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A Multienzyme Complex for CO_2 Fixation†

Jack S. Wolpert and Mary Lou Ernst-Fonberg*

ABSTRACT: Acetyl-coenzyme A carboxylase from *Euglena gracilis* strain Z was isolated as a component of a multienzyme complex which includes phosphoenolpyruvate carboxylase and malate dehydrogenase. The multienzyme complex was shown to exist in crude extracts and was purified to a homogeneous protein with a molecular weight of 360,000 by gel filtration. The ratio of the activities of the constituent enzymes was acetyl-CoA carboxylase:phosphoenolpyruvate carboxylase:malate dehydrogenase, 1:25:500. The complex is proposed to operate in conjunction with malic enzyme, which is present in *Euglena,* to facilitate the formation of substrates, malonyl-CoA, and NADPH, for fatty acid biosynthesis. The interaction of the enzymes may represent a means of control of acetyl-CoA carboxylase activity in organisms which do not possess an enzyme subject to allosteric regulation. The acetyl-CoA carboxylase activity from *Euglena* is unaffected by citrate and isocitrate.

Acetyl-coenzyme A carboxylases obtained from different organisms vary in their organization. The enzymes from bacteria and plants (Alberts and Vagelos, 1968; Dimroth et al., 1970; Burton and Stumpf, 1966; Heinstein and Stumpf, 1968) do not respond to molecules which have been shown to cause modifications in the physical structure and activity of the same enzymes obtained from animal tissues (Gregolin et al., 1966a,b; Numa et al., 1966, 1967; Kleinschmidt et al., 1969; Miller and Levy, 1969; Shrago et al., 1969). Since *Euglena gracilis* readily adapts to heterotrophic or photoauxotrophic modes of growth, it was of interest to ascertain whether the organism contained an acetyl-CoA carboxylase resembling the plant and bacterial form or the animal form. *Euglena* possesses two fatty acid synthetases (Delo et al., 1971; Ernst-Fonberg and Bloch, 1971; Ernst-Fonberg, 1973), one organized in a manner resembling the enzyme systems from bacteria and plants (Overath and Stumpf, 1964; Brooks and Stumpf, 1966; Simoni et al., 1967; Alberts et al., 1963; Goldman et al., 1963; Lennarz et al., 1962), the other resembling the form from yeast and animals (Lynen, 1961; Burton et al., 1968; Larrabee et al., 1965; Smith and Abraham, 1970). The expression of these two forms in the cell depends on conditions of light or darkness. The possibility that there was an acetyl-CoA carboxylase corresponding to each of the two fatty acid synthetases was investigated.

Euglena has been shown to have an unusual pattern of heterotrophic CO_2 fixation (Lynch and Calvin, 1953; Moses et al., 1959). Available data appear to describe two direct routes of CO_2 incorporation in darkness (Levedahl, 1968). The first is a reaction by way of phosphogluconic acid proposed by Moses and colleagues (1959), while the second is proposed to be incorporation into oxaloacetate possibly through phosphoenolpyruvate (Levedahl, 1968). However, the enzymology of these reactions in *Euglena* is not well understood. During the investigation of the acetyl-CoA carboxylase from the phytoflagellate, it became apparent that the structure and possibly the function of this enzyme were related intimately to the second of the two primary routes of heterotrophic CO_2 fixation in this organism. Furthermore, this relationship which is described in the subsequent text may be a means of control of fatty acid biosynthesis in an organism which does not possess an allosteric acetyl-CoA carboxylase.

Experimental Section

Materials and Reagents. Sigma Chemical Co. was the source of *d*-biotin, grade I ammonium sulfate, oxaloacetate, pyruvate kinase, phosphoenolpyruvate, bovine serum albumin, lactate dehydrogenase, chymotrypsinogen A, thyroglobulin, catalase, malate dehydrogenase, and avidin. Apoferritin, lysozyme, and ovalbumin were from Calbiochem. Fisher was the source of dimethylformamide which was redistilled prior to use. Dicyclohexylcarbodiimide was from Aldrich while 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide · HCl was from Pierce. Bio-Rad Laboratories was the source of Bio-Gel A-1.5 and Affi-Gel 102, and DEAE was Whatman DE-52. Acetyl-CoA was obtained from P-L Biochemicals. Acrylamide and bisacrylamide were from Eastman Organic Chemicals, and Cyanogum was from E-C Apparatus. New England Nuclear was the source of

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$NaH^{14}CO_3$ and [2-^{14}C]malonyl-CoA. Imidazole was recrystallized from acetone–petroleum ether.

Growth of Cells. Euglena gracillis strain Z were grown in large quantities as described by DiNello and Ernst-Fonberg (1973). The harvested packed cells were stored at −50° until use.

