Proceedings of the National Academy of Sciences
Vol. 67, No. 3, pp. 1353–1360, November 1970

Enzymatic Carboxylation of Biotin: Molecular and Catalytic Properties of a Component Enzyme of Acetyl CoA Carboxylase*

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Communicated by Albert L. Lehninger, August 21, 1970

Abstract. The biotin carboxylase component of acetyl CoA carboxylase has been purified approximately 2000 times from *Escherichia coli*. This protein, which catalyzes the carboxylation of free *d*-biotin, is free of the biotin-containing carboxyl carrier protein, is homogeneous by polyacrylamide gel electrophoresis and analytical ultracentrifugation, and has been crystallized. Biotin carboxylase, with a molecular weight of approximately 100,000, is composed of two 50,000-dalton subunits. The catalytic capacity of biotin carboxylase is markedly enhanced by ethanol (11 times at 15% v/v), and certain other organic solvents; this may mimic an effector-mediated response. The kinetic effect is exclusively on the maximal velocity of the reaction. Activation by ethanol is reversible and not accompanied by aggregation or disaggregation of the enzyme.

Acetyl CoA carboxylase, a key enzyme in the biosynthesis of fatty acids and other acetogenins, catalyzes the ATP-dependent formation of malonyl CoA from acetyl CoA and bicarbonate (Reaction 3). After the recognition that enzyme-bound biotin was involved in this reaction,[1] investigations with a number of acyl CoA carboxylases[2] clarified the role of biotin and showed that the overall process could be partitioned into the following half-reactions:

$$\text{Enzyme-biotin} + HCO_3^- + \text{ATP} \overset{Me^{2+}}{\rightleftharpoons} \text{enzyme-biotin-}CO_2^- + \text{ADP} + P_i \quad (1)$$

$$\text{Enzyme-biotin-}CO_2^- + \text{acetyl CoA} \rightleftharpoons \text{malonyl CoA} + \text{enzyme-biotin} \quad (2)$$

$$\text{Overall: } HCO_3^- + \text{ATP} + \text{acetyl CoA} \overset{Me^{2+}}{\rightleftharpoons} \text{malonyl CoA} + \text{ADP} + P_i \quad (3)$$

In contrast to the biotin enzymes from animal tissues[2–5] and yeast,[6] which can be isolated as stable multisubunit structures, the acetyl CoA carboxylase from *Escherichia coli* dissociates during fractionation into two protein components.[7] One of these components, E_a, contains covalently-linked biotin and can be carboxylated in the presence of bicarbonate, ATP, and divalent metal ion to give rise to carboxybiotinyl enzyme (Reaction 1). The other protein component, E_b, is presumed to catalyze transcarboxylation from the carboxybiotin pros-

thetic group to acetyl CoA to form malonyl CoA (Reaction 2). As reported by Alberts *et al.*,[8] E_a can be further resolved into a biotin-containing polypeptide (carboxyl carrier protein) and a biotin-free enzyme (biotin carboxylase) which catalyzes the carboxylation of the carboxyl carrier protein. In addition, biotin carboxylase catalyzes the ATP-dependent carboxylation of free biotin. This model reaction was first demonstrated in Lynen's laboratory[9,10] with bacterial β-methylcrotonyl CoA carboxylase and was subsequently found by Stoll *et al.*[11] to occur with liver acetyl CoA carboxylase.

While citrate is well-established as an allosteric activator of acetyl CoA carboxylase from animal tissues,[2-5,12] activators of the carboxylase from *E. coli* or other bacteria are not known. A novel activation of the biotin carboxylase-catalyzed partial reaction by ethanol, and certain other organic solvents, reported in this communication may implicate this component enzyme of the *E. coli* acetyl CoA carboxylase in the regulation of the overall reaction. Biotin carboxylase has been purified to homogeneity; certain of its molecular, and catalytic characteristics are described in this report.

Materials. *E. coli* B (full log, enriched medium) were obtained from Grain Processing Corp. The biotin auxotroph, *E. coli* strain SA 283, obtained from Drs. S. Adhya and A. Campbell, Stanford University, was grown in the presence of [^{14}C]biotin (57 Ci/mol; 1.3 μg/ml). After reaching logarithmic phase of growth, the cells were harvested and stored at -20°C. *E. coli* DNA polymerase[13] was provided by Dr. P. Englund and ribulose diphosphate carboxylase[22] by Mr. M. Siegel.

