosis. On the other hand, the glucose transport defect could only be shown in adult patients with diabetes mellutus and vascular involvement but not Juvenile diabetes.

There are number of growth factors in platelet some of them are released from α-granules. Growth factors stimulate smooth muscle cell proferation. Some of growth factors derived from platelet Platelet-Derived Growth Factor (PDGF), Some of derived endothelial cell derived, EDGF, and some of them derived monocyte-macrophage MDGF. The PDGF released from α-granules of platelets is a strong mitogen. PDGF does not produce hyperplasia of the endothelium, although it causes proliferation of arterial smooth muscle cells and fibroblasts. In contrast, MDGF can cause proliferation of endothelial cells, smooth muscle cells and fibroblasts. Most of the mitogenic effect of serum can be attributed to PDGF. It is highly cationic protein with a molecular wlight about 13.000 to 16.000 daltons. The presence of a receptor for PDGF has been reported in smooth muscle cells of vessels.

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Coagulation and Plasma Coagulation Proteins

There are multicomponent enzyme system, cofactors and inhibitors in the coagulation sytem. In normal hemostasis following vascular injury fibrin network forms after convertion of fibrinogen to fibrin by thrombin. This fibrin network occurs around the primary hemostatic platelet plug, thereby preventing it from being swept away by the bloodstream. Coagulation has been termed secondary hemostasis. If fibrin formation is impaired due to an abnormality of the blood coagulation mechanism bleeding is seen. Most of the caagulation factors now recognised by Roman numerals, in the order in which they were discovered. The coagulation reaction is shown schematically in figure 39.

As shown in schema coagulation is activated two different pathways, an intrinsic system and extrinsic system. **Intrinsic system initiated by contact activation involving** Factors XII, Fletcher factor, Williams Factor, Factors XI, IX, XIII and phospholipid (platelet factor 3).

The complex interaction of Factor XII and high molecular weight kininogen, which are absorbed onto the negatively charged endothelial surface, and the binding of prekallikrein and factor XI to high molecular weight kininogen lead to the activation of Factor XI. Then follows a series of conversions of inert precursors into active serine proteases leading to fibrin formation.

Diffusion of tissue juices into the circulation **will activate the Extrinsic pathaway** by converting factor VII into factor VIIa which subsequently activates Factor X, there by extrinsic system, triggered by tissue factor activation of Factor VII. Both pathways feed into a common pathiways involving Factors X, V, platelet factor 3, prothrombin and fibrinogen.

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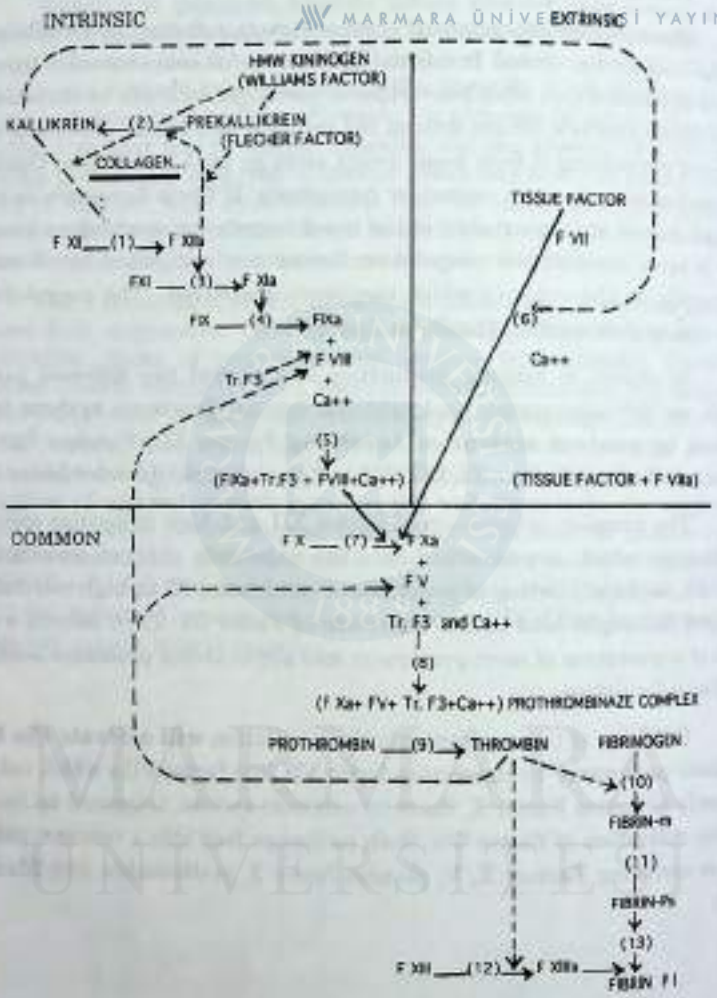

Figure 39 : Intrinsic and extrinsic coagulation mechanism

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The first step in the triggering of coagulation via the intrinsic pathway (reaction 1) involves the binding of factor XII to an active foreign surface such as Collagen, Other circulating activators such as endotoxins or fatty acids activate the Factor XII. Binding to such surfaces is thought to be associated with a conformational change in the factor XII molecule, which may expose the active site of the enzyme. Factor XIIa generated by surface contact then converts prekallikrein to kallikrein, which appears to require a cofactor, HMW kininogen and leads to direct further activation of factor XI by a positive feedback mechanism. (reaction 2) Factor XII activation not only represents the first reaction, (Figure 39) but also appears to serve a key role in the activation of at least 3 other important biologic enzyme system these are:

1) The plasminogen plasmin system, which capable of proteolytic degradation and removal of intravascular fibrin deposits and thus may serve as an important hemostatic control mecahism

2) The kallikrein bradykinin system, which plays an important role in the inflamatory process.

3) The complement system, which is involved in humoral and cellular immunologic defense mechanisms

Factor XII_a activates F XI_a, (reaction 3) then it acts on its natural substrate factor IX to form FIXa. the final reaction of the intrinsic pathway consists of a nonenzymatic interaction between Factor IXa, PF3, factor VIII and calcium ions to form a highly active complex that is capable of activating factor X. Although factor IXa alone can activate factor X in the presence of phospholipid and calcium ions, this reaction is accelerated 1000-fold in the persence of F VIII (reaction 5).

In the extrinsic pathway; proenzyme factor VII is activated by tissue factor which is a lipoprotein and present in all tissues. Conversion of FVII to F VIIa may be induced by kallikrein formed from prekallikrein by factor XIIa. This reaction may provide a link between the intrinsic and extrinsic pathway.

Formation of prothrombin converting complex is known common pathway. The first reaction in this way is conversion of factor X to Xa, in the presence of Ca ions. (reaction 7) Almost all reactions require Ca^{++} in the coagulation system. Then FXa, which is a powerful serine protease, forms a reversible complex with FV, PF_3, and the complex also called

prothrombinase, which is a powerful activator of activator of prothrombin. (reaction 8) Then this activator acts on its substrate, prothrombin (reaction 9). There is evidence that this reaction involves the binding of prothrombin to the lipoprotein complex, that Factor Xa is the enzyme in this reactinon. This reaction is markedly accelerated in the presence of FV. then thrombin is released from the activation complex during the reaction.

The activation of prothrombin to thrombin appears to occur in several steps with at least 3 intermediate proteolytic products. Figure 40 shows schematic representation of prothrombin activation which is called protrombinase complex.

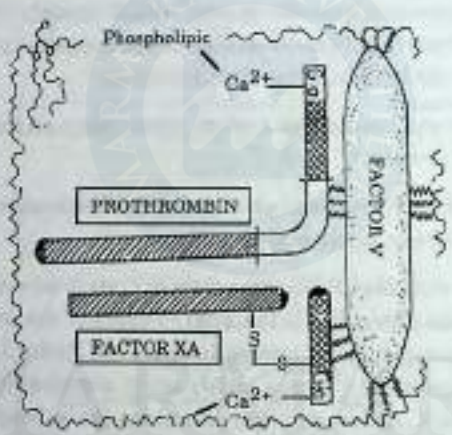

Figure 40: Schematic representation of prothrombinase complex (From ref. 69)

Following prothrombin activation the active enzyme, thrombin, converts fibrinogen to fibrin monomer (inreaction 10) which involve the cleavage of two low-molecular-weight peptides (fibrinopeptide A and B) from each of the two pairs of α and β-chains at the N-terminal and of the molecule. Once the fibrin monemer (Fibrin M) is formed it undergoes spontaneous polymerization involving both end to end and side to-side aggregation.

The fibrin monomers participating in this reacion are held together by noncovalent weak bonds, which can be readily dissociated by expeaure of the polymer to 5M Urea. Fibrin monomer formed in the circulating blood during intravascular coagulation may also bind to fibrinogen to form soluble fibrin complexs. Fibrin polymer (Fibrin Pi) formed (reaction 13) in Figure This reaction is cotalyzed by an enzyme, Factor XIIa, which is formed from an inactive precursor by thrombin and requires calcium ions. There is covalant cross-linking bonds in the fibrin polymer molecules. Fibrin monomer is soluble in 5 M urea hence the term soluble fibrin polymer (Fibrin Ps) is used. After activation of FXIIIa insoluble fibrin polymer formed (Fibrin Pi). This is much more resistant to proteolysis by the fibrinolytic enzyme plasmin than (Fibrin Ps.) This is the end product of coagulation cascade.

The coagulation factors that have been represented Roman numerals are listed in table 6. Some of the properties of these coagulation factors are also shown in this table Several additional factors have been recognized in recent years, which have not been assigned Roman numerals by the International Committee on nomeclature. Some of the properties of these factors and their functions in the coagulation system will also be discussed summarised form below.

Fibrinogen (Factor I)

Fibrinogen is one of the major plasma proteins (normal concentration: 180-400 mg/dl) the elucidation of the structure of fibrinogen by using; electron microscopy, chemical and enzymatic degredation, immunologic probing, amino acid sequencing x-ray diffraction, calorimetry, light scattering and measurements of hydrodynamic properties, has occupied scientists for 50 years. Some properties of fibrinogen molecule is shown

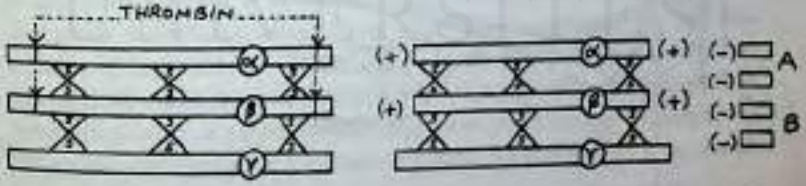

Figure 41: Schamatic representation of fibrinogen and fibrin monomes following prothrombin activation

in table 6. Fibrinogen is a globular shaped glycoprotein with a molecular weight of about 340.000. Its structure has not been definitely established although it has been the subject of intensive investigation. However, it is now generally accepted that fibrinogen circulates as a dimer consisting of a pair of 3 polypeptide chains, termed α, β and γ chains, held together by disulfide bridges. Convertion of fibrinogen to fibrin by thrombin is associated with the loss of two small peptides, termed, fibrinopeptide A (from the A α-chain) from the N-terminal part of the fibrinogen molecule. (Figure 41) Fibrin monomers resulting from this reaction are insoluble in plasma and under normal circumstances will spontaneously polymerize to form a fibrin gel. Fibrinogen is synthesized in the hepatocytes of the liver at a rate of 1.7-5.0 g/d. Its rate of synthesis may be increased 10 to 20 fold in acute inflammatory disease, after tissue injury, or following injection of endotoxin (acute phase response). Some other plasma proteins such as; α_1 , protease inhibitor, α_2, -acid glycoprotein, seroloplasmin, C-reactive protein, and haptoglobulin give acute phase response. Hence these proteins are called Acute Phase Reactans. In acute tissue damage, due to accidents, major surgery, infanction (e.g. myocardial infarction), in infections and burns, in collagen diseases and malignant diseases acute phase reactans concentretions increase in plasma.

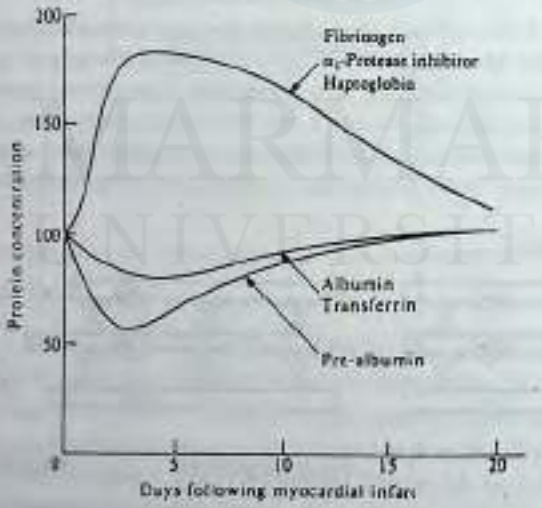

Figure 42: Acute phase reactans after myocardial infarction. (From ref. 1)

Figure 42 shows changes in plasma protein concentrations after myocardial infarction. Acute phase reactants are also synthesized in increased amounts in pregnancy. Following an acute injury plasma acute phase reactants starts to increase within 24 hours and usually reach their peak concentration after 3-5 days. The increase amounts, in most cases, to not more than a 2 to 3 fold increase in concentration, but C-reactive protein shows 10 to 100-fold increase. Some of the other plasma proteins (e.g. albumin) show fall in concentration after the injury.

The biologic half-life of fibrinogen has been estimated to be from 77-108 hours. Fibrinogenolysis, i.e., degradation of fibrinogen to lower molecular weight derivatives by the fibrinolytic enzme system (plasminogen-plasmin) appears to account for a major proportion of the physiologic fibrinogen catabolism.

Factor II (Prothrombin)

Prothrombin is a plasma glycoprotein with a molecular weight of 68.000-72.000. It composed of a single polypeptide chain. Its normal concentration in plasma is 10-15 mg/dl. It is synthesized in the liver parenchyma, vitamin K an essential reguirement. In the absence of vitamin K, a founctionally impaired prothrombin molecule is synthesized that lacks a tryptic peptide containing γ-carboxyglutamic acid residues. These residues are essential for the binding of prothrombin to phospholipid and calcium ions. The biologic half-life of prothrombin has been estimated at 68 hours.

Prothrombin molecule which consists of a single polypeptide chain, can be divided in to two parts, the "Pro" portion and "Thrombin forming" portion the proportion, which is derived from the amino terminal end of prothrombin consists of a polypeptide of 274 amino acids and two oligosaccharide chains attached at asparagine 77 and asparagine 101, for a total mass of approximately 35.000 the pro half of prothrombin may be further divided into two structurally similar, but functionally distinct. The thrombin portion of prothrombin designated prethrombin 2, consists of 308 amino acids and a single oligosaccharide chain attached to an asparagine residue (Figure. 43)

Thrombin differs from prethrombin 2 in that a cleavage of a single arginyl-isoleacyl peptide bound in prethrombin 2 creates thrombin and permits expression of proteoalytic activity. Becouse of the disulfide bond betwen residue 22 and 168, thrombin posseses two polypeptide chains A and B.

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Figure 43 : Schemic structures of the prothrombin molecule (From ref. 123)

Under physiologic conditions, rapid prothrombin activation to form thrombin involves some components. Although clear evidence in existence since the mid-1950s indicated that only the proteolytic enzyme factor Xa was necessary for thrombin generation factor Va, phospholipids and Ca^{++} are also necessary for protrombin activation. As it is shown in figure during prothrombinase complex formation phospholipids provide a cell membrane - like surface to which the protein molecules bind. Calcium ions are required for both factor Xa and prothrombin to bind to the phospholipid membrane surface. This Ca^{2+} ion-mediated binding requires the presence of severol residues of a modified form of glutamic acid (γ-carboxy-glutamic acid) in each protein. This reaction is releation with vitamin K action during their biosynthesis in the liver. The functional consequence of oral anticoagulant is elimination of the Ca^{++} ion-mediated association of the vitamin K-dependent clotting factors with the phospholipids of the cell membrane.

In citrated or oxalated plasma stored at room temperature, slow activation of prothrombin will occur. During clotting of normal blood, prothrombin is virtually completely lost so that little is detectable in serum. Prothrombin is readily removed from oxalated plasma by barium sulphate, from which it can be cluted with sodium citrate solution.

Factor III (Tissue factor, thromboplastin)

Tissue factor is a lipoprotein that can be extracted from virtually all organ tissues and has endopeptidase and exopeptidase activity. It binds to Factor VII and exopeptidase activity. It binds to Factor VII and converts it to Factor VIIa. Its precise molecular structure has not been conclusively elucidated and may vary from tissue to tissue. Brain, lung are particularly rich in tissue factor and is the major source of tissue

thromboplastin preparations used in the Quick one and two stage prothrombin time.

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Factor V (proaccelerin accelerator globulin, AcG)

Factor V is a cofactor that accelerates the conversion of prothrombin to thromin by factor Xa in the presence of phospholipid and calcium ion as was mentioned above. It is a glycoprotein with a molecular weight of over 300.000. It is the most unstable of all coagulation facors so it is also called labile factor. In oxalated plasma 50% of its activity is lost at 23 C in 2 hours, and rapid loss of activity is also observed at 4 C and in citrated plasma. Because of this exceptional instabilitiy, which is enhanced during purification, the exact physical properties have yet to be determined. Factor V is synthesized in the liver. Its biologic half-life has been estimated from 12-36 hours Factor V activity is also inhibited by plasmin.

Factor VII (Proconvertin, autoprothrombin I)

In contrast to factor V, Factor VII is stable factor in serum. It is a glycoprotein with a molecular weight of 63.000 It is synthesized in the liver with vitamin K being an esential requirement the role of vitamin K in factor VII synthesis is assumed to be similar to that shown in prothrambin biosynthesis. Factor VII is activated by tissue factor (tissue lipoprotein) in the extrinsic coagublation pathway in the presence of calcium ions. It was shown that FVII may also be activated by Factor XIIa and XIa and by plasmin.

Factor VIII (Antihemophilic Factor, (AHF)

Factor VIII is a glycoprotein complex with several different activities these are;

- Factor VIII:C or F VIII_{AHF}
- Factor VIII antigen (F VIII: Ag)
- Von Willebrand Factor (vWF)

Factor VIII is thrombin sensitive protein. Other thrombin sensitive proteins are fibrinogen, factor XIII, factor V.

Factor VIII: C : This is a proenzyme in plasma. It is cleaved by thrombin to give rise to active cofactor VIIIa. It is necessary for optimal

activation of factor x by factor IX. Further celavega by thrombin leads to inactivation factor VIII:c and vWF circulate in plasma as a complex and probably stabilize and protect each other. Factor VIII activity corrects the defect in classic hemophilia A this factor is also called $FVIII_{AHF}$ Molecular weight is 1000.000 - 200.000 Under physiologic conditions, VIII_{AHF} is bound to a protein with a molecular weight greater than 1.200.000 (FVIII: Ag) from which it can be dissociated in vitro in the presence of high salt concentration. Factor VIII: C is absent in the plasma of severe classic hemophilia A is measured in standard coagulation assays. This factor plays as a cofactor in the conversion of factor X to Factor Xa by factor IXa in the presence of phospholipid and calcium ions. Factor VIII_{AHF} is unstable in citrated or oxalated plasma at 23 C. It can be greatly poteniated by exposure to thrombin following which its activity is lost. It can also be destroyed by plasmin and other proteolytic enzymes. Factor VIII complex is probably synthesized in endothelial cells. Its biologic haf-life has been estimated to range from 9 to 15 hours.

Factor VIII antigen (VIII: Ag)

This is quantitated by immunoassays. Factor VIII antigens are stable during coagulation, even though Factor VIII:C (coagulant activity) is inactivated. It was recognised that some hemophilic plasmas contain immunoreactive, non functional, Factor VIII-like protein. Thrombin and plasmin also affect the FVIII: Ag content. In contrast, activated proten C rapidly inactivates VIII:C but does not affect VIII:Ag levels. FVIII antigenic determinants are also more stable than is procoagulant activity when plasma is held at 4 C or 37 C. Temperatures above 45 C rapidly inactivate VIII:C, as do hydrogen ion concentrations outside of the range of pH 6 to 8.

Von Willebrand Factor ($FVIII_{AHF}$) It is necessary for normal platelet adherence to surfaces. Platelet have the property of adhering to foreign surfaces, forming agregation and releasing their substances to the medium. These 3 fonction of platelets are separete and independent phenomena. Under normal conditions, they take place together or following each other. According to some authors these phenomena are the different phases of the same reaction. The first findings about the adhening ability of the platelets go as for back as. In 1873 authors observed that the platelets can adhere to glass by the phase contrast microscope. Later (1959) authors observed that platelets can adhere to collagen and non-

collagenous subendothelial substances.

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For adhesion to glass and non-collagenous subendothelial substances, Ca^{++}, fibrinogen and vWF are necessary. In hemophilia, the adhesion to glass is normal, where as in vWF disease it is defective. In vWF the adhesion to the non-collagenous subendotheliol substances is also decreased.

When a blood vessel is injured, platelets immediately adhere at the site of injury to form a haemostatic plug after a complex series of reactions. Formation of adhesive complex between collagen of endothelium and specific glycoproteins located on the pletelet cell surface is shown in figure.

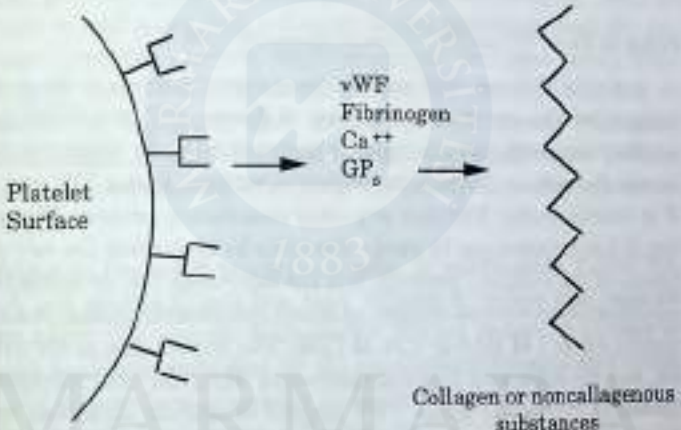

Figure 44 : Hypothetical steps in platelet adhesion to collagen or non collagenous subendothelium substance.

Factor VIII has a close, but noncovalent, association with von willebrand factor (VWF) in normal human plasma the synthesis of this protein takes place in the endothelial cell and megakaryocyte, But factor VIII synthesis has not known yet. vWF is large polymeric glycoprotein.

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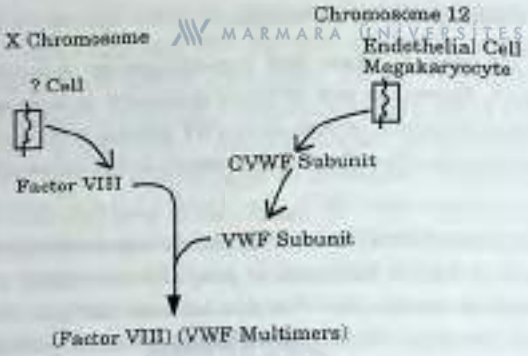

Figure 45: Factor VIII and vWF complex in plasma (From ref. 123)

Factor VIII and vWF association in normal plasma is the most likely basis for the excellent correlation of Factor VIII and VWF content, whether measured immunologically or functionally by ristocetin cofactor assays. In contrast, there is no corelation between Factor VIII and Factor V or betwen Factor VIII and any other coagulation protein. The association of two proteins can be easily disrupted by increasing the salt concentration of the solution, however, and the seperation can be detected when gel-filtration or sucrose-density gradient ultracentrifugation studies are carried out in 1-M Nacl or 0.24 M $CaII_2$. The dissociation is not irreversible, and the separated proteins reassociate when the ionic strength is returned to physiologic levels. vWF coupled to agarose binds and removes Factor VIII from normal plasma.

Factor IX (Christmas Factor)

Factor IX is a glycoprotein in plasma at a concentration of less than 10mg/ml with a molecular weight of 72.000 consisting of a single polypeptide chain. It is a proenzyme that is converted to the active enzyme by factor XIa in the presence of calcium ions. It is missing or defective in hemophilia B Factor IX activity is stable in citrated or oxalated plasma at 23 C and 37 C. It is readily absorbed to barium sulfate or aliminyum hydroxide. Factor IX is synthesized in the liver parenchyma, it is vitamin K dependent protein.

Hemophilia B, like hemophilia A, can occur in a severe, moderate, or mild form, according to the level of factor IX activity in plasma. The

severe form has a factor IX level of less than 1 % and half of the hemophilia B patients belong to this group the clotting time in these patients is usually longer than 1 hour. In moderate hemophilia B, the Factor IX level is 1 % to 4 %, and clotting time is about 20 to 30 minutes. The factor activity in mild hemophilia B is 5 % to 25 % of normal, these patients have only slightly prolonged clotting times. Lower levels of Factor IX have been found in carriers of hemophilia B, but the results have been somewhat contradictory. In a study of 28 definite carriers and 36 possible carriers of hemophilia B perfermed in Melmo, Sweden, 26 of 28 definite carriers were found to have clearly decreased levels of Factor IX activity.

Two genetic types of hemophilia B were described in 1956, at which time it was found that a barium sulfate adsorbate from one patient's blood inhibited a specific inhibitor against Christmas factor, while barium sulfate adsorbate from another patient did not. Approximately 10 % of hemophilia B patients have meterial in their plasma that cross-reacts with Factor IX antibody (CRM^+), while the remaining 90 % either lack such cross-reacting material (CRM^-) or possess it in clearly reduced amounts (CRM^{-R}).

Factor IX has many properties similar to prothrombin, Factor VII, Factor X and protein C and has been difficult to isolate and separate from the latter four vitamin K-dependent clotting factors. A number of techniques have been published for the purification of human and bovine Factor IX Column chromatography on heparin-Sepharose has been particularly helpful in the isolation of this protein.

