PROPERTIES AND MORPHOLOGY OF BARLEY EMBRYO ACETYL CoA CARBOXYLASE

by

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Acetyl CoA carboxylase was isolated and purified from barley embryos. The purified enzyme fixed CO_2 at the rate of 7 to 7.4 μmoles per min per mg protein. It had a molecular weight of 610 000 daltons and one mole of biotin per mole of enzyme. The purified enzyme aggregates during sepharose 6 B gel filtration. Aggregation of the enzyme can be prevented by using 1% sodium chloride in the elution buffer. The biotin carboxyl carrier protein of the enzyme was identified as a small polypeptide with a molecular weight of 21 000 daltons. This peptide behaves identically to barley chloroplast biotin carboxyl carrier protein during polyacrylamide disc electrophoresis in phenol: acetic acid: urea. The morphology of purified acetyl CoA carboxylase was studied by electron microscopy using a negative staining technique. This enzyme is a globular protein with a size of 275 ± 8 Å × 192 ± 42 Å and displays characteristic cavities.

1. INTRODUCTION

Acetyl CoA carboxylase catalyses the formation of malonyl CoA from acetyl CoA and bicarbonate. This enzyme has been extracted and examined in animal tissues (15, 6, 7, 21), microorganisms (1, 16), and higher plants (8, 10, 11). The acetyl CoA carboxylase obtained from liver tissues has a molecular weight of 7.8×10^6 daltons. In its active form the enzyme is filamentous. These filaments are made up of homogeneous subunits of molecular weight 410 000 daltons (17). In higher plants, the enzyme was first demonstrated in wheat germ (8). It was later obtained in an over 1000 fold purified form and was shown to have a molecular weight of 630 000 ± 25 000 daltons (10). Wheat germ acetyl CoA carboxylase is a soluble protein which separates into two

protein components, with S_{w20} values of 7.3 ± 0.6 and 9.4 ± 0.6, during analytical ultracentrifugation. Acetyl CoA carboxylase has also been investigated in photosynthetic tissues (11, 12). The enzyme in chloroplasts resembles the carboxylase of *Escherichia coli* (1, 2) where the enzyme is made up of three easily separable proteins, biotin carboxyl carrier protein, biotin carboxylase and transcarboxylase. In *E. coli* all three components of the enzyme are present in the soluble fraction of the cell. By contrast biotin carboxyl carrier protein of the chloroplast enzyme is localized in the insoluble membrane fraction (11, 12). We investigated acetyl CoA carboxylase of barley embryos with the aim to determine the nature and location of biotin carboxyl carrier protein in this enzyme. The present communication presents evidence that barley embryo acetyl CoA carboxylase is closely similar to the wheat germ enzyme. Barley embryo carboxylase is morphologically different from the enzyme obtained from liver tissues. The biotin containing polypeptide of the soluble embryo enzyme appears to be similar to the membrane bound chloroplast biotin carboxyl carrier protein.

2. MATERIALS AND METHODS

2.1 Isolation of Embryos

Seeds of barley, cultivar Tern were dehusked essentially as described previously (3). Seeds were soaked in 50% by vol. sulphuric acid at 25° for 2-3 hours with periodic stirring after which time the seeds were washed thoroughly in cold water to remove all traces of acid and husk fragments. Any remaining acid was neutralized by soaking the grain in ice cold water containing $CaCO_3$ (50 g $CaCO_3$/kg seed) for 30 min. The grain was then washed to remove $CaCO_3$. During this washing, the seeds were rubbed together and against the wall of the container to loosen adhering husks. Washing was continued until no further husk fragments were released from the grain.

Dehusked barley seeds were germinated on damp filter paper for 16-24 hours. Subsequent isolation of embryos was as described by MURPHY AND BRIGGS (18). The isolated embryos were washed with 0.1 M potassium phosphate buffer pH 8.3 and stored frozen at -20°.