Preparation of biotin carboxylase: Extracts (Step 1) of *E. coli* B were prepared in 0.1 M potassium phosphate buffer, pH 7.0, with a Manton-Gaulin laboratory homogenizer. The enzyme was purified by: ammonium sulfate fractionation (Step 2), adsorption and elution from calcium phosphate gel (Step 3), DEAE-cellulose chromatography (Step 4), and cellulose phosphate chromatography (Step 5).

Biotin carboxylase assays: (*a*) [^{14}*C*]*bicarbonate fixation:* The rate of [^{14}C]-bicarbonate incorporation into 1′-*N*-carboxybiotin was determined with a reaction mixture (0.5 ml, pH 8) containing 100 mM triethanolamine·HCl buffer, 1 mM ATP, 8 mM $MgCl_2$, 3 mM glutathione, 8 mM [^{14}C]$KHCO_3$ (300 cpm per nmol), 0.3 mg bovine serum albumin, 50 mM potassium-*d*-biotin and 0.1–1 milliunits of biotin carboxylase. After a 10 min incubation at 30°C, the reaction was terminated and ^{14}C-activity in the form of carboxybiotin was determined.[11]

(*b*) *Spectrophotometric:* A coupled spectrophotometric assay was used with highly-purified biotin carboxylase preparations. The reaction mixture (1 ml) was the same as that for assay (*a*) except that bicarbonate was unlabeled and 2–20 milliunits of biotin carboxylase, 2.5 mM P-enolpyruvate, 0.2 mM NADH, 15 μg of lactate dehydrogenase, and 20 μg of pyruvate kinase were added. The rate of NADH oxidation, coupled to biotin-dependent ADP formation, was followed spectrophotometrically at 340 nm and 30°C. The ratio of bicarbonate fixed to ADP formed, estimated by the two assay methods (*a*/*b*), was 0.9 when "CO_2 free" assay reagents were used. The ^{14}C-labeled carboxylation product was identified as 1′-*N*-carboxybiotin.[9-11]

One unit of biotin carboxylase catalyzes the carboxylation of 1 μmol of biotin per min under the conditions described for the spectrophotometric assay. Protein concentration of impure enzyme preparations was estimated by the biuret method.[14] The absorbance at 280 nm of homogeneous biotin carboxylase is converted to refractometrically-determined[15] protein concentration by the relation: mg protein/ml $= 1.6 \times A^{1cm}_{280nm}$.

Resolution of biotin carboxylase: The cell-free extract (Step 1) and ammonium sulfate-fractionated (Step 2) enzyme catalyzes the carboxylation of both acetyl CoA and free biotin. In the subsequent step (calcium phosphate gel, Step 3), acetyl CoA carboxylase activity is quantitatively lost, due to the resolution of biotin carboxylase and carboxyl carrier protein from the transcarboxylase component, E_b. The biotin car-

boxylase preparation at this point still contains a considerable amount of carboxyl carrier protein. The gel-fractionated enzyme, prepared from cells of the *E. coli* biotin auxotroph (see above) grown in the presence of [^{14}C]biotin, contained substantial amounts of protein-bound [^{14}C]biotin. Complete resolution of the biotin carboxylase component from the [^{14}C]biotin-containing carboxyl carrier protein has been achieved by DEAE-cellulose chromatography, a routine step (4) in our purification procedure. Calcium phosphate gel-purified (Step 3) enzyme prepared from a mixture of unlabeled and [^{14}C] biotin-labeled cells was subjected to DEAE-cellulose chromatography as described in Fig. 1. The elution pattern (Fig. 1) shows that biotin carboxylase, which appears early