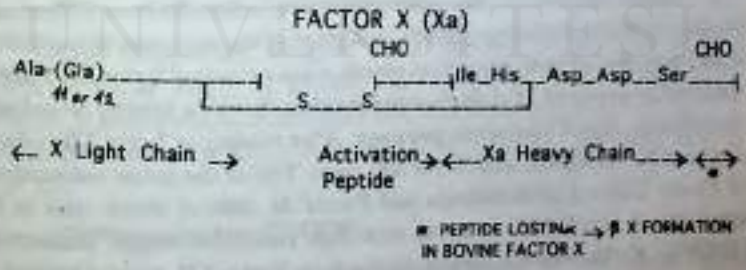

Figure 48: Schematic representation of Factor molecule (From ref. 123)

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Factor X

Factor X is a glycoprotein in plasma at a concentration of 12 µg/ml, with a molecular weight of 55.000 consisting of 2 polypeptide chains. (Figure 46.) It is a proenzyme that is converted to Factor Xa by Factor IXa in the intrinsic pathway and Factor VIIa in the extrinsic pathway.

Factor X can also be activated by other proteolytic enzymes such as trypsin or a protease from Russell's viper venom (Stypven). Activation of factor X by all these different enzymes appears to involve a similar molecular change. It is synthesized in the liver, with Vitamin K an essential requirement. Bovine Factor X has been purified in a number of laboratories using DEAE- Sephedex Chromotography or other techniques. But first the protein is eluated from plasma by barium citrate and precipitated by ammonium sulphate.

Factor XI

Factor XI is a glycoprotein, with a molecular weight of 164.000 consisting of 2 polypeptide chains. It is activated by Factor XIIa. Factor XI is stable in plasma, but once activated it is rapidly inactivated by various plasma inhibitors.

Factor XII (Hageman Factor)

Factor XII is a glycoprotein with a molecular weight of 80.000 the plasma concentration is 27-40 µg/ml. Binding of Factor XII to an active surface such as collagen, sulfatides, dextran sulfaye, glass celite, kaolin leads to its activation and conformational change in the molecule.

Hageman Factor (FXII) is the first protein in the series of coagulation reactions designated **a waterfall or cascade.** As shown in figure 39. It binds to negatively changed surface as mentioned above. Following binding to these activating surface, initiation of the sequence of reactions is achieved by autoactivation that occurs when activated factor XII (XIIa) cleaves the zymogen at a single bond inside a disulfide bridge, revealing the catalytic site of this serine protease. After binding of factor XII to foreign surface kallikrein and factor XIa form. Two of the major substrates of Factor XIIa are prekallikrein and Factor XI, both of which exist in a noncovalent bimolecular complex with high molecular weight kininogen (HMWK). Kallikrein has positive feedback on Factor XII, which is repeated elsewhere in the coagulation system thereby it accelerates contact activation. In addition, kallikrein cleaves HMWK to liberate the nonapeptide bradykinin (Figure 47).

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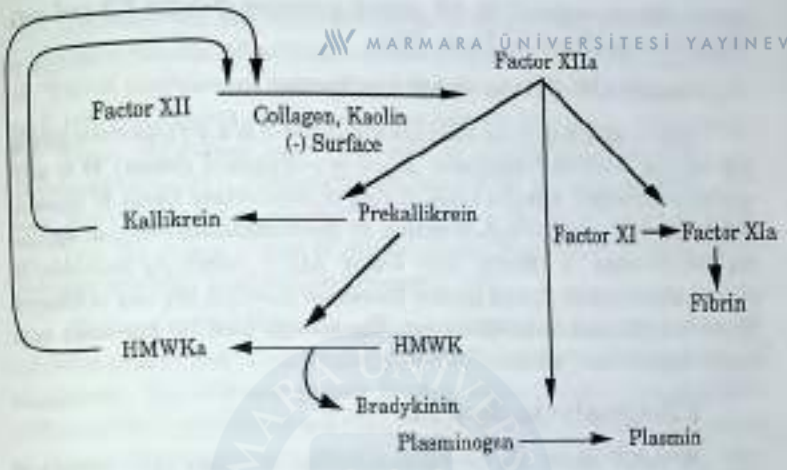

Figure 47: Interaction of Hageman factor to other haemostatic proteins.

Factor XIIa also activates the fibrinolytic system by activating plasminogen

Factor XII is stable in noncontact plasma stored a 23 or frozen. Once activated, the molecule undergoes (fragmentation) and inhibition by several plasma inhibitors (C1 esterase inhibitor, Antithrom bin III). The site of Factor XII biosyntheeis is not known. The biologic half life has been estimated from 52 to 60 hours.

Fletcher Factor (Prekallikrein)

This factor is not assigned romen numeral. It is a glycoprotein with molecular weight of 108.000 Active form kallikrein. Occurs after Factor XIIa activation. If activates HMWK and it also has pasitive feedback effect on Factor XII. It is capable of direct further activation of Factor XII.

Williams Factor (HMWK)

This Factor also known as Fitzgerald Factor or Washington factor. It is a glycoprotein of molecular weight 200.000 This factor, which has been shown to be deficient in several patients recently studied, appears

to be a cofactor required for full contact activation of factor XII and activation of prekallikrein by Factor XIIa.

Factor XIII (Fibrin stabilising Factor)

This is also known as Laki Lorand factor it is a glycoprotein molecular weight of 320.000 (tetremer, 2 pair of polypeptide chains). It is activated by thrombin, induces cross-linking of polymerized fibrin at sites on the γ and, to a lesser extent, α-chains, by transamidation reaction requiring the presence of calcium ions. Factor XIII is relatively unstable in plasma stored at 23 C and greatly decreased in serum the site of biosynthesis has not been established yet. The biologic half life has been estimated from 4.5 to 7 days.

Fibrinolytic System

As stated above, the coagulation system is normally in a state of dynamic equilibrium in which fibrin clots are constantly being laid down and dissolved. Plasmin circulates the form of its inactive zymogen plasminogen, degrades fibrin clots. Various mechanism of plasminogen activation have been described, and there are also a variety of inhibiters and modulators of the system. Antiactivator and antiplasmin in the fibrinolytic system is shown in figure 48.

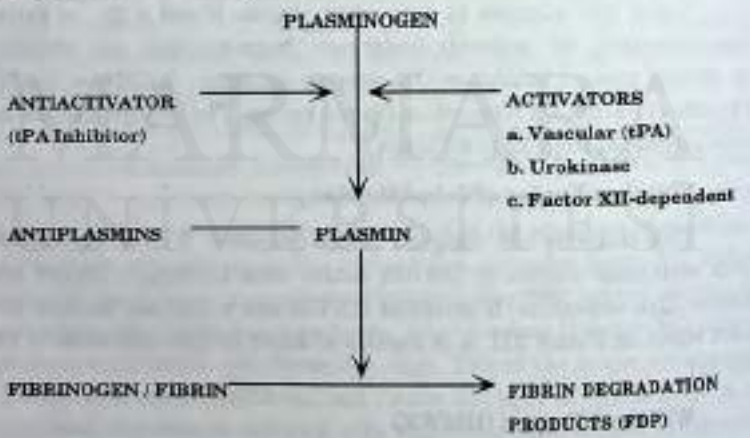

Figure 48 : Fibrinolytic sytem; activators and inhibitors involved in the conversion of plasminogen to plasmin

Patients with decreased fibrinolytic activities, either for congenital or acquired reasons, often have a increased incidence of venous thrombosis. Tissue plasminogen activator (tPA) has been produced by recombinant DNA technology, while urokinase and streptokinase readily purified. These substances have been used as thrombolytic agents for the treatment of massive pulmonary embolus and for the restoration of coronary blood flow after myocardial infarct and occlusion of coronary arteries. There are also a variety of profibrinolytic drugs which promote plasminogen activator synthesis and release in vivo.

Three systems of plasminogen activation have been described, an extrinsic system and two intrinsic systems (Factor XII-dependent and independent). The extrinsic system involves tissue plasminogen activator (tPA) secreted by vascular endothelial cells. tPA is the major component of plasminagen activator activity measured by euglobulin lysis time (ELT) and fibrin plates, but may also be measured more specifically by immunological assays or functional assays using chromogenic substrates. The intrinsic pathways of plasminogen activation account for about 50 % of the total activity. The Factor XII dependent pathway involves the activation of Factor XII an prekallikrein, with the liberation of kallikrein. This reaction is catalysed by high molecular weight kininogen and occurs most readily at surfaces. The Factor XII-independent pathway is due to urokinase (uPA) activity, the origin of which is unknown, although leucocytes are able to release the protein uPA has a direct action of plasminogen. Plasmin cleaves fibrin, yielding a number of fibrin degradation products (FDP) of varying sizes. It was shown that there is a correlation between the stage of malignant disease and the level of FDP. Morover there are some observations about anti-metastatic influence of coumarin derivatives. Warfarin did appear to exert some immunopotentiating action.

Inhibitors in Haemostatic System

Hemostasis is a well - maintained biological system, containing self-regulating enzyme and in inhibitors of various steps. There are procoagulant pathways as well as anticoagulant and fibrinolytic pathways on the other hand there are also specific inhibitors of procoagulant enzymes and inhibitors of fibrinolytic enzymes. The inhibitors may be divided into;

-) Enzyme inhibitors which block the active site by forming a complex with enzyme (e.g. antithrombin III, those which complete and interfere with reactions FDPs in fibrin monomer polymerisation.

-) Inhibitors which act by degrading a substance, such as protein C.

There are also various cofactors of these inhibitors, e.g. heparin for Antithrombin III, protein S for protein C.

Table 7: Plasma protease inhibitors of the blood coagulation and fibrinolytic system

Inhibitor	Concentration in plasma g/l	Mol. wt	Main Substrate
α_1 Proteinase inhibitor	1.3	53.000	Non haemostatic
α_2 Macroglobulin	2.5	725.000	Thrombin, Plasmin
Antithrombin III	0.35	58.000	F Xa, Thrombin, Plasmin
C_1 inhibitor	0.18	105.000	Contact Factors
α_2 Plasmin inhibitor	0.06	67.000	Plasmin
Heparin cofactor	0.09	66.000	Thrombin
Act. protein C inhibitor	0.05	57.000	F VIIIa, FVa
Plasminogen activator inhibitor	variable	57.000	Plasminogen, Plasmin

The clinical importance of these substances is in their deficiency states. Antithrombin and protein C, deficiencies causing venous thrombosis. C_1 inhibitor deficiency causing angioneurotic oedema. The biochemistry of many of these inhibitory systems are poorly understood. In recent years our knowledge of the inhibitors have expanded dramatically, but many problembs remain unresolved yet.

Investigation of the Haemostatic Mechanism

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The causes of haemostatic failure are numerous, the initial problem is to determine whether bleeding is due to a local factor, such as a peptic ulcer, or to an underlying haemostatic abnormality. Often a mild abnormality only becomes apparent after trauma or a local precipitating lesion. Any bleeding disorder may be inherited or acquired. This may result from one of the following disease;

- Thrombocytopenia
- Functional platelet abnormality
- Blood vessel defect
- Coagulation factor or factors defect
- Excess fibrinolysis
- Combined defect

In a haemostatic failure, initially a series of simple screening tests which are easy to perform and give reliable results should be undertaken. These tests are named firstline tests of haemostatic failure. If the screening procedures suggest a disorder, further tests should be organised.

Haemostatic mechanism disorders are may be investigated in a various ways. These are;

- Disorders of vascular haemostasis,
- Pletelet function,
- Blood coagulation,
- Fibrinolytic system and,
- Investigation of a thrombotic tendency.

Test of defective vascular function are difficult to perform and interpret. The only test of value is the **Bleeding Time** and its modifications. Tests of capillary resistance are of limited value. In some cases skin biopsy with specific staining or even biochemical analysis of the micro-sample may be helpful.

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The Beeding Time:

A standard incision is made on the volar surface of the forearm and the time the incision bleeds is measured. Cessation of bleeding indicates the formation of haemestatic plugs which are also releated to pletelets number and functions.

IVY's Method:

Two seperate puncture are made, 5-10 cm apart in quick succession using a disposable lancet. Any microlance with a cutting depth of 2.5 mm and width of just over 1 mm is suitable. First clean the volar surface of the forearm or finger with 70 % ethanol. Blot off gently but completely with filter paper at 15 second intervals the blood exuding from the cut.

Normal range:

2-7 minutes, but ideally, each laboratory should determine its own normal range. A prolonged bleeding time may be due to:

- Thrombocytopenia. Patients with a platelet count below $50x10^9/L$ may have a very long bleeding time and the bleeding may be difficult to arrest.

- Disorders of platelet function. They may be congenital, such as thrombasthenia, storage pool defect, etc, or acquired, due to drugs, the presence of a paraprotein, or platelet abnormalities.

- Von willebrand's disease, due to dafective platelet adherence to the subendothelium in the absence of a von willebrand Factor (vWF).

- Vascular abnormalities, as found in Ehler-Danlos's syndrome or in Pseudoxanthoma elasticum.

- Occasionally in severe deficiency of factore V and XI.

Investigation of the Platelet Disorders

The peripheral blood platelet count and the skin bleeding time, as mentioned above, are the first line tests of platelet function. If the screening procedures suggest a disorder of platelet function, **further tests** should be organised. Drugs and certain foods as shown in table may affect platelet function tests and the patient must be asked to avoid taking such substances for at least seven days before the test.

Table 8: Substances which commonly affect platelet function

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1. Agents which affect prostanoid synthesis Asperin and other preparations containing ASA. Non-steroidal anti-inflammatory drugs corticosteroids
2. Antibiotics
   - Penicilin
   - Cephalosporins
   - β - lactam derivatives
3. Membrane stabilising agents
   - β - blockers
   - α - antagonists
   - Local anesthetics (procaine)
   - Antihistamines
   - Tricyclic antidepressants
   - Frusemide
4. Agents which increase c-AMP levels
   - Dipyridamole
   - Aminophyline
   - Prostanoids
5. Food stuffs
   - Alcohol
   - Garlic
   - Certain chinese foods
6. Others
   - Heparin
   - Dextrans
   - Ethanol

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Phenothiazine

Clofibrate

Papaverine

Further platelet function tests can be summurised as follows:

- Inspecting a fresh blood film may show abnormalities of platelet size or morphology

- Platelet adhesion tests (Retention in a glass-bead column)

- Platelet aggregation tests

Turbidometric technique using

ADP, Collagen, Ristocetin or others.

- Investigation of granular content and release

- Investigation of prostaglandin pathways

- Investigation of platelet membrane glycoproteins

- Investigation of platelet coagulant activity

Platelet adhesion tests are rarely used because of the lack of specificity. In contrast, platelet aggregation tests commonly used in specialist laboratory. The granular content of the platelets can be assessed by electron microscopy or by measuring the substances released. Adenine nucleotides, serotonin, β-thromboglobulin and platelet factor 4 can be measured. The release from the granules is mostly used as a marker of in vivo platelet activation and thrombotic tendency.

Platelet Aggregation

Platelet aggregation is the process which platelets interact with one another to form a hemostatic plug. Platelet aggregation begins after platelet adherence on vascular subendothelial connective tissue exposed by endothelial injury. Collagen contained in the basement membrane induces a change in shape from discoid to spheroid with pseudopodic projections. After secretion dense and α-granules contents such as ADP, serotonin and fibrinogen interplatelets contact and agregation occurs. Reagents such as collegen, thrombin and ADP bind to specific platelet membrane receptors activating platelets and triggening a series of reac-

tions which can culminate in shape change, granule release, and aggregation.

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There are different basic pathways of platelet aggregation; ADP causes a chain of events involving phosphatidyl inositol metabolism leading to the exposure of fibrinogen binding sites on the membrane and causes to aggregation. Fibrinogen has essential role in platelet aggregation fibrinogen is believed to bind to a calcium complex of platelet membrane glycoproteins IIb and IIIa. Aggregation does not occur in thrombasthenia because of lacking or abnormal membrane glycoproteins. Fibrinogen makes a molecular bridge, from platelet to platelet due to its molecular structure. Low dose of collagen causes arachidonic acid mobilisation from the membrane, and this is converted to TXA_2, which is potent stimulator of aggregation, causing ADP release and calcium flux. Higher doses of collagen will also cause release of ADP directly. Low concentrations of thrombin cause aggregation through ADP and arachidonate release, but high doses of thrombin, can induce aggregation through a mechanism independent of TXA_2 and ADP, which may be due to PAF.

Platelet Aggregation Test

Several methods have been desribed to measure platelet aggregation. The prinsible is the sticking of platelets to each other, in vitro. In the turbidimetric method developed by Born and O'Brien, citrated platelet rich plasma (PRP) is stirred at 37 C and a constant speed in a siliconized glass cuvette contained in a instrument known as an aggregometer. As shown in Figure 49 light transmission through the PRP, which is continuously monitored by means of a suitable recorder, changes on addition of an agonist such as ADP, adrenalin (epinephrine), thrombin, collagen due to formation of platelet aggregates. After aggregation the transmission increases and is detected by movement of a pen on the chart recorder.

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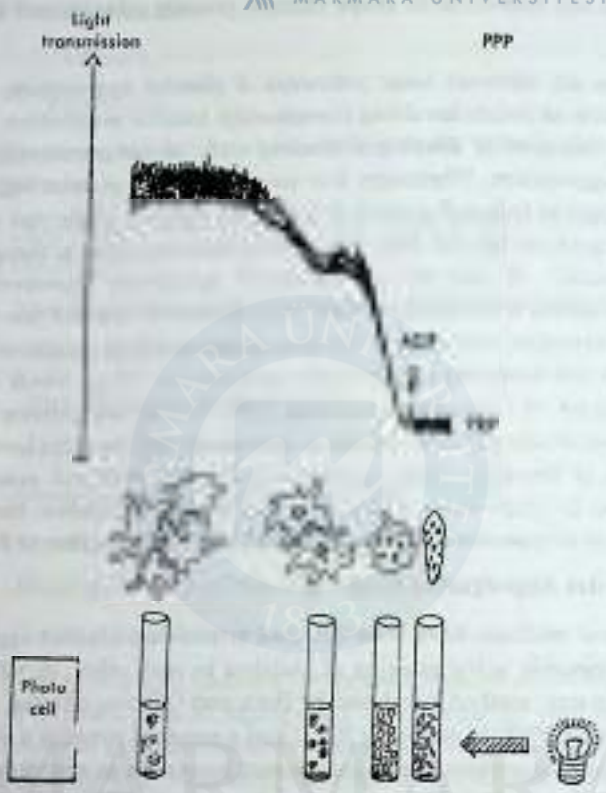

Figür 49: Measurement of platelet aggregation by turbidometric method (From ref..2)

In normal PRP the aggregation response with ADP is biphasic, the first phase is due to the direct effect of ADP on platelets. This first phase is followed by a second phase that is associated with release of endogenous platelet ADP. But these reactions depends on ADP concentration which added to PRP during the measurement of aggregation In contrast to ADP collagen represents a monophasic aggregation curve the antibiotic Ristocetin also induces a biphasic platelet aggregation response if the PRP contains an adequate concentration of vWF which is called ristocetin cofactor.

Routinely used agonists for platelet aggregation studies are as follows;

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	Final concentration
ADP	0.5 - 10.0 µM
Ristocetin	0.5 - 1.2 mg/ml
Collagen	1.0 - 4.0 µg/ml

Collagen concentretion varies according to species and tissue source of collagen. Preparation of sample for ADP induced platelet aggregation is as follows:

- PRP is diluted with autologous platelet poor plasma (PPP) to give a final platelet count of 200 x 10^9/L.

- ADP-prepare 10,5, 2.5 µM solutions in saline on ice (ADP Grade III, Sigma Chemical Co-Ltd 10 m M solution in saline, stored in aliquots at - 20 C).

- Allow aggregometer to warm to 37 C and set strirred speed to 900 rpm.

- Set chart recorder to 10 mV and 3 cm/minute

- 300 µl PPP is placed in one cuvette and 270 µl PRP in another, these are used to calibrate the aggregometer signal on the chart recorder to 10 % and 95 % settings, using the output and zero controls respectivelly.

- 270 µl of undiluted PRP is placed in a cuvette in the aggregometer, and stirred for 15 minutes to check for spontaneous aggregation.

- If spontaneity is present, dilute the PRP in PPP and repeat the test, until a dilution is found where spontaneous aggregation disappears. If this point is found at or above a platelet count of $200x10^9$ aggregation tests may proceed.

- Place 270 µl dilute PRP in a cuvette and warm until a steady baseline is obtained.

- Add 30 µl of aggregating reagent and record response. ADP normally gives a secondary wave at 2.5 µM or above, with no disaggregation.

Only primary wave should be obtained below 1 µM. The length of the lag phase, aggregation rate, and amplitude of the aggregation wave may be measured for diagnosis. Platelet aggregation defects in some disease induced by ADP and collagen and ristocetin is shown in table 9.

Table 9: ADP, collagen and Ristocetin induced aggregation in some disease

	ADP	**Collagen**	**Ristocetin**
Von Willebrand Disease	Normal	Normal	Absent or Abnormal
Thrombasthenia (Glanzman's)	Absent	Absent	Primary
Storage pool defect	Primary	Abnormal	Normal or primary
Cyclo-oxygenase defect (such as aspririn intake)	Abnormal or Normal	Absent	Normal
TXA_2 synthetase defect	Abnormal or Normal	Abnormal	Normal
TXA_2 reseptor defect	Abnormal or Normal	Abnormal	Normal
Other membrane proteins defect	Normal or Abnormal	Abnormal	Normal or Abnormal
Ehler's Danlos defect	Normal	Abnormal	Normal

Blood Coagulation Tests:

Two separate pathways of coagulation, intrinsic and extrinsic, are defined by the two commonly used coagulation tests, the activated partial thromboplastin time and the prothrombin time. The coagulation proteins can conveniently be divided into three groups on the basis of their biochemical properties and functions;

- Vitamin K-dependent proteins
- Contact proteins
- Thrombin sensitive proteins

Table 6 shows some biochemical properties of the three families of coagulation proteins.

Table 10: Techniques used for the measurement of coagulation factors

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Factor	Coagulation assay	Immunoassay	Chromogenic assay	Others
Fibrinogen	Clot capacity Clot weight	ID IE		Chemical tests
Prothrombin	Two-stage	ID	Specific substrate	Taipan venom
Factor V	One-stage		Non-specific	
Factor VII	One-stage ELISA	ID substrate	Specific	
Factor VIII:C	One-stage Two-stage	IRMA	Non-specific	
von Willebrnad factor		IE, CIE, ELISA, IRMA		Ristocetin cofactor
Factor IX	One-stage	IRMA, ELISA	Non-specific	
Factor X	One-stage	ID, IE, ELISA	Specific substrate	Russell viper venom (RVV)
Factor XI	One-stage	ID		
Factor XII	One-stage	IE	Non-specifi	
Prekallikrein	One-stage		Specific substrate	
HMWK	One-stage	ID		
Factor XIII	Clot solubility	IE		Incorporation of synthetic amines

IE = Immuno-electrophoresis; CIE = crossed immuno-electrophoresis;
ID = immunodiffusion; IRMA = immunoradiometric assay;

The first line coagulation tests are; activated partial thromboplastin time (APTT), One stage prothrombin time (PT) and measurement of fibrinogen by one of the techniques listed in table 10. A platelet count should always be carried out and the skin bleeding time performed whenever von willebrand's disease is suspected.

Every laboratory should be capable of performing screening or first-line coagulation tests coagulation tests are necessary in patient with an acute or chronic haemorrhagic tendency as wal as in those with a family history of coagulation factor defect or deficiency. The first step, however, in the diagnostic process is to obtain a detailed history, including the family history. The type, location frequency, duration and severity of bleeding, whether spontaneous or traumatic must be established.

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Partial Thromboplastin Time with Kaolin (PTTK)

This test is also known as the Activated Partial Thromboplastin Time (APTT) and the kaolin cephalin clotting Time (KCCT).

The test measures the clotting time of plasma after the activating of contact factors but without added tissue thromboplastin and so indicates the overall efficiency of the intrinsic pathway. To standardize the activation of contact factors, the plasma is first preincubated with kaolin. A standardized phospholipid is provided to allow the test to be performed on platelet poor plasma the test depends not only on the contact factors and on factors VIII and IX, test also on the reactions with factors X,V prothrombin and fibrinogen. (Figure 50)

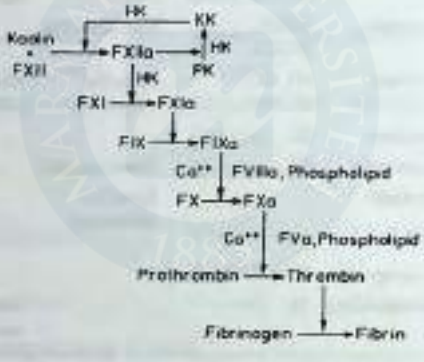

Figure 50: Reactions taking place in the partial thromboplastin time carried out with kaolin (APTT)

Normal values and interpretation:

The test is also sensitive to the presence of circulating anticoagulants (inhibitors) and heparin Normal range for APTT is 30-40 seconds the actual times depend on the reagents used. The difference between the patient's and the control means should not exceed 6 seconds. If the difference is greater than 10 seconds the patient's plasma is clearly abnormal.

The common causes of a prolonged PTTK are:

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- Deficiences of contact factors, FVIII, FIX, FI and FII
- Disseminated intravascular coagulation
- Liver disease
- Massive transfusion with stored blood.
- Adminstration of heparin or contamination with heparin
- A circulating anticoagulant

The PTTK is also moderately prolonged in patients on oral anticoagulant drugs and in the presence of vitamine K deficiency.