Analytical Procedures. Acrylamide gel electrophoresis was done in Tris-borate–EDTA,[1] 0.18 *M*, pH 8.4, 0.001 *M* in 2-mercaptoethanol in an E-C 470 Vertical Gel Cell. The composition of the gels was 5% acrylamide, 0.25% bisacrylamide, 0.1% tetramethylethylenediamine, and 0.1% ammonium persulfate. Following polymerization at room temperature, the gels were subjected to electrophoresis for 30 min prior to sample application. Protein, 10–100 μg in volumes up to 90 μl, was applied to the gels along with 10 μl of 0.03% Bromophenol Blue in 33% sucrose. Current was applied until the dye had migrated at least 10 cm. The dye front was marked with a dot of india ink from a 22 gage needle. A discontinuous system was run in a Shandon Polyanalyst where the gels were 5% acrylamide with 2% stacking gels, pH 7.7 (Tris-sodium dihydrogen phosphate, 0.0658 *M*), with a running buffer of pH 8.3 (Tris-glycine, 0.0433 *M*, 0.002 *M* in dithiothreitol). The gels were subjected to electrophoresis prior to sample application for 30 min in gel buffer containing dithiothreitol, but the latter was not present during gel polymerization. Protein bands were detected by staining with 0.05% Coomassie Blue in 7% acetic acid and 30% methanol solution for 12 hr followed by destaining in 7% acetic acid and 30% methanol. For subunit determination, proteins (1 mg/ml) were dialyzed overnight against 200 vol of the pH 8.4 buffer described above, which contained 1.0% sodium dodecyl sulfate, and then were heated at 100° for 5 min. Samples of 25–100 μg were applied in the presence of sucrose and Bromophenol Blue as described. Gels of 7% and 10% acrylamide were prepared and run in the pH 8.4 buffer which was 0.2% sodium dodecyl sulfate. After the run, gels were rinsed twice in distilled water, washed free of sodium dodecyl sulfate for 24 hr in 30% methanol–7% acetic acid, and stained as described. Protein standards run under identical conditions included: apoferritin, bovine serum albumin, phosphorylase *a*, lysozyme, α-chymotrypsinogen A, ovalbumin, and pyruvate kinase. Subunit molecular weights were determined by their mobilities on a plot of the log molecular weight vs. mobility of the standards.

Protein was measured by the method of Lowry et al. (1951) as modified by Eggstein and Kreutz (1955) and by absorbance (Kalckar, 1947).

Assay of Acetyl-CoA Carboxylase Activity by $H^{14}CO_3$ Fixation. The assay solution, final volume 0.5 ml, contained: imidazole buffer, pH 7.5, 100 m*M*; $MgCl_2$, 8 m*M*; dithiothreitol, 2 m*M*; acetyl-CoA, 0.66 m*M*; ATP, 4 m*M*; bovine serum albumin, 0.2 mg; and $NaH^{14}CO_3$, 40 m*M* (specific activity 0.05). The reaction was carried out at 30°, and, following a 2-min warming period, reaction was initiated by the addition of $NaH^{14}CO_3$. After 15 min, the reaction was terminated with 0.1 ml of 12 *N* HCl and purged with air for 15 min. Two traps of 40% KOH captured the $^{14}CO_2$ effluent. Denatured protein was sedimented by centrifugation in a clinical centrifuge. An aliquot of the supernatant was spotted on Whatman #1 filter paper, 1.4 cm × 3.3 cm, and dried under an infrared lamp. The papers were placed in counting vials containing 15 ml of toluene which contained 15.1 g of 2,5-diphenyloxazole/gal. The radioactivity was measured in a Packard Model 3320 liquid scintillation counter, and quench correction was made by the channels ratio method. On occasions where enzymes catalyzing CO_2 incorporation other than acetyl-CoA carboxylase were measured, the appropriate substrates in the concentrations indicated were substituted for acetyl-CoA.

Spectrophotometric Assay of Acetyl-CoA Carboxylase Activity. The assay was basically that described by Gregolin and coworkers (1968). It included in a final volume of 0.25 ml: $NaHCO_3$, 25 m*M*; lactate dehydrogenase, 0.01 mg; pyruvate kinase, 0.01 mg; phosphoenolpyruvate, 0.5 m*M*; NADH, 0.18 m*M*; and the components listed in the radioactive assay. The reaction was carried out at 30° and, following a 5-min warming period, was initiated by the addition of enzyme followed by rapid stirring with a thin stream of N_2. The decrease in absorbance at 340 nm was monitored.

Preparation of Biotin–Affi-Gel 102. Affi-Gel 102, 100 ml, containing 8–10 μmol of ω-amino alkyl groups/ml was mixed with 4.8 mmol of *d*-biotin, a 4.8-fold excess, dissolved in 400 ml of dimethylformamide–water (6:4). The pH was adjusted to 4.7, and at room temperature, with constant stirring, 5 mmol of dicyclohexylcarbodiimide in 13 ml of dimethylformamide was added. Similar additions of the carbodiimide were made at 48 and 120 hr of reaction. After 161 hr of reaction, the mixture was filtered with a Büchner funnel and Whatman #13 filter paper. The coupled gel was washed extensively with dimethylformamide–water until the absorbance at 262 nm measured against the same solvent was 0. Following a wash with 2 l. of distilled water, the gel was poured into a glass column where it was washed with 95% ethanol until the absorbance at 220 nm was less than 0.01. This was followed by 4 l. of 0.5 *M* NaCl (pH 10.0) and finally by 4 l. of distilled water.

The extent of coupling was determined by a hydroxamate assay described by Wolpert and Ernst-Fonberg (1973) which indicated that the gel contained 4–5 μmol of bound biotin/ml. The coupled gel was chilled to 4°, mixed with an equal volume of water, poured into a glass column, and settled to a final bed size of 2.5 × 11 cm. For use, the gel column was equilibrated in 0.02 *M* imidazole buffer (pH 7.0), 0.007 *M* in 2-mercaptoethanol and 0.04 *M* in NaCl. The void volume and internal volume were estimated by blue dextran and cytochrome *c*, respectively. The former showed some tendency to absorb to the gel. Biotin-liganded gel also was prepared as described by Wolpert and Ernst-Fonberg (1973) using a water-soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide · HCl, as the coupling reagent. A small column, 1 × 4.5 cm, was prepared from this material. Both carbodiimides gave the same extent of coupling so the use of the water-soluble compound is preferred.