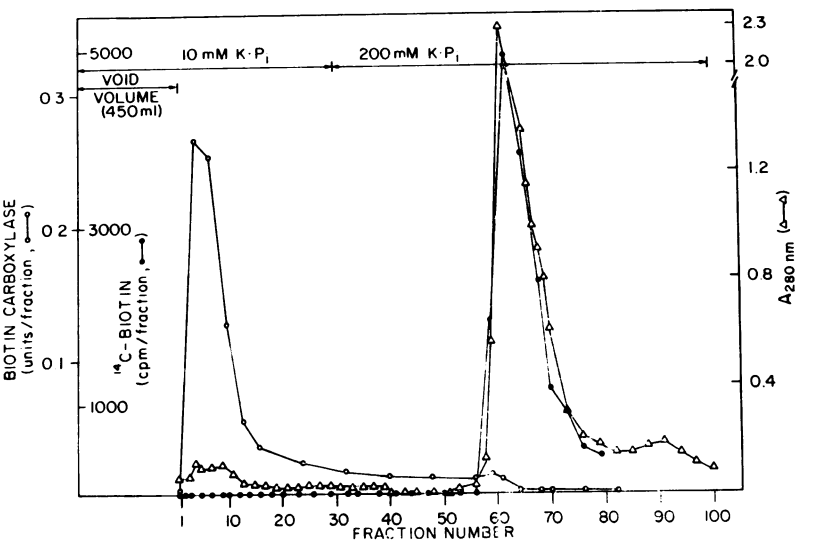

FIG. 1. Resolution of biotin carboxylase from carboxyl carrier protein containing [^{14}C]biotin by chromatography on DEAE-cellulose. Biotin carboxylase was purified through the calcium phosphate gel fractionation step from a 1 to 20 mixture of cell-free extracts of the *E. coli* biotin auxotroph grown on [^{14}C]biotin-containing media and of unlabeled wild-type *E. coli* cells, respectively. The enzyme preparation (812 mg of protein in 25 ml of 10 mM potassium phosphate (pH 7)-1 mM EDTA-5 mM 2-mercaptoethanol) was applied to a 4.5×40 cm column of DEAE-cellulose. The column was eluted with 10 mM, followed by 200 mM, potassium phosphate, pH 7, containing 1 mM EDTA and 5 mM 2-mercaptoethanol. 20-ml fractions were collected, then assayed for biotin carboxylase activity by method (*a*), [^{14}C]biotin content, and for protein by measurement of absorbance at 280 nm.

in the "break-through" peak, is well-separated from the fractions containing [^{14}C]biotin (carboxyl carrier protein) and the bulk of the protein applied to the column. The chromatographic behaviour of biotin carboxylase is dramatically altered upon dissociation from the biotin-containing peptide. After resolution, biotin carboxylase is firmly bound at low ionic strength (10 mM K·P$_i$, pH 7) to cellulose phosphate in the subsequent purification step (Step 5). In contrast, before DEAE-cellulose chromatography the biotin carboxylase-carboxyl carrier protein complex is not retained by cellulose phosphate. Partial resolution of the complex does occur in this procedure; however, only that fraction of the carboxylase which is free of carboxyl carrier protein is retained by the column. The biotin carboxylase-carboxyl carrier protein complex may also be resolved with excess avidin, which has four biotin-binding sites per molecule, followed by affinity chromatography on a column of biocytin Sepharose.[16] The carboxyl carrier protein remains firmly bound by its biotin prosthetic group through an avidin cross-linkage, whereas the biotin carboxylase is quantitatively eluted. It is evident that the interaction between biotin carboxylase and carboxyl carrier protein is relatively weak.

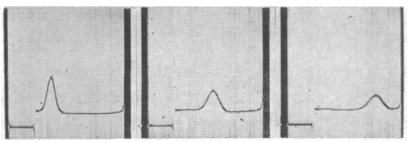

FIG. 2. Sedimentation pattern of purified biotin carboxylase. Purified carboxylase (7.7 mg per ml), dialyzed to equilibrium against 50 mM potassium phosphate buffer, pH 7-1 mM EDTA-2 mM dithiothreitol, was centrifuged at 52,000 rpm at 4.5°C. Photographs were taken at 32, 96, and 160 min, and a bar angle of 70°. Direction of sedimentation is from left to right.

Results. Purity and molecular characteristics: The overall purification of biotin carboxylase by the procedure outlined is approximately 2000 times from the cell-free extract (specific activity 3–5 $\times$ 10^{-4} units per mg of protein) and gives rise to a preparation that catalyzes the carboxylation of 1 μmol of *d*-biotin per min per mg of refractometrically-determined protein. Examination of purified carboxylase in the analytical ultracentrifuge (Fig. 2) revealed a single, symmetrical sedimenting boundary with no evident inhomogeneity. Disc gel electrophoresis of the purified enzyme on 7.5% acrylamide gels at pH 8.9[17] gave a single stained protein band. As indicated below, dissociation of the carboxylase gives rise to weight-homogeneous subunits. We have recently crystallized the carboxylase in the form of elongated prisms, ranging in length from 5–25 μm.