One Stage Prothrombin Time (PT)

The test measures the clotting time of plasma in the presence of tissue extract (thromboplastin) and indicates the overall efficiency of the extrinsic clotting system. In in vivo condition tissue factor can be released from a wide range of cell types following rupture or damage, and activates the extrinsic pathway of coagulation. In the in vitro test, tissue factor and calcium ions are added to plasma resulting in the activation of extrinsic clotting factors. Thrombin generation and formation of a fibrin is indicated in figure 51. The method of Quick one stage prothrombin time is the most suitable for investigation of an inhherited or acquired coagulotion defect of the extrinsic seystem. Tbe test is sensitive to the vitamin K-dependent factors (Factor II, VII and X) but insensitive to Factor V deficiency. This test is also used in the monitoring of anticoagulant therapy

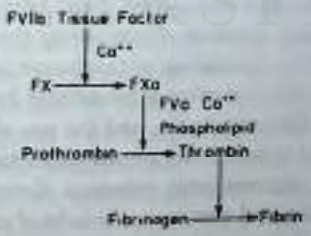

Figure 51 : Reactions involved in the prothrombin time test

Reagents

- Patient's and control platelet poor plasma

- Thromboplastin: Thromboplastins are tissue extracts obtained from different species and different organs. The majority of thromboplastins now in use are extracts of rabbit brain or lung. Each preparation has a different sensitivity of clotting factor deficiencies and defects, in particular to the defect induced by oral anticoagulants.

- $CaCl_2$ - 0.025 mol/L

Method

- Deliver 0.1 ml of plasma into a glass tu be placed in a water- bath (37 C)

- Add 0.1 ml thromboplastin

- Wait 1 to 3 min to allow the mixture to warm.

- Add 0.1 ml of wormed $Cacl_2$ and mix the contents of the tube

- Start the stop-watch and record the end-point

Normal Values

Normal values depends on the thromboplastin used, with most rabbit thromboplastins the normal range or prothrombin time is between 11 and 16 seconds. The results are expressed as the mean of the duplicate readings in seconds or as the ratio of the mean patient's time to the mean normal control time. The control value is obtained from 20 normal men and women (non pregnant and not on oral contraceptives).

The one-stage prothrombin time of Quick is the most commonly used test. However, lack of standardization of the thromboplastin preparations used in this simple test has caused great discrepancies in the results. In addition, different methods of expressing the prothrombin time results have led to varying intensities of treatment in different parts of world. The use of ISI (the International Sensitivity Index, to assess the sensitivity of any given thromboplastin, and the use of INR (the International Normalized Ratio) which is the prothrombin time ratio expressed in terms of a common international reference thromboplastin, should minimize these difficulties and ensure uniformity of anticoagulation and interpretation throughout the world.

Reporting for prothrombin time normal value varies from laboratory to laboratory. In its simplest form the result can be reported as patients time in seconds and control time in seconds, e.g. patient 24 s, control 12s. This is usually reported as a ratio which is the result with the test plasma divided by the result with the control plasma, e.g. in this case 2.0

Many laboratories are using INR for reporting the results, i.e. The prohrombin time ratio which would have been obtained with the patients plasma, had the primary reference thromboplastin been used to perform the test. Some laboratories report the results as a percentage of normal clotting activity derived from a graph prepared by the manufacturer or locally. This graph is prepared by plotting dilutions of the normal plasma against the clotting times. There are serious drawbacks to this method because the diluted normal plasma does not reflect the anticoagulant defect in the anticoagulant plasma and its use may lead to either bleeding or thrombotic complications in patients whose treatment is controlled in this way.

Anticoagulant therapy prevents thrombasis, therefcre, laboratory control of anticoagunt therapy is very important the most commonly used test for laboratory control of oral anticoagulant therapy is prothrombin time test. Standardization of thromboplastin, used in this test should be performed. There are a number of commercial thromboplastins of animal origin. Human brain thromboplastin is no longer used for the prothrombin time because of the potential danger of the transmission of retroviruses. Each thromboplastin gives a different ratio or percentage on the same test and control plasma, even different batches of the same thromboplastin be have differently. It is not possible to define the therapeutic range unless the thromboplastin used is also specified or calibrated in terms of the International Sensitivity Index (ISI).

The calibration of thromboplastin is achieved by comparing the results from the test thromboplastin with those given by a reference thromboplastin calibrated in accordance with method recommended by the WHO Expert Committee on Biologic Standardization. Table 11 indicate comparison of different ways of reporting the prothrombin time using a thromboplastin with international Sensitivity Index. The therapeutic range in this table lies between 2.0 and 4.0 according to International Normalized Ratio (INR)

Table 11: Different reporting ways for prothrombin time (From ref. 120)

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PT time	PT ratio	PT index	% activity	INR
12	1.0	100	100	1.0
13.2	1.1	91	74	1.2
14.4	1.2	83	57	1.5
15.6	1.3	77	48	1.8
16.8	1.4	71	41	2.2
18	1.5	67	35	2.7
19.2	1.6	62	31	2.9
20.5	1.7	59	28	3.4
21.6	1.8	56	25	3.9
22.9	1.9	53	23	4.4
24	2.0	50	21	4.9
25.5	2.1	48	20	5.5
26.5	2.2	45	18.5	6.1
27.6	2.3	43	17.4	6.8
28.8	2.4	42	16b4	7.5
30	2.5	40	15.4	8.2
36	3.0	33	12	12.5

PT: prothrombin time
INR: International Normalized Ratio.

Factor VIII Assay Test

Factor VIII assay is the most common used test after first line investigation of intrinsic pathway, deficiency or defect of Factor VIII:C or vWF, Factor IX or Factor XI.

Different methods are described for Factor VIII:C assay; one stage assay where is assay and thromboplastin generation will be described in detail.

One-stage Assay Of Factor VIII: C

The one-stage assay for Factor VIII coagulant activity is based on the APTT (PTTK). It consists of comparing the ability of dilutions of the patient's plasma and of a standard plasma to correct the PTTK of a plasma known to be severely deficient in factor VIII:C, but containing all other factors required for normal coagulation. An identical, principle applies to the assays of factors IX, XI and XII.

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Reagents

- Pletelet poor plasma from the patient

- Standard plasma from normal donor (prepared from between 6 and 15 donors, include individuals of both sexes, aged between 20 and 60 women taking oestrogen-containing oral contraceptives should not contribute to the standard pool).

- Factor VIII deficient plasma (substrate plasma) F VIII: C concentration should be less than 1 % and the plasma should be free of inhibitors.

- Kaolin: 5 mg/ml in barbitone buffered saline

- Barbitone buffered saline

- Phospholipid solution (platelet substitute)

- $CaCl_2$ - 0.025 mol/l.

- Ice-bath

Place the reagents in the ice-bath until used. Make 1 and 10 dilutions of the test and standard plasma in buffered saline in plastic tubes in the ice-bath. Add to each dilution 0.1 ml of freshly reconstituted or thawed substrate plasma and warm up at 37 C. Add 0.2 ml of the phospholipid/kaolin mixture, mix the contents of the tube and start a master. Stop-watch. At 10 min exactly add the $CaCl_2$ and start a second stopwatch. Record the clotting time.

Plot the clotting times of the test and standard against the concentration of factor VIII:C on 4 cycle x 3 cycle log paper. Read the % activity from graph paper. Normal range 50-200 iu/dl

Factor VIII: C Assay by Thromboplastin Generetion Test

In this method, the amount of "intrinsic thromboplastin" generated when serum, calcium, phospholipid and absorbed plasma are incubated is directly proportional to the amount of factor VIII in the plasma sample. Generated thromboplastin is measured by its shortening effect on hemophilic substrate plasma.

Some clinically normal people have factor VIII:C concentrations of

35-50 iu/dl. Values below 30 iu/dl are abnormal, values below 50 iu/dl are carriers. A reduced and increased concentrations of Factor VIII:C and Factor IX are indicated below:

Factor	Normal range	Decreased	Increased
Factor VIII:C	50-200 iu/dl	-Hemophilia A	-Acute phase reaction
		-Some Carriers of haemophilia A	-Stress -Exercise
		-vWF deficiency	-Pregnancy
		-DIC	-Chronic liver disease
		-Circulating Anticoagulant	-Vascalitic diseases
Factor IX	40-160 iu/dl	-Hemophilia B	-Pregnancy
			-Women taking oestrogen-containing contraceptives.

Investigation of Fibrinalysis

Fibrinolitic potential is releated to plasminogen activators and inhibitors. The tests used are, firstly, the assay of plasminogen activators, using a fibrin substrate (Euglobulin lysis time, fibrin plate and many others) or chromogenic substrate or ELISA techniquis, and secondly, assays of inhibitors. The commonly used tests for inhibitors are the chromogenic assays of plasminogen activator inhibitor (PAI) and α_2 antiplasmin (AP). The euglobulin lysis time is a global test of fibrinolite system.

Euglobulin Lysis time (ELT)

When plasma is diluted and acidified the precipitate which forms contains plasminogen activator (mostly tPA) plasminogen and fibrinogen. Most of the inhibitors are left in the solution. The precipitate is redissolved, the fibrinogen clotted with thrombin and the time for clot lysis measured. Blood should be collected with minimal venous stasis and taken into plastic tubes containing trisodium citrate with 1:9 ratio, the blood should be kept in ice until processing, which should be as rapid as possible, then it is centrifuged at 2000 *g* for 10 minutes at 4 C to obtain platelet-poor plasma, which is seperated and kept in ice again.

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Reagents

- 0,27 M Acetic acid (3.2 ml of 1 % acetic acid diluted to 200 ml.)

- Phosphate buffer pH 7.4

1.515 g Na_2HPO_4

0.483g KH_2PO_4

6.8 g NaIl, 1 litre distilled water

- Bovine thrombin 4 NIH u/ml (Parke-Davis)

Method

- 9.5 ml of diluted acetic acid is placed in each of two plastic centrifuge tubes in ice, and 0.5 ml of plasma is added to each.

- After 15 minutes the tubes are centrifuged at 4 C for 10 minutes at 1000 g.

- The supernatant is poured off and the tubes inverted onto tissues, and then carefully dried inside using further paper tissues, before replacing in ice.

- 0,5 ml of cold phosphate buffer is added to each tube, and the precipitate redissolved by drawing it up and down in a plastic pipette.

- The redissolved precipitates are transferred to glass test tubes in ice, and 0,5 ml thrombin is added.

- After a few minutes, the solution clots and should then be transferred to a 37 C bath.

The tubes are observed at intervals of about 15 minutes for the first two hours and then every 30-40 minutes until the clots have completely lysed.

Normal values should be determined in each laboratory, but will usually lie within the range 60-170 minutes. The lysis time varies with age, sex, exercise, alcohol and smoking habits.

Investigation of a thrombotic tendency

The hemostatic process is carefully balanced so that hemorhage is promptly arrested and inappropriate thrombosis does not occur. An acquired thrombotic tendency is common and occurs in many conditions.

Over the last decade important advances have been made in clinicol diagnostic techniques in arterial thrombotic disease and venous thrombo-embolism. There are several important laboratory approaches to the in vestigation of thrombotic or hypercoagulable state as shown in table 12).

Table 12: Tests for a thrombotic or hypercoagulability conditions.

1. Coagulation system

Prothrombin time

Thrombin time

Activated partial thromboplastin time

Antithrombin III

Protein C

Protein S

Lupus anticoagulant test

2. Platelets

Platelet count

Platelet aggregation studies

Plasma β-thromboglobulin

Platelet adhesion

Platelet granules content (PF-4, PF-3 ADP, fibrinogen)

Platelet c-AMP

Platelet membrane phospholipid

Platelet membrane glycoproteins

Platelet membrane transport

Half life of platelets

Platelet prostanoid

3. Fibrinolytic system

Euglobin lysis time

Tissue plasminogen activator

Platelets play an important role in thrombus formation due to they adhere on the damaged vessel walls and form a platelet island. The findings concerning the platelet adhesion and platelet thrombus formation in vessel injury areas has been known for a long time. In various experiments done on animals, it was shown that in the walls of damaged vessels, first a platelet adhesion, then a permeable and fragile platelet island is formed and finally, this island is converted to a thrombus. These observations was studied by several investigators. It was shown in damaged arteries, the platelets adhere to a subendothelial non-collagenous substance and collagen fibers . Alterations of platelet function and biochemistry in patients with atherosclerosis and thromboembolism are well known. A number of alterations have been observed in the hemostatic parameters of patients with atherosclerosis.

Heparin, which is a sulfated polysaccharide with anticoagulant and antithrombotic properties, decreases in atherosclerotic patients. May be due to this finding there are a number of reports positive effects of intermittent heparin treatment. Fibrinolytic activity is decreased in atherosclerote subjects. In dog experiments, fibrinalitic therapy has shown that it prevent vascular wall alterations caused by experimental thrombosis.

An increase in platelet adhesion and aggregation in atheroselerosis and thromboembolism was demonstrated both in vitro and in vivo studies. (Figure 52) Smoking stimulates the coagulation mechanism and platelet aggregation and that it shortens the platelet survival time. Numerous studies show that an increase of β-thromboglobulin (BTG) and PF_4 level in atherosclerosis and thrombotic conditions. Amino acid transport systems across platelet membranes were found to be normal but glucose transport was shown to be defective. Galactose incorporation into platelet proteins and some of the membrane glycoproteins are increased in atherosclerosis,this may be explain why there is hyperagregation in atherosclerotic patients.

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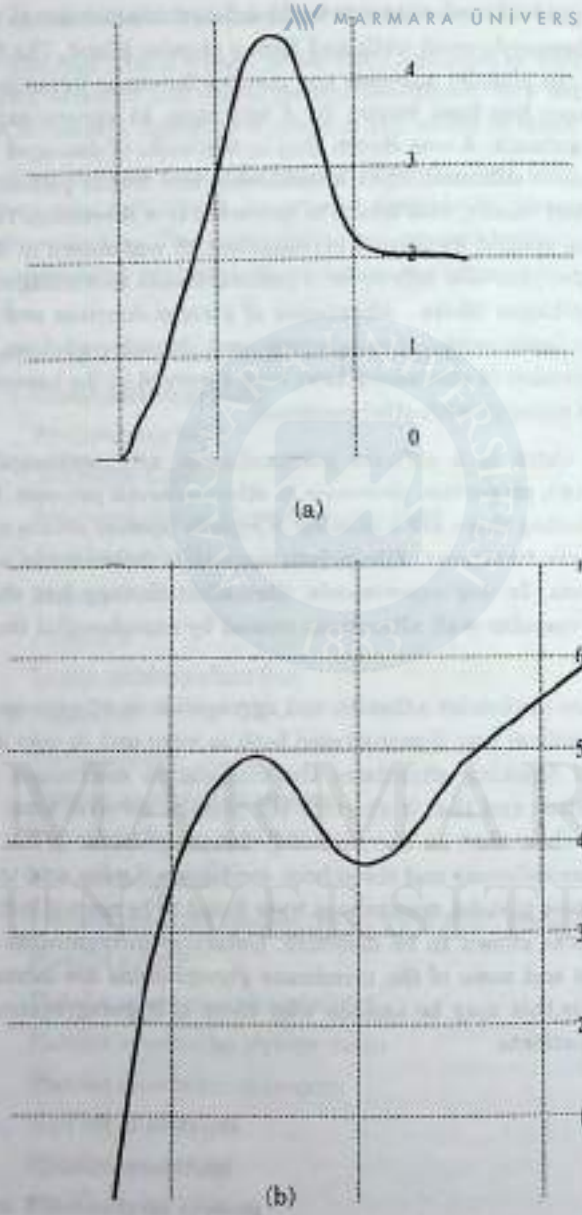

Figure 52: Characteristic aggregation curves indued by 0.25 µg/ml ADP with normal (a) and atherosclerotic platelets (b) (From ref. 80)

There are different phosholipids distribution in the platelet membrane of atherosclerosis. The total amount of membrane phospholipids was increased, but each phospholipid increased at a different rate. Prostaglandin pathway was also enhanced in platelets of atherosclerosis. Glucose transport was shown to be defective however amino asid transport across platelet membrane were found to be normal in atherosclerosis.

A list of causes of acquired thrombotic disease and other haemostatic abnormalities and probable mechanism is represented in table 13.

Table.13: Acquired thrombotic tendency. Aetiology, mechanisms of hypercoagulability and effects on haemostasis (From ref.120)

Aetiology	Mechanisms of hypercoagulability	Effect on first-line tests	Other haemostatic abnormalities
Acute phase reaction	Increase in VIII:C, vWF, fibrinogen and PAI	PTTK and PT often	Increased plasma concentration of acute reactants
Malignancy	Acute phase reaction and/or DIC due to release of tumour products	PTTK, PT and TT may be long	Increased FDP, signs of activation of coagulation and of consumption
Myeloproliferative diseases	Increased blood viscosity and high platelet count with activation of haemostasis	PTTK and PT may be short	Increased TG and increased acute reactants
Oestrogen-containing pill	Increased levels of vitamin-K dependent factors, reduced ATIII; dose related	PT may be short	Increased plasma conc. of VII, IX, and X; reduced ATIII concentration
Lupus anticoagulant	Anti-phospholipid antibodies interfering with coagulation	PTTK long, PT variable	Tests for lupus anticoagulant positive; ATIII and PC conc. may be low
Nephrotic syndrome	Loss of ATIII in urine	Normal	Reduced plasma ATIII
Vasculitic diseases and angiopathies	Stimulation of endothelium acute phase reaction, fibrinolytic shut-down and platelet activation	Variable	Very high plasma vWF, VIII:C and fibrinogen; increased PAI, FDP, βTG and PF4
ARDS	Low grade DIC	Variable	Reduced ATIII, PC, increased FDP, variable levels of acute reactants

ARDS: Adult Respiratory Distress syndrome. PAI: plasminogen activator inhibitor
vWF: von Willebrand factor; β-TG: B-thromboglobulin. PF4: platelet factor 4.
ATIII: antithrombin III; PC: protein C

NORMAL BLOOD PLASMA OR SERUM VALUES

(Values vary with procedure used)

Glucose	60-100 mg/dl	3.3-5.5 mmol/l
Protein: Total	5-8 g/dl	60-80 g/l
Albumin	3.5-5.5 g/dl	0.54-0.84 mmol/l
Globulin	1.5-3 g/dl	15-30 g/l
Sodium	136-145 mEq/L; 310-340 mg/dl	136-145 mmol/l
Potassium	2.5-5 mEq/L; 14-20 mg/dl	2.5-5.0 mmol/l
Chloride	100-106 mEq/l; 350-375 mg/dl	100-106 mmol/l
Sulfate	0.5-1.5 mEq/l	50-150 µmol/l
Transaminases:		
Glutamic-oxaloacetic (SGOT)	5-40 units	
Glutamic-pyruvic (SGPT)	5-35 units	
Triglycerides	<165 mg/dl	<18 mmol/l
Cholesterol	150-280 mg/dl	3.9-7.3 mmol/l
Cholesteryl esters	50-65% of total cholesterol	
Phospholipid	145-200 mg/dl	1.87-2.58 mmol/l
Phosphorus, inorganic	3-4.5 mg/dl (children, 4-7 mg)	
Total lipid	500-800 mg/dl	
Urea	20-40 mg/dl	
Urea nitrogen	8-20 mg/dl	2.86-7.14 mmol/l
Amino acid nitrogen	3-5.5 mg/dl	2.1-3.9 mmol/l
Creatinine	0.7-1.5 mg/dl	60-130 µmol/l
Ammonia	40-70 µg/dl	22.16-38.78 µmol/l
Uric acid	3-7.5 mg/dl	0.18-0.29 mmol/l
Acetone bodies	0.3-2 mg/dl	3-20 mg/l
Aldosterone	0.003-0.01 µ g/dl	0.03-0.1 µg/l
Amylase	0.8-3.2 IUK	
Lipase	0.2-1.5 units (ml of 0.1N NaOH)	
Ascorbic acid	0.4-1.5 mg/dl (fasting)	23-85 µmol/l
	25-40 mg/dl (W.C.B.)	1420-2272 µmol/l
Bilirubin	Direct: 0.1-0.4 mg/dl	1.71-6.84 µmol/l
	Indirect: 0.2-0.7 mg/dl	3.42-11.97 µmol/l
Calcium	9-10.6 mg/dl, 4.5-5.3 mEq/l (varies with protein concentration)	2.25-2.65 mmol/l

TABLE: Continue

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Carbon dioxide: Content	24-29 mEq/l, 55-65 vol %	24-29 mmol/l
Carotenoids	50-300 µg/dl	
Vitamin A	24-60 IU/dl; 24-60µg/dl	0.84-2.10µmol/l
Copper	100-200 µg/dl	16-31 µmol/l
Cortisol (free)	4-18 µg/dl	110-497 nmol/l
Iodine, protein-bound	4-8µg/dl	0.32-0.63 µmol/l
Iron	65-175 µg/dl	11.6-31.3 µmol/l
Iron-binding capacity	250-410 µg/dl	44.8-73.3 µmol/l
Pyruvic acid	0.7-2 mg/dl	79.8-228 µmol/l
Lactic acid	4-16 mg/dl	0.44-1.28 µmol/l
Lactic dehydrogenase	90-200 IU/l	
Magnesium	1-3 mg/dl	0.75-1.25 mmol/l
Oxygen: Capacity	16-24 vol % (varies with Hb concentration)	0.16-0.24 of volume
Arterial content	15-23 vol%(varies with Hb content)	0.15-0.23 of volume
Venous content	10-16 vol %	0.1-0.16 of total
Acid Phosphatase	1-5 units (King-Armstrong); 0.5-2 units (Bodansky); 0.5-2 units (Gutman);	
Alkaline Phosphatase	5-13 units (King-Armstrong); 2-4.5 units (Bodansky);	

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Erythrocytes (red blood cells) count per microlitre:

Neonate	6.000.000
Child	4.000.000 - 5.500.000
Man	4.800.000 - 6.000.000
Woman	4.600.000 - 5.600.000

High normal values may be present in states of excitation, during muscular activity, and at high altitudes.

Hematocrit

Males	0.40 - 0.52
Females	0.37 - 0.47

Hemoglobin Content (g/l)

Adult males	155 25
Adult females	140 25
Newborn infants	165 30
Children (3-6 years)	130 10
15.000 feet above sea level	208

Colour index

$$\text{Calculation: } \frac{\text{haemoglobin \% (= units)}}{20 \times \text{erythrocytes (millions/µl)}}$$

$$\text{Example: } \frac{100}{20 \times 5} = 1.0$$

Normal values: 0.9 - 1.1

Colour coefficient Hb or mean corpuscular haemoglobin M.C.H. (haemoglobin content of individual erythrocyte in picograms [10^{-12} g/l)

$$\text{Calculation: } \frac{\text{Hb in g/100 ml} \times 10}{\text{erythrocytes (millions/µl}}$$

Example: $\frac{16 \times 10}{5} = 32$

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Normal values: 28 - 36

Saturation index

Calculation: $\frac{\text{Haemoglobin \% (= units)}}{\text{packed cell volume/100 ml}}$

Example: $\frac{100}{45} = 2.22$

Normal values: about 2.22

Mean corpuscular haemoglobin concentration M.C.H.C. (number of grams of Hb in 100 ml of red blood cells)

Calculation: $\frac{\text{Hb in g/100 ml} \times 100}{\text{packed cell volume/100 ml}}$

Example: $\frac{16 \times 100}{45} = 35.5$ g

Normal values:

Neonates	about 45 g
Children	34-41 g
Adults	33-37 g

Mean volume of erythrocyte or mean corpuscular volume M.C.V.

Calculation: $\frac{\text{packed cell volume/100 ml} \times 10}{\text{erythrocytes (millions/µl)}}$

Example: $\frac{46 \times 10}{5.4} = 85\ \mu m^3$

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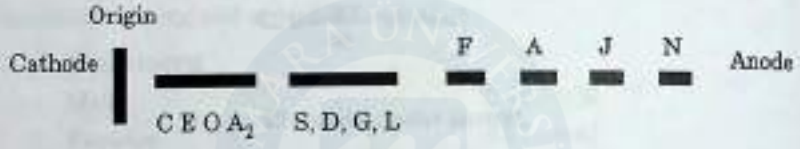
Normal values:

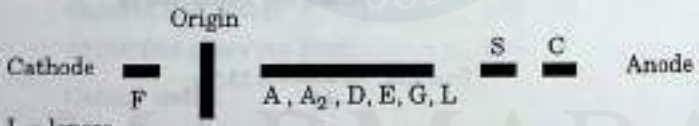
Infant	105 μm^3
Child	73-87 μm^3
Adult	82-92 μm^3

Hemoglobin migrate on cellulose acetate electrophoresis



L = lepore

Hemoglobin migrate on citrate agar electrophoresis



L = lepore

Hb A_2	2.2 - 3.2	%
HbF	0.1 - 0.5	%
2, 3 DPG (red cell)	4.0 - 5.0	m moles/l RBC
Serum Folate	3 - 25	ng/ml
Serum B_{12}	170 - 1000	pg/ml
GSH	83.3 11.8	mg/dl **RBC**
GSH	8.6 1.3	µ moles/g **Hb**

Erythrocyte sedimentation rate (upper limit range mm/hour)

Males

17 - 20 years	10
51 - 60 years	12
> 60 years	14

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Females	
17 - 50 years	12
51 - 60 years	19
> 60 years	20

White cell count (absolute count per microlitre)

Adult	4 - 11
Infants at birth	10 - 24
Infants 1 year	6 - 18
Children < 10 years	5 - 14

White cell differentiation

	Children up to 8 years		Adults	
	Relative (%)	Absolute per 10.000 WBC	Relative (%)	Absolute per 7000 W.B.C.
Basophils	0 - 2	0-200	0 - 2	0-140
Eosinophils	0 - 5	0-500	0 - 3	0-210
Neutrophils				
with staff nuclei	0 - 10	0-1000	0 - 5	0-350
with segmented nuclei	25 - 65	2500-6500	45 - 70	3150-4900
Lymphocytes	30 - 70	3000-7000	20 - 45	1400-3150
Monocytes	0 - 8	0-800	0 - 8	0-560
Plasma cells	0 - 5	0-500	0 - 2	0-140

Blood platelets (Absolute count per microlitre)

150.000 - 400.000

The various methods of determination yield widely varying results, and there are also marked variations between individuals.

