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Structural Relationship of Biotin-Containing Enzymes

Acetyl-CoA Carboxylase and Pyruvate Carboxylase from Yeast

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Acetyl-CoA carboxylase from yeast was isolated in homogeneous form and compared in its properties with pyruvate carboxylase from yeast. Both enzymes have very similar sedimentation coefficients and molecular weights. Both enzymes are composed of four protomers. Acetyl-CoA carboxylase and pyruvate carboxylase split under identical conditions into a variety of aggregates; besides the protomer, dimeric, trimeric and polymeric forms are found. Patterns of the dissociated enzymes obtained by sedimentation in sucrose density gradients and by electrophoresis in polyacrylamide are almost identical.

Within the limits of sensitivity of the immunochemical techniques used in this study no cross reaction could be observed between anti-acetyl-CoA carboxylase and pyruvate carboxylase. This indicates that the substructures catalyzing the ATP-dependent carboxylation of biotin, common to both enzymes are not based on identical primary structures. From these results it is proposed that the genes for acetyl-CoA carboxylase and pyruvate carboxylase may have been derived from a common ancestor.

All known biotin-containing enzymes [1] catalyze a carboxylation of their substrates. The overall reactions catalyzed by these enzymes proceed in two steps. The carboxylated biotinyl prosthetic group plays a central role in the reaction sequence. The initial step involves the formation of this *N*-carboxy-biotinyl intermediate. Regarding the source of this carboxyl group, the biotin-containing enzymes can be divided into two classes. Enzymes of class I utilize bicarbonate as carboxyl donor and require ATP to drive the formation of the new carbon-nitrogen bond. Biotin-containing enzymes belonging to this group include acetyl-CoA carboxylase, propionyl-CoA carboxylase, 3-methylcrotonyl-CoA carboxylase and pyruvate carboxylase. Enzymes of class II catalyze the formation of the carboxy-biotinyl intermediate by an ATP-independent trans-carboxylation with either a 3-oxo acid or a malonyl-CoA derivative serving as carboxyl donor. Enzymes belonging to this class include oxaloacetate decarboxylase and methylmalonyl-CoA : pyruvate transcarboxylase.

Enzymes. Acetyl-CoA carboxylase (EC 6.4.1.2); pyruvate carboxylase (EC 6.4.1.1); propionyl-CoA carboxylase (EC 6.4.1.3); methylcrotonyl-CoA carboxylase (EC 6.4.1.4); methylmalonyl-CoA carboxyltransferase (EC 2.1.3.1); pyruvate decarboxylase (EC 4.1.1.1.1); aldolase or fructose-1,6-bisphosphate D-glyceraldehyde-3-phosphate-lyase (EC 4.1.2.13); catalase (EC 1.11.1.6); malate dehydrogenase (EC 1.1.1.37).

In the second step enzymes of class I and II transfer the carboxyl group from the carboxy-biotinyl intermediate to an appropriate acceptor substrate. The chemical nature of this acceptor depends on the specific enzyme involved.

Recently it has been demonstrated with a bacterial biotin-containing enzyme [2–7] that the two catalytic steps are catalyzed by two distinct subunits. There is in addition a small polypeptide chain carrying the biotinyl prosthetic group [4, 5, 8, 9]. Hence each of the two partial steps in the action of biotin-containing enzymes from procaryotic organisms seems to be catalyzed by a particular subunit enzyme. The chemical reaction catalyzed by a given biotin-containing enzyme may therefore result from the specific combination of such subunit enzymes.

ATP-dependent carboxylations of the biotinyl prosthetic group is a partial reaction common to all biotin enzymes of class I. It is possible that for a given organism this part structure of class I enzymes is similar or perhaps identical. If this is true, then also the different part structures catalyzing the second steps within class I enzymes should be similar to some extent, irrespective of their different substrate specificity; at least that region of the three-dimensional structure responsible for the association with the first part structure must be similar.

Pursuing this idea, we isolated two biotin-containing enzymes, acetyl-CoA carboxylase and

pyruvate carboxylase from the same organism, yeast, and investigated whether a structural relationship between these two enzymes exists.

MATERIALS AND METHODS

Chemicals

ATP, CoA, NADH, NADPH, sodium pyruvate, malate dehydrogenase, aldolase, catalase, cytochrome *c*, bovine serum albumin and pyruvate decarboxylase were purchased from Boehringer Mannheim GmbH (Mannheim, Germany). D-[carbonyl-^{14}C]Biotin, [8-^{14}C]adenosine 5′-diphosphate and [1-^{14}C]pyruvic acid were obtained from the Radiochemical Centre (Amersham, England), DEAE-cellulose, cellulose phosphate, acrylamide and *N*,*N*′-methylene-bisacrylamide from Serva (Heidelberg, Germany). Agarose was purchased from Behring-Werke (Marburg, Germany). All other chemicals were of analytical grade.

Acetyl-CoA was prepared as described by Shemin [10] and determined according to Buckel *et al.* [11]. Protein was determined by the biuret method of Beisenherz *et al.* [12].

Assay of Enzymes

Acetyl-CoA carboxylase was determined according to the procedure (assay B) of Matsuhashi *et al.* [13, 14]. One unit of acetyl-CoA carboxylase is defined as the amount of enzyme which catalyzes the carboxylation of 1 μmole acetyl-CoA per minute (corresponding to 2 μmoles NADPH consumed).

Pyruvate carboxylase activity was assayed as described by Young *et al.* [15]. Units are expressed as μmoles NADH oxidized per minute at 25 °C and pH 7.8.

Exchange of [8-^{14}C]ADP with ATP was assayed according to the procedure of Lynen *et al.* [16].

For the assay of exchange of [1-^{14}C]pyruvate with oxaloacetate, catalyzed by pyruvate carboxylase, the following substances were mixed in a test tube: 50 μmol Tris-HCl pH 7.5, 1.0 μmol [1-^{14}C]-pyruvate (7000 counts/min), 1.0 μmol oxaloacetate and water to a final volume of 0.50 ml. The reaction was initiated by the addition of enzyme and after incubation at 30 °C for various times (2—10 min) aliquots of 0.15 ml were mixed with 0.025 ml of trichloroacetic acid (12%) at 0 °C to terminate the reaction. The pH of the mixture was adjusted to 6 by the addition of 1 N KOH and 0.05 ml of 1 M citrate pH 6.0 was added. The radioactive label of [1-^{14}C]pyruvate was removed as $^{14}CO_2$ by addition of 2 U pyruvate decarboxylase and incubation at 30 °C. After 10 min the solution was acidified with acetic acid at 0 °C and transferred quantitatively to a strip of Whatman-1 paper (3×7 cm). After drying (to remove $^{14}CO_2$ derived from [1-^{14}C]pyruvate) the stable radioactivity ([1-^{14}C]oxaloacetate) was counted in a liquid scintillation counter.