2.2 Extraction and Purification

Two methods were employed for extraction and purification of barley embryo acetyl CoA carboxylase. The first procedure has been described in detail by HEINSTEIN and STUMPF (10). Only the second procedure will be described here. The embryos were homogenized in 55 mM imidazole-HCl buffer pH 8.3 containing 1 mM dithiothreitol. The homogenate was filtered through mira cloth and centrifuged at 30 000 g for 20 min in a Sorvall centrifuge. Manganese chloride (0.025 volumes of 1 M $MnCl_2$) was added to the supernatant with rapid stirring and the precipitate formed was removed by centrifugation. The clarified supernatant thus obtained was centrifuged at 120 000 g for 5 hours in a Beckman model L5/75 ultracentrifuge. This caused the enzyme to concentrate at the bottom of the centrifuge tube as illustrated in Fig. 1. The fractions containing the highest acetyl CoA carboxylase activity were collected, diluted (1:6 fold) with the homogenization buffer and recentrifuged for further 5 hours at the same speed. This

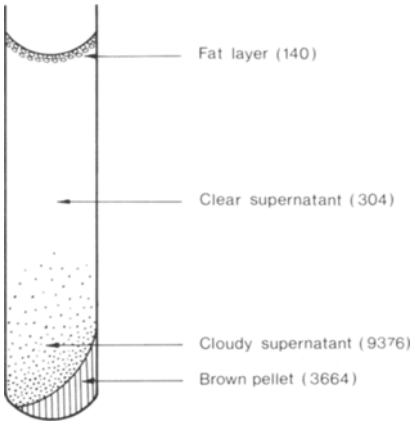

Figure 1. *Distribution of acetyl CoA carboxylase activity in the tube after ultracentrifugation.*
A $MnCl_2$ treated extract of barley embryos in 55 mM imidazole buffer pH 8.3 containing 1 mM dithiothreitol was centrifuged for 5 hours at 120 000 g and 4°C. The numbers in brackets refer to carboxylase activity given as $^{14}CO_2$ (cpm) fixed/min/mg protein.

procedure was repeated twice more. The enzyme preparation was then loaded onto a sepharose 6 B column (40 x 2.5 cm). Elution was carried out with 55 mM imidazole buffer pH 8.0 containing 1 mM dithiothreitol and 1% NaCl. Fractions (2.6 ml) were collected from the column and assayed for carboxylase activity. All procedures of extraction and purification were carried out at 0–4°C.

2.3 Acetyl CoA carboxylase assay

The reaction mixture (total volume 0.5 ml) contained the following in μmoles: Tris-HCl buffer pH 8.3, 0.05; ATP, 2.5; $MgCl_2$, 5.0; $MnCl_2$, 0.5; dithiothreitol, 1.0; acetyl CoA, 0.15; $NaH^{14}CO_3$, 1.17 (3 μCi). The amount of enzyme used in an assay varied from 20-200 μg protein. Control tubes were set up for each assay by omitting acetyl CoA. Incubations were carried out at 30°C for 20 min. The reactions were terminated by adding 0.2 ml of conc. HCl. Aliquots (50 μl) were placed on filter paper discs and dried for 20 min. at 80°C. Acid stable radioactivity on filter paper discs was counted in a Packard model 2002 Tricarb scintillation spectrometer using 10 ml of Packard's »Dimilume« scintillation fluid. Acid stable radioactivity observed in the control tubes was subtracted from the values obtained in the respective test tubes to determine $^{14}CO_2$ fixation due to acetyl CoA carboxylase activity.

2.4 Other assays

Biotin was measured by the method of GREEN (4), while protein was estimated by the Folin-Ciacalteau technique of LOWRY *et al.* (14). Biotin carboxyl carrier protein was assayed as described previously (11, 12, 13).

Polyacrylamide disc gel electrophoresis in an asparagine system was performed according to HEDRICK and SMITH (9) and phenol: acetic acid: urea disc gel electrophoresis according to NIELSEN (20).

2.5 Electron microscopy

The method of VALENTINE *et al.* (23) was employed to examine molecules of acetyl CoA carboxylase in