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Bleeding time (Duke's test)		1 - 3	minutes
(Template test)		2.5 - 9.5	minutes
	Total	**Release**	
Platelet ATP	41-61	8-20	nmol/10^9 Pts
Platelet ADP	19-38	18-28	nmol/10^9 Pts
Platelet B-TG	< 50	ng/ml	
Coagulation time			
Schultz's hollow-bead capillary method			
in capillary blood		4 - 9	minutes
in venous blood	up to	20	minutes
Prothrombin time (Quick's test)		12-13	seconds
thrombin time		within 2	seconds
Fibrinogen		1.5 - 4.0 g/l	
FDP		< 200 ng/ml	
Factor IX		0.50 - 1.50	IU/ml
Factor VIII:c		0.50 - 2.00	IU/ml
Factor XII		0.73 - 1.45	U/ml
vWF (Elisa)		45 - 160	IU/dl
Euglobulin Lysis time		90 - 240	minutes
AT III (İmmunologic)		0.80 - 1.20	IU/ml
Protein C (Elisa)		0.70 - 1.3	u/ml
Protein S (Elisa)		0.70 - 1.3	u/ml
α_2 macroglobulin		0.67 - 1.51	u/ml
α_1 antitrypsin		0.69 - 1.53	u/ml
Kallikrein inhibitors		0.85 - 1.29	u/ml
Peasminogen		80 - 120	u/ml

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PART IV

L I P I D S

General Description

The term lipid applies to a class of compounds that are soluble in organic solvents and nearly insoluble in water. Chemically, lipids include compounds that yield fatty acids on hydrolysis and complex alcohols that can combine with fatty acids to form esters.

In the organism fat serves as an efficient source of energy, both directly and potentially when stored in adipose tissue. It serves as a thermal insulator in the subcutaneous tissues and around certain organs, and nonpolar lipids act as electrical insulators allowing rapid propagation of depolarization waves along myelinated nerves. The fat content of nerve tissue is particularly high. Combinations of fat and protein which called lipoproteins are important cellular constituents.

Lipids are a heterogeneous group of compounds related more by their physical rather than by their chemical properties. Lipids include fats, oils, steroids, waxes and related compounds.

Lipids are important dietary constituents not only because of their high energy value but also because of the fat soluble vitamins and the essential fatty acids contained in the fat of natural foods. They serve as hormones or hormone precursors, aid in digestion.

In the last decade, however, lipids in clinical chemistry have become associated with lipoprotein metabolism and a form of arterial disease known as atherosclerosis (see page 453).

Classification of Clinically Important Lipids

Some lipids are more complex, containing groups that tend to increase the solubility of lipids in polar solvents. Lipids can be broadly subdivided into five groups based on their chemical structure as shown below:

1-Fatty acids

Short chain (2-4 carbon atoms)

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Medium chain (6-10 carbon atoms)

Long chain (12-26 carbon atoms)

Prostaglandins

2-Sterol derivatives

Cholesterol and cholesterol esters

Steroids hormones

Bile acids

Vitamin D

3-Glycerol esters

Triglycerides (Nötral lipids)

Phosphotriglycerides (phospholipid)

4-Sphingosine derivatives

Sphingomyelin

Glycosphingolipids

5-Terpenes (Isoprene polymers)

Vitamin A, E, K

FATTY ACIDS

Fatty acids are aliphatic carboxylic acids. Fatty acids (FA) are simple molecular forms of lipids depicted by the chemical formula RCOOH, where R stands for an alkyl chain. The most common classification is based on chain length:2-4 carbon atoms (short chain), 6-10 carbon atoms (medium chain) and 12-26 carbon atoms (long chain).

Fatty acids can be further classified according to their degree of saturation. Saturated fatty acids contain an alkyl chain without any double bonds between carbon atoms, monounsaturaded fatty acids contain one double bond, and polyunsaturated fatty acid have more than one double bond. In mammals, all naturally occurring unsaturated fatty acids are of the cis-variety. Trans-fatty acids are a result of catalytic hydrogenation a process used to "harden" fats in the manufacture of certain foods such as margarine.

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Most fatty acids can synthesize in humans except linoleic acid (18 and polyunsature fatty acid)

Linoleic acid is vital for maintenance of health, as well as for growth and development. Because of this it is termed an assential fatty acid. Linoleic acid plays an important role in prostaglandin synthesis and perhaps in myelinization of the central nervous system.

Fatty acids occur mainly as esters in natural fat and oils but do occur in the unesterified form as free fatty acids.

Fatty acids that occur in natural fats are usually straight-chain derivatives and contain an even number of carbon atoms because they are synthesized from two carbon units. The chain may be saturated (containing no double bonds) or unsaturated (containing one or more double bonds).

Fatty acids are named after corresponding hydrocarbons. Saturated fatty acids end in-**anoic**

Unsaturated acids with double bonds end in **–enoic**, eg.octadecenoic acid (oleic acid).

Saturated fatty acids contain no double bonds example are shown in table 1. Unsaturated fatty acids contain one or more double bonds are shown in table 2.

Carbon atoms are numbered from the carboxyl carbon (carbon no 1). The carbon atom adjacent to the carboxyl carbon (No.2) is also known as the alfa carbon. Carbon atoms No. 3 and No 4 are the beta and gamma carbons, respectively and the terminal methyl carbon is known as the omega carbon or n-carbon atom. Various conventions are in use for indicating the number and position of the double bonds. W^9 indicates a double bond on the ninth carbon counting from the w-carbon atom.

TABLE 1: Saturated fatty acids.

Common Name	Number of C Atoms	
Formic[1]	1	Takes part in the metabolism of "C_1" units (formate)
Acetic	2	Major end product of carbohydrate fermentation by rumen organisms[2]
Propionic	3	An end product of carbohydrate fermentation by rumen organisms[2]
Butyric	4	In certain fats in small amounts (especially butter). An end product of carbohydrate fermentation by rumen organisms[2]
Valeric	5	
Caproic	6	
Caprylic (octanoic)	8	In small amounts in many fats (including butter), especially those of plant origin
Capric (decanoic)	10	
Lauric	12	Spermaceti, cinnamon, palm kernel, coconut oils, laurels, butter
Myristic	14	Nutmeg, palm kernel, coconut oils, myrtles, butter
Palmitic	16	Common in all animal and plant fats
Stearic	18	
Arachidic	20	Peanut (arachis) oil
Behenic	22	Seeds
Lignoceric	24	Cerebrosides, peanut oil

[1]Strictly, not an alkyl derivative.
[2]Also formed in the cecum of herbivorse and to a lesser extent in the colon of humans.

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TABLE 2: Unsaturated fatty acids.

Number of C Atoms and Number and Position of Double Bonds	Series	Common Name	Systematic Name	Occurrence
Monoenoic acids (one double bond)				
16:1;9	ω7	Palmitoleic	cis-9-Hexadecenoic	In nearly all fats
18:1;9	ω9	Oleic	cis-9-Octadecenoic	Possibly the most common fatty acid in natural fats
18:1;9	ω9	Elaidic	trans-9-Octadecenoic	Hydrogenated and ruminant fats
22:1;13	ω9	Erucic	cis-13-Docosenoic	Rape and mustard seed oils
24:1;15	ω9	Nervonic	cis-15-Tetracosenoic	In cerebrosides
Dienoic acids (two double bonds)				
18:2;9,12	ω6	Linoleic	all-cis-9,12-Octadecadienoic	Corn, peanut, cottonseed, soybean, and many plant oils
Trienoic acids (three double bonds)				
18:3;6,9,12	ω6	γ-Linolenic	all-cis-6,9,12-Octadecatrienoic	Some plants, eg, oil of evening primrose, borage oil; minor fatty acid in animals
18:3;9,12,15	ω3	α-Linolenic	all-cis-9,12,15-Octadecatrienoic	Frequently found with linoleic acid but particularly in linseed oil
Tetraenoic acids (four double bonds)				
20:4;5,8,11,14	ω6	Arachidonic	all-cis-5,8,11,14-Eicosatetraenoic	Found in animal fats and in peanut oil; important component of phospholipids in animals
Pentaenoic acids (five double bonds)				
20:5;5,8,11,14,17	ω3	Timnodonic	all-cis-5,8,11,14,17-Eicosapentaenoic	Important component of fish oils, eg, cod liver, mackerel, menhadden, salmon oils
22:5;7,10,13,16,19	ω3	Clupanodonic	all-cis-7,10,13,16,19-Docosapentaenoic	Fish oils, phospholipids in brain
Hexaenoic acids (six double bonds)				
22:6;4,7,10,13,16,19	ω3	Cervonic	all-cis-4,7,10,13,16,19-Docosahexaenoic	Fish oils, phospholipids in brain



Linoleic acid, a polyunsaturated fatty acid. Both double bonds are *cis*.

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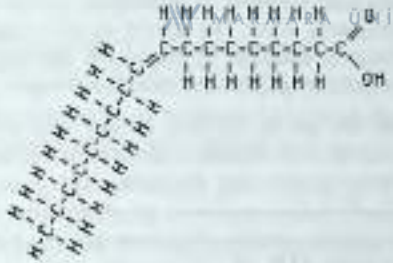
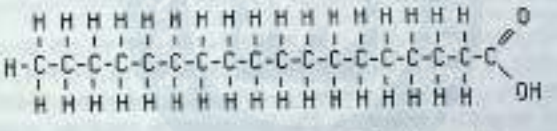


Oleic acid, a monounsaturated fatty acid. Note that the double bond is *cis*; this is the common natural configuration.



Stearic acid, a saturated fatty acid

Fatty Acid Synthesis

One might predict that the pathway for the synthesis of fatty acids would be the reversal of the oxidation pathway. However, this would not allow distinct regulation of the two pathways to occur even given the fact that the pathways are separated within different cellular compartments.

The pathway for fatty acid synthesis occurs in the cytoplasm, whereas, oxidation occurs in the mitochondria. The other major difference is the use of nucleotide co-factors. Oxidation of fats involves the reduction of $FADH^+$ and NAD^+. Synthesis of fats involves the oxidation of NADPH. However, the essential chemistry of the two processes are reversals of each other. Both oxidation and synthesis of fats utilize an activated two carbon intermediate, acetyl-CoA. However, the acetyl-CoA in fat synthesis exists temporarily bound to the enzyme complex as malonyl-CoA.

The synthesis of malonyl-CoA is the first committed step of fatty acid synthesis and the enzyme that catalyzes this reaction, ***acetyl-CoA carboxylase*** **(ACC)**, is the major site of regulation of fatty acid synthesis. Like other enzymes that transfer CO_2 to substrates, ACC requires a biotin co-factor.

The rate of fatty acid synthesis is controlled by the equilibrium between monomeric ACC and polymeric ACC. The activity of ACC requires polymerization. This conformational change is enhanced by citrate and inhibited by long-chain fatty acids. ACC is also controlled through hormone mediated phosphorylation (see below).

The acetyl groups that are the products of fatty acid oxidation are linked to CoASH. As you should recall, CoA contains a **phosphopantetheine** group coupled to AMP. The carrier of acetyl groups (and elongating acyl groups) during fatty acid synthesis is also a phosphopantetheine prosthetic group, however, it is attached a serine hydroxyl in the synthetic enzyme complex. The carrier portion of the synthetic complex is called **acyl carrier protein, ACP**. This is somewhat of a misnomer in eukaryotic fatty acid synthesis since the ACP portion of the synthetic complex is simply one of many domains of a single polypeptide. The acetyl-CoA and malonyl-CoA are transferred to ACP by the action of ***acetyl-CoA transacylase*** and ***malonyl-CoA transacylase***, respectively. The attachment of these carbon atoms to ACP allows them to ***enter*** the fatty acid synthesis cycle.

The synthesis of fatty acids from acetyl-CoA and malonyl-CoA is carried out by ***fatty acid synthase***, **FAS**. The active enzyme is a dimer of identical subunits.

All of the reactions of fatty acid synthesis are carried out by the multiple enzymatic activities of FAS. Like fat oxidation, fat synthesis involves 4 enzymatic activities. These are, ***-keto-ACP synthase***, ***-keto-ACP reductase***, ***3-OH acyl-ACP dehydratase*** and ***enoyl-CoA reductase***. The two reduction reactions require NADPH oxidation to $NADP^+$.

The primary fatty acid synthesized by FAS is palmitate. Palmitate is then released from the enzyme and can then undergo separate elongation and/or unsaturation to yield other fatty acid molecules.

De novo Synthesis of Fatty Acids

Overview: Tissue locations

Fatty acid synthesis occurs primarily in the cytoplasm of these tissues.

- liver
- adipose (fat)
- central nervous system
- lactating mammary gland

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Sum of the reactions:

8 acetyl CoA + 7 ATP + 14 (NADPH + H^+) -> palmitate (16:0) + 8 CoA + 7 (ADP + P_i) + 14 $NADP^+$ + 6 H_2O

This is the overall process for fatty acid synthesis. Acetyl CoA for fatty acid synthesis comes mostly from glycolytic breakdown of glucose.

Origin of Cytoplasmic Acetyl-CoA

Acetyl-CoA is generated in the mitochondria primarily from two sources, the pyruvate dehydrogenase (PDH) reaction and fatty acid oxidation. In order for these acetyl units to be utilized for fatty acid synthesis they must be present in the cytoplasm. The shift from fatty acid oxidation and glycolytic oxidation occurs when the need for energy diminishes. This results in reduced oxidation of acetyl-CoA in the TCA cycle and the oxidative phosphorylation pathway. Under these conditions the mitochondrial acetyl units can be stored as fat for future energy demands.

Acetyl-CoA enters the cytoplasm in the form of citrate via the **tricarboxylate transport system** as diagrammed. In the cytoplasm, citrate is converted to oxaloacetate and acetyl-CoA by the ATP driven ***ATP-citrate lyase*** reaction. This reaction is essentially the reverse of that catalyzed by the TCA enzyme ***citrate synthase*** except it requires the energy of ATP hydrolysis to drive it forward. The resultant oxaloacetate is converted to malate by ***malate dehydrogenase*** (MDH).

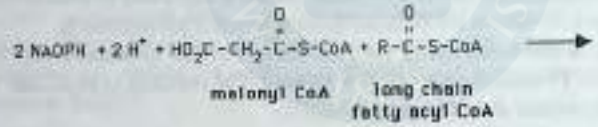
The malate produced by this pathway can undergo oxidative decarboxylation by ***malic enzyme***. The co-enzyme for this reaction is $NADP^+$ generating NADPH. The advantage of this series of reactions for converting mitochondrial acetyl-CoA into cytoplasmic acetyl-CoA is that the NADPH produced by the ***malic enzyme*** reaction can be a major source of reducing co-factor for the ***fatty acid synthase*** activities.

Elongation and Desaturation

The fatty acid product released from FAS is palmitate (via the action of ***palmitoyl thioesterase***) which is a 16:0 fatty acid, i.e. 16 carbons and no sites of unsaturation. Elongation and unsaturation of fatty acids occurs in both the mitochondria and endoplasmic reticulum (microsomal membranes). The predominant site of these processes is in the ER membranes. Elongation involves condensation of acyl-CoA groups with malonyl-CoA. The resultant product is two carbons longer (CO_2 is released from malonyl-CoA as in the FAS reaction) which undergoes reduction, dehydration and reduction yielding a saturated fatty acid. The reduction reactions of elongation require NADPH as co-factor just as for the similar reactions catalyzed by FAS. Mitochondrial elongation involves acetyl-CoA units and is a reversal of oxidation except that the final reduction utilizes NADPH instead of $FADH_2$ as co-factor.

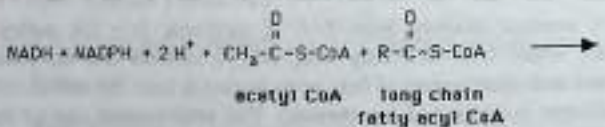
Desaturation occurs in the ER membranes as well and in mammalian cells involves 4 broad specificity ***fatty acyl-CoA desaturases*** (non-heme iron containing enzymes). These enzymes introduce unsaturation at C4, C5, C6 or C9. The electrons transferred from the oxidized fatty acids during desaturation are transferred from the desaturases to cytochrome b5 and then ***NADH-cytochrome b5 reductase***. These electrons are un-coupled from mitochondrial oxidative-phosphorylation and, therefore, do not yield ATP.

Since these enzymes cannot introduce sites of unsaturation beyond C9 they cannot synthesize either **linoleate** ($18:2^{9,12}$) or **linolenate** ($18:3^{9,12,15}$). These fatty acids must be acquired from the diet and are, therefore, referred to as **essential fatty acids**. Linoleic is especially important in that it required for the synthesis of **arachidonic acid**. As we shall encounter later, arachindorate is a precursor for the eicosanoids (the **prostaglandins** and **thromboxanes**). It is this role of fatty acids in eicosanoid synthesis that leads to poor growth, wound healing and dermatitis in persons on fat free diets. Also, linoleic acid is a constituent of epidermal cell sphingolipids that function as the skins water permeability barrier





fatty acyl CoA
lengthened by two carbons





fatty acyl CoA
lengthened by two carbons

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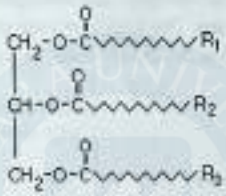
Storage in adipocytes as triacylglycerol

Fatty acids are stored primarily in adipocytes as triacylglycerol. Triacylglycerol must be hydrolyzed to release the fatty acids.

- Adipocytes are found mostly in the abdominal cavity and subcutaneous tissue.
- Adipocytes are metabolically very active; their stored triacylglycerol is constantly hydrolyzed and resynthesized.



A triacylglycerol

R_1 is often palmitate.
R_2 is often oleate.
R_3 is often oleate or a polyunsaturated fatty acyl group.

Adipocytes release nonesterified fatty acids.

Nonesterified fatty acid release from the adipocytes is initiated by the action of hormone sensitive lipase (HSL), which begins to hydrolyze the stored triglyceride.

The final products of triacylglycerol hydrolysis are glycerol and nonesterified fatty acids.

- HSL is activated by epinephrine, norepinephrine, ACTH and glucagon, acting via phosphorylation of the enzyme.
- It is inhibited by insulin.

Nonesterified fatty acids are bound to serum albumin for transport to other tissues, where they are used.

- Major target tissues are muscle and liver.
- At the target cells nonesterified fatty acids are taken up passively. Within the target cells they are bound to fatty acid binding protein. Next they must be activated.

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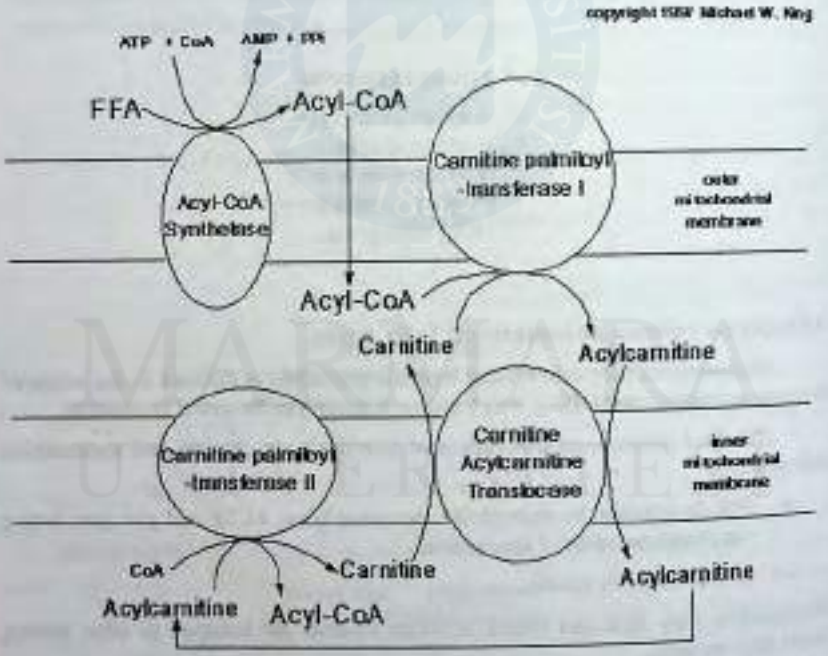
Fatty Acid Oxidation

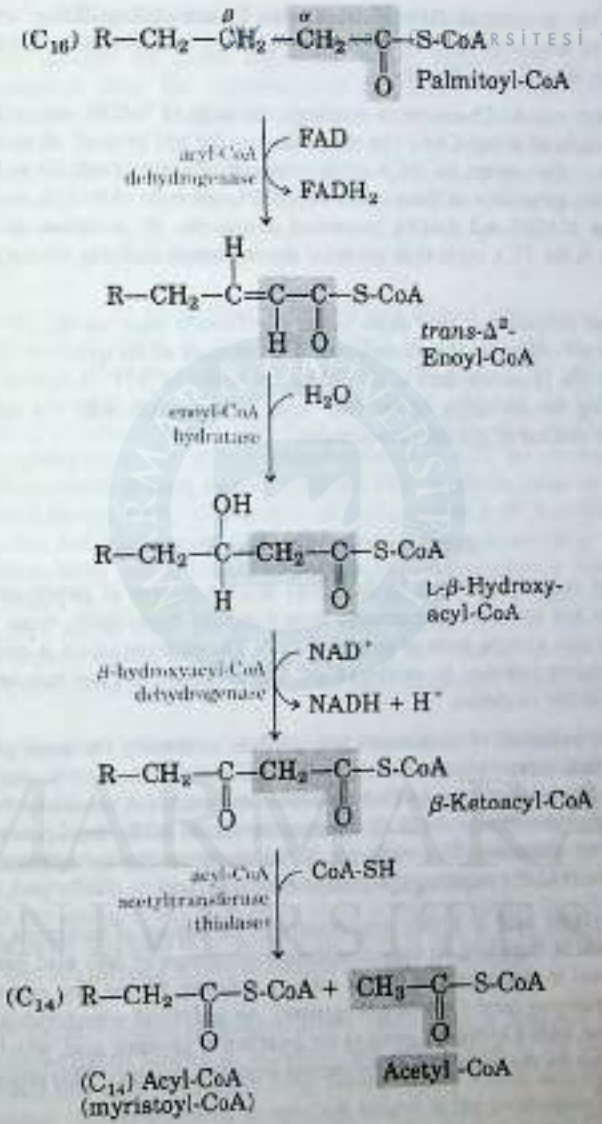
Fatty acids must be activated in the cytoplasm before being oxidized in the mitochondria. Activation is catalyzed by ***fatty acyl-CoA ligase*** (also called ***acyl-CoA synthetase*** or ***thiokinase***). The net result of this activation process is the consumption of 2 molar equivalents of ATP.

Fatty acid + ATP + CoA -------> Acyl-CoA + PP_i + AMP

Oxidation of fatty acids occurs in the mitochondria. The transport of fatty acyl-CoA into the mitochondria is accomplished via an **acyl-carnitine** intermediate, which itself is generated by the action of ***carnitine acyltransferase I***, an enzyme that resides in the outer mitochondrial membrane. The acyl-carnitine molecule then is transported into the mitochondria where ***carnitine acyltransferase II*** catalyzes the regeneration of the fatty acyl-CoA molecule.



Transport of fatty acids from the cytoplasm to the inner mitochondrial space for oxidation. Following activation to a fatty-CoA, the CoA is exchanged for carnitine by ***carnitine-palmitoyltransferase I***. The fatty-carnitine is then transported to the inside of the mitochondrion where a reversal exchange takes place through the action of ***carnitine-palmitoyltransferase II***. Once inside the mitochondrion the fatty-CoA is a substrate for the b-oxidation machinery.



β oxidation pathway (24)

The process of fatty acid oxidation is termed b-oxidation since it occurs through the sequential removal of 2-carbon units by oxidation at the b-carbon position of the fatty acyl-CoA molecule.

Each round of b-oxidation produces one mole of NADH, one mole of $FADH_2$ and one mole of acetyl-CoA. The acetyl-CoA--- the end product of each round of b-oxidation--- then enters the TCA cycle, where it is further oxidized to CO_2 with the concomitant generation of three moles of NADH, one mole of $FADH_2$ and one mole of ATP. The NADH and $FADH_2$ generated during the fat oxidation and acetyl-CoA oxidation in the TCA cycle then can enter the respiratory pathway for the production of ATP.

The oxidation of fatty acids yields significantly more energy per carbon atom than does the oxidation of carbohydrates. The net result of the oxidation of one mole of oleic acid (an 18-carbon fatty acid) will be 146 moles of ATP (2 mole equivalents are used during the activation of the fatty acid), as compared with 114 moles from an equivalent number of glucose carbon atoms.

Alternative Oxidation Pathways

The majority of natural lipids contain an even number of carbon atoms. A small proportion that contain odd numbers; upon complete b-oxidation, these yield acetyl-CoA units plus a single mole of propionyl-CoA. The propionyl-CoA is converted, in an ATP-dependent pathway, to succinyl-CoA. The succinyl-CoA can then enter the TCA cycle for further oxidation.

The oxidation of unsaturated fatty acids is essentially the same process as for saturated fats, except when a double bond is encountered. In such a case, the bond is isomerized by a specific ***enoyl-CoA isomerase*** and oxidation continues. In the case of linoleate, the presence of the D-12 unsaturation results in the formation of a dienoyl-CoA during oxidation. This molecule is the substrate for an additional oxidizing enzyme, the NADPH requiring ***2,4-dienoyl-CoA reductase***.

Phytanic acid is a fatty acid present in the tissues of ruminants and in dairy products and is, therefore, an important dietary component of fatty acid intake. Because phytanic acid is methylated, it cannot act as a substrate for the first enzyme of the b-oxidation pathway (acyl-CoA dehydrogenase). An additional mitochondrial enzyme, ***a-hydroxylase***, adds a hydroxyl group to the a-carbon of phytanic acid, which then serves as a substrate for the remainder of the normal oxidative enzymes. This process is termed a-oxidation.