PURIFICATION OF ENZYMES

Acetyl-CoA Carboxylase from Yeast

All operations were carried out at 4 °C. Brewer's yeast (4 kg, wet weight), from Löwenbräu (Munich) was washed twice with about 6 l 0.1 M potassium phosphate pH 6.5 and cells were collected by centrifugation.

Step 1. Disruption of the Cells. The washed cells (4 kg) were suspended in 6 l of 0.2 M potassium phosphate pH 6.5, containing 1 mM EDTA and each 250 ml suspension was agitated with 250 g glass beads (size 31/10; Dragonwerk Wild, Bayreuth), in the cell homogenizer of Merkenschlager *et al.* [17], operated at 0 °C to 5 °C and run for 40 sec. The combined crude extracts were centrifuged at 15000×*g* for 40 min.

Step 2. Ammonium-Sulfate Fractionation. The crude extract from step 1 was taken to 40% saturation by the addition of ammonium sulfate. The precipitate, which contained the acetyl-CoA carboxylase activity was collected by centrifugation at 15000×*g* for 60 min.

Step 3. Ultracentrifugation. The precipitate was dissolved in 1000 ml of 0.1 M potassium phosphate pH 6.5, containing 1 mM 2-mercaptoethanol. The cloudy liquid was centrifuged at 100000×*g* for 90 min and the supernatant liquid which contained the acetyl-CoA carboxylase activity was saved.

Step 4. Ammonium-Sulfate Fractionation (0 to 35%). The supernatant fraction from stage 3 was taken to 35% saturation by the addition of ammonium sulfate. After stirring for 20 min, the precipitate was collected by centrifugation at 15000×*g* for 40 min.

Step 5. Polyethylene-Glycol Fractionations. The collected precipitate from step 4 was dissolved in 0.1 M potassium phosphate pH 6.5 containing 1 mM 2-mercaptoethanol. The protein concentration was lowered to 15—20 mg/ml by addition of buffer (about 2000 ml). 230 ml of 50% (w/w) aqueous polyethylene glycol (average molecular weight: 1500) per 1000 ml protein solution were added with stirring and the solution was stirred for a further 30 min. The resulting precipitate was removed by centrifugation at 15000×*g* for 40 min. An additional 220 ml of 50% aqueous polyethylene glycol solution per 1000 ml initial volume of the protein solution was added to the supernatant. The resulting precipitate was collected by centrifugation at 15000×*g* for 40 min. The pellet, which contained the enzyme, was dissolved in 0.05 M potassium phosphate pH 6.5 to a protein concentration of about 10 mg/ml. Fore each 100 ml of protein solution, 5 g solid ammonium sulfate and 10 ml 50% (w/w) aqueous

solution of polyethylene glycol (average molecular weight: 6000) was added. The resulting precipitate was removed by centrifugation at 15000 × *g* for 30 min. The acetyl-CoA carboxylase was then precipitated by further addition of 10 ml polyethylene glycol solution per 100 ml initial volume. The sample was stirred for 30 min and the resulting precipitate was collected by centrifugation at 15000 × *g* for 40 min.

Step 6. DEAE-Cellulose Chromatography. The pellet from step 5 was dissolved in approximately 20 ml 0.02 M potassium phosphate pH 7.5, containing 20% glycerol and 2 mM $MgCl_2$. Water, containing 20% glycerol was added to the solution until its conductance was equal to that of the equilibrating buffer. The solution was applied to a DEAE-cellulose column (4.6 × 30 cm) equilibrated with the above buffer. Elution was carried out with a linear concentration gradient established between 1.5 l buffer containing 50 mM NaCl and 1.5 l containing 200 mM NaCl. The fractions of high specific activity (corresponding to those between approximately 1400 and 2100 ml of effluent volume) were taken to 50% saturation with solid ammonium sulfate and centrifuged at 15000 × *g* for 60 min. The precipitate was dissolved in a minimum volume of 20 mM potassium phosphate pH 6.5 containing 20% glycerol.

Step 7. Cellulose-Phosphate Chromatography. Water containing 20% glycerol was added to the resulting protein solution until its conductance was equal to that of the equilibrating buffer: 20 mM potassium phosphate pH 6.5 containing 20% glycerol. This solution was applied to a column (3.6 × 20 cm) of cellulose phosphate. Elution was carried out with a linear concentration gradient established between 1.2 l 50 mM KCl in buffer and 1.2 l of 220 mM KCl in buffer. The acetyl-CoA carboxylase appeared in the eluate after about 1600 ml. The fractions containing acetyl-CoA carboxylase were pooled and the protein was precipitated by addition of solid ammonium sulfate.

Storage. The pure enzyme could be kept at least for 4 months without any loss of activity when stored in 0.3 M potassium phosphate pH 6.5 containing 50% glycerol at −15 °C.

Purification of Pyruvate Carboxylase

All operations were carried out at room temperature. Yeast cells were cultured under the conditions of Young *et al.* [15] with the following changes made: ergosterol was omitted, the amount of yeast extract reduced to 5 g/l and the amount of lactate (60%) increased to 10 ml/l. The purification procedure was carried through with about 400 g (wet weight) yeast cells.

The enzyme purification throughout steps 1 to 4 preparation of the crude extract, heat denaturation, protamine sulfate treatment and ammonium sulfate fractionation) was performed using the procedure of Young *et al.* [15]. Further purification was achieved by the following steps.

Step 5. Polyethylene-Glycol Fractionation. The combined extracts of step 4 containing the pyruvate carboxylase activity were taken to 45% saturation by the addition of solid ammonium sulfate. The precipitate, which contained all the pyruvate carboxylase activity was collected by centrifugation at 15000 × *g* for 20 min and the pellet was dissolved in 80 ml standard buffer [15]. 40 ml of a 50% (w/w) aqueous solution of polyethylene glycol (average molecular weight 1500) was added and the resulting precipitate was removed by centrifugation at 15000 × *g* for 30 min.

Step 6. DEAE-Cellulose Chromatography. The clear supernatant solution was dialyzed overnight against 1 l 20 mM potassium phosphate pH 7.5, containing 2 mM $MgCl_2$ and 20% glycerol with a change of buffer after 2 h. The dialyzed solution was applied to a DEAE-cellulose column (2.5 × 32 cm) equilibrated previously with the dialysis buffer and the enzyme then eluted with 700 ml of linear gradient of NaCl (0.05 M to 0.20 M) in the same buffer. Carboxylase appeared in the eluate when the NaCl concentration reached approximately 0.15 M. Fractions containing activity were combined and the enzyme precipitated by addition of solid ammonium sulfate to a final concentration of 60% saturation.

Step 7. Sucrose Density-Gradient Centrifugation. The precipitate from step 6 was collected by centrifugation, dissolved in about 4 ml of the standard buffer [15] and dialyzed against 400 ml of the same buffer for 2—4 h. The dialyzed solution was layered over 4 tubes of the Spinco L 2-65 SW 27 rotor containing a 10—25% sucrose density gradient in the standard buffer. The gradient tubes were centrifuged for 36 h at 27000 rev./min. The fractions containing pyruvate carboxylase were pooled and the enzyme was precipitated by ammonium sulfate as described above (step 6). The enzyme was dissolved in 1 ml 50 mM potassium phosphate pH 7.5 containing 1 mM 2-mercaptoethanol, 0.02% sodium azide and 20% glycerol.