A Stokes radius for the undissociated biotin carboxylase of 35 Å was obtained by gel filtration on a Sephadex G-200 column, calibrated with several reference proteins,[18,19] as shown in Fig. 3. Using this value for the Stokes radius, a sedi-

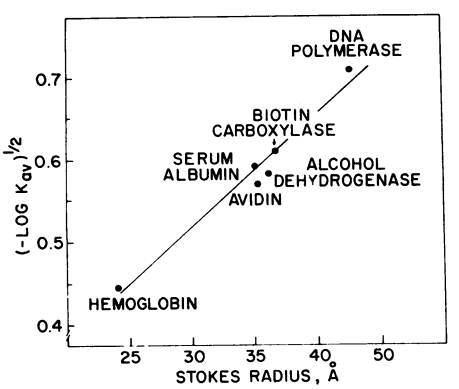

FIG. 3. Estimation of the Stokes radius of biotin carboxylase by gel filtration on Sephadex G-200. Biotin carboxylase (0.3 mg) or marker proteins (2–5 mg) were applied in 0.5 ml to a 1.5 $\times$ 67 cm Sephadex G-200 column, previously equilibrated with elution buffer, pH 7, containing 50 mM potassium phosphate-5mM 2-mercaptoethanol-1mM EDTA. The Stokes radii of the marker proteins were calculated[18,19] from their molecular weights, sedimentation coefficients, and partial specific volumes. The data were plotted according to Siegel and Monty.[19]

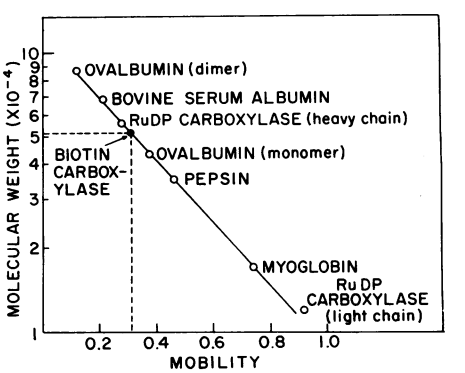

FIG. 4. Estimation of the subunit molecular weight of biotin carboxylase by dodecyl sulfate-polyacrylamide gel electrophoresis.[21] Protein samples were incubated at 30°C for 2 hr in 0.01 M sodium phosphate buffer, pH 7.0, containing 1% sodium dodecyl sulfate and 1% 2-mercaptoethanol. After incubation the protein solutions were diluted to bring the dodecyl sulfate concentration to 0.1%. Aliquots (12.5 μg of protein in 10–25 μl) containing tracking dye, 2-mercaptoethanol, and glycerol were applied to the gels. A constant current of 8 mA per gel was used for 4 hr at room temperature. Gels were stained with 0.25% Coomassie brilliant blue. The molecular weight of the spinach-leaf ribulose diphosphate (RuDP) carboxylase heavy chain is 56,000 and light chain is 12,000.[22]

mentation coefficient ($s_{20,w}$ = 5.7 S) determined by analytical ultracentrifugation, and an estimated partial specific volume of 0.75, a molecular weight of 95,000 was calculated.[18] This value agrees well with that of 98,000 daltons determined in preliminary sedimentation equilibrium experiments,[20] although the latter method gave some indication of dissociation into subunits at low protein concentration. Dissociation of biotin carboxylase into subunits was demonstrated and the subunit molecular weight determined by the dodecyl sulfate-polyacrylamide gel electrophoresis method.[21] The carboxylase gave rise to a single protein band, with a mobility corresponding to a subunit of 51,000 daltons (see Fig. 4). Since the molecular weight of biotin carboxylase is about 100,000, this enzyme component must be composed of two subunits of identical weight.

Specificity and kinetics: Biotin carboxylase exhibits a high degree of specificity for the naturally-occurring isomer of biotin, as indicated by the relative carboxylation rates of a number of analogues summarized in Table 1. Due to the high K_m (170 mM) for free *d*-biotin, and the limited supply of certain analogues, it was not possible to make this comparison at saturating concentrations of the analogues. Nevertheless, it is evident that the configuration about the 2-position, the point at which the side-chain joins the thiophane ring, is critical since *l*-biotin is completely inactive. Furthermore, the importance of the thiophane ring is indicated by the inactivity of dethiobiotin and 2-imidazolidone, as well as the greatly reduced activity caused by substitution of O for S in the ring as with O-heterobiotin. Shortening or lengthening of the side-chain by one methylene group (norbiotin or homobiotin) or addition of a terminal substituent (biotin methyl ester and ε-*N*-*d*-biotinyl-L-lysine) also markedly reduces, but does not abolish activity. Modification of the ureido group by replacing O with S, as with 2′-thiobiotin, completely blocks activity. The specificity pattern is similar to that of intact bacterial β-methylcrotonyl CoA carboxylase,[9] but differs markedly from that of avian liver acetyl CoA carboxylase where, for example, biocytin is considerably more active than biotin.[11,12] This, and the fact that the