Regulation of Fatty Acid Metabolism

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One must consider the global organismal energy requirements in order to effectively understand how the synthesis and degradation of fats (and also carbohydrates) needs to be exquisitely regulated. The blood is the carrier of triacylglycerols in the form of VLDLs and chylomicrons, fatty acids bound to albumin, amino acids, lactate, ketone bodies and glucose. The pancreas is the primary organ involved in sensing the organisms dietary and energetic states via glucose concentrations in the blood. In response to low blood glucose, glucagon is secreted, whereas, in response to elevated blood glucose insulin is secreted.

The regulation of fat metabolism occurs via two distinct mechanisms. One is **short term regulation** which is regulation effected by events such as substrate availability, allosteric effectors and/or enzyme modification. ACC is the rate limiting (committed) step in fatty acid synthesis. This enzyme is activated by citrate and inhibited by palmitoyl-CoA and other long chain fatty acyl-CoAs. ACC activity also is affected by phosphorylation. The primary phosphorylation of ACC occurs through the action of ***AMP-regulated protein kinase*, AMPRK** (this is not the same as ***cAMP-dependent protein kinase*, PKA**). Glucagon stimulated increases in PKA activity result in phosphorylation and inhibition of ACC. Additionally, glucagon activation of PKA leads to phosphorylation and activation of ***phosphoprotein phosphatase inhibitor-1*, PPI-1** which results in a reduced ability to dephosphorylate ACC maintaining the enzyme in a less active state. On the other hand insulin leads to activation of ***phosphatases***, thereby leading to dephosphorylation of ACC that results in increased ACC activity. These forms of regulation are all defined as short term regulation.

Control of a given pathways' regulatory enzymes can also occur by alteration of enzyme synthesis and turn-over rates. These changes are long term regulatory effects. Insulin stimulates ACC and FAS synthesis, whereas, starvation leads to decreased synthesis of these enzymes. Adipose tissue lipoprotein lipase levels also are increased by insulin and decreased by starvation. However, in contrast to the effects of insulin and starvation on adipose tissue, their effects on heart lipoprotein lipase are just the inverse. This allows the heart to absorb any available fatty acids in the blood in order to oxidize them for energy production. Starvation also leads to increases in the levels of fatty acid oxidation enzymes in the heart as well as a decrease in FAS and related enzymes of synthesis.

Adipose tissue contains ***hormone-sensitive lipase***, that is activated by PKA-dependent phosphorylation leading to increased fatty acid release to the blood. This leads to increased fatty acid oxidation in other tissues such as muscle and liver. In the liver the net result (due to increased acetyl-CoA levels) is the production of ketone bodies. This would occur under conditions where insufficient carbohydrate stores and gluconeogenic precursors were available in liver for increased glucose production. The increased fatty acid availability in response to glucagon or epinephrine is assured of being completely oxidized since PKA also phosphorylates (and as a result inhibits) ACC, thus inhibiting fatty acid synthesis.

Insulin, on the other hand, has the opposite effect to glucagon and epi leading to increased glycogen and triacylglyceride synthesis. One of the many effects of insulin is to lower cAMP levels which leads to increased dephosphorylation through the enhanced activity of protein phosphatases such as PP-1. With respect to fatty acid metabolism this yields dephosphorylated and inactive hormone sensitive lipase. Insulin also stimulates certain phosphorylation events. This occurs through activation of several cAMP-independent kinases. Insulin stimulated phosphorylation of ACC activates this enzyme.

Regulation of fat metabolism also occurs through **malonyl-CoA** induced inhibition of ***carnitine acyltransferase I***. This functions to prevent the newly synthesized fatty acids from entering the mitochondria and being oxidized.

In order to understand how the synthesis and degradation of fats needs to be exquisitely regulated, one must consider the energy requirements of the organism as a whole. The blood is the carrier of triacylglycerols in the form of VLDLs and chylomicrons, fatty acids bound to albumin, amino acids, lactate, ketone bodies and glucose. The pancreas is the primary organ involved in sensing the organism's dietary and energetic states by monitoring glucose concentrations in the blood. Low blood glucose stimulates the secretion of glucagon, whereas, elevated blood glucose calls for the secretion of insulin.

The metabolism of fat is regulated by two distinct mechanisms. One is **short-term regulation**, which can come about through events such as substrate availability, allosteric effectors and/or enzyme modification. The other mechanism, **long-term regulation**, is achieved by alteration of the rate of enzyme synthesis and turn-over.

ACC is the rate-limiting (committed) step in fatty acid synthesis. This enzyme is activated by citrate and inhibited by palmitoyl-CoA and other long-chain fatty acyl-CoAs. ACC activity can also be affected by phosphorylation. For instance, glucagon-stimulated increases in PKA activity result in the phosphorylation of certain serine residues in ACC leading to decreased activity of the enzyme. By contrast, insulin leads to PKA-independent phosphorylation of ACC at sites distinct from glucagon, which bring about increased ACC activity. Both of these reaction chains are examples of short-term regulation.

Insulin, a product of the well-fed state, stimulates ACC and FAS synthesis, whereas starvation leads to a decrease in the synthesis of these enzymes. Adipose tissue levels of lipoprotein lipase also are increased by insulin and decreased by starvation. However, the effects of insulin and starvation on lipoprotein lipase in the heart are just the inverse of those in adipose tissue. This sensitivity allows the heart to absorb any available fatty acids in the blood in order to oxidize them for energy production. Starvation also leads to increases in the levels of cardiac enzymes of fatty acid oxidation, and to decreases in FAS and related enzymes of synthesis.

Adipose tissue contains ***hormone-sensitive lipase***, which is activated by PKA-dependent phosphorylation; this activation increases the release of fatty acids into the blood. This in turn leads to the increased oxidation of fatty acids in other tissues such as muscle and liver. In the liver, the net result (due to increased acetyl-CoA levels) is the production of ketone bodies (see below). This would occur under conditions in which the carbohydrate stores and gluconeogenic precursors available in the liver are not sufficient to allow increased glucose production. The increased levels of fatty acid that become available in response to glucagon or epinephrine are assured of being completely oxidized, because PKA also phosphorylates ACC; the synthesis of fatty acid is thereby inhibited.

Insulin has the opposite effect to glucagon and epinephrine: it increases the synthesis of triacylglycerols (and glycogen). One of the many effects of insulin is to lower cAMP levels, which leads to increased dephosphorylation through the enhanced activity of protein phosphatases such as PP-1. With respect to fatty acid metabolism, this yields dephosphorylated and inactive hormone-sensitive lipase. Insulin also stimulates certain phosphorylation events. This occurs through activation of several cAMP-independent kinases, one of which phosphorylates and thereby stimulates the activity of ACC.

Fat metabolism can also be regulated by malonyl-CoA-mediated inhibition of carnitine acyltransferase I. Such regulation serves to prevent *de novo* synthesized fatty acids from entering the mitochondria and being oxidized.

Clinical Significance of Fatty Acids

The majority of clinical problems related to fatty acid metabolism are associated with processes of oxidation. These disorders fall into four main groups:

- **1. Deficiencies in Carnitine:** Deficiencies in carnitine lead to an inability to transport fatty acids into the mitochondria for oxidation. This can occur in newborns and particularly in pre-term infants. Carnitine deficiencies also are found in patients undergoing hemodialysis or exhibiting organic aciduria. Carnitine deficiencies may manifest systemic symptomology or may be limited to only muscles. Symptoms can range from mild occasional muscle cramping to severe weakness or even death. Treatment is by oral carnitine administration.
- 2. Carnitine Palmitoyltransferase I (CPT I) Deficiency: Deficiencies in this enzyme affect primarily the liver and lead to reduced fatty acid oxidation and ketogenesis. Carnitine Palmitoyltransferase II (CPT II) deficiency results in recurrent muscle pain and fatigue and myoglobinuria following strenuous exercise. Carnitine acyltransferases may also be inhibited by sulfonylurea drugs such as **tolbutamide** and **glyburide**.

- **3. Deficiencies in Acyl-CoA Dehydrogenases:** A group of inherited diseases that impair b-oxidation result from deficiencies in acyl-CoA dehydrogenases. The enzymes affected may belong to one of four categories:
  - **very long-chain acyl-CoA dehydrogenase (VLCAD)** Disease description
  - **long-chain acyl-CoA dehydrogenase (LCAD)** Disease description
  - **medium-chain acyl-CoA dehydrogenase (MCAD)** Disease description
  - **short-chain acyl-CoA dehydrogenase (SCAD)** Disease description

  MCAD deficiency is the most common form of this disease. In the first years of life this deficiency will become apparent following a prolonged fasting period. Symptoms include vomiting, lethargy and frequently coma. Excessive urinary excretion of medium-chain dicarboxylic acids as well as their glycine and carnitine esters is diagnostic of this condition. In the case of this enzyme deficiency, taking care to avoid prolonged fasting is sufficient to prevent clinical problems.
- **4. Refsum's Disease:** Refsum's disease is a rare inherited disorder in which patients lack the mitochondrial a-oxidizing enzyme. As a consequence, they accumulate large quantities of phytanic acid in their tissues and serum. This leads to severe symptoms, including cerebellar ataxia, retinitis pigmentosa, nerve deafness and peripheral neuropathy. As expected, the restriction of dairy products and ruminant meat from the diet can ameliorate the symptoms of this disease.

Ketogenesis

During high rates of fatty acid oxidation, primarily in the liver, large amounts of acetyl-CoA are generated. These exceed the capacity of the TCA cycle, and one result is the synthesis of ketone bodies, or **ketogenesis**. The ketone bodies are **acetoacetate**, **b-hydroxybutyrate**, and **acetone** *see page 230*.

The formation of acetoacetyl-CoA occurs by condensation of two moles of acetyl-CoA through a reversal of the ***thiolase*** catalyzed reaction of fat oxidation. Acetoacetyl-CoA and an additional acetyl-CoA are converted to **b-hydroxy-b-methylglutaryl-CoA (HMG-CoA)** by ***HMG-CoA synthase***, an enzyme found in large amounts only in the liver. Some of the HMG-CoA leaves the mitochondria, where it is converted to mevalonate (the precursor for cholesterol synthesis) by ***HMG-CoA reductase***. HMG-CoA in the mitochondria is converted to acetoacetate by the action of ***HMG-CoA lyase***. Acetoacetate can undergo spontaneous decarboxylation to acetone, or be enzymatically converted to b-hydroxybutyrate through the action of ***b-hydroxybutyrate dehydrogenase***. When the level of glycogen in the liver is high the production of b-hydroxybutyrate increases.

When carbohydrate utilization is low or deficient, the level of oxaloacetate will also be low, resulting in a reduced flux through the TCA cycle. This in turn leads to increased release of ketone bodies from the liver for use as fuel by other tissues. In early

stages of starvation, when the last remnants of fat are oxidized, heart and skeletal muscle will consume primarily ketone bodies to preserve glucose for use by the brain. Acetoacetate and b-hydroxybutyrate, in particular, also serve as major substrates for the biosynthesis of neonatal cerebral lipids.

Ketone bodies are utilized by extrahepatic tissues through the conversion of b-hydroxybutyrate to acetoacetate and of acetoacetate to acetoacetyl-CoA. The first step involves the reversal of the b-hydroxybutyrate dehydrogenase reaction, and the second involves the action (shown below) of ***acetoacetate:succinyl-CoA transferase***, also called ***ketoacyl-CoA-transferase***.

Acetoacetate + Succinyl-CoA <——> Acetoacetyl-CoA + succinate

The latter enzyme is present in all tissues except the liver. Importantly, its absence allows the liver to produce ketone bodies but not to utilize them. This ensures that extrahepatic tissues have access to ketone bodies as a fuel source during prolonged starvation.

Regulation of Ketogenesis

The fate of the products of fatty acid metabolism is determined by an individual's physiological status. Ketogenesis takes place primarily in the liver and may by affected by several factors:

- 1. Control in the release of free fatty acids from adipose tissue directly affects the level of ketogenesis in the liver. This is, of course, substrate-level regulation.
- 2. Once fats enter the liver, they have two distinct fates. They may be activated to acyl-CoAs and oxidized, or esterified to glycerol in the production of triacylglycerols. If the liver has sufficient supplies of glycerol-3-phosphate, most of the fats will be turned to the production of triacylglycerols.
- 3. The generation of acetyl-CoA by oxidation of fats can be completely oxidized in the TCA cycle. Therefore, if the demand for ATP is high the fate of acetyl-CoA is likely to be further oxidation to CO_2.
- 4. The level of fat oxidation is regulated hormonally through phosphorylation of ACC, which may activate it (in response to glucagon) or inhibit it (in the case of insulin).

Clinical Significance of Ketogenesis

The production of ketone bodies occurs at a relatively low rate during normal feeding and under conditions of normal physiological status. Normal physiological responses to carbohydrate shortages cause the liver to increase the production of ketone bodies from the acetyl-CoA generated from fatty acid oxidation. This allows the heart and skeletal muscles primarily to use ketone bodies for energy, thereby preserving the limited glucose for use by the brain.

The most significant disruption in the level of ketosis, leading to profound clinical manifestations, occurs in untreated insulin-dependent diabetes mellitus. This physiological state, **diabetic ketoacidosis (DKA)**, results from a reduced supply of glucose (due to a significant decline in circulating insulin) and a concomitant increase in fatty acid oxidation (due to a concomitant increase in circulating glucagon). The increased production of acetyl-CoA leads to ketone body production that exceeds the ability of peripheral tissues to oxidize them. Ketone bodies are relatively strong acids (pK_a around 3.5), and their increase lowers the pH of the blood. This acidification of the blood is dangerous chiefly because it impairs the ability of hemoglobin to bind oxygen.

Triglycerides

Fatty acids are stored for future use as triacylglycerols in all cells, but primarily in adipocytes of adipose tissue. Triacylglycerols constitute molecules of glycerol to which three fatty acids have been esterified. The fatty acids present in triacylglycerols are predominantly saturated. The major building block for the synthesis of triacylglycerols, in tissues other than adipose tissue, is glycerol. Adipocytes lack *glycerol kinase*, therefore, dihydroxyacetone phosphate (DHAP), produced during glycolysis, is the precursor for triacylglycerol synthesis in adipose tissue. This means that adipocytes must have glucose to oxidize in order to store fatty acids in the form of triacylglycerols. DHAP can also serve as a backbone precursor for triacylglycerol synthesis in tissues other than adipose, but does so to a much lesser extent than glycerol

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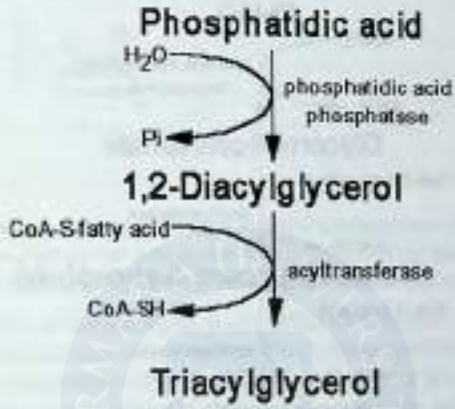

The glycerol backbone of triacylglycerols is activated by phosphorylation at the C-3 position by ***glycerol kinase***. The utilization of DHAP for the backbone is carried out through the action of ***glycerol-3-phosphate dehydrogenase***, a reaction that requires NADH (the same reaction as that used in the glycerol-phosphate shuttle). The fatty acids incorporated into triacylglycerols are activated to acyl-CoAs through the action of ***acyl-CoA synthetases***. Two molecules of acyl-CoA are esterified to glycerol-3-phosphate to yield 1,2-diacylglycerol phosphate (commonly identified as **phosphatidic acid**). The phosphate is then removed, by ***phosphatidic acid phosphatase***, to yield 1,2-diacylglycerol, the substrate for addition of the third fatty acid. Intestinal monoacylglycerols, derived from the hydrolysis of dietary fats, can also serve as substrates for the synthesis of 1,2-diacylglycerols.

Phospholipids

Phospholipids are synthesized by the addition of a basic group (predominantly a nitrogenous base) to phosphatidic acid or 1,2-diacylglycerol. Most phospholipids have a saturated fatty acid on C-1 and an unsaturated fatty acid on C-2 of the glycerol backbone. The major classifications of phospholipids are:

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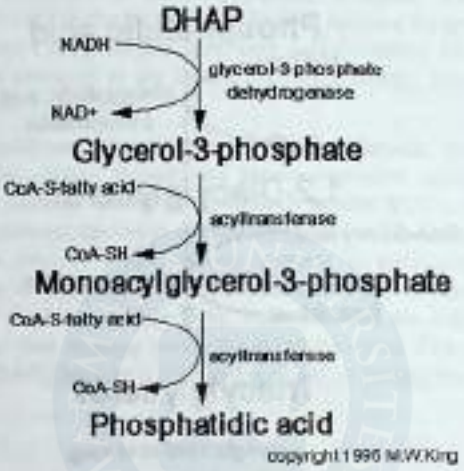
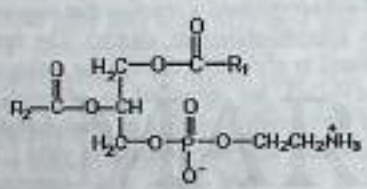
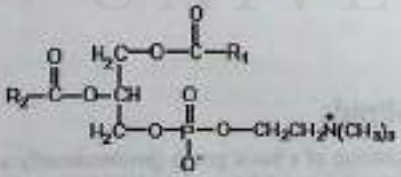
Phosphatidic Acid Synthesis



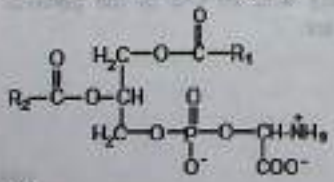
Phosphatidylcholine (PC)



Phosphatidylethanolamine (PE)



Phosphatidylserine (PS)



Phosphatidylinositol (PI)

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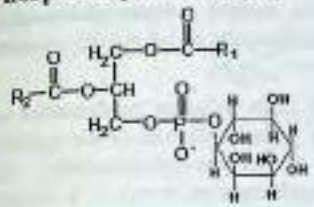

Phospholipids can be synthesized by two mechanisms. One utilizes a CDP-activated polar head group for attachment to the phosphate of phosphatidic acid. The other utilizes CDP-activated 1,2-diacylglycerol and an inactivated polar head group.

PC: This class of phospholipids is also called the **lecithins.** At physiological pH, phosphatidylcholines are neutral zwitterions. They contain primarily palmitic or stearic acid at carbon 1 and primarily oleic, linoleic or linolenic acid at carbon 2. The lecithin **dipalmitoyllecithin** is a component of **lung** or **pulmonary surfactant.** It contains palmitate at both carbon 1 and 2 of glycerol and is the major (80%) phospholipid found in the extracellular lipid layer lining the pulmonary alveoli.

Choline is activated first by phosphorylation and then by coupling to CDP prior to attachment to phosphatidic acid. PC is also synthesized by the addition of choline to CDP-activated 1,2-diacylglycerol. A third pathway to PC synthesis, involves the conversion of either PS or PE to PC. The conversion of PS to PC first requires decarboxylation of PS to yield PE; this then undergoes a series of three methylation reactions utilizing S-adenosylmethionine (SAM) as methyl group donor.

PE: These molecules are neutral zwitterions at physiological pH. They contain primarily palmitic or stearic acid on carbon 1 and a long chain unsaturated fatty acid (e.g. 18:2, 20:4 and 22:6) on carbon 2.

Synthesis of PE can occur by two pathways. The first requires that ethanolamine be activated by phosphorylation and then by coupling to CDP. The ethanolamine is then transferred from CDP-ethanolamine to phosphatidic acid to yield PE. The second involves the decarboxylation of PS.

PS: Phosphatidylserines will carry a net charge of -1 at physiological pH and are composed of fatty acids similar to the phosphatidylethanolamines.

The pathway for PS synthesis involves an exchange reaction of serine for ethanolamine in PE. This exchange occurs when PE is in the lipid bilayer of the u membrane. As indicated above, PS can serve as a source of PE through a decarboxylation reaction.

PI: These molecules contain almost exclusively stearic acid at carbon 1 and arachidonic acid at carbon 2. Phosphatidylinositols composed exclusively of non-phosphorylated inositol exhibit a net charge of -1 at physiological pH. These molecules

exist in membranes with various levels of phosphate esterified to the hydroxyls of the inositol. Molecules with phosphorylated inositol are termed **polyphosphoinositides**. The polyphosphoinositides are important intracellular transducers of signals emanating from the plasma membrane.

The synthesis of PI involves CDP-activated 1,2-diacylglycerol condensation with *myo*-inositol. PI subsequently undergoes a series of phosphorylations of the hydroxyls of inositol leading to the production of polyphosphoinositides. One polyphosphoinositide (**phosphatidylinositol 4,5-bisphosphate, PIP_2**) is a critically important membrane phospholipid involved in the transmission of signals for cell growth and differentiation from outside the cell to inside.

PG: Phosphatidylglycerols exhibit a net charge of -1 at physiological pH. These molecules are found in high concentration in mitochondrial membranes and as components of **pulmonary surfactant**. Phosphatidylglycerol also is a precursor for the synthesis of cardiolipin.

PG is synthesized from CDP-diacylglycerol and glycerol-3-phosphate. The vital role of PG is to serve as the precursor for the synthesis of diphosphatidylglycerols (DPGs).

DPG: These molecules are very acidic, exhibiting a net charge of -2 at physiological pH. They are found primarily in the inner mitochondrial membrane and also as components of **pulmonary surfactant**.

One important class of diphosphatidylglycerols is the **cardiolipins**. These molecules are synthesized by the condensation of CDP-diacylglycerol with PG.

The fatty acid distribution at the C-1 and C-2 positions of glycerol within phospholipids is continually in flux, owing to phospholipid degradation and the continuous phospholipid remodeling that occurs while these molecules are in membranes. Phospholipid degradation results from the action of ***phospholipases***. There are various phospholipases that exhibit substrate specificities for different positions in phospholipids.

In many cases the acyl group which was initially transferred to glycerol, by the action of the acyl transferases, is not the same acyl group present in the phospholipid when it resides within a membrane. The remodeling of acyl groups in phospholipids is the result of the action of ***phospholipase A_1*** and ***phospholipase A_2***.

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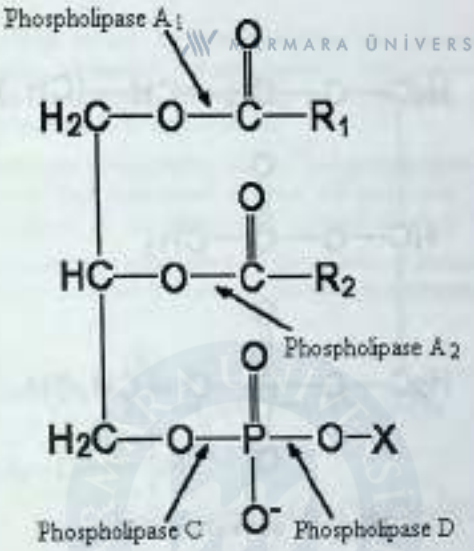

Sites of action of the phospholipases A_1, A_2, C and D

The products of these phospholipases are called **lysophospholipids** and can be substrates for acyl transferases utilizing different acyl-CoA groups. Lysophospholipids can also accept acyl groups from other phospholipids in an exchange reaction catalyzed by ***lysolecithin:lecithin acyltransferase*** **(LLAT).**

Phospholipase A_2 is also an important enzyme, whose activity is responsible for the release of arachidonic acid from the C-2 position of membrane phospholipids. The released arachidonate is then a substrate for the synthesis of the prostaglandins and leukotrienes.

Plasmalogens

Plasmalogens are glycerol ether phospholipids. They are of two types, alkyl ether and alkenyl ether. Dihydroxyacetone phosphate serves as the glycerol precursor for the synthesis of glycerol ether phospholipids. Three major classes of plasmalogens have been identified: **choline, ethanolamine** and **serine plasmalogens.** Ethanolamine plasmalogen is prevalent in myelin. Choline plasmalogen is abundant in cardiac tissue.

One particular choline plasmalogen (1-alkyl, 2-acetyl phosphatidylcholine) has been identified as an extremely powerful biological mediator, capable of inducing cellular responses at concentrations as low as 10^{-11} M. This molecule is called **platelet activating factor, PAF.**

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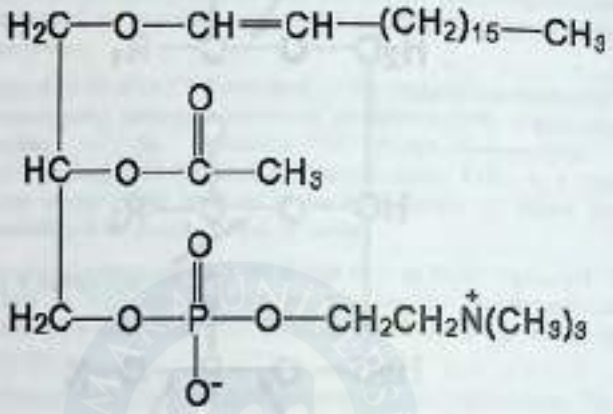


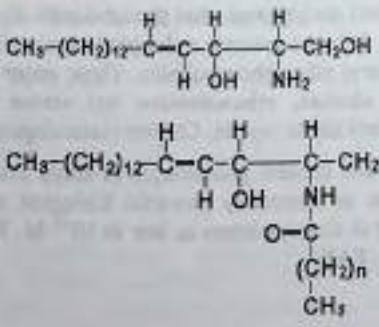
Platelet activating factor

PAF functions as a mediator of hypersensitivity, acute inflammatory reactions and anaphylactic shock. PAF is synthesized in response to the formation of antigen-IgE complexes on the surfaces of basophils, neutrophils, eosinophils, macrophages and monocytes. The synthesis and release of PAF from cells leads to platelet aggregation and the release of serotonin from platelets. PAF also produces responses in liver, heart, smooth muscle, and uterine and lung tissues.