Storage. The enzyme could be stored over a month at 4 °C without loss of activity. Storage at lower temperatures resulted in loss of activity.

PREPARATION OF [^{14}C]BIOTIN-LABELLED ENZYMES

[^{14}C]Biotin-Labelled Acetyl-CoA Carboxylase

Aspartic acid [18] and fatty acids, added to the growth medium of *Saccharomyces cerevisiae* are able to replace biotin for growth. With these compounds added *de novo* synthesis of C_4-dicarboxylic and fatty acids becomes unnecessary and therefore the activity of the biotin-containing enzymes acetyl-CoA carb-

oxylase and pyruvate carboxylase is not required for growth under these conditions. On the basis of this observation [^{14}C]biotin-labelled acetyl-CoA carboxylase was prepared.

Yeast was growth aerobically at 30 °C for 36 h in a medium containing per liter of solution: 50 g glucose, 0.55 g K_2HPO_4, 0.125 g $MgSO_4$, 0.125 g $CaCl_2$, 0.4 g KCl, 2.5 mg $MnSO_4$, 2.5 mg $FeCl_3$, 5 g potassium citrate, 1 g citric acid, 3.75 g $(NH_4)_2SO_4$, 1 mg pantothenic acid, 0.2 mg *p*-aminobenzoic acid, 0.4 mg nicotinic acid, 0.4 mg pyridoxine, 0.4 mg thiamine, 25 mg inositol, 10 mg L-histidine, 20 mg DL-methionine, 20 mg DL-tryptophan, 10 ml Tween 40, 2 ml oleic acid and 1.8 g aspartic acid. The cells were harvested by centrifugation and then resuspended (about 30 g cells per liter) in the same medium (without oleic and aspartic acid and detergent) containing in addition 20 μg to 100 μg [^{14}C]biotin. This suspension was then incubated with shaking on a rotatory flask agitator for 2 h at 30 °C. The rate of appearance of acetyl-CoA carboxylase activity was not inhibited by the addition of cycloheximide to the medium, indicating that acetyl-CoA carboxylase pre-exists as apoenzyme in the yeast cells. After harvesting and washing the cells, the purification of the radioactive enzyme was carried through as described above for the nonlabelled acetyl-CoA carboxylase.

The same procedure was applied for the preparation of the acetyl-CoA carboxylase apoenzyme, omitting the biotin treatment after growth. The apoenzyme was isolated in the same way described above for the holoenzyme.

[^{14}C]Biotin-Labelled Pyruvate Carboxylase

One liter of the culture medium already decribed for the nonlabelled enzyme contained in addition 2 μg [^{14}C]biotin of high specific radioactivity (about 30—50 mCi/mmol).

OTHER METHODS

Preparation of Antibodies

The purified and concentrated acetyl-CoA carboxylase preparation from the cellulose phosphate step was diluted with 0.3 M potassium phosphate pH 6.5 to give a protein concentration of 5 mg/ml. The following inoculating schedule was used: 1 ml acetyl-CoA carboxylase solution was mixed with an equal volume of Freund's adjuvant and injected subcutaneously in several portions over the back of rabbits. Two weeks later 5 mg enzyme without adjuvant were injected intraperitoneally. This series was repeated twice, starting two weeks later. The rabbits were bled from the central artery of the ear and serum precipitin titers were followed. 8 days after the last injection blood was removed by cardiac puncture.

The same inoculating schedule was applied for the preparation of antibody to pyruvate carboxylase, except that the amount of enzyme given per injection was 1 mg.

γG-Immunoglobulin fractions were prepared from the sera by precipitation from 40% saturated ammonium sulfate at 4 °C; after dialysis of the redissolved precipitate the antibody preparation was chromatographed on DEAE-cellulose as described in [19].

Electrophoretic Methods

Double diffusion (Ouchterlony) and immunoelectrophoresis plates were prepared using 1% agarose in buffers as indicated in the legends, and adding 20 mg % sodium azide.

Buffer system 1 described by Maurer [20] was used in analytical electrophoresis in 6% polyacrylamide gel.

RESULTS

Purification and Purity of Acetyl-CoA Carboxylase from Brewer's Yeast

Matsuhashi and Lynen [13] described a purification procedure for acetyl-CoA carboxylase from yeast. Enzyme preparations obtained by this procedure contain about 10% of pure acetyl-CoA carboxylase in the best preparations. To obtain a homogeneous enzyme a new purification procedure was developed.

Acetyl-CoA carboxylase was isolated from fresh brewer's yeast. This source contains a 5 to 10-fold higher enzyme activity in the crude extract than bakers' yeast, as was found by Rominger [21]. This fact is due to a low content of biotin in the growth medium of bakers' yeast [21].

The acetyl-CoA carboxylase was purified by ammonium sulfate fractionation, ultracentrifugation, fractionation by polyethylene glycols and by chromatography on DEAE-cellulose and on cellulose phosphate, as described in the experimental part. The results of a typical purification are summarized in Table 1. The specific activity obtained was generally 6 U/mg protein at 25 °C. No activation of the enzyme by citrate was observed.

No higher specific activity was achieved by further purification procedures. Neither gel filtration with Sephadex G-200 nor chromatography on hydroxyapatite resulted in a further fractionation of the protein. Acetyl-CoA carboxylase which had been submitted to the purification procedure described in the experimental part sedimented as a single, symmetrical protein peak with no evidence of impurity.

No enzymatic activity of other biotin-containing enzymes could be detected in the acetyl-CoA carboxylase preparations. Pyruvate carboxylase was

Table 1. *Purification of acetyl-CoA carboxylase from yeast*
4 kg (wet weight) yeast were used. Enzyme activity was determined at 25 °C by the spectrophotometric assay. In the crude extract, no reliable results could be obtained using the spectrophotometric assay

Fraction	Protein	Total activity	Specific activity	Recovery
	mg	units	mU/mg	%
Crude extract	—	—	—	—
1st $(NH_4)_2SO_4$	115000	(3100)	27	100
Ultracentrifugation	60000	3000	50	96
2nd $(NH_4)_2SO_4$	20400	2650	130	84
Polyethylene glycol	2100	2100	1000	68
DEAE-cellulose	400	1450	3600	47
Phosphocellulose	175	1050	6000	34

removed at step 5 (first polyethylene glycol fractionation), where this enzyme remained in the supernatant.

Reversible Inactivation of Acetyl-CoA Carboxylase

Acetyl-CoA carboxylase was inactivated by low ionic strength and alkaline pH. High ionic strength and pH values between 5.5 and 7.0 stabilised the enzyme activity.