Detection of Malic Enzyme. Freshly grown cells, 1.0 g, were suspended in 1 ml of 0.1 *M* imidazole (pH 7.0) which was 7 m*M* in 2-mercaptoethanol, 5 m*M* in sodium pyruvate, and 5 m*M* in $MgCl_2$. After sonication at 4°, the mixture was brought to 10% saturation with ammonium sulfate and immediately centrifuged at 10,000 *g* for 10 min. Following desalting on Sephadex G-25 (equilibrated and eluted with 0.02 *M* imidazole buffer (pH 7.0) with the additions listed above) the protein effluent was measured for the presence of malic enzyme. The assay solution was 0.25 ml final volume and contained: imidazole, pH 7.5, 0.1 *M*; $MgCl_2$, 8

[1] Abbreviations used are: ACP, acyl carrier protein; EDTA, ethylenediaminetetraacetic acid.

m*M*; dithiothreitol, 2 m*M*; $NaH^{14}CO_3$, 25 m*M* (specific activity 0.6); sodium pyruvate, 20 m*M*; and NADH or NADPH, 0.15 m*M*. The reaction was carried out as described for other $^{14}CO_2$ incorporation experiments.

Purification of Enzyme Complex. Cells grown in the light, 50 g, were mixed with an equal volume of 0.1 *M* imidazole buffer (pH 7.0), 0.007 *M* in 2-mercaptoethanol, and disrupted with a Branson S-125 Sonifier or with a Parr Nitrogen Bomb at 1000 psi. Multiple short bursts of sound were administered until breakage was complete by microscopic examination. The temperature was kept below 4° with a methanol–ice bath. All subsequent manipulations were done at 4°. The mixture was centrifuged at 100,000 *g* for 1 hr. The supernatant was collected, and the pellet was washed with another volume of buffer. Following centrifugation, the supernatants were combined. An aliquot for assay of protein and enzyme activity was run through a Sephadex G-25 column, 1.3 × 7 cm, in buffer A.[2] The remainder was brought to 60% saturation with ammonium sulfate. After stirring for 30 min, the precipitate was collected and dissolved in 100 ml of buffer A. A small aliquot was desalted on Sephadex G-25 for assay of enzyme activity.

The protein solution was applied to a DEAE-cellulose column (10 mg of protein/ml column bed) equilibrated in buffer A. The enzyme activity was washed through the column in the breakthrough peak with the same buffer, and the fractions comprising the protein peak were pooled and concentrated by precipitation in 60% saturated ammonium sulfate solution. The precipitate was collected by centrifugation and dissolved in 15 ml of buffer A, and then desalted on a G-25 column. The enzyme was then frozen in 1-ml aliquots in liquid N_2 and stored at −60°. Following thawing and centrifugation, the enzyme solution was applied to the biotin-affinity column. The column was eluted with the equilibration buffer (0.02 *M* imidazole (pH 7.0), 0.007 *M* in 2 mercaptoethanol and 0.04 *M* in NaCl) until no more discrete peaks of protein were eluted, and protein began to trail from the column. At this point, in order to hasten this slow release of enzyme, the ionic strength of the buffer was raised to 0.29 with NaCl, and a discrete peak of protein was eluted, the fractions of which were pooled and concentrated in an Amicon Filtration cell using a UM 20 E membrane. A 1.4-ml (10 mg) aliquot of this material was chromatographed on a Bio-Gel A-1.5 column, 2.5 × 30 cm, and the fractions comprising the major protein peak which was coincident with enzyme activity were pooled and concentrated by Amicon Filtration.

Product Identification. The enzyme assay employing radioactive substrates was carried out as described. After completion of the entire reaction protocol, the solutions were brought to pH 1, 4, or 12 with 45% KOH. After 0–16 hr at 4°, 5–50-μl aliquots (2 μl at a time) were applied to Eastman 13254 cellulose chromogram sheets with fluorescent indicator, and the chromatogram was developed in isobutyric acid–NH_3–H_2O (66:1:30). Standards were [2-^{14}C]malonyl-CoA, malonic acid, malic acid, oxaloacetic acid, pyruvic acid, citric acid, and phosphoenolpyruvic acid. Compounds were detected with I_2 vapors, ultraviolet light, or by cutting the chromatogram in 1 × 2 cm strips and measuring radioactivity in a liquid scintillation counter as described earlier.

[2] Buffer A was 0.02 *M* imidazole (pH 7.0), 0.007 *M* in 2-mercaptoethanol.