Sphingolipids

The sphingolipids, like the phospholipids, are composed of a polar head group and two nonpolar tails. The core of sphingolipids is the long-chain amino alcohol, **sphingosine**. Amino acylation, with a long chain fatty acid, at carbon 2 of sphingosine yields a **ceramide**

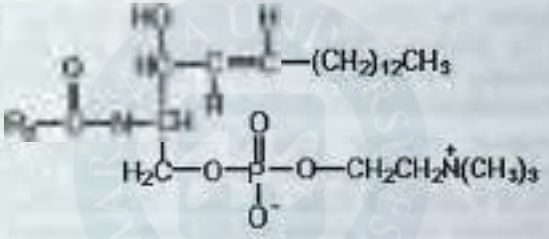
Top: Sphingosine
Bottom: Ceramide



The sphingolipids include the **sphingomyelins** and **glycosphingolipids** (the cerebrosides, sulfatides, globosides and gangliosides). Sphingomyelins are the only sphingolipid that are phospholipids. Sphingolipids are a component of all membranes but are particularly abundant in the myelin sheath.

Sphingomyelins are sphingolipids that are also phospholipids. Sphingomyelins are important structural lipid components of nerve cell membranes. The predominant sphingomyelins contain palmitic or stearic acid N-acylated at carbon 2 of sphingosine.

The sphingomyelins are synthesized by the transfer of phosphorylcholine from phosphatidylcholine to a ceramide in a reaction catalyzed by ***sphingomyelin synthase***.



A sphingomyelin

Defects in the enzyme ***acid sphingomyelinase*** result in the lysosomal storage disease known as Niemann-Pick disease. There are at least 4 related disorders identified as Niemann-Pick disease Type A and B (both of which result from defects in ***acid sphingomyelinase***), Type C1 and a related C2 and Type D. Types C1, C2 and D do not result from defects in ***acid sphingomyelinase***. More information on Niemann-Pick sub-type C1 is presented below in the section on Clinical Significances of Sphingolipids.

Glycosphingolipids, or glycolipids, are composed of a ceramide backbone with a wide variety of carbohydrate groups (mono- or oligosaccharides) attached to carbon 1 of sphingosine. The four principal classes of glycosphingolipids are the **cerebrosides, sulfatides, globosides** and **gangliosides**.

Cerebrosides have a single sugar group linked to ceramide. The most common of these is galactose (**galactocerebrosides**), with a minor level of glucose (**glucocerebrosides**). Galactocerebrosides are found predominantly in neuronal cell membranes. By contrast glucocerebrosides are not normally found in membranes, especially neuronal membranes; instead, they represent intermediates in the synthesis or degradation of more complex glycosphingolipids.

Galactocerebrosides are synthesized from ceramide and UDP-galactose. Excess accumulation of glucocerebrosides is observed in Gaucher's disease.

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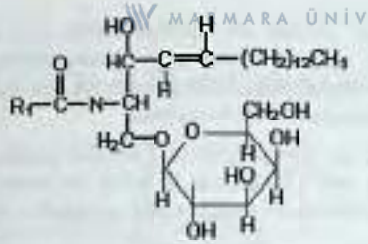

A Galactocerebroside

Sulfatides: The sulfuric acid esters of galactocerebrosides are the sulfatides. Sulfatides are synthesized from galactocerebrosides and activated sulfate, 3'-**phosphoadenosine 5'-phosphosulfate (PAPS)**. Excess accumulation of sulfatides is observed in sulfatide lipidosis (metachromatic leukodystrophy).

Globosides: Globosides represent cerebrosides that contain additional carbohydrates, predominantly galactose, glucose or GalNAc. Lactosyl ceramide is a globoside found in erythrocyte plasma membranes. Globotriaosylceramide (also called ceramide trihexoside) contains glucose and two moles of galactose and accumulates, primarily in the kidneys, of patients suffering from Fabry's disease.

Gangliosides: Gangliosides are very similar to globosides except that they also contain NANA in varying amounts. The specific names for gangliosides are a key to their structure. The letter G refers to ***ganglioside***, and the subscripts M, D, T and Q indicate that the molecule contains mono-, di-, tri and quatra(tetra)-sialic acid. The numerical subscripts 1, 2 and 3 refer to the carbohydrate sequence that is attached to ceramide; 1 stands for GalGalNAcGalGlc-ceramide, 2 for GalNAcGalGlc-ceramide and 3 for GalGlc-ceramide.

Deficiencies in lysosomal enzymes, which normally are responsible for the degradation of the carbohydrate portions of various gangliosides, underlie the symptoms observed in rare autosomally inherited diseases termed lipid storage diseases, many of which are listed below.

Clinical Significances of Sphingolipids

One of the most clinically important classes of sphingolipids are those that confer antigenic determinants on the surfaces of cells, particularly the erythrocytes. The **ABO blood group antigens** are the carbohydrate moieties of glycolipids on the surface of cells as well as the carbohydrate portion of serum glycoproteins. When present on the surface of cells the ABO carbohydrates are linked to sphingolipid and are therefore of the glycosphingolipid class. When the ABO carbohydrates are associated with protein in the form of glycoproteins they are found in the serum and are referred to as the

secreted forms. Some individuals produce the glycoprotein forms of the ABO antigens while others do not. This property distinguishes **secretors** from **non-secretors**, a property that has forensic importance such as in cases of rape.

A significant cause of death in premature infants and, on occasion, in full term infants is respiratory distress syndrome (RDS) or hyaline membrane disease. This condition is caused by an insufficient amount of pulmonary surfactant. Under normal conditions the surfactant is synthesized by type II endothelial cells and is secreted into the alveolar spaces to prevent atelectasis following expiration during breathing. Surfactant is comprised primarily of dipalmitoyllecithin; additional lipid components include phosphatidylglycerol and phosphatidylinositol along with proteins of 18 and 36 kDa (termed surfactant proteins). During the third trimester the fetal lung synthesizes primarily sphingomyelin, and type II endothelial cells convert the majority of their stored glycogen to fatty acids and then to dipalmitoyllecithin. Fetal lung maturity can be determined by measuring the ratio of lecithin to sphingomyelin (L/S ratio) in the amniotic fluid. An L/S ratio less than 2.0 indicates a potential risk of RDS. The risk is nearly 75-80% when the L/S ratio is 1.5.

The carbohydrate portion of the ganglioside, G_{M1}, present on the surface of intestinal epithelial cells, is the site of attachment of cholera toxin, the protein secreted by *Vibrio cholerae*.

These are just a few examples of how sphingolipids and glycosphingolipids are involved in various recognition functions at the surface of cells. As with the complex glycoproteins, an understanding of all of the functions of the glycolipids is far from complete.

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Disorders Associated with Abnormal Sphingolipid Metabolism

Disorder	Enzyme Deficiency	Accumulating Substance	Symptoms
Tay-Sachs disease see below table	HEXA	G_{M2} ganglioside	rapidly progressing mental retardation, blindness, early mortality
Sandhoff-Jatzkewitz disease see below table	HEXB	Globoside, G_{M2} ganglioside	same symptoms as Tay-Sachs, progresses more rapidly
Tay-Sachs AB variant see below table	G_{M2} activator (GM2A)	G_{M2} ganglioside	same symptoms as Tay-Sachs
Gaucher's disease	Glucocerebrosidase	Glucocerebroside	hepatosplenomegaly, mental retardation in infantile form, long bone degeneration
Fabry's disease	α-Galactosidase A	Globotriacsylceramide; also called ceramide trihexoside (CTH)	kidney failure, skin rashes
Niemann-Pick disease, more info below Types A and B Type C1 Type C2 Type D	Sphingomyelinase see info below see info below	Sphingomyelin LDL-derived cholesterol LDL-derived cholesterol	all types lead to mental retardation, hepatosplenomegaly, early fatality potential
Krabbe's disease; globoid leukodystrophy	Galactocerebrosidase	Galactocerebroside	mental retardation, myelin deficiency
G_{M1} gangliosidosis	G_{M1} ganglioside:β-galactosidase	G_{M1} ganglioside	mental retardation, skeletal abnormalities, hepatomegaly
Sulfatide lipodosis; metachromatic leukodystrophy	Arylsulfatase A	Sulfatide	mental retardation, metachromasia of nerves
Fucosidosis	α-L-Fucosidase	Pentahexosylfucoglycolipid	cerebral degeneration, thickened skin, muscle spasticity
Farber's lipogranulomatosis	Acid ceramidase	Ceramide	hepatosplenomegaly, painful swollen joints

The G_{M2} gangliosidoses include Tay-Sachs disease, the Sandhoff diseases and the G_{M2} activator deficiencies. G_{M2} ganglioside degradation requires the enzyme ***b-hexosaminidase*** and the G_{M2} activator protein (GM2A). ***Hexosaminidase*** is a dimer composed of 2 subunits, either a and/or b. The HexS protein is aa, HexA is ab and HexB is bb. It is the a-subunit that carries out the catalysis of G_{M2} gangliosides. The activator first binds to G_{M2} gangliosides followed by hexosaminidase and then digestion occurs.

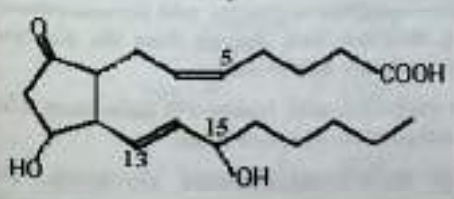
Based upon genetic linkage analyses as well as enzyme studies and the characterization of accumulating lysosomal substances, Niemann Pick disease should be divided into type I and type II; type I has 2 subtypes, A and B (NPA and NPB), which show deficiency of ***acid sphingomyelinase***. Niemann Pick disease type II likewise has 2 subtypes, type C1 and C2 (NPC) and type D (NPD). It is obviously confusing to use the abbreviation NPD for Niemann Pick disease in some cases and for subtype D of Niemann Pick disease in other cases.

Recent studies identified the gene for NPC1. This gene contains regions of homology to mediators of cholesterol homeostasis suggesting why LDL-cholesterol accumulates in lysosomes of afflicted individuals. The encoded protein product of *NPC1* gene is 1278 amino acids long. Within the protein are regions of homology to the transmembrane domain of the morphogen receptor **PATCHED** (of *Drosophila melanogaster*), the putative sterol-sensing regions of **SREBP (sterol regulatory element binding protein) cleavage-activating protein (SCAP)** and ***HMG-CoA reductase***.

Eicosanoids

The eicosanoids consist of the **prostaglandins (PGs)**, **thromboxanes (TXs)** and **leukotrienes (LTs)**. The PGs and TXs are collectively identified as prostanoids. Prostaglandins were originally shown to be synthesized in the prostate gland, thromboxanes from platelets (thrombocytes) and leukotrienes from leukocytes, hence the derivation of their names *see page 34*.

PGE_2



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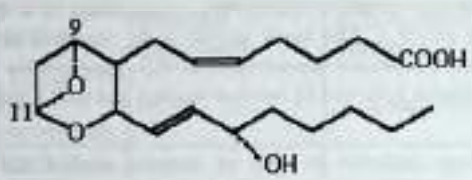
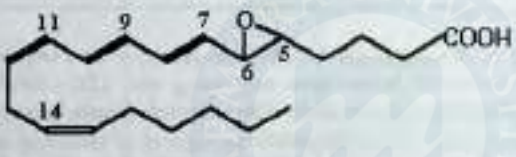
TXA_2



LTA_4

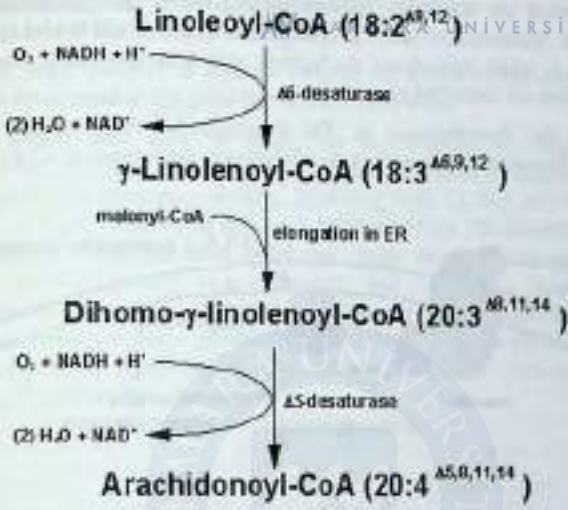


The eicosanoids produce a wide range of biological effects on inflammatory responses (predominantly those of the joints, skin and eyes), on the intensity and duration of pain and fever, and on reproductive function (including the induction of labor). They also play important roles in inhibiting gastric acid secretion, regulating blood pressure through vasodilation or constriction, and inhibiting or activating platelet aggregation and thrombosis.

The principal eicosanoids of biological significance to humans are a group of molecules derived from the C_{20} fatty acid, **arachidonic acid**. Minor eicosanoids are derived from eicosopentaenoic acid which is itself derived from α-linolenic acid obtained in the diet. The major source of arachidonic acid is through its release from cellular stores. Within the cell, it resides predominantly at the C-2 position of membrane phospholipids and is released from there upon the activation of ***phospholipase*** A_2 (see diagram above). The immediate dietary precursor of arachidonate is linoleic acid. Linoleic acid is converted to arachidonic acid through the steps outlined in the figure below. Linoleic acid (arachidonate precursor) and α-linolenic acid (eicosapentaenoate precursor) are essential fatty acids, therefore, their absence from the diet would seriously threaten the body's ability to synthesize eicosanoids.

Pathway from linoleic acid to arachidonic acid. Numbers in parentheses refer to the fatty acid length and the number and positions of unsaturations.

All mammalian cells except erythrocytes synthesize eicosanoids. These molecules are extremely potent, able to cause profound physiological effects at very dilute concentrations. All eicosanoids function locally at the site of synthesis, through receptor-mediated G-protein linked signaling pathways leading to an increase in cAMP levels.



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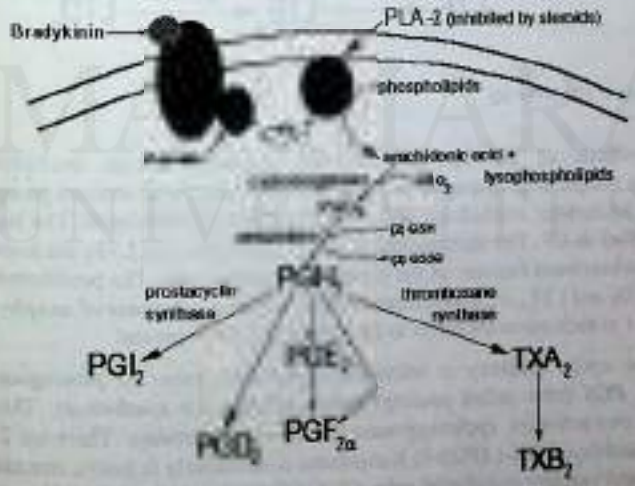
Two main pathways are involved in the biosynthesis of eicosanoids. The prostaglandins and thromboxanes are synthesized by the cyclic pathway, the leukotrienes by the linear pathway.



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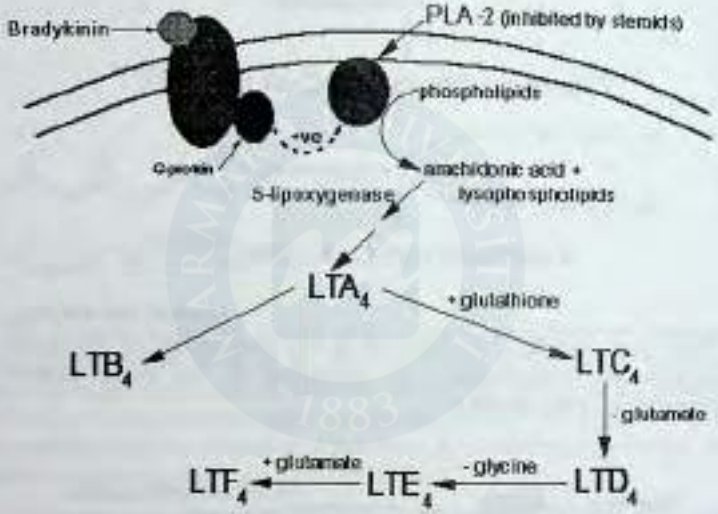
Synthesis of the clinically relevant prostaglandins and thromboxanes from arachidonic acid. Numerous stimuli (e.g. epinephrine, thrombin and bradykinin) activate ***phospholipase*** A_2 which hydrolyzes arachidonic acid from membrane phospholipids. The prostaglandins are identified as

PG and the thromboxanes as TX. Prostaglandin PGI_2 is also known as **prostacyclin**. The subscript 2 in each molecule refers to the number of -C=C- present.



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Synthesis of the clinically relevant leukotrienes from arachidonic acid. Numerous stimuli (e.g. epinephrine, thrombin and bradykinin) activate ***phospholipase*** A_2 which hydrolyzes arachidonic acid from membrane phospholipids. The leukotrienes are identified as LT. The leukotrienes, LTC_4, LTD_4, LTE_4 and LTF_4 are known as the **peptidoleukotrienes** because of the presence of amino acids. The peptidoleukotrienes, LTC_4, LTD_4 and LTE_4 are components of **slow-reacting substance of anaphylaxis** The subscript 4 in each molecule refers to the number of -C=C- present.

The cyclic pathway is initiated through the action of ***prostaglandin G/H synthase, PGS*** (also called ***prostaglandin endoperoxide synthetase***). This enzyme possesses two activities, ***cyclooxygenase*** (**COX**) and ***peroxidase***. There are 2 forms of the COX activity. COX-1 (PGS-1) is expressed constitutively in gastric mucosa, kidney, platelets, and vascular endothelial cells. COX-2 (PGS-2) is inducible and is expressed in macrophages and monocytes in response to inflammation. The primary trigger for COX-2 induction in monocytes and macrophages is **platelet-activating factor, PAF**

and interleukin-1, IL-1. Both COX-1 and COX-2 catalyze the 2-step conversion of arachidonic acid to PGG_2 and then to PGH_2.

The linear pathway is initiated through the action of ***lipoxygenases***. It is the enzyme, ***5-lipoxygenase*** that gives rise to the leukotrienes.

A widely used class of drugs, the non-steroidal anti-inflammatory drugs (**NSAIDs**) such as ibuprofen, indomethacin, naproxen, phenylbutazone and aspirin, all act upon the cyclooxygenase activity, inhibiting both COX-1 and COX-2. Because inhibition of COX-1 activity in the gut is associated with NSAID-induced ulcerations, pharmaceutical companies have developed drugs targeted exclusively against the inducible COX-2 activity (e.g. celecoxib and rofecoxib). Another class, the corticosteroidal drugs, act to inhibit ***phospholipase*** A_2, thereby inhibiting the release of arachidonate from membrane phospholipids and the subsequent synthesis of eicosanoids. Some properties of significant eicosanoide are seen below. SRS-A = slow-reactive substance of anaphylaxis.

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Properties of Significant Eicosanoids

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Eicosanoid	Major site(s) of synthesis	Major biological activities
PGD_2	mast cells	inhibits platelet and leukocyte aggregation, decreases T-cell proliferation and lymphocyte migration and secretion of IL-1α and IL-2; induces vasodilation and production of cAMP
PGE_2	kidney, spleen, heart	increases vasodilation and cAMP production, enhancement of the effects of bradykinin and histamine, induction of uterine contractions and of platelet aggregation, maintaining the open passageway of the fetal ductus arteriosus; decreases T-cell proliferation and lymphocyte migration and secretion of IL-1α and IL-2
$PGF_{2\alpha}$	kidney, spleen, heart	increases vasoconstriction, bronchoconstriction and smooth muscle contraction
PGH_2		precursor to thromboxanes A_2 and B_2, induction of platelet aggregation and vasoconstriction
PGI_2	heart, vascular endothelial cells	inhibits platelet and leukocyte aggregation, decreases T-cell proliferation and lymphocyte migration and secretion of IL-1α and IL-2; induces vasodilation and production of cAMP
TXA_2	platelets	induces platelet aggregation, vasoconstriction, lymphocyte proliferation and bronchoconstriction
TXB_2	platelets	induces vasoconstriction
LTB_4	monocytes, basophils, neutrophils, eosinophils, mast cells, epithelial cells	induces leukocyte chemotaxis and aggregation, vascular permeability, T-cell proliferation and secretion of INF-γ, IL-1 and IL-2
LTC_4	monocytes and alveolar macrophages, basophils, eosinophils, mast cells, epithelial cells	component of SRS-A, induces vasodilation, vascular permeability and bronchoconstriction and secretion of INF-γ
LTD_4	monocytes and alveolar macrophages, eosinophils, mast cells, epithelial cells	predominant component of SRS-A, induces vasodilation, vascular permeability and bronchoconstriction and secretion of INF-γ
LTE_4	mast cells and basophils	component of SRS-A, induces vasodilation and bronchoconstriction

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Cholesterol

Cholesterol is an extremely important biological molecule that has roles in membrane structure as well as being a precursor for the synthesis of the steroid hormones and bile acids. Both dietary cholesterol and that synthesized *de novo* are transported through the circulation in lipoprotein particles. The same is true of cholesteryl esters, the form in which cholesterol is stored in cells.

Role of Cholesterol in Atherosclerosis

High serum cholesterol is a major cause of coronary atherosclerosis leading to coronary heart disease (CHD). Although evidence to support a cholesterol atherosclerosis link has been accumulating for the past century , the past three decades have witnessed a geometrical growth in information about this link. The evidence comes from many sources. It comes from studies in laboratory animacls epidemiologic surveys, studies of genetic disorders of cholesterol metabolism. Clinical investigations and large scale clinical trials. The evidence that high cholesterol levels are a cause of CHD is widely accepted as proof by most investigatios in the field. There is also a growing concensus that therapeutic loweringz of serum cholesterol levels will reduce the risk for CHD.

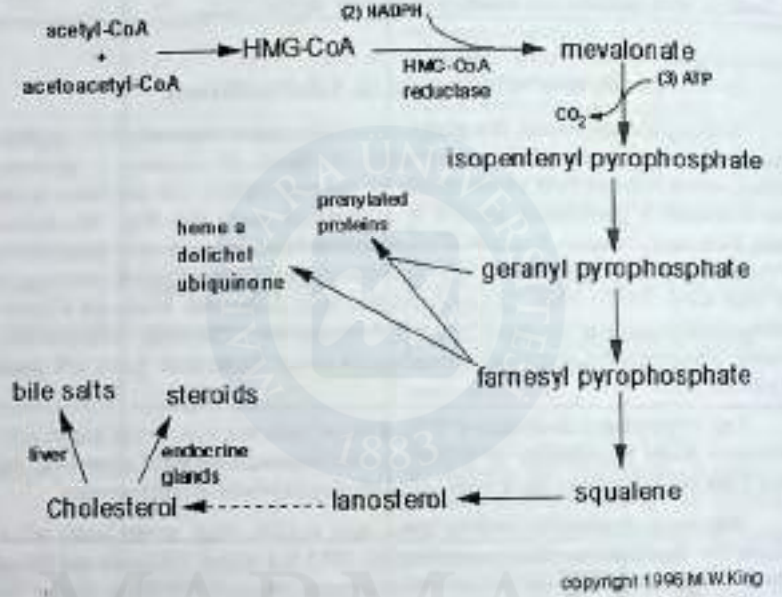
One of the major discoveries in the field of lipid research is that cholesterol is transported in the bloodstream by particles called lipoproteins.(See Lipoproteins Part) Three types of lipoproteins have emerged as playing key roles in atherogenesis.

The major cholesterol carrying lipoprotein is LDL. High serum levels of LDL promote the development of atherosclerosis. In 1985 Drs Joseph Goldstein and Michael Brown, of the University of Texas Southwestern Medical Center at Dallas were awarded the Nobel Prize in Medicine for de discovery of LDL receptors. These proteins, located on the surface of liver cell, remove LDL from the circulation and in this way regulate serum concentrations of LDL. They are thus important determination of the atherosclerotic process. The research of Drs. Goldstein and Brown has helped bring cholesterol research into the molecular age, and has provided a rational foundation for the management of high serum cholesterol levels. Another lipoprotein intimately related to atherogenesis is HDL . In contrast to LDL, H may actually protect against the development of atherosclerosis. The way in which a high HDL level protects against CHD has not yet been determined.

The synthesis and utilization of cholesterol must be tightly regulated in order to prevent over-accumulation and abnormal deposition within the body.

Biosynthesis and regulation of cholesterol

Slightly less than half of the cholesterol in the body derives from biosynthesis *de novo*. Biosynthesis in the liver accounts for approximately 10%, and in the intestines approximately 15%, of the amount produced each day. Cholesterol synthesis occurs in the cytoplasm and microsomes from the two-carbon acetate group of acetyl-CoA.



The process has five major steps:

- 1. Acetyl-CoAs are converted to 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA)
- 2. HMG-CoA is converted to mevalonate
- 3. Mevalonate is converted to the isoprene based molecule, isopentenyl pyrophosphate (IPP), with the concomitant loss of CO_2
- 4. IPP is converted to squalene
- 5. Squalene is converted to cholesterol.

The acetyl-CoA utilized for cholesterol biosynthesis is derived from an oxidation reaction (eg, fatty acids or pyruvate) in the mitochondria and is transported to the cytoplasm by the same process as that described for fatty acid synthesis. Acetyl-CoA can also be derived from cytoplasmic oxidation of ethanol by ***acetyl-CoA synthetase***. All the reduction reactions of cholesterol biosynthesis use NADPH as a cofactor. The

isoprenoid intermediates of cholesterol biosynthesis can be diverted to other synthesis reactions, such as those for dolichol (used in the synthesis of N-linked glycoproteins), coenzyme Q (of the oxidative phosphorylation) pathway or the side chain of heme *a*. Additionally, these intermediates are used in the lipid modification of some proteins.