Addition of glycerol to enzyme solutions prevented inactivation. As shown below, inactivation at alkaline pH was accompanied by change in physical properties of the enzyme, indicating dissociation into subunits. Acetyl-CoA carboxylase, inactivated by low ionic strength and alkaline pH, could be reactivated to about 40% of its original activity upon transfer to concentrated potassium phosphate buffer pH 6.5 containing 20% glycerol and 10 mM dithiothreitol. The reactivation of acetyl-CoA carboxylase to an active enzyme was temperature dependent. The best reactivation occurred at 0 °C. At 25 °C scarcely any reactivation was observed. Up to the present, a systematic study of the parameters involved hat not been undertaken. Table 2 describes the results of a typical experiment.

Purification of Pyruvate Carboxylase from Yeast

Pyruvate carboxylase was isolated from brewer's yeast grown on a lactate-containing medium as described by Young *et al.* [15]. The purification procedure of these authors was changed in the last three steps, as described in the experimental part. The specific activity obtained was usually 25 U/mg protein. No higher specific activity was achieved by an additional chromatography on cellulose phosphate [15]. The enzyme appeared homogeneous in the ultracentrifuge and showed no contamination in immunoelectrophoresis as documented below (Fig. 12). No enzymatic activity of acetyl-CoA carboxylase could be detected in the pyruvate carboxylase preparations.

Table 2. *Inactivation and reactivation of acetyl-CoA carboxylase*
Enzyme solution (20 mg/ml in 50% glycerol) was diluted 1:25 with 0.1 M Tris-HCl pH 8.5 and incubated at 0 °C. After 7 h the enzyme activity was reactivated by adding an equal volume of 1 M potassium phosphate pH 6.5 containing 20% glycerol and 10 mM dithiothreitol to the inactivated enzyme solution. The incubation was at 0 °C

Enzyme	Specific activity	Activity *cf.* original
	U/mg	%
Native enzyme	6.1	100
Inactivation at pH 8.5		
2 h	0.9	15
7 h	0.3	5
Reactivation at pH 6.5		
12 h	1.3	21
24 h	1.9	31
72 h	2.8	46

Comparative Studies of the Biotin-Containing Enzymes Acetyl-CoA Carboxylase and Pyruvate Carboxylase

To study possible structural relationships of acetyl-CoA carboxylase and pyruvate carboxylase the following characteristics were investigated: molecular weight, dissociation into subunits, sedimentation and electrophoretic behaviour of the subunits and immunochemical assays of the enzymes and their subunits.

Sedimentation Coefficients and Molecular Weights

The sedimentation coefficient of acetyl-CoA carboxylase was measured by sucrose density gradient centrifugation according to the method of Martin and Ames [22]. The average value of eight experiments was 15.5 ± 0.1 S, when catalase was used as reference ($s_{20,w}$ = 11.3 S [23]). For pyruvate carboxylase from yeast Young *et al.* [15] found a sedimentation coefficient of 15.6 S, which is in good agreement with the above value found for acetyl-CoA carboxylase. To compare both enzymes directly acetyl-CoA carboxylase and pyruvate carboxylase were centrifuged together in the same tube. Fig. 1 shows the result of this experiment. After centrifugation at 39000 rev./min in a SW-40 swinging-bucket rotor for 15 h no separation of the two enzymes could be observed. This means that the sedimentation coefficients of acetyl-CoA carboxylase and pyruvate carboxylase are identical within the experimental error of ± 0.2 S.

For pyruvate carboxylase from yeast Young *et al.* [24] determined a molecular weight of 600000.

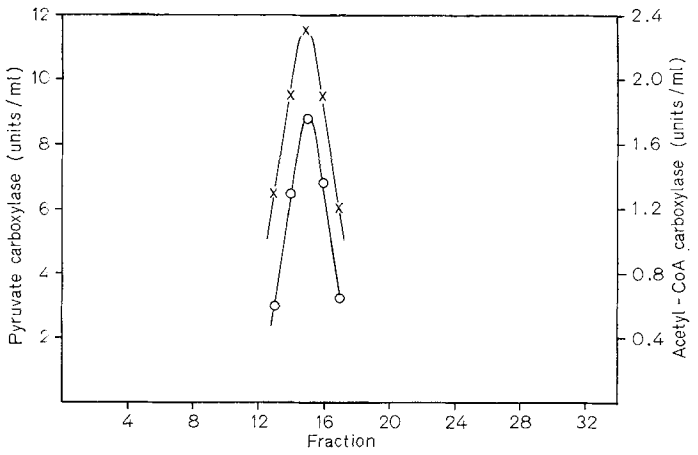

Fig. 1. *Comparison of the sedimentation behaviour of acetyl-CoA carboxylase and pyruvate carboxylase.* 2 mg pyruvate carboxylase and 3 mg acetyl-CoA carboxylase were mixed together and layered over a linear sucrose gradient (5—20%) in 0.3 M potassium phosphate pH 6.5. Centrifugation was at 39000 rev./min (SW-40 rotor, L2-ultracentrifuge) for 15 h at 8 °C. The gradient was fractionated from the bottom. Pyruvate carboxylase (○); acetyl-CoA carboxylase (×)

From the finding of identical sedimentation behaviour in sucrose density gradient centrifugation it can be estimated that both enzymes have very similar molecular weights [22].

Dissociation of Acetyl-CoA Carboxylase and Pyruvate Carboxylase

A rapid inactivation of acetyl-CoA carboxylase occurred at pH values above 7.5. This loss of enzymatic activity was accompanied by a change of sedimentation behaviour (Fig. 2A). To demonstrate this effect acetyl-CoA carboxylase was dialyzed against 0.1 M ammonia containing 10 mM 2-mercaptoethanol at 20 °C for 5 h and then centrifuged in a sucrose density gradient. Fig. 2A shows the dissociation of acetyl-CoA carboxylase into subunits with sedimentation coefficients of approximately 12, 9 and 6 S. These values were estimated by comparison with catalase [23], aldolase [25] and cytochrome *c* as reference proteins.

Pyruvate carboxylase, treated in the same way, showed a sedimentation pattern of remarkable similarity to that of acetyl-CoA carboxylase. The different subunits of acetyl-CoA carboxylase and pyruvate carboxylase sedimented within the experimental error at identical rates (Fig. 2B). In both cases, subunits with sedimentation coefficients less than 15 S could no longer catalyze the carboxylation of acetyl-CoA and pyruvate, respectively.

Both in the case of acetyl-CoA carboxylase and in the case of pyruvate carboxylase, the relative amounts of the 12-S, 9-S and 6-S subunits in the sedimentation patterns were affected by temperature.