Results

Acetyl-CoA Carboxylase Reaction. The enzyme required acetyl-CoA, HCO_3^-, ATP, and Mg^{2+} for activity and was completely inhibited by avidin as measured by $H^{14}CO_3^-$ incorporation. Propionyl-CoA was carboxylated 11% of the extent to which acetyl-CoA was. This was less than that reported for acetyl-CoA carboxylases from animals (Gregolin et al., 1968), whereas no carboxylation of propionyl-CoA was catalyzed by the *Escherichia coli* acetyl-CoA carboxylase (Alberts and Vagelos, 1968). Unlike the enzyme from animal tissues (Kleinschmidt et al., 1969), the *Euglena* acetyl-CoA carboxylase was unaffected by incubation with citrate or isocitrate prior to initiation of reaction. A similar lack of response to effectors has been reported for the enzymes obtained from *E. coli* (Alberts and Vagelos, 1968; Dimroth et al., 1970) and plant materials (Burton and Stumpf, 1966; Heinstein and Stumpf, 1968). The reaction rate was linear with time and with enzyme concentration providing that the total protein concentration in the assay solution was greater than 0.2 mg. The latter was the reason for the addition of bovine serum albumin to all assays. The pH optimum was sharply defined at 7.5 in imidazole buffer. Unlike the rat liver enzyme studied by Gregolin and colleagues (1968), the presence of phosphate was not important for stability.

The product of the reaction was malonyl-CoA, R_f 0.67, the thiolester bond of which was readily hydrolyzed when the pH was adjusted to 12 after completion of the assay. The R_f of the radioactive label after alkaline hydrolysis was 0.35, identical with malonic acid. The product of the reactions served as a substrate for the ACP-dependent[1] fatty acid synthetase obtained from *Euglena*. Fatty acid biosynthesis was measured by the incorporation of [^{3}H]acetyl-CoA into long-chain fatty acids as described by Ernst-Fonberg (1973).

Biotin-Affinity Chromatography. The biotin-liganded Affi-Gel 102 proved to be useful only within a narrow range of ionic strengths, partly for reasons which are inherent in the low affinity between the ligand and protein (Steers et al., 1971) and partly due to the structure of the 3,3′-diaminodipropylamine side chains on the gel. A profile of the protein and enzyme activity eluted from the affinity column is shown in Figure 1 where it is seen that the acetyl-CoA carboxylase interacted with the bound biotin. There was a slightly retarded peak of enzyme activity which trailed until the eluent ionic strength was increased speeding the release of the remaining bound enzyme. As pointed out by Cuatrecasas and Anfinsen (1971) protein specifically absorbed to a liganded gel will eventually emerge from the column without altering the eluent if the affinity for the ligand is not too great. This type of elution, however, gives a very dilute solution of the protein. For this reason, the increase in ionic strength was used to increase the rate of release of the acetyl-CoA carboxylase from the biotin-liganded solid support. Upon elution beyond the internal volume of the column (76 ml) the profiles of acetyl-CoA carboxylase and protein coincide. Rechromatography under the same conditions of enzyme from any point in the elution profile generated a similar chromatogram. This indicated that the elution pattern was a function of enzyme–ligand interaction rather than representing two different states of the enzyme.

When the column loading conditions were increased to an ionic strength of 0.15, there was no retardation of the acetyl-CoA carboxylase activity which limited the system to use at relatively low ionic strengths. This is unfortunate be-

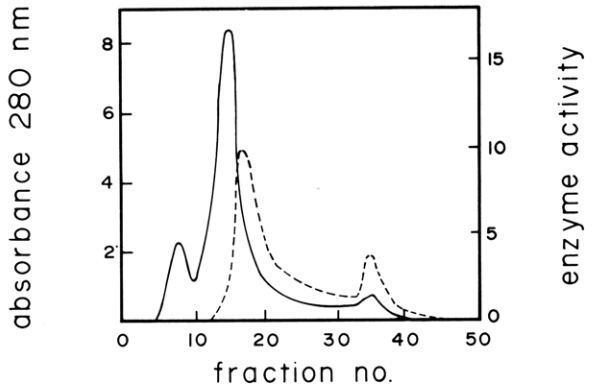

FIGURE 1: Biotin affinity chromatography of acetyl-CoA carboxylase. The preparation and use of the column are detailed in the text. Absorbance at 280 nm is shown by the solid line, and the dotted line gives the profile of enzyme activity which was determined by the incorporation of $^{14}CO_2$ (nmol/15 min per ml of enzyme) in the presence of acetyl-CoA as described in the text. Fractions of 5 ml were collected. The void volume of the column determined with blue dextran was 26–28 ml, and cytochrome *c* appeared at 76 ml of effluent. Enzyme washed through a DEAE-cellulose column without binding was concentrated, frozen in small aliquots in liquid N_2, and stored at −60°. Prior to affinity chromatography, it was thawed, centrifuged, and applied to the column which was eluted as described in the text with the salt increment started at fraction 23. Fractions 26–42 were pooled and concentrated in an Amicon Filtration Cell with a UM 20E membrane.

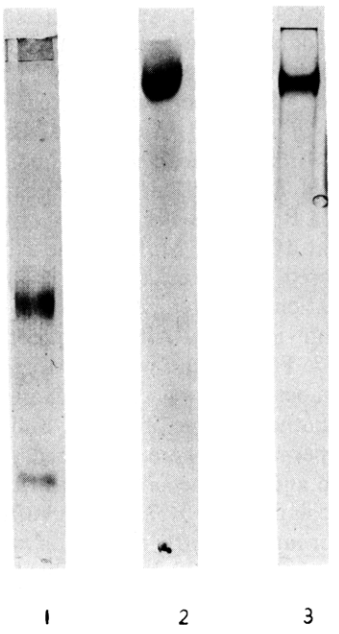

FIGURE 2: Acrylamide gel electrophoresis: (1) pure complex, 25 μg, run in the pH 8.4 system which contained 0.2% sodium dodecyl sulfate; (2) pure complex, 40 μg, run in the discontinuous system with gel pH 7.7 and running buffer pH 8.3; (3) pure complex, 80 μg, run in the pH 8.4 system. Experimental details are in the text.

cause the Affi-Gel 102 has some capacity for ion exchange, and we suspect that a significant portion of this occurs by way of the secondary amino groups on the side arm. At column loading ionic strengths of 0.02 *M* and lower, ion exchange was evident. In systems where ligand–protein affinity is relatively strong, this is a minor problem as relatively high ionic strength medium can be used throughout the experiment, but in situations similar to the one here where dissociation constants are of the order of 5 m*M* (Steers et al., 1971; Moss and Lane, 1971) options are limited. Further experiments to couple biotin to Sepharose containing a diaminohexyl side arm are in progress with the objective of expanding the useful range of ionic strength.