Acetyl-CoA units are converted to mevalonate by a series of reactions that begins with the formation of **HMG-CoA**. Unlike the HMG-CoA formed during ketone body synthesis in the mitochondria, this form is synthesized in the cytoplasm. However, the pathway and the necessary enzymes are the same as those in the mitochondria. Two moles of acetyl-CoA are condensed in a reversal of the *thiolase* reaction, forming acetoacetyl-CoA. Acetoacetyl-CoA and a third mole of acetyl-CoA are converted to HMG-CoA by the action of ***HMG-CoA synthase***. HMG-CoA is converted to **mevalonate** by ***HMG-CoA reductase***, **HMGR** (this enzyme is bound in the endoplasmic reticulum, ER). **HMGR** absolutely requires NADPH as a cofactor and two moles of NADPH are consumed during the conversion of HMG-CoA to mevalonate.

The reaction catalyzed by **HMGR** is the rate limiting step of cholesterol biosynthesis, and this enzyme is subject to complex regulatory controls. Mevalonate is then activated by three successive phosphorylations, yielding **5-pyrophosphomevalonate**. In addition to activating mevalonate, the phosphorylations maintain its solubility, since otherwise it is insoluble in water. After phosphorylation, an ATP-dependent decarboxylation yields **isopentenyl pyrophosphate**, IPP, an activated isoprenoid molecule. Isopentenyl pyrophosphate is in equilibrium with its isomer, **dimethylallyl pyrophosphate, DMPP**. One molecule of IPP condenses with one molecule of DMPP to generate **geranyl pyrophosphate, GPP**. GPP further condenses with another IPP molecule to yield **farnesyl pyrophosphate, FPP**. Finally, the NADPH-requiring enzyme, ***squalene synthase*** catalyzes the head-to-tail condensation of two molecules of FPP, yielding squalene (squalene synthase also is tightly associated with the endoplasmic reticulum). Squalene undergoes a two step cyclization to yield **lanosterol**. The first reaction is catalyzed by ***squalene monooxygenase***. This enzyme uses NADPH as a cofactor to introduce molecular oxygen as an epoxide at the 2,3 position of squalene. Through a series of 19 additional reactions, lanosterol is converted to cholesterol.

Regulating Cholesterol Synthesis

Normal healthy adults synthesize cholesterol at a rate of approximately 1g/day and consume approximately 0.3g/day. A relatively constant level of cholesterol in the body (150 - 200 mg/dL) is maintained primarily by controlling the level of *de novo* synthesis. The level of cholesterol synthesis is regulated in part by the dietary intake of cholesterol. Cholesterol from both diet and synthesis is utilized in the formation of membranes and in the synthesis of the steroid hormones and bile acids (see below). The greatest proportion of cholesterol is used in bile acid synthesis.

The cellular supply of cholesterol is maintained at a steady level by three distinct mechanisms:

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- 1. Regulation of **HMGR** activity and levels
- 2. Regulation of excess intracellular free cholesterol through the activity of ***acyl-CoA:cholesterol acyltransferase*, ACAT**
- 3. Regulation of plasma cholesterol levels via LDL receptor-mediated uptake and HDL-mediated reverse transport.

Regulation of **HMGR** activity is the primary means for controlling the level of cholesterol biosynthesis. The enzyme is controlled by three distinct mechanisms: control of gene expression, rate of enzyme degradation and phosphorylation-dephosphorylation.

The first two control mechanisms are exerted by cholesterol itself. Cholesterol acts as a feed-back inhibitor of pre-existing **HMGR** as well as inducing rapid turn-over of the enzyme. In addition, when cholesterol is in excess the amount of mRNA for **HMGR** is reduced as a result of decreased expression of the gene. The exact mechanism for this cholesterol-induced regulation of gene activity is not known.

Regulation of **HMGR** through covalent modification occurs as a result of phosphorylation and dephosphorylation. The enzyme is most active in its unmodified form. Phosphorylation of the enzyme decreases its activity. **HMGR** is phosphorylated by ***AMP-regulated protein kinase*, AMPRK** (this is not the same as ***cAMP-dependent protein kinase*, PKA**). AMPRK itself is activated via phosphorylation. The phosphorylation of AMPRK is catalyzed by ***kinase kinase***.

The activity of **HMGR** is further controlled by the cAMP signaling pathway. Increases in cAMP lead to activation of ***cAMP-dependent protein kinase*, PKA**. In the context of **HMGR** regulation, PKA phosphorylates ***phosphoprotein phosphatase inhibitor-1* (PPI-1)** leading to an increase in its' activity. PPI-1 can inhibit the activity of numerous ***phosphatases*** including ***protein phosphatase 2C*** and ***HMG-CoA reductase phosphatase*** which remove phosphates from AMPRK and **HMGR**, respectively. This maintains AMPRK in the phosphorylated and active state, and **HMGR** in the phosphorylated and inactive state. As the stimulus leading to increased cAMP production is removed, the level of phosphorylations decreases and that of dephosphorylations increases. The net result is a return to a higher level of **HMGR** activity.

Since the intracellular level of cAMP is regulated by hormonal stimuli, regulation of cholesterol biosynthesis is hormonally controlled. Insulin leads to a decrease in cAMP, which in turn activates cholesterol synthesis. Alternatively, glucagon and epinephrine, which increase the level of cAMP, inhibit cholesterol synthesis.

The ability of insulin to stimulate, and glucagon to inhibit, **HMGR** activity is consistent with the effects of these hormones on other metabolic pathways. The basic function of these two hormones is to control the availability and delivery of energy to all cells of the body.

Long-term control of **HMGR** activity is exerted primarily through control over the synthesis and degradation of the enzyme. When levels of cholesterol are high, the

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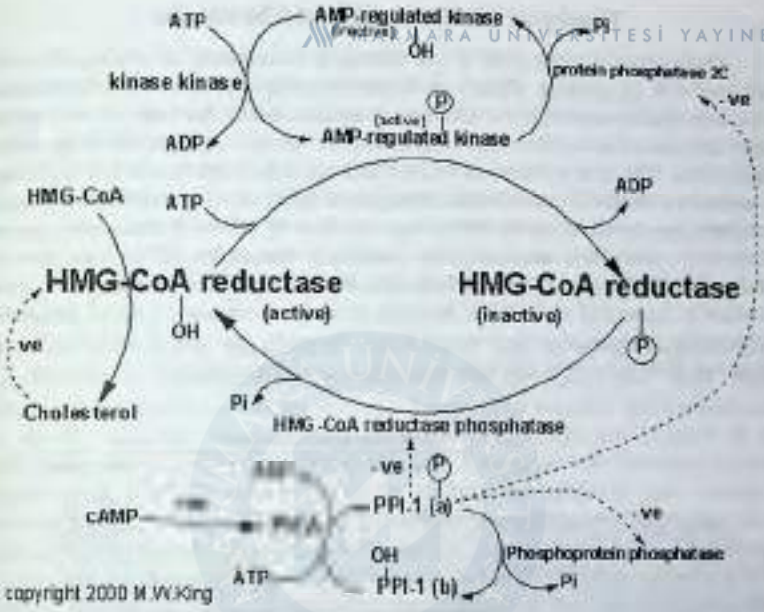

level of expression of the **HMGR** gene is reduced. Conversely, reduced levels of cholesterol activate expression of the gene. Insulin also brings about long-term regulation of cholesterol metabolism by increasing the level of **HMGR** synthesis.

Proteolytic Regulation of HMG-CoA Reductase

The stability of **HMGR** is regulated as the rate of flux through the mevalonate synthesis pathway changes. When the flux is high the rate of **HMGR** degradation is also high. When the flux is low, degradation of **HMGR** decreases. This phenomenon can easily be observed in the presence of the statin drugs.

HMGR is localized to the ER and like SREBP (see below) contains a sterol-sensing domain, SSD. When sterol levels increase in cells there is an concommitant increase in the rate of **HMGR** degradation. The degradation of **HMGR** occurs within the proteosome, a multiprotein complex dedicated to protein degradation. The primary signal directing proteins to the proteosome is ubiquitination. Ubiquitin is a 7.6kDa protein that is covalently attached to proteins targeted for degradation by ubiqitin ligases. These enzymes attach multiple copies of ubiquitin allowing for recognition by the proteosome. **HMGR** has been shown to be ubiquitinated prior to its degradation. The primary sterol regulating **HMGR** degradation is cholesterol itself. As the levels of free cholesterol increase in cells, the rate of **HMGR** degradation increases.

Transport and Utilization of Cholesterol

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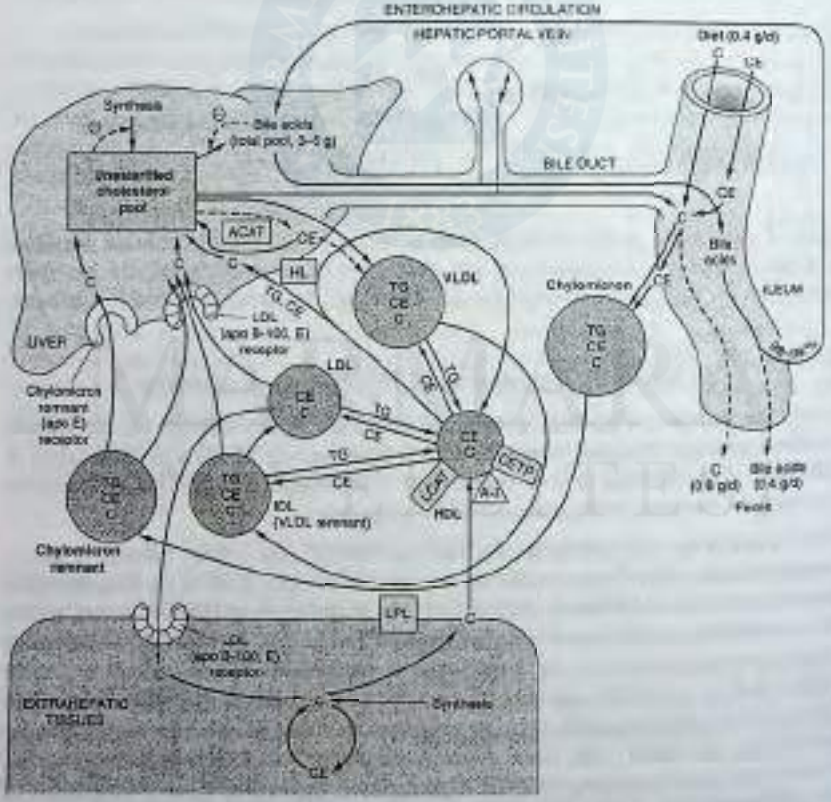
Cholesterol is transported in the plasma predominantly as cholesteryl esters associated with lipoproteins. Dietary cholesterol is transported from the small intestine to the liver within chylomicrons. Cholesterol synthesized by the liver, as well as any dietary cholesterol in the liver that exceeds hepatic needs, is transported in the serum within LDLs. The liver synthesizes VLDLs and these are converted to LDLs through the action of endothelial cell-associated lipoprotein lipase. Cholesterol found in plasma membranes can be extracted by HDLs and esterified by the HDL-associated enzyme LCAT. The cholesterol acquired from peripheral tissues by HDLs can then be transferred to VLDLs and LDLs via the action of cholesteryl ester transfer protein (apo-D) which is associated with HDLs. **Reverse cholesterol transport** allows peripheral cholesterol to be returned to the liver in LDLs. Ultimately, cholesterol is excreted in the bile as free cholesterol or as bile salts following conversion to bile acids in the liver.



Cholesterol transport by the lipoproteins (21).

Regulation of Cellular Sterol Content

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The continual alteration of the intracellular sterol content occurs through the regulation of key sterol synthetic enzymes as well as by altering the levels of cell-surface LDL receptors. As cells need more sterol they will induce their synthesis and uptake, conversely when the need declines synthesis and uptake are decreased. Regulation of these events is brought about primarily by sterol-regulated transcription of key rate limiting enzymes and by the regulated degradation of **HMGR**. Activation of transcriptional control occurs through the regulated cleavage of the membrane-bound transcription factor **sterol regulated element binding protein, SREBP**. As discussed above, degradation of **HMGR** is controlled by the ubiquitin-mediated pathway for proteolysis.

Sterol control of transcription affects more than 30 genes involved in the biosynthesis of cholesterol, triacylglycerols, phospholipids and fatty acids. Transcriptional control requires the presence of an octamer sequence in the gene termed the **sterol regulatory element, SRE-1**. It has been shown that SREBP is the transcription factor that binds to SRE-1 elements. It turns out that there are 2 distinct SREBP gene, SREBP-1 and SREBP-2. In addition, the SREBP-1 gene encodes 2 proteins, SREBP-1a and SREBP-1c as a consequence of alternative exon usage. All 3 proteins are proteolytically regulated by sterols. Full-length SREBPs have several domains and are embedded in the membrane of the endoplasmic reticulum (ER). The N-terminal domain contains a transcription factor motif of the basic helix-loop-helix (bHLH) type that is exposed to the cytoplasmic side of the ER. There are 2 transmembrane spanning domains followed by a large C-terminal domain also exposed to the cytosolic side. When sterols are scarce, cleavage of the full-length SREBP takes place with the result being that the N-terminla bHLH motif is released into the cytosol. The bHLH domian then migrates to the nucleus to direct transcription. Conversely, when sterols are abundant, cleavage of SREBP is inhibited. To control the level of SREBP-mediated transcription, the soluble bHLH domain is itself subject to rapid proteolysis.

The cleavage of SREBP is carried out by 2 distinct enzymes, one of which is regulated by sterols. The regulated cleavage occurs in the lumenal loop between the 2 transmembrane domains. This cleavage is catalyzed by **site-1 protease, S1P**. High sterol content blocks the activity of S1P. The second cleavage, catalyzed by **site-2 protease, S2P**, occurs in the first transmembrane span, leading to release of active SREBP. In order for S2P to act on SREBP site-1 must already have been cleaved.

Additional studies on sterol-regulated gene expression demonstrated that cleavage of SREBP by S1P is controlled by the level and action of an additional protein termed, **SREBP cleavage-activating protein, SCAP**. SCAP is a large protein also found in the ER membrane and contains at least 8 transmembrane spans. The C-terminal portion, which extends into the cytosol, has been shown to interact with the C-terminal domain of SREBP. This C-terminal region of SCAP contains 4 motifs called **WD40 repeats**. The WD40 repeats are required for interaction of SCAP with SREBP. Interestingly, the N-terminus of SCAP, including membrane spans 2-6, resembles

HMGR which itself is subject to sterol-stimulated degradation (see above). This shared motif is called the **sterol sensing domain, SSD**. Several proteins whose functions involve sterols also contain the SSD. These include *patched*, an important development regulating receptor whose ligand, *hedgehog*, is modified by attachment of cholesterol and the Neimann Pick C1 (NPC1) protein which is involved in cholesterol transport in the secretory pathway.

The function of SCAP is to positively stimulate S1P-mediated cleavage of SREBP. The function of sterols is to inhibit this positive action of SCAP. The activity of SCAP involves movement from the ER to the Golgi and back. Because the C-terminus of SCAP interacts with SREBP, movement of SCAP takes SREBP along for the ride. When sterols are low, SCAP and SREBP move to the Golgi. This transit is required for SREBP cleavage as S1P is Golgi-localized. When sterols are high, movement of SCAP is halted. Thus, the overall effect of sterols is to regulate the ability of SCAP to present SREBP to S1P.

Bile Acids

The end products of cholesterol utilization are the bile acids, synthesized in the liver. Synthesis of bile acids is one of the predominant mechanisms for the excretion of excess cholesterol. However, the excretion of cholesterol in the form of bile acids is insufficient to compensate for an excess dietary intake of cholesterol.

The most abundant bile acids in human bile are **chenodeoxycholic acid** (45%) and **cholic acid** (31%). These are referred to as the **primary bile acids**. Within the intestines the primary bile acids are acted upon by bacteria and converted to the **secondary bile acids**, identified as deoxycholate (from cholate) and lithocholate (from chenodeoxycholate). Both primary and secondary bile acids are reabsorbed by the intestines and delivered back to the liver via the portal circulation.

Within the liver the carboxyl group of primary and secondary bile acids is conjugated via an amide bond to either glycine or taurine before their being resecreted into the bile canaliculi. These conjugation reactions yield **glycoconjugates** and **tauroconjugates**, respectively. The bile canaliculi join with the bile ductules, which then form the bile ducts. Bile acids are carried from the liver through these ducts to the gallbladder, where they are stored for future use. The ultimate fate of bile acids is secretion into the intestine, where they aid in the emulsification of dietary lipids. In the gut the glycine and taurine residues are removed and the bile acids are either excreted (only a small percentage) or reabsorbed by the gut and returned to the liver. This process of secretion from the liver to the gallbladder, to the intestines and finally reabsorbtion is termed the **enterohepatic circulation**.

Clinical Significance of Bile Acids

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Bile acids perform four physiologically significant functions:

1. their synthesis and subsequent excretion in the feces represent the only significant mechanism for the elimination of excess cholesterol.
2. bile acids and phospholipids solubilize cholesterol in the bile, thereby preventing the precipitation of cholesterol in the gallbladder.
3. they facilitate the digestion of dietary triacylglycerols by acting as emulsifying agents that render fats accessible to pancreatic lipases.
4. they facilitate the intestinal absorption of fat-soluble vitamins.

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INTESTINAL UPTAKE OF LIPIDS

In order for the body to make use of dietary lipids, they must first be absorbed from the small intestine. Since these molecules are oils, they are essentially insoluble in the aqueous environment of the intestine. The solubilization (or emulsification) of dietary lipids is thereforeaccomplished by means of bile salts, which are synthesized from cholesterol in the liver and then stored in the gallbladder; they are secreted following the ingestion of fat.

The emulsification of dietary fats renders them accessible to pancreatic lipases (primarily ***lipase*** and ***phospholipase*** A_2). These enzymes, secreted into the intestine from the pancreas, generate free fatty acids and a mixtures of mono- and diacylglycerols from dietary triacylglycerols. Pancreatic lipase degrades triacylglycerols at the 1 and 3 positions sequentially to generate 1,2-diacylglycerols and 2-acylglycerols. Phospholipids are degraded at the 2 position by pancreatic phospholipase A_2 releasing a free fatty acid and the lysophospholipid. The products of pancreatic lipases then diffuse into the intestinal epithelial cells, where the re-synthesis of triacyglycerols occurs.

LIPOPROTEINS

Since lipids are insoluble in water, they are transported in the plasma as lipoproteins. Four major lipid classes are present in lipoproteins. These are triacylglycerols, phospholipids, cholesterol and cholesterol esters.

Dietary triacylglycerols and cholesterol, as well as triacylglycerols and cholesterol synthesized by the liver, are solubilized in lipid-protein complexes. These complexes contain triacylglycerol lipid droplets and cholesteryl esters surrounded by the polar phospholipids and proteins identified as apolipoproteins. These lipid-protein complexes vary in their content of lipid and protein. Fat absorbed from the diet and lipids synthesized by the liver and adipose tissue must be transported between the various tissues and organs for utilization and storage.

Composition of the Major Lipoprotein Complexes

Complex	Source	Density (g/ml)	%Protein	%TG[a]	%PL[b]	%CE[c]	%C[d]	%FFA[e]
Chylomicron	Intestine	<0.95	1-2	85-88	8	3	1	0
VLDL	Liver	0.95-1.006	7-10	50-55	18-20	12-15	8-10	1
IDL	VLDL	1.006-1.019	10-12	25-30	25-27	32-35	8-10	1
LDL	VLDL	1.019-1.063	20-22	10-15	20-24	37-48	8-10	1
*HDL_2	Intestine, liver (chylomicrons and VLDLs)	1.063-1.125	33-35	5-15	32-41	20-30	5-10	0
*HDL_3	Intestine, liver (chylomicrons and VLDLs)	1.125-1.21	55-57	3-13	26-46	15-30	2-6	6
Albumin-FFA	Adipose tissue	>1.281	99	0	0	0	0	100

[a]Triacylglycerols, [b]Phospholipids, [c]Cholesteryl esters, [d]Free cholesterol, [e]Free fatty acids

*HDL2 and HDL3 derived from nascent HDL as a result of the acquisition of cholesteryl esters

APOLIPOPROTEINS

The protein moiety of a lipoprotein is known as an apolipoprotein or apoprotein. The apoprotein contents of lipoproteins are different as it is shown in tables.

One or more protein or polypeptides are present in each lipoprotein. The apoproteins are designated according to the ABC nomenclature. The major apoprotein of HDL is designated A. Apoprotein B is the main lipoprotein of LDL and VLDL. Apoprotein B of chylomicrons (Apo B-48) is smaller than Apo B-100. Apoprotein B-48 is synthesized in the intestine and apoprotein B-100 in the liver.

Apo B-100 is one of the longest single polypeptide chains known, having 4536 amino acids. Apo B-48 (48 % as large as B-100) is formed from the same mRNA as Apo B-100.

Apoprotein C –1th different subgroups are smaller polypeptides freely transferable between several different lipoproteins. Carbohydrates content of apoproteins approximately 5 % for Apo B and include mannose, galactose, fucose, glucose, glucosamine and sialic acid.

Apolipoproteins carry out several roles;

-They are enzyme cofactors.

-They can form part of the structure of the lipoproteins.

-They act as ligands for interaction with lipoprotein receptors in tissues. Eg: Apo B-100 and Apo E for the LDL receptor, Apo A-1 for the HDL receptor.

Some properties and function of Apoproteins and lipoproteins are shown below.

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Apoprotein Classifications

Apoprotein - MW (Da)	Lipoprotein Association	Function and Comments
apoA-I - 29,016	Chylomicrons, HDL	major protein of HDL, activates lecithin:cholesterol acyltransferase, LCAT
apoA-II - 17,400	Chylomicrons, HDL	primarily in HDL, enhances hepatic lipase activity
apoA-IV - 46,000	Chylomicrons and HDL	present in triacylglycerol rich lipoproteins
apoB-48 - 241,000	Chylomicrons	exclusively found in chylomicrons, derived from apoB-100 gene by RNA editing in intestinal epithelium; lacks the LDL receptor-binding domain of apoB-100
apoB-100 - 513,000	VLDL, IDL and LDL	major protein of LDL, binds to LDL receptor; one of the longest known proteins in humans
apoC-I - 7,600	Chylomicrons, VLDL, IDL and HDL	may also activate LCAT
apoC-II - 8,916	Chylomicrons, VLDL, IDL and HDL	activates lipoprotein lipase
apoC-III - 8,750	Chylomicrons, VLDL, IDL and HDL	inhibits lipoprotein lipase
apoD, 33,000	HDL	closely associated with LCAT
cholesterol ester transfer protein, CETP	HDL	exclusively associated with HDL, cholesteryl ester transfer
apoE - 34,000 (at least 3 alleles [E_2, E_3, E_4] each of which have multiple isoforms)	Chylomicron remnants, VLDL, IDL and HDL	binds to LDL receptor, apoE_4 allele amplification associated with late-onset Alzheimer's disease
apoH - 50,000 (also known as -2-glycoprotein I)	Chylomicrons	triacylglycerol metabolism
apo(a) - at least 19 different alleles; protein ranges in size from 300,000 - 800,000	LDL	disulfide bonded to apoB-100, forms a complex with LDL identified as lipoprotein(a), Lp(a); strongly resembles plasminogen; may deliver cholesterol to sites of vascular injury, high risk association with premature coronary artery disease and stroke

Chylomicrons

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Chylomicrons are assembled in the intestinal mucosa as a means to transport dietary cholesterol and triacylglycerols to the rest of the body. Chylomicrons are, therefore, the molecules formed to mobilize dietary (exogenous) lipids. The predominant lipids of chylomicrons are triacylglycerols (see Table above). The apolipoproteins that predominate before the chylomicrons enter the circulation include apoB-48 and apoA-I, -A-II and IV. ApoB-48 combines only with chylomicrons.

Chylomicrons leave the intestine via the lymphatic system and enter the circulation at the left subclavian vein. In the bloodstream, chylomicrons acquire apoC-II and apoE from plasma HDLs. In the capillaries of adipose tissue and muscle, the fatty acids of chylomicrons are removed from the triacylglycerols by the action of ***lipoprotein lipase*** **(LPL)**, which is found on the surface of the endothelial cells of the capillaries. The apoC-II in the chylomicrons activates LPL in the presence of phospholipid. The free fatty acids are then absorbed by the tissues and the glycerol backbone of the triacylglycerols is returned, via the blood, to the liver and kidneys. Glycerol is converted to the glycolytic intermediate dihydroxyacetone phosphate (DHAP). During the removal of fatty acids, a substantial portion of phospholipid, apoA and apoC is transferred to HDLs. The loss of apoC-II prevents LPL from further degrading the **chylomicron remnants**.

Chylomicron remnants--- containing primarily cholesterol, apoE and apoB-48--- are then delivered to, and taken up by, the liver through interaction with the chylomicron remnant receptor. The recognition of chylomicron remnants by the hepatic remnant receptor requires apoE. Chylomicrons function to deliver dietary triacylglycerols to adipose tissue and muscle and dietary cholesterol to the liver.

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VLDL & LDL

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The dietary intake of both fat and carbohydrate, in excess of the needs of the body, leads to their conversion into triacylglycerols in the liver. These triacylglycerols are packaged into VLDLs (see page 478) and released into the circulation for delivery to the various tissues (primarily muscle and adipose tissue) for storage or production of energy through oxidation. VLDLs are, therefore, the molecules formed to transport endogenously derived triacylglycerols to extra-hepatic tissues. In addition to triacylglycerols, VLDLs contain some cholesterol and cholesteryl esters and the apoproteins, apoB-100, apoC-I, apoC-II, apoC-III and apoE. Like nascent chylomicrons, newly released VLDLs acquire apoCs and apoE from circulating HDLs.