TABLE 1. *Specificity of enzymatic carboxylation of free biotin.*

Analogue	Concentration (mM)	Carboxylation rate relative to *d*-biotin* [^{14}C]bicarbonate fixation assay (%)	Carboxylation rate relative to *d*-biotin* Spectrophotometric assay (%)
d-Biotin	10	100	100
l-Biotin	10	0	0
d-Homobiotin	10	10	10
d-Norbiotin	10	4	8
d-Biotin methyl ester	10	5	9
Biocytin	10	15	19
d,l-O-Heterobiotin	20	16	21
d,l-Dethiobiotin	20	0.2	7
d-2′-Thiobiotin	10	0	0
1′-*N*-carboxy-*d*-biotin dimethyl ester	10	1	0
2-Imidazolidone	10–400	0	0
Urea	10–400	0	0

Carboxylation rates were determined as described except that biotin analogues (potassium salts where applicable) were substituted for *d*-biotin and reaction mixtures contained 10% ethanol.

* The rate of carboxylation at a *d*-biotin concentration of 10 mM is 1.5 μmol/min per mg of refractometrically-determined protein.

rates of carboxylation of free biotin catalyzed by both bacterial carboxylases are about 1000 times higher than with the animal carboxylase, indicate that the carboxylation sites of the bacterial and animal enzymes are significantly different.

The K_m for free *d*-biotin, i.e., about 170 mM, is the same for the unresolved biotin carboxylase-carboxyl carrier protein complex (ammonium sulfate-fractionated enzyme; Step 2) as for the homogeneous biotin-carboxylase component. In the case of the unresolved complex, the covalently attached biotinyl prosthetic group remains carboxylated in the absence of acyl CoA acceptor and might be expected to compete with free biotin at the carboxylation site. Since this does not occur, the carboxylated prosthetic group of the carboxyl carrier protein subunit must be displaced from the specific carboxylation site on the biotin carboxylase subunit. This is consistent with our earlier suggestion[23] that carboxylation of the biotinyl prosthetic group, hence acquisition of an anionic site, may promote its expulsion from the carboxylation site and facilitate translocation to a site on the transcarboxylase subunit where carboxyl transfer to acetyl CoA occurs.

The K_m values for ATP-Mg complex and HCO_3^- were 0.11 and 2.9 mM, respectively.

Activation by organic solvents: Ethanol markedly activates the biotin carboxylase-catalyzed reaction. As illustrated in Fig. 5, the rate of carboxylation of free *d*-biotin increases linearly with ethanol concentration, reaching a maximum at 15% (v/v) ethanol, which corresponds to an 11-fold rate enhancement. Activation by ethanol (10%) is readily reversible, is independent of pH over the range 6.5–8.5, and is the same whether Mg^{2+} or Mn^{2+} is the source of divalent metal ion. The extent of activation is substantially increased by lowering the assay temperature to 2°C, at which point the activation by 15% ethanol is >50-fold. Activation is not specific for ethanol since other organic solvents, namely, methanol, *n*-propanol, tetrahydrofuran, dioxane, and ethyleneglycol monomethylether produce similar effects at 30°C. The effect of ethanol is exclusively on the

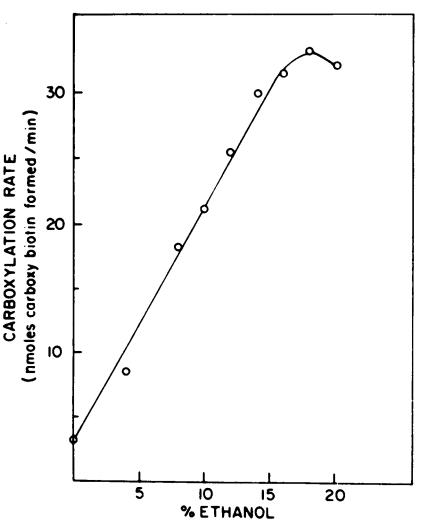

FIG. 5. Activation of biotin carboxylase by ethanol. Biotin carboxylase activity was determined by the [^{14}C]bicarbonate fixation assay at the concentrations (v/v) of ethanol indicated. The carboxylation reaction was initiated with enzyme. Similar results are obtained with the coupled spectrophotometric assay except that at ethanol concentrations above 10%, significant inactivation of the coupling enzymes occurs.