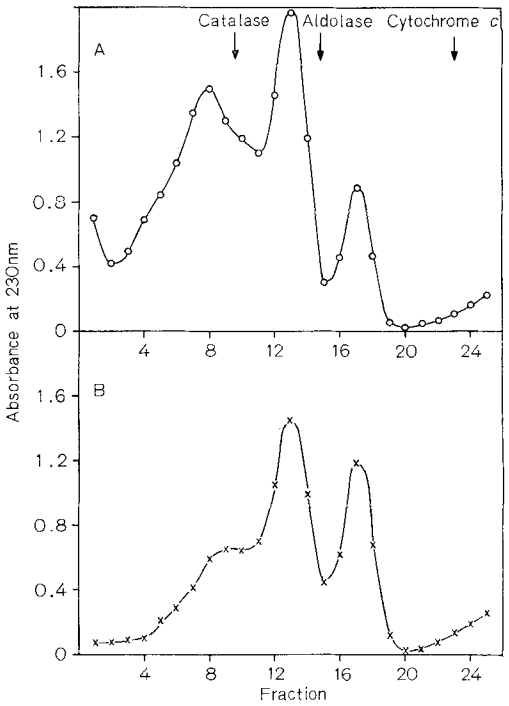

Fig. 2. *Sucrose density-gradient centrifugation of dissociated acetyl-CoA carboxylase (A) and pyruvate carboxylase (B).* The enzymes were dialyzed against 0.1 M ammonia containing 10 mM 2-mercaptoethanol for 5 h at 20 °C. Protein concentration during dissociation was 15 mg/ml. The enzyme solutions were layered over a linear sucrose gradient (5—20%) in 0.1 M Tris-HCl pH 9.0 and centrifuged for 16.5 h at 39000 rev./min (SW-40 rotor, L2-ultracentrifuge). The temperature was 6 °C. Markers were included in separate tubes. The gradients were fractionated from the bottom

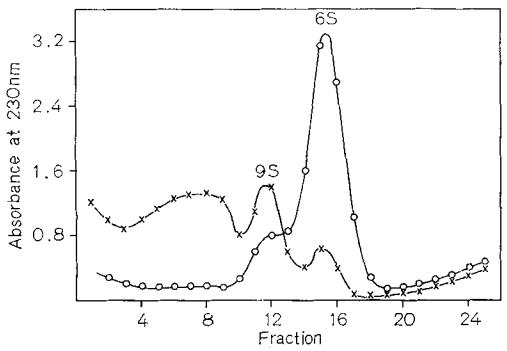

Fig. 3. *Sedimentation behaviour of pyruvate carboxylase dissociated at 25 °C and 4 °C, respectively.* Two samples of pyruvate carboxylase (15 mg/ml) were dialyzed against 0.1 M ammonia, containing 10 mM 2-mercaptoethanol at 25 °C and at 4 °C for 5 h. The dialyzed solutions were layered over sucrose gradients (5—20%) in 0.1 M Tris-HCl pH 9.0 and centrifuged for 19 h at 39000 rev./min (SW-40 rotor, L2-ultracentrifuge). The temperature during centrifugation was 6 °C. The gradients were fractionated from the bottom. ×, Dissociation at 25 °C; ○, dissociation at 4 °C

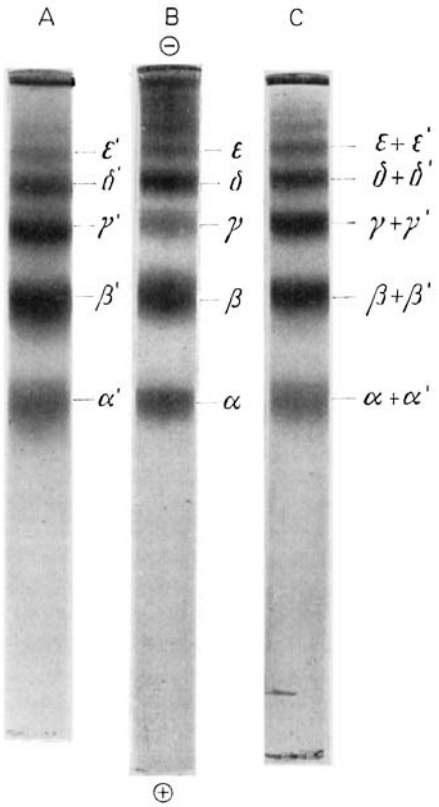

Fig. 4. *Polyacrylamide-gel electrophoresis of dissociated acetyl-CoA carboxylase and pyruvate carboxylase.* The enzymes were dialyzed against 0.1 M ammonia containing 10 mM 2-mercaptoethanol for 3 h at 20 °C. (A) Pyruvate carboxylase; (B) acetyl-CoA carboxylase; (C) pyruvate carboxylase and acetyl-CoA carboxylase. Approximately 50 µg enzyme was layered onto the gels. For details see Materials and Methods

If the enzymes were dissociated at lower temperatures the amount of the 12-S component decreased in favour of the 9-S and 6-S components. The pattern of pyruvate carboxylase was particularly strongly influenced by temperature. At 2 °C to 4 °C this enzyme yielded only the 6-S subunit upon dissociation. Fig. 3 compares the sedimentation patterns of pyruvate carboxylase dissociated at 25 °C and 4 °C, respectively.

Furthermore, as found for acetyl-CoA carboxylase the distribution of protein in the 12-S, 9-S and 6-S components was influenced by the biotin coenzyme. Apoenzyme of acetyl-CoA carboxylase (no differene in the sedimentation behaviour was observed compared with the holoenzyme) yielded upon dissociation no 12-S but mainly the 9-S and the 6-S components in nearly equal amounts. Compared with the holoenzyme, dissociated under identical conditions, the distribution had shifted in favour of the smaller components. Fig. 5 demonstrates this effect in the electrophoretic pattern (see also below). Pyruvate carboxylase apoenzyme was not available for this comparison.

Dissociation of acetyl-CoA carboxylase and pyruvate carboxylase could also be followed by electrophoresis in polyacrylamide gels. Fig. 4 presents the electrophoretic behaviour of both enzymes dissociated under identical conditions at alkaline pH. The patterns of acetyl-CoA carboxylase and pyruvate carboxylase were again very similar. The corresponding protein bands migrate at virtually identical rates as shown by coelectrophoresis of dissociated acetyl-CoA carboxylase and pyruvate carboxylase (Fig. 4C): no separation of the corresponding bands of acetyl-CoA carboxylase and pyruvate carboxylase, α and α', β and β' *etc.* could be observed.

By comparison of the electrophoretic behaviour of the isolated 12-S, 9-S and 6-S subunits (isolated by sucrose density gradient centrifugation) with the electrophoretic pattern of the dissociated enzymes it could be shown that the 12-S component is identical with band γ, the 9-S component is identical with protein band β and the 6-S component is identical with band α. Fig. 6 shows this experiment for the pyruvate carboxylase pattern. The same result was obtained for the subunits of acetyl-CoA carboxylase.