The fatty acid portion of VLDLs is released to adipose tissue and muscle in the same way as for chylomicrons, through the action of lipoprotein lipase. The action of lipoprotein lipase coupled to a loss of certain apoproteins (the apoCs) converts VLDLs to intermediate density lipoproteins (IDLs), also termed VLDL remnants. The apoCs are transferred to HDLs. The predominant remaining proteins are apoB-100 and apoE. Further loss of triacylglycerols converts IDLs to LDLs.

The cellular requirement for cholesterol as a membrane component is satisfied in one of two ways: either it is synthesized *de novo* within the cell, or it is supplied from extra-cellular sources, namely, chylomicrons and LDLs. As indicated above, the dietary cholesterol that goes into chylomicrons is supplied to the liver by the interaction of chylomicron remnants with the remnant receptor. In addition, cholesterol synthesized by the liver can be transported to extra-hepatic tissues if packaged in VLDLs. In the circulation VLDLs are converted to LDLs through the action of lipoprotein lipase. LDLs are the primary plasma carriers of cholesterol for delivery to all tissues.

The exclusive apolipoprotein of LDLs is apoB-100. LDLs are taken up by cells via LDL receptor-mediated endocytosis, as described above for IDL uptake. The uptake of LDLs occurs predominantly in liver (75%), adrenals and adipose tissue. As with IDLs, the interaction of LDLs with LDL receptors requires the presence of apoB-100. The endocytosed membrane vesicles (endosomes) fuse with lysosomes, in which the apoproteins are degraded and the cholesterol esters are hydrolyzed to yield free cholesterol. The cholesterol is then incorporated into the plasma membranes as necessary. Excess intracellular cholesterol is re-esterified by ***acyl-CoA-cholesterol acyltransferase*** (**ACAT**), for intracellular storage. The activity of ACAT is enhanced by the presence of intracellular cholesterol.

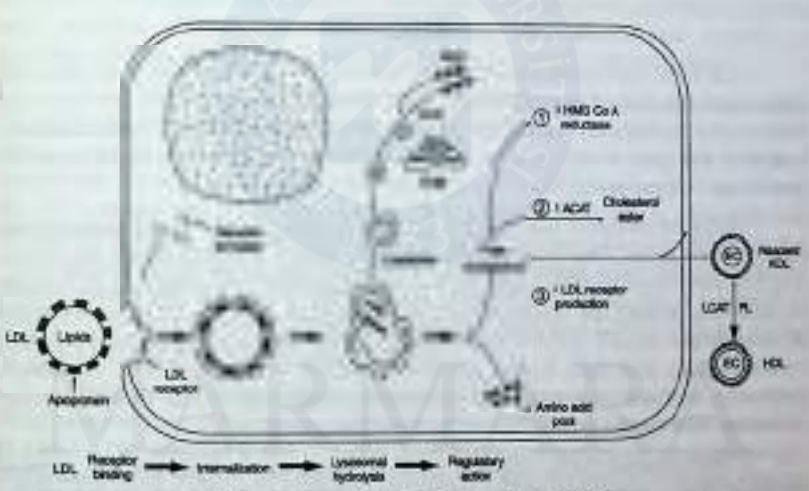
Insulin and tri-iodothyronine (T3) increase the binding of LDLs to liver cells, whereas glucocorticoids (e.g., dexamethasone) have the opposite effect. The precise mechanism for these effects is unclear but may be mediated through the regulation of apoB degradation. The effects of insulin and T3 on hepatic LDL binding may explain the hypercholesterolemia and increased risk of atherosclerosis that have been shown to be associated with uncontrolled diabetes or hypothyroidism.

An abnormal form of LDL, identified as lipoprotein-X (Lp-X), predominates in the circulation of patients suffering from ***lecithin-cholesterol acyl transferase*** (LCAT,

see HDL discussion for LCAT function) deficiency or cholestatic liver disease. In both cases there is an elevation in the level of circulating free cholesterol and phospholipids.

LDL Receptors

LDLs are the principal plasma carriers of cholesterol delivering cholesterol from the liver (via hepatic synthesis of VLDLs) to peripheral tissues, primarily the adrenals and adipose tissue. LDLs also return cholesterol to the liver. The cellular uptake of cholesterol from LDLs occurs following the interaction of LDLs with the LDL receptor (also called the apoB-100/apoE receptor). The sole apoprotein present in LDLs is apoB-100, which is required for interaction with the LDL receptor as seen in below.



LDL uptake and catabolism by the cell (29)

The LDL receptor is a polypeptide of 839 amino acids that spans the plasma membrane. An extracellular domain is responsible for apoB-100/apoE binding. The intracellular domain is responsible for the clustering of LDL receptors into regions of the plasma membrane termed **coated pits**. Once LDL binds the receptor, the complexes are rapidly internalized (**endocytosed**). ATP-dependent proton pumps lower the pH in the endosomes, which results in dissociation of the LDL from the receptor. The portion of the endosomal membranes harboring the receptor are then recycled to the plasma membrane and the LDL-containing endosomes fuse with lysosomes. Acid hydrolases of

the lysosomes degrade the apoproteins and release free fatty acids and cholesterol. As indicated above, the free cholesterol is either incorporated into plasma membranes or esterified (by ACAT) and stored within the cell.

The level of intracellular cholesterol is regulated through cholesterol-induced suppression of LDL receptor synthesis and cholesterol-induced inhibition of cholesterol synthesis. The increased level of intracellular cholesterol that results from LDL uptake has the additional effect of activating ACAT, thereby allowing the storage of excess cholesterol within cells. However, the effect of cholesterol-induced suppression of LDL receptor synthesis is a decrease in the rate at which LDLs and IDLs are removed from the serum. This can lead to excess circulating levels of cholesterol and cholesteryl esters when the dietary intake of fat and cholesterol exceeds the needs of the body. The excess cholesterol tends to be deposited in the skin, tendons and (more gravely) within the arteries, leading to **atherosclerosis**.

HDL

HDLs are synthesized *de novo* in the liver and small intestine, as primarily protein-rich disc-shaped particles. These newly formed HDLs are nearly devoid of any cholesterol and cholesteryl esters. The primary apoproteins of HDLs are apoA-I, apoC-I, apoC-II and apoE. In fact, a major function of HDLs is to act as circulating stores of apoC-I, apoC-II and apoE.

HDLs are converted into spherical lipoprotein particles through the accumulation of cholesteryl esters. This accumulation converts nascent HDLs to HDL_2 and HDL_3. Any free cholesterol present in chylomicron remnants and VLDL remnants (IDLs) can be esterified through the action of the HDL-associated enzyme, ***lecithin:cholesterol acyltransferase*, LCAT**. LCAT is synthesized in the liver and so named because it transfers a fatty acid from the C-2 position of lecithin to the C-3-OH of cholesterol, generating a cholesteryl ester and lysolecithin. The activity of LCAT requires interaction with apoA-I, which is found on the surface of HDLs.

Cholesterol-rich HDLs return to the liver, where they are endocytosed. Hepatic uptake of HDLs, or **reverse cholesterol transport**, may be mediated through an HDL-specific apoA-I receptor or through lipid-lipid interactions. Macrophages also take up HDLs through apoA-I receptor interaction. HDLs can then acquire cholesterol and apoE from the macrophages; cholesterol-enriched HDLs are then secreted from the macrophages. The added apoE in these HDLs leads to an increase in their uptake and catabolism by the liver.

HDLs also acquire cholesterol by extracting it from cell surface membranes. This process has the effect of lowering the level of intracellular cholesterol, since the cholesterol stored within cells as cholesteryl esters will be mobilized to replace the cholesterol removed from the plasma membrane.

The cholesterol esters of HDLs can also be transferred to VLDLs and LDLs through the action of the HDL-associated enzyme, ***cholesterol ester transfer protein***

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(CETP). This has the added effect of allowing the excess cellular cholesterol to be returned to the liver through the LDL-receptor pathway as well as the HDL-receptor pathway.

Clinical Significances of Lipoprotein Metabolism

Fortunately, few individuals carry the inherited defects in lipoprotein metabolism that lead to hyper- or hypolipoproteinemias (see Tables below for brief descriptions). Persons suffering from diabetes mellitus, hypothyroidism and kidney disease often exhibit abnormal lipoprotein metabolism as a result of secondary effects of their disorders. For example, because ***lipoprotein lipase*** (LPL) synthesis is regulated by insulin, LPL deficiencies leading to Type I hyperlipoproteinemia may occur as a secondary outcome of diabetes mellitus. Additionally, insulin and thyroid hormones positively affect hepatic LDL-receptor interactions; therefore, the hypercholesterolemia and increased risk of atherosclerosis associated with uncontrolled diabetes or hypothyroidism is likely due to decreased hepatic LDL uptake and metabolism.

Of the many disorders of lipoprotein metabolism, familial hypercholesterolemia (FH) may be the most prevalent in the general population. Heterozygosity at the FH locus occurs in 1:500 individuals, whereas, homozygosity is observed in 1:1,000,000 individuals. FH is an inherited disorder comprising four different classes of mutation in the LDL receptor gene. The class 1 defect (the most common) results in a complete loss of receptor synthesis. The class 2 defect results in the synthesis of a receptor protein that is not properly processed in the Golgi apparatus and therefore is not transported to the plasma membrane. The class 3 defect results in an LDL receptor that is incapable of binding LDLs. The class 4 defect results in receptors that bind LDLs but do not cluster in coated pits and are, therefore, not internalized.

FH sufferers may be either heterozygous or homologous for a particular mutation in the receptor gene. Homozygotes exhibit grossly elevated serum cholesterol (primarily in LDLs). The elevated levels of LDLs result in their phagocytosis by macrophages. These lipid-laden phagocytic cells tend to deposit within the skin and tendons, leading to **xanthomas**. A greater complication results from cholesterol deposition within the arteries, leading to **atherosclerosis**, the major contributing factor of nearly all cardiovascular diseases.

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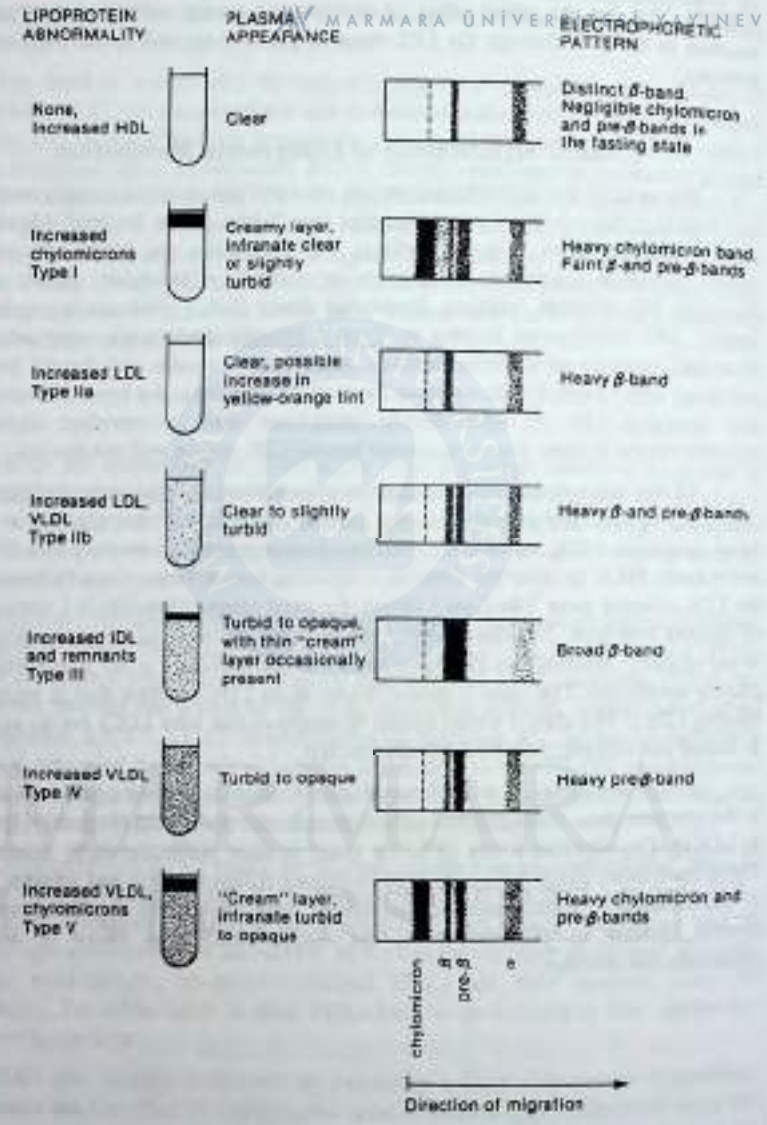

Electroforetic Migration of Hiperlipoproteinemias (36)

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Hyperlipoproteinemias

Disorder	Defect	Comments
Type I (familial LPL deficiency, familial hyperchylomicronemia)	(a) deficiency of LPL; (b) production of abnormal LPL; (c) apoC-II deficiency	Slow chylomicron clearance, reduced LDL and HDL levels; treated by low fat/complex carbohydrate diet; no increased risk of coronary artery disease
Type II (familial hypercholesterolemia, FH)	4 classes of LDL receptor defect	Reduced LDL clearance leads to hypercholesterolemia, resulting in atherosclerosis and coronary artery disease
Type III (familial dysbetalipoproteinemia, remnant removal disease, broad beta disease, apolipoprotein E deficiency)	hepatic remnant clearance impaired due to apoE abnormality; patients only express the $apoE_2$ isoform that interacts poorly with the apoE receptor	Causes xanthomas, hypercholesterolemia and atherosclerosis in peripheral and coronary arteries due to elevated levels of chylomicrons and VLDLs
Type IV (familial hypertriacylglycerolemia)	elevated production of VLDL associated with glucose intolerance and hyperinsulinemia	Frequently associated with type-II non-insulin dependent diabetes mellitus, obesity, alcoholism or administration of progestational hormones; elevated cholesterol as a result of increased VLDLs
Type V familial	elevated chylomicrons and VLDLs due to unknown cause	Hypertriacylglycerolemia and hypercholesterolemia with decreased LDLs and HDLs
Familial hyperalphalipoproteinemia	increased level of HDLs	a rare condition that is beneficial for health and longevity
Type II Familial hyperbetalipoproteinemia	increased LDL production and delayed clearance of triacylglycerols and fatty acids	Strongly associated with increased risk of coronary artery disease
Familial ligand-defective apoB	2 different mutations: Gln for Arg (amino acid 3500) or Cys for Arg (amino acid 3511); both lead to reduced affinity of LDL for LDL receptor	Dramatic increase in LDL levels; no affect on HDL, VLDL or plasma triglyceride levels; significant cause of hypercholesterolemia and premature coronary artery disease
Familial LCAT deficiency	absence of LCAT leads to inability of HDLs to take up cholesterol (reverse cholesterol transport)	Decreased levels of plasma cholesteryl esters and lysolecithin; abnormal LDLs (Lp-X) and VLDLs; symptoms also found associated with cholestasis
Wolman's disease (cholesteryl ester storage disease)	defect in lysosomal *cholesteryl ester hydrolase*; affects metabolism of LDLs	Reduced LDL clearance leads to hypercholesterolemia, resulting in atherosclerosis and coronary artery disease
Heparin-releasable hepatic triglyceride lipase deficiency	deficiency of the lipase leads to accumulation of triacylglycerol-rich HDLs and VLDL remnants (IDLs)	Causes xanthomas and coronary artery disease

Hypolipoproteinemias

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Disorder	Defect	Comments
Abetalipoproteinemia (acanthocytosis, Bassen-Kornzweig syndrome)	no chylomicrons, VLDLs or LDLs due to defect in apoB expression	rare defect: intestine and liver accumulate, malabsorption of fat, retinitis pigmentosa, ataxic neuropathic disease, erythrocytes have *thorny* appearance
Familial hypobetalipoproteinemia	at least 20 different apoB gene mutations identified, LDL concentrations 10-20% of normal, VLDL slightly lower, HDL normal	mild or no pathological changes
Familial alpha-lipoprotein deficiency (Tangier disease, Fish-eye disease, apoA-I and -C-III deficiencies)	all of these related syndromes have reduced HDL concentrations, no effect on chylomicron or VLDL production	Tendency to hypertriacylglycerolemia; some elevation in VLDLs; Fish-eye disease characterized by severe corneal opacity

Durg Therapy of Hyperlipidemia

Drug treatment to lower plasma lipoproteins and/or cholesterol is primarily aimed at reducing the risk of atherosclerosis and subsequent coronary artery disease that exists in patients with elevated circulating lipids. Drug therapy usually is considered as an option only if non-pharmacologic interventions (altered diet and exercise) have failed to lower plasma lipids

- **Mevinolin, Mevastatin, Lovastatin:** These drugs are fungal HMG-CoA reductase inhibitors. The net result of treatment is an increased cellular uptake of LDLs, since the intracellular synthesis of cholesterol is inhibited and cells are therefore dependent on extracellular sources of cholesterol. However, since mevalonate (the product of the HMG-CoA reductase reaction) is required for the synthesis of other important isoprenoid compounds besides cholesterol, long-term treatments carry some risk of toxicity.
- **Nicotinic acid:** Nicotinic acid reduces the plasma levels of both VLDLs and LDLs by inhibiting hepatic VLDL secretion, as well as suppressing the flux of FFA release from adipose tissue by inhibiting lipolysis. Because of its ability to cause large reductions in circulating levels of cholesterol, nicotinic acid is used to treat Type II, III, IV and V hyperlipoproteinemias.
- **Clofibrate, Gemfibrozil, Fenofibrate:** These compounds are derivatives of fibric acid and promote rapid VLDL turnover by activating lipoprotein lipase. They also induce the diversion of hepatic free fatty acids from esterification reactions to those of oxidation, thereby decreasing the liver's secretion of triacylglycerol- and cholesterol-rich VLDLs.

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- **Probucol:** Probucol increases the rate of LDL metabolism and may block the intestinal transport of cholesterol. The net result is a significant reduction in plasma cholesterol levels.
- **Cholestyramine or colestipol (resins):** These compounds are nonabsorbable resins that bind bile acids which are then not reabsorbed by the liver but excreted. The drop in hepatic reabsorption of bile acids releases a feedback inhibitory mechanism that had been inhibiting bile acid synthesis. As a result, a greater amount of cholesterol is converted to bile acids to maintain a steady level in circulation. Additionally, the synthesis of LDL receptors increases to allow increased cholesterol uptake for bile acid synthesis, and the overall effect is a reduction in plasma cholesterol. (This treatment is ineffective in homozygous FH patients, since they are completely deficient in LDL receptors.

LİPOPROTEINS OF BLOOD COAGULATION

Coagulation of mammalian blood is a multi stage process involving a variety of physical and chemical changes in substances derived both from the blood itself and from surrounding tissues.

Lipoproteins have been shown to influence platelets, coagulation mechanism and fibrinolysis. The effects of lipoproteins on platelets function have been studied in a number of laboratories.

A profound effect of LDL in increasing platelet intracellular calcium and inositol triphosphate concentrations was discovered in 1988. These observations led these and other authors to propose a hormonal effect of LDL on platelets and other cell types.

Although platelets play a key role in haemostatic mechanisms, the soluble coagulation cascade is also very important, especially in the development of the thrombi, which are commonly associated with myocardial infarction. There is now strong evidence that haemostatic factors such as fibrinogen and factor VIIc are associated with and increased risk. Procoagulant activity is enhanced by lipid peroxides, but particularly when associated with triglyceride rich lipoproteins which appear to promote thrombin generation and the inhibition of antithrombin III.

Furthermore tissue factor pathway inhibitor (TFPI), a protein associated with plasma lipoproteins (particularly LDL and HDL), appears to be active against factor Xa. The major site of synthesis of TFPI is the endothelium. This suggests another direct action of lipoproteins on the function of the endothelium which may influence profoundly the performance of platelets.

Lipoprotein interractions may occur both at the level of coagulation and with fibrinolysis and the mechanism may be quite diverse , affecting both the extrinsic and intrinsic pathways.

History of tissue factor

In 1834 it had been reported that intravenous injection of a suspension of brain tissue was immediately lethal, occluding the animal's blood vessels with clots. Later it was confirmed the clot-promoting effect of tissue extracts. The injection of a variety of tissue extracts into different animals was reproduced many times at the turn of the nineteenth centruy and was one of the models used to advance the understanding of clotting at that time. In 1862 Alexander Schmidt suggested that tissues provide a zymoplastic substance which converts prothrombin to thrombin Another observations from Morawitz in 1905, had reported that after cellular damage thrombokinase (today its called tissue factor, TF) effects protrombin to produce thrombin and it effects fibrinogen and fibrin occur. This is called Morawitz 's clotting theory.

The classical concept of the clotting theory of Morawitz enabled Quick to develop his quantitative prothrombin test (PT) in 1935. This test is based on the assumption that clotting time of blood plasma in the presence of optimal calcium concentration and excess tissue thrombokinase (TF) is directly dependent upon the prothrombin concentration.

The first applied work about TF was determited by Bülent Berkarda, Uğur Derman and Cemi Demiroğlu in 1963 in Türkiye. Detailed biochemical studies of thromboplastic material were carried out by Ulutin and coworkers for long years. TF is still being studied today by the new generation young scientists. The Picture (see page 479) shows the TF investigation group as four generation following each others.

It is possible to obtain tissue thromboplastin (TF) from various tissues, notably lung, brain, placenta, thymus and vascular tissue as well as body fluids such as saliva and urine. All tissue thromboplastin have different property of accelerating the coagulation of blood.

The extrinsic pathway is initiated by the exposure of TF following injury to a variety of cell types. TF is a receptor for coagulation factor VII which is rapidly activated in the resultant complex by selected proteases including factor Xa. In normal endothelium, the synthesis of TF may be induced by exposure to minimally oxidized LDL.

The extrinsic pathway of the coagulation is activated by the expression of thromboplastin (TF) upon disruption of the symmetry of the phospholipid bilayer in the endothelial cell plasma membrane. This membrane reorganization involves the exposure of negatively charged lipids, particularly phosphatidylserine, on the outer surface of the membrane bilayer, which enhances thromboplastic activity. The procoagulant activity of thromboplastin arises from its ability to act as a cofactor for

factor VII, providing a surface for the approximation and activation of factor X by factor VII.

Followign clot formation, the cofactor activity of thromboplastin is suppressed by a number of circulating inhibitors, including TFPI, a serine protease inhibitor. TFPI is known to be secreted by the liver and to be expressed on endothelial cells. It is carried mainly in association with plasma lipoproteins, principally LDL and HDL. However, more recently ith has been shown that LDL and HDL, are inhibitory toward TF, at least in assays performed in vitro. This corresponds to earlier observations that both LDL and HDL exhibit anticoagulant activity. VLDL appears to have procoagulant activity, which accords with the observation that increased factor VIIc i s related to hypertriglyceridaemia. In contrast, native LDL and HDL are directly anticoagulant in relation to standard test for TF activity. The anticoagulant effects of HDL and LDL appear to be related principally to their apolipoproteins although at least in the case of LDL lipids may modulate this effect. Apo A-II appears to be much more effective than Apo A-I in this respect, but the reasons for this have not been well researched and require further investigation. The effects of apo B-100 are maximal at concentrations seen in normocholesterolemic individuals and therefore , there is no advantage in individuals with higher concentrations of these lipoproteins. Indeed, oxidation of LDL leads to a loss of these inhibitory qualities. The nature of the interactions of LDL and TF has been investigated in more depth.

Interaction of tissue factor with apolipoprotein B-100 The inhibitory action of LDL arises from its protein moiety, Apo B-100, and is distinct from the effect of TFPI. Isolated Apo B-100 free of TFPI and solubilized with sodium deoxycholate and soybean phospholipid (recorstituted LDL), inhibits the procoagulant activity of thromboplastin by 98% of the original value. The inhibition of the extrinsic pathway by LDL occurs by direct interaction of the protein component of thromboplastin and apo B-100, not by supresion of the proteolytic action of factor VII and X as is the case for TFPI.

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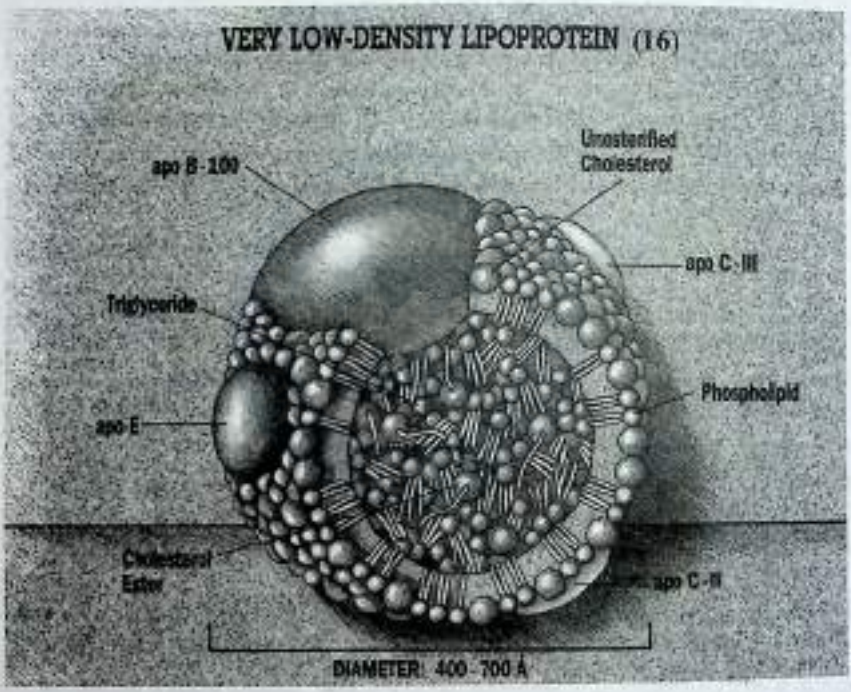

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Our coagulation and TF investigation group as four generation following each others

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