Purification of a Multienzyme Complex Containing Acetyl-CoA Carboxylase. The purification procedure is summarized in Table I. Figure 2 shows that the purified protein yielded a single band on acrylamide gel electrophoresis done under two different sets of conditions. When an apparently homogeneous protein had been achieved, attempts were made to measure acetyl-CoA carboxylase activity by the spectrophotometric assay described earlier. This assay had given anomalous results when used with crude preparations of the enzyme where the presence of other ATPase's or other NADH oxidizing systems could not be ruled out. However, the use of this assay with the pure protein preparation indicated that the reaction measured was dependent only on the presence of phosphoenolpyruvate, NADH, and HCO_3^-. There was no need for any of the other components, including the enzymes for the coupled reaction, lactate dehydrogenase and pyruvate kinase, as well as acetyl-CoA. The assay with the cited substrates completely paralleled the assay measuring the incorporation of $^{14}CO_2$ into acetyl-CoA. This indicated that the homogeneous protein preparation contained other enzymic activities in addition to acetyl-CoA carboxylase. Figure 3 shows the coincidence of protein and enzymic activity obtained in the final step of purification when the protein retarded by the biotin-liganded affinity column was chromatographed on Bio-Gel A-1.5. Again, both the modified spectrophotometric and ^{14}C assay results were coincidental with the protein profile at a molecular weight of about 360,000 on the calibrated column.

Table I: Purification Scheme.

Step	Total Protein (mg)	Acetyl-CoA Carboxylase Total Act. (Units[a])	P-enolpyruvate Carboxylase Total Act. (Units[a])	Malate Dehydrogenase Total Act. (Units × 10^{-3}[a])
Crude extract[b]	4359	418		
100,000*g* 60-min supernatant of crude extract	1568	526	12,834	
0–60% $(NH_4)_2SO_4$ fraction	790	516	11,920	7291
DEAE-cellulose	714	578	14,856	7037
0–60% $(NH_4)_2SO_4$ fraction	636	546	13,213	6716
Biotin affinity chromatography	11	130	3,029	66
Bio-Gel A-1.5	10	127	3,156	64

[a] A unit of activity is the production of 1 nmol of product/min. [b] Obtained from the disruption of 50 g of *Euglena*.

Initially, EDTA, 1 m*M*, was used in all buffers in the handling of acetyl-CoA carboxylase. However, omission of EDTA led to a considerable improvement of the stability of the activity. This was contrary to the findings of Gregolin and colleagues (1968) for the rat liver enzyme. Repeated freezing and thawing and extensive dialysis were also detrimental to enzyme activity.

The best way of storing the preparation at various stages was as an ammonium sulfate precipitate at 4°.

Enzymes Present in Addition to Acetyl-CoA Carboxylase. The spectrophotometric assay indicated a requirement for phosphoenolpyruvate and HCO_3^- in order that the enzyme could catalyze the oxidation of NADH; however, the route followed for this reaction was not clear. A scheme of possible metabolic routes in this area indicated that the presence of the following enzymes had to be considered: phosphoenolpyruvate carboxylase, phosphoenolpyruvate carboxykinase, pyruvate kinase, pyruvate carboxylase, malate dehydrogenase, and malic enzyme.

Experiments which identified the enzymes present are summarized in Table II. Phosphoenolpyruvate alone (Table II, reaction 2) resulted in a large incorporation of $H^{14}CO_3^-$ with oxaloacetate identified as the product, R_f 0.37, indicating the presence of phosphoenolpyruvate carboxylase. Reactions 3 and 4 demonstrated that this activity was unaffected by ADP at the concentration indicated but was stimulated about 150% by the presence of ATP. The product of reaction 3 was identified as oxaloacetate. Sanwall and Maeba (1966) have shown that phosphoenolpyruvate carboxylase from *Salmonella* was stimulated by nucleotides. Reaction 5 shows the same as well as indicating that oxaloacetate did not cause inhibition under the conditions used; the incorporation of $^{14}CO_2$ was similar in reactions 3 and 5. Reaction 6 contained NADH so that if malate dehydrogenase were present, radioactive malate would be formed. Thin-layer chromatography of the products of this reaction gave radioactive spots R_f 0.37 and 0.32; oxaloacetate and malate have R_f values of 0.37 and 0.32, respectively. The latter reaction as well as comparison of reactions 2 and 7 indicated that acetyl-CoA stimulated phosphoenolpyruvate carboxylase. Maeba and Sanwall (1969) have shown that acetyl-CoA was a positive effector of the phosphoenolpyruvate carboxylase from *Salmonella*. Stimulation of phosphoenolpyruvate carboxylase by NADH as well as by ATP would not be distinguished in reaction 6, but further study has made this distinction which is described in the following article (Wolpert and Ernst-Fonberg, 1975). Reaction 8 showed no incorporation of $H^{14}CO_3^-$ when pyruvate and NADPH were offered as substrates; therefore, malic enzyme was not present in the complex. Reaction 10 showed the absence of pyruvate carboxylase. Reaction 1 indicated the presence of low levels of acetyl-CoA carboxylase activity. This enzyme activity was the least stable of the three catalytic activities in the complex, and the enzyme used in these experiments had been stored for 2 months at 4° as an ammonium sulfate precipitate. During this time the acetyl-CoA carboxylase had a 20-fold decrease in activity. In the 0–70% saturation with ammonium sulfate fraction of crude extracts of *Euglena* as well as in the freshly purified complex, the ratio of phosphoenolpyruvate carboxylase activity to that of acetyl-CoA carboxylase was about 25:1. However, with aging, the latter enzyme lost activity while the malate dehydrogenase and phosphoenolpyruvate carboxylase were relatively stable. The ratio of the latter two enzy-