Dissociation in the Presence of p-*Chloromercuribenzoate*

p-Chloromercuribenzoate caused a rapid loss of enzymatic activity of acetyl-CoA carboxylase and pyruvate carboxylase. It appeared possible that *p*-chloromercuribenzoate might also influence the dissociation behaviour. To test this possibility, both enzymes were dialyzed against 0.1 M Tris pH 8.5 containing 0.25 mM *p*-chloromercuribenzoate for 3 h at room temperature. The degree of dissociation

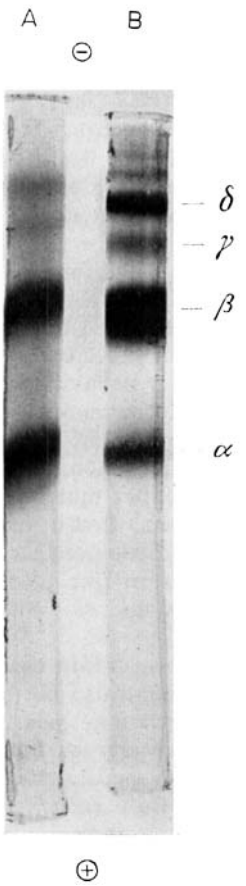

Fig. 5. *Polyacrylamide-gel electrophoresis of dissociated acetyl-CoA carboxylase apoenzyme and holoenzyme.* Apoenzyme and holoenzyme were dialyzed against 0.1 M ammonia containing 10 mM 2-mercaptoethanol for 3 h at 20 °C. Electrophoresis of aliquots (30--60 μg) was carried out as described in the experimental part. (A) Apoenzyme; (B) holoenzyme

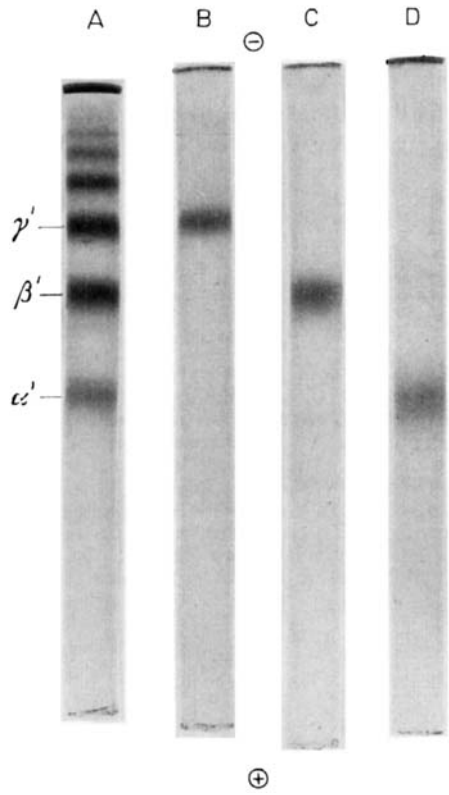

Fig. 6. *Polyacrylamide-gel electrophoresis of dissociated pyruvate carboxylase and the 12-S, 9-S and 6-S components (isolated by sucrose density-gradient centrifugation).* (A) Dissociated pyruvate carboxylase; (B) 12-S subunit; (C) 9-S subunit; (D) 6-S subunit. Electrophoresis were carried out as described in the experimental part

(and aggregation, see below) was followed by electrophoresis in polyacrylamide gels. Fig. 7 presents the patterns obtained, which indicate an extensive, very similar splitting of acetyl-CoA carboxylase and pyruvate carboxylase into seven protein bands. Incubation of the enzymes without *p*-chloromercuribenzoate caused only little splitting under the same conditions. The similarity of the electrophoretic patterns formed by treating acetyl-CoA carboxylase and pyruvate carboxylase with *p*-chloromercuribenzoate or by incubating at alkaline pH suggests that the enzymes split into the same subunit species under these different sets of conditions. Compared with the dissociation pattern at alkaline pH (0.1 M ammonia) the intensity distribution between the protein bands was changed in favour of the slow migrating bands *γ*, *δ*, *ε etc.* (especially for pyruvate carboxylase).

Characterization of the Subunits of Acetyl-CoA Carboxylase and Pyruvate Carboxylase

As shown above, the distribution of protein between the various subunits of acetyl-CoA carboxylase and pyruvate carboxylase was by no means a constant relation. This distribution was in contrast highly dependent on the conditions of dissociation used. Especially in the case of pyruvate carboxylase the heavy 12-S and 9-S components were nearly quantitatively convertible into the 6-S subunit (Fig. 3). Because all of the protein of the native enzyme can be converted to the 6-S subunit this species must be the smallest unit containing the complete primary structure of the native enzyme. Also the acetyl-CoA carboxylase apoenzyme split preferably into the small 9-S and 6-S subunits. These observations may be interpreted to mean that the heavier components of the dissociated enzymes are aggregates of different numbers of 6-S subunits. To investigate this possibility acetyl-CoA carboxylase and pyruvate carboxylase labelled with [^{14}C]biotin were dissociated by alkaline pH and the

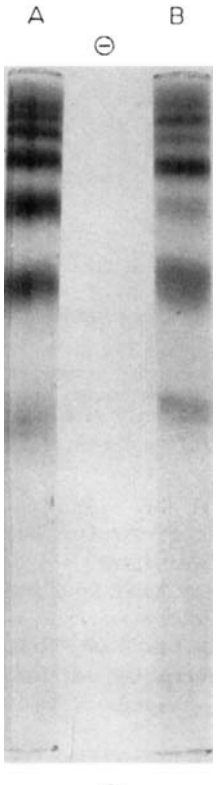

Fig. 7. *Polyacrylamide-gel electrophoresis of acetyl-CoA carboxylase and pyruvate carboxylase, dissociated in the presence of* p-*chloromercuribenzoate.* Acetyl-CoA carboxylase (2 mg/ml) and pyruvate carboxylase (2 mg/ml) were dialyzed against 0.1 M Tris-HCl pH 8.5 containing 0.25 mM *p*-chloromercuribenzoate for 3 h at 20 °C and then layered onto the gels. (A) Pyruvate carboxylase; (B) acetyl-CoA carboxylase. For details see Materials and Methods

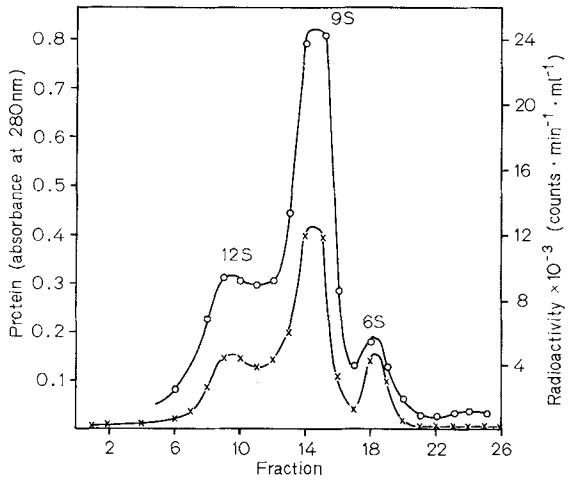

Fig. 8. *Distribution of protein and radioactivity of dissociated acetyl-CoA carboxylase following density-gradient centrifugation.* Acetyl-CoA carboxylase (55000 counts × min^{-1} × mg^{-1}) labelled with [^{14}C]biotin was dialyzed against 0.1 M ammonia containing 10 mM 2-mercaptoethanol for 6 h at 15 °C. The enzyme solution (about 1.5 mg protein) was layered over a linear sucrose gradient (5—20%) in 0.1 M Tris-HCl pH 9.0 and centrifuged for 18 h at 39000 rev./min (SW-40 rotor, L2-ultracentrifuge). The temperature was 6 °C. The gradient was fractionated from the bottom and each fraction was monitored for protein concentration (×) and radioactivity (○)