maximal velocity of the carboxylation reaction. Lineweaver-Burk analysis reveals that 10% (v/v) ethanol increases V_{max} 6–7 times with no detectable change in the K_m values for ATP-Mg, HCO_3^-, or *d*-biotin. The sedimentation velocity of biotin carboxylase (5.8 S) in sucrose density gradients containing all components of the assay reaction mixture is not affected by the presence of 10% (v/v) ethanol, indicating that activation by ethanol is not accompanied by aggregation or disaggregation of the carboxylase.

Biotin carboxylase is activated by ethanol to the same extent before and after its resolution from complexes containing E_b, the presumed transcarboxylase component, and/or carboxyl carrier protein. Despite this, the acetyl CoA carboxylase activity of the cell-free extract and ammonium sulfate-fractionated enzyme, which require the presence of all three components, is inhibited by ethanol. Apparently, the productive conformational changes in the biotin carboxylase component induced by ethanol are offset by an unfavorable conformational change in the transcarboxylase component or carboxyl carrier protein, or by disruption of the interactions among components.

Discussion. Dissociation of acetyl CoA carboxylase from animal tissues[12,23] requires relatively drastic conditions and gives rise to catalytically-inactive subunits. Reconstitution of active enzyme from these subunits has not yet been accomplished. On the other hand, acetyl CoA carboxylase from *E. coli* is easily resolved into three protein components, biotin carboxylase, a biotin-containing carboxyl carrier protein, and a component presumed to be a transcarboxylase, and can be reconstituted to catalytically-active acetyl CoA carboxylase.[8] The biotin carboxylase component catalyzes the carboxylation of free biotin, a model for the first half-reaction (Reaction 1), and exhibits a high degree of specificity for *d*-biotin. Preliminary investigations on the mechanism of this partial reaction reveal that biotin carboxylase catalyzes neither ATP–$^{32}P_i$ nor ATP–[^{14}C]-ADP exchanges at rates compatible with "β-γ P-O cleavage" of ATP prior to carboxybiotin formation. Further study will be necessary to determine whether a stepwise or concerted mechanism is involved in carboxybiotin formation.

All acetyl CoA carboxylases from animal tissues described thus far are activated by citrate,[12] which appears to regulate fatty acid synthesis as a feed-forward allosteric effector. In contrast, metabolite effectors of acetyl CoA carboxylase from *E. coli* have not yet been found. The facts that this is the committed step in fatty acid synthesis and that carboxylase activity measured *in vitro* is insufficient to account for the rate of fatty acid synthesis in *E. coli*,[24] suggest that the carboxylase is probably also regulated in this organism. The observation that the biotin carboxylase component of this acetyl CoA carboxylase system is markedly activated by ethanol demonstrates that its catalytic capacity can be enhanced to a level compatible with the overall rate of fatty acid synthesis. By analogy, the regulatory enzyme, P-enolpyruvate carboxylase from *E. coli*, is activated >50-fold by 15% ethanol, as well as by other organic solvents (unpublished results). Moreover, activation by ethanol completely replaces the requirement for acetyl CoA as allosteric effector. A similar effect of organic solvents on the dependence of P-enolpyruvate carboxylase from *Salmonella* upon fructose diphosphate as allosteric activator was observed by

Sanwal *et al.*[25] It is possible that biotin carboxylase undergoes a productive conformational change by an increase in the hydrophobicity of the environment produced by ethanol, or certain other organic solvents, which may mimic an effector-mediated response. Unlike the effect of citrate on the avian liver acetyl CoA carboxylase which promotes polymerization, activation by ethanol has no effect on the state of aggregation of the *E. coli* biotin carboxylase. However, like citrate activation of the liver and adipose tissue enzymes,[12] the kinetic effect of ethanol is due entirely to an increased maximal velocity, with no detectable effect on the K_m values for substrates.

We thank Mrs. Sylvia Himmelfarb, Mr. Jack Halsey, and Dr. Morris Burke of the McCollum-Pratt Institute for conducting the sedimentation velocity and equilibrium experiments and Mr. Eberhard Zwergel for competent technical assistance.

* Supported by research grants from the National Institutes of Health, USPHS (AM-14574 and AM-14575), and the American Heart Association, Inc.

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