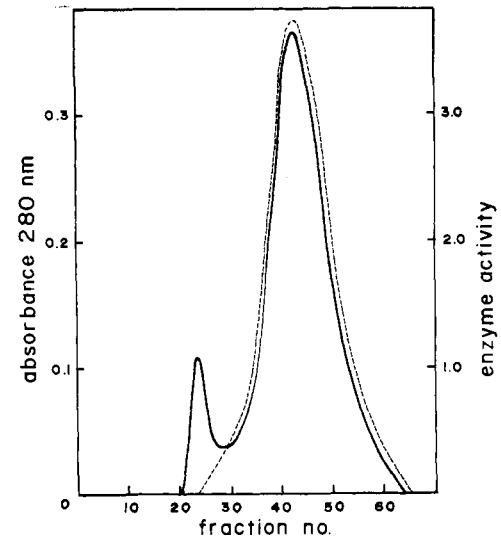

FIGURE 3: Gel filtration chromatography of enzyme activity retained by the biotin affinity chromatography. Enzyme, 10 mg, retained by the biotin-liganded solid support was concentrated to 1.2 ml, and applied to a Bio-Gel A-1.5 column, 2.5 × 30 cm, equilibrated in and eluted with buffer A; flow rate was 10 ml/hr per cm^2. Fractions of 2 ml were collected and measured for absorbance at 280 nm and enzyme activity (nmol/15 min per ml of enzyme). The latter was measured by the incorporation of $H^{14}CO_3$ into acetyl-CoA and by the change in absorbance at 340 nm in the presence of the phosphoenolpyruvate and NADH subtrates as described in the text. The void volume was 46 ml.

Table II: Demonstration of Enzyme Activities Present in the Protein Complex.[a]

Reaction	NADPH	ATP	ADP	P-enol-pyruvate	NADH	Oxalo-acetate	Acetyl-CoA	Pyruvate	nmol of $^{14}CO_2$ Fixed
1	0	4	0	0	0	0	0.3	0	1
2	0	0	0	0.5	0	0	0	0	239
3	0	4	0	0.5	0	0	0	0	360
4	0	0	0.02	0.5	0	0	0	0	272
5	0	4	0.02	0.5	0	1	0	0	352
6	0	4	0.02	0.5	0.2	1	0.3	0	566
7	0	0	0	0.5	0	0	0.24	0	294
8	0.3	0	0	0	0	0	0	0.5	0
9	0	4	0	0	0	0	0.24	0.5	1
10	0	4	0	0	0	0	0	0.5	0

[a] Enzyme activities were assayed by the incorporation of $H^{14}CO_3$ into acid stable product as described in the text. The indicated substrates were used in the millimolar concentrations shown. The source of enzymes was the purified complex which had been stored as an ammonium sulfate precipitate at 4°. Assays contained 200 μg of enzyme/assay.

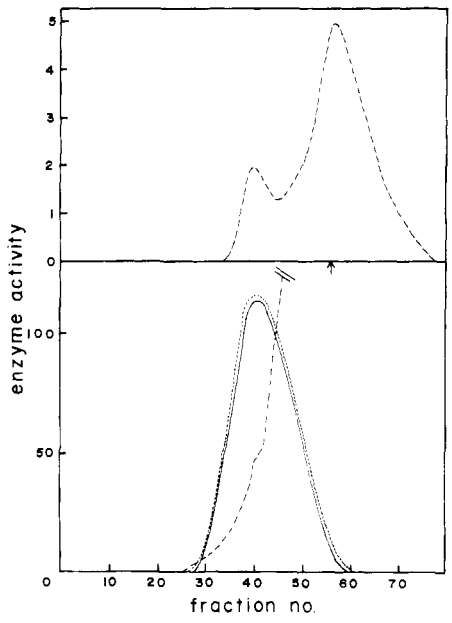

FIGURE 4: Demonstration of complex of enzymes in a crude preparation of *Euglena* extract. *Euglena* were processed as described in the text and the extract was applied to a Bio-Gel A-1.5 column, 2.5 × 30 cm. Fractions of 2 ml were collected and analyzed for acetyl-CoA carboxylase activity and phosphoenolpyruvate carboxylase activity by measuring $H^{14}CO_3$ incorporation in the presence of the appropriate substrates as described. Malate dehydrogenase activity was measured spectrally by following the oxidation of NADH (0.176 m*M*) at 340 nm in the presence of oxaloacetate (0.8 m*M*) in a final volume of 0.25 ml, pH 7.0 (imidazole, 0.1 *M;* dithiothreitol, 2 m*M;* $MgCl_2$, 8 m*M*). The upper portion of the figure is the rechromatography of pooled and concentrated fractions 32–48. The arrow indicates the position of the peak of noncomplexed malate dehydrogenase activity in the initial chromatography. Malate dehydrogenase activity is shown by the dashed line (μmol of NADH oxidized/15 min per ml of enzyme). Phosphoenolpyruvate carboxylase activity (nmol/15 min per ml of enzyme × 10^{-1} is represented by the dotted line. Acetyl-CoA carboxylase activity (nmol/15 min per ml of enzyme × 2.5) is shown by the solid line. The void volume was 46 ml.