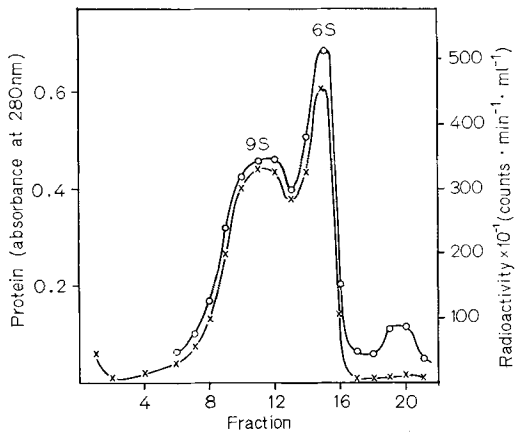

Fig. 9. *Distribution of protein and radioactivity of dissociated pyruvate carboxylase following density-gradient centrifugation.* Pyruvate carboxylase (10000 counts × min^{-1} × mg^{-1}) labelled with [^{14}C]biotin was dialyzed against 0.1 M ammonia containing 10 mM 2-mercaptoethanol for 4 h at 15 °C. The enzyme solution (about 2.5 mg) was layered over a linear sucrose gradient (5—20%) in 0.1 M Tris-HCl pH 9.0 and centrifuged for 17 h at 39000 rev./min (SW-40 rotor, L2-ultracentrifuge). The temperature was 7 °C. The gradient was fractionated from the bottom and each fraction was monitored for protein concentration (×) and radioactivity (○)

distribution of radioactivity in the subunits was determined by sucrose density gradient centrifugation. With the assumption of identical 6-S subunits existing as monomers, dimers, trimers *etc.* one would predict a constant specific radioactivity across the sedimentation pattern. Fig. 8 and 9 show that indeed the patterns of protein and radioactivity correspond to each other. Only for the 6-S subunit of acetyl-CoA carboxylase the specific radioactivity was lower than expected. Usually acetyl-CoA carboxylase preparations contain some apoenzyme [21] which dissociates in contrast to the holoenzyme preferably into the 6-S subunit (Fig. 5). Therefore a lower specific radioactivity of the 6-S subunit is found.

Further evidence for the aggregation of identical 6-S subunits was provided by immunochemical techniques. Antibodies against acetyl-CoA carboxylase and pyruvate carboxylase were prepared by injecting rabbits with the purified enzymes. Using the double diffusion Ouchterlony technique, an immunochemical comparison of the 12-S, 9-S and 6-S subunits of both enzymes was undertaken. Fig. 10A

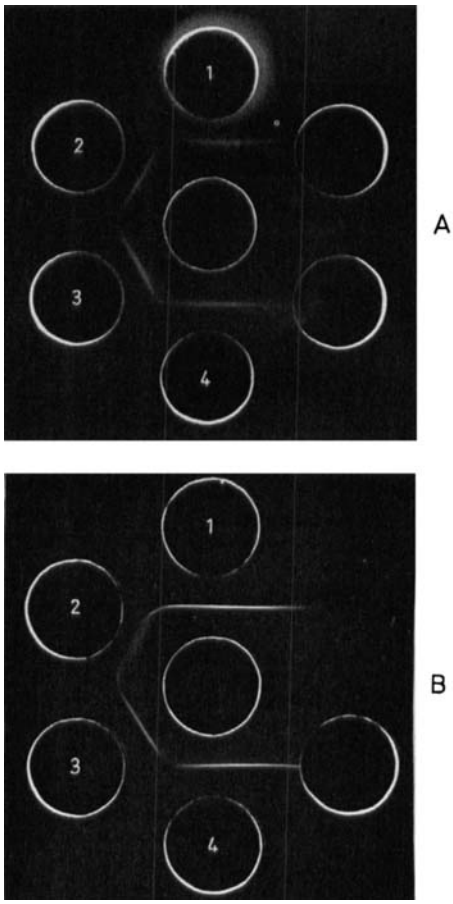

Fig. 10. *Immunodiffusion of the separable subunits of acetyl-CoA carboxylase and pyruvate carboxylase.* (A) Agarose gel (0.6%) contained 100 mM potassium phosphate pH 7.5. The plate was developed at 20 °C for 24 h. Well (1), native acetyl-CoA carboxylase; well (2), 12-S component; well (3), 9-S component; well (4), 6-S component. The center well contained antiacetyl-CoA carboxylase γ-globulin. (B) Agarose gel (0.6%) contained 50 mM Tris-HCl pH 7.5. The plate was developed at 20 °C for 48 h. Well (1), native pyruvate carboxylase (1.5 mg/ml); well (2). 12-S component; well (3), 9-S component; well (4), 6-S component. The center well contained antipyruvate carboxylase γ-globulin

shows the pattern obtained when antiacetyl-CoA carboxylase γ-globulin was applied to the centre well and the various subunits, previously separated by sucrose density gradient centrifugation, and the native enzyme were arranged peripherally. A single connecting band of precipitation was observed both in the case of acetyl-CoA carboxylase (Fig. 10 A) and in the case of pyruvate carboxylase (Fig. 10 B). The absence of spurs indicates that there are no antigenic sites present on the native enzymes that are not present on the 12-S, 9-S and 6-S subunits. These antigenic data support the concept that the native enzymes, the 12-S and the 9-S components are composed of identical 6-S subunits, aggregated into forms of different molecular weights. Only after prolonged incubation times (> 5 days) of the Ouchterlony plates a very weak spur between the 9-S and 6-S fractions was observed. This observation would be explained by the possibility that the first aggregation of the monomeric 6-S subunits into the dimers creates some few antigenic sites not present in the monomers.

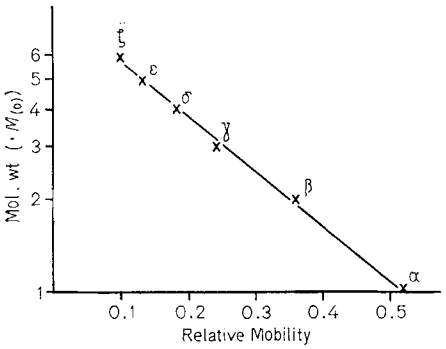

Fig. 11. *Electrophoretic mobility of the protein bands* α, β, γ etc. *of dissociated acetyl-CoA carboxylase as a function of their molecular weights (as multiples of* $M_{(0)}$ = *molecular weight of the 6-S subunit* α*)*

Using the relationship (1) between sedimentation coefficients (s) and molecular weights (M) which is approximately correct [26]

$$\frac{s_1}{s_2} = \left(\frac{M_{(1)}}{M_{(2)}}\right)^{2/3} \qquad (1)$$

an estimation of the sedimentation coefficients of the suggested dimers, trimers and tetramers of the 6-S subunit could be obtained. With the molecular weights 2 $M_{(0)}$, 3 $M_{(0)}$ and 4 $M_{(0)}$ ($M_{(0)}$ = molecular weight of the 6-S subunit) the following sedimentation coefficients were calculated: 9.5 S for a dimer, 12.0 S for a trimer and 15.1 S for a tetramer of the 6-S subunit. These values are in good agreement with the experimentally found sedimentation coefficients. According to this estimation a tetrameric structure of 6-S subunits for acetyl-CoA carboxylase and pyruvate carboxylase from yeast is very probable.

It is noteworthy in this context that the relative mobilities of the protein bands α, β, γ *etc.* (or α', β', γ' *etc.*) in the electrophoresis (Fig. 4) plotted against the logarithm of their molecular weights (as multiples of $M_{(0)}$ = molecular weight of the 6-S subunit, α and α') gave a linear relationship. Obviously the separation of these components in polyacrylamide gels is strongly dependent on their size. Similar effects in polyacrylamide gel electrophoresis were shown previously by Ornstein [27] and Davis [28] for other proteins. This interpretation of the data indicates that the slower migrating components, ε, ζ, . . . are higher aggregations with a pentamer, hexamer *etc.* arrangement of the 6-S subunit (Fig. 11).

Table 3. *Inhibition of pyruvate carboxylase by antipyruvate carboxylase γ-globulin*
Pyruvate carboxylase preparations, inhibited by antibody to 55, 70 and 90% of its original activity in the overall reaction, were assayed for partial activities. For details see Materials and Methods

Expt	Inhibition of the overall reaction	Inhibition of [^{14}C]ADP ⇌ ATP exchange reaction	Inhibition of [^{14}C]pyruvate ⇌ oxaloacetate exchange reaction
	%	%	%
1	55	50	10
2	70	75	15
3	90	—	20

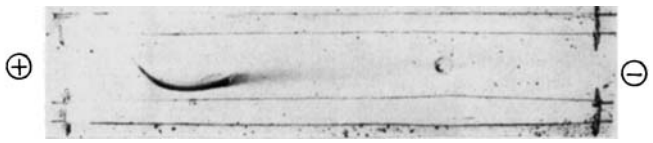

Fig. 12. *Immunoelectrophoresis of pyruvate carboxylase.* Agarose gel (1%) contained 40 mM potassium phosphate pH 7.5. Electrophoresis was performed at a constant current of 4 mA per plate for 3 h. Top trough contained antiacetyl-CoA carboxylase, lower trough contained antipyruvate carboxylase (antisera were 1:4 concentrated by precipitation from 40% saturated ammonium sulfate)

Immunochemical Comparison of Acetyl-CoA Carboxylase and Pyruvate Carboxylase

The enzyme activities of both enzymes were inhibited completely by their corresponding antisera. In order to establish insight into which step of the overall reaction was blocked by antibody action, the partial activities of pyruvate carboxylase were assayed. Table 3 demonstrates the result. The inhibition of ATP-dependent carboxylation of biotin parallels the overall inhibition, whereas the second step, namely the carboxylation of pyruvate, is only slightly influenced.

To check whether the observed similarities of acetyl-CoA carboxylase and pyruvate carboxylase are also reflected in the immunochemical properties, the reaction of antiacetyl-CoA carboxylase γ-globulin with pyruvate carboxylase was studied. Even a 20-fold excess of antibody, necessary to inhibit completely the acetyl-CoA carboxylase, has no effect on the pyruvate carboxylase activity.

In a second experiment cross reaction of antiacetyl-CoA carboxylase with pyruvate carboxylase was checked by immunoelectrophoresis. As shown in Fig. 12 no line of precipitation was formed between pyruvate carboxylase and antiacetyl-CoA carboxylase. It is therefore concluded that the first partial reaction (ATP-dependent carboxylation of biotin) common to both enzymes is catalyzed by protein substructures which are not identical in primary structure.

DISCUSSION

The data presented indicate close structural similarities between the biotin-containing enzymes acetyl-CoA carboxylase and pyruvate carboxylase from yeast. Both enzymes have very similar or identical sedimentation coefficients (and molecular weights). Both enzymes are composed of four protomers. Comparing the protomer of acetyl-CoA carboxylase with the protomer of pyruvate carboxylase, no difference was found in their sedimentation behaviour nor in their electrophoretic mobilities in polyacrylamide. Both enzymes show upon treatment with alkaline pH or *p*-chloromercuribenzoate a very similar spectrum of aggregates. Besides the protomer, dimeric, trimeric and polymeric forms are found.

Within the limits of sensitivity of the immunological techniques applied in this study no cross reaction could be observed between antiacetyl-CoA carboxylase and pyruvate carboxylase. It may therefore be concluded that the protein parts responsible for the ATP-dependent carboxylation are different in their primary structure, although having identical catalytic functions in both enzymes. On the basis of these results, we assume the genes which control the sequences of acetyl-CoA carboxylase and pyruvate carboxylase are derived from a common ancestor. By a process of one or more duplications, the ancestral gene gave rise to two or more genes which subsequently evolved independently to code for biotin enzymes with different but still similar functions. In particular the biotin enzymes acetyl-CoA carboxylase, propionyl-CoA carboxylase, 3-methylcrotonyl-CoA carboxylase and geranyl-CoA carboxylase appear to be a very closely related group, because their substrates are all homologues or vinylogues of acetyl-CoA.

Examples of gene duplication are well known [29]. An especially interesting case of duplication followed by separate evolution to different functional proteins was found for bovine α-lactalbumin and egg-white lysozyme [30, 31]. There is little doubt about the ancestral homology of these two proteins.

The idea of ancestral homology of biotin enzymes is supported by another experimental fact. All of the two-step reactions of the biotin enzymes can be accounted for by appropriate combinations of a few basic types of partial reactions. For instances the second step in the pyruvate carboxylase reaction, namely the carboxylation of pyruvate to oxaloacetate (a) and the second step in the propionyl-CoA carboxylase reaction, namely the carboxylation of propionyl-CoA to methylmalonyl-CoA (b) are combined to the overall reaction catalyzed by transcarboxylase:

$$\text{Pyruvate} + \text{E} \sim \text{CO}_2 \rightleftharpoons \text{oxaloacetate} + \text{E} \quad \text{(a)}$$

$$\text{Methylmalonyl-CoA} + \text{E} \rightleftharpoons \text{E} \sim \text{CO}_2 + \text{propionyl-CoA} \quad \text{(b)}$$

$$\text{Transcarboxylase: methylmalonyl-CoA} + \text{pyruvate} \rightleftharpoons \text{propionyl-CoA} + \text{oxaloacetate}.$$

Further evidence for the close structural relationship of biotin enzymes is obtained from the fact, that biotin:apoenzyme ligase can catalyze the acylation by biotin of apoenzymes of various types which can even be from different organisms [32].

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