mic activities was 20:1. Thus, the activity ratios in the multienzyme complex were 1:25:500 for acetyl-CoA carboxylase:phosphoenolpyruvate carboxylase:malate dehydrogenase.

In addition to the data in Table II, reaction 8, a spectrophotometric assay including malic acid and $NADP^+$ indicated that malic enzyme was not present within the complex (Wolpert, 1975). A similar assay containing NADPH or NADH verified the presence of malate dehydrogenase and showed that it was specific for the cofactor NADH. In summary, the data presented in Table II and the subsequent text indicate that acetyl-CoA carboxylase, phosphoenolpyruvate carboxylase, and malate dehydrogenase were constituents of the complex of proteins.

Acrylamide gel electrophoresis of the sodium dodecyl sulfate treated multienzyme complex (Figure 2) showed that subunits, presumably nonidentical, of about 12,000 and 51,000 molecular weight were present. The limited number of protein bands obtained by this technique makes it unlikely that the complex contained additional proteins which were not detected by the enzyme assays employed.

Although malic enzyme was shown not to be present in the complex, its presence in extracts of *Euglena* was verified. Execution of the procedure described in the Experimental Section showed that $H^{14}CO_3^-$ was incorporated into pyruvate in the presence of NADPH. There was no incorporation if NADH was the cofactor. The same enzyme from maize was shown by Johnson and Hatch (1970) to have an absolute requirement for NADPH as a cofactor.

Demonstration of the Enzyme Complex in a Crude Preparation of Euglena Extract. Light grown cells, 15 g, were disrupted and processed through ammonium sulfate fractionation as described in the Experimental Section. The protein solution, 4 ml, was applied immediately to a Bio-Gel A-1.5 column, and the chromatogram of enzyme activities is shown in Figure 4, lower half. The protein profile of the crude preparation was nondescript so it was not included in the figure. The peaks of the acetyl-CoA carboxylase activity and the phosphoenolpyruvate carboxylase activity coincided at a molecular weight of about 360,000. The malate dehydrogenase activity evidenced a discrete shoulder at the same molecular weight; the peak was obscured in the crude preparation owing to the presence of three forms of this activity in *Euglena* (Chancellor-Maddison and Noll, 1963; Wolpert and Ernst-Fonberg, 1975). In order to minimize the presence of the lower molecular weight malate dehydrogenase, fractions 32 through 48 were pooled, concentrated, and rechromatographed (upper portion of Figure 4). A discrete peak of malate dehydrogenase activity appeared coincidental with the other two enzyme activities at a molecular weight of 360,000.

Discussion

An apparently homogeneous protein preparation obtained by biotin-affinity chromatography and verified by acrylamide gel electrophoresis and gel filtration chromatography was shown to contain three separate enzymic activities: acetyl-CoA carboxylase, phosphoenolpyruvate carboxylase, and malate dehydrogenase. The molecular weight of the multienzyme complex was shown to be about 360,000. The molecular weights of the constituent enzymes of the complex have been shown to be well below this value (Wolpert and Ernst-Fonberg, 1975). It is unlikely that the association of the three enzymes into a complex was an artifact of the preparative procedure since similar results were obtained when the cells were disrupted by N_2 pressure as well as by ultrasound, and the complex was shown repeatedly to be present in relatively crude extracts of cells.

Pathways of CO_2 fixation other than the Calvin cycle must exist in various living systems in order to account for observed rapid CO_2 incorporation into non-Calvin cycle compounds (Moses et al., 1959; Levedahl, 1968; Black, 1973; Hatch and Slack, 1970). In particular, an alternative cycle, the Hatch-Slack cycle (Hatch and Slack, 1970), has been demonstrated although some aspects of it remain to be clarified. In it, CO_2 is introduced by the carboxylation of phosphoenolpyruvate, and the resulting oxaloacetate is rapidly interconverted with pools of malate and aspartate. Hatch and Slack (1970) proposed that the C_4 carboxyl of a dicarboxylic acid, probably oxaloacetate, is transferred into an acceptor in the Calvin cycle and appears as the C_1 of 3-phosphoglyceric acid. The pyruvate remaining is phosphorylated to regenerate phosphoenolpyruvate (Hatch and Slack, 1967). An independent study of the nature of phosphoenolpyruvate carboxylase was done by Maruyama and colleagues (1966) from which they concluded that it may serve as an excellent scavenger for trapping CO_2 for introduction into metabolic pathways. Although the logic of the scheme in conjunction with the proposed role of phosphoenolpyruvate carboxylase is appealing, there is little direct experimental evidence to support the existence of the necessary in vivo relationships among the enzymes involved. The

findings reported herein of a complex of enzymes, two of which are involved in carboxylation reactions, are the first direct evidence suggesting that such enzymic schemes may exist and function within cells.

We suggest that the incorporation of CO_2 into acetyl-CoA to form malonyl-CoA for fatty acid biosynthesis is expedited by the existence of the observed complex of enzymes: phosphoenolpyruvate carboxylase, malate dehydrogenase, and acetyl-CoA carboxylase. The ratio of K_m values for HCO_3^- for the acetyl-CoA carboxylase and phosphoenolpyruvate carboxylase in the complex from *Euglena* was about 6 (Wolpert and Ernst-Fonberg, 1975). In terms of affinity for substrate, the phosphoenolpyruvate carboxylase is at an advantage. Moreover, the catalytic efficiency (Dixon and Webb, 1952) of the latter enzyme was about 25 times greater than that of the acetyl-CoA carboxylase. Thus, the phosphoenolpyruvate carboxylase captures CO_2 more readily. The resulting oxaloacetate is reduced by malate dehydrogenase, which is specific for NADH, to yield malate which is then acted upon by malic enzyme to generate the CO_2 substrate for the acetyl-CoA carboxylase and NADPH, Figure 5. Although the spatial arrangement of the enzymes within the complex is not known, it is conceivable that their arrangement is such that a microenvironment of relatively high HCO_3^- concentration is created by malic enzyme catalysis in the region of the active site of the acetyl CoA carboxylase. In this way, the CO_2 captured by the phosphoenolpyruvate carboxylase is channelled specifically to the acetyl-CoA carboxylase. Experimental models have demonstrated the validity of microenvironmental compartmentation in other systems (Srere et al., 1973). Malic enzyme in *Euglena* is specific for $NADP^+$. The malonyl-CoA and NADPH resulting from the reactions are then used to form fatty acids. Although it is not present within the complex, malic enzyme may occupy a key position in controlling the availability of substrates for fatty acid biosynthesis, NADPH, and malonyl-CoA. Therefore, it is conceivable that keener metabolic control and efficiency could be realized by keeping this enzyme separate from the CO_2 handling system until the need for fatty acid biosynthesis did arise. It is possible that similar schemes provide a control mechanism for regulating the substrates for fatty acid biosynthesis in plants and bacteria which do not appear to have acetyl-CoA carboxylases which are subject to any demonstrated controls like those found in animal tissues (Lane and Moss, 1971).

There is ample evidence that malic enzyme is important in photosynthesis in plants with the C_4 dicarboxylic acid pathway (Johnson and Hatch, 1970) so that its interaction with the enzymes present in the complex has been implied. Malic enzyme is currently considered a major source of the reduced cofactor NADPH which provides H for the de novo biosynthesis of fatty acids (Rous, 1971). Young and colleagues (1964) provided biochemical confirmation of the hypothesis that oxaloacetate generated by cleavage of citrate following its diffusion out of mitochondria is immediately converted to malate by the use of NADH. Then malate is acted upon by malic enzyme with the reduction of $NADP^+$ to NADPH, and the latter provides H for fatty acid biosynthesis. They found that the activity of malic enzyme correlated with the changes in the rate of lipogenesis induced by fasting or refeeding.

Thus, there is a great deal of biochemical evidence from diverse sources to support our explanation of the observed association of phosphoenolpyruvate carboxylase, malate dehydrogenase, and acetyl-CoA carboxylase within a single complex and its relationship to malic enzyme. Furthermore, the present work is the first evidence, to the authors' knowledge, for proposing a means of regulating fatty acid biosynthesis through control of acetyl-CoA carboxylase activity in living materials where the latter enzyme, unlike those isolated from animal tissues, has not been shown to be responsive to effectors.

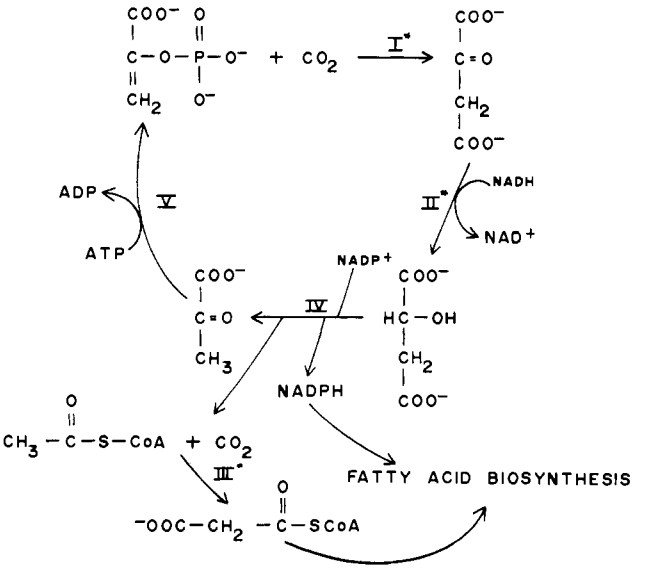

FIGURE 5: Metabolic scheme for CO_2 fixation to provide substrates for fatty acid biosynthesis. I*, II*, and III* are the enzymes found within the complex, phosphoenolpyruvate carboxylase, malate dehydrogenase, and acetyl-CoA carboxylase, respectively. IV represents malic enzyme which has been shown to be present in *Euglena*. V is pyruvate phosphokinase which was detected in *Euglena* by Drobnica and Ebringer (1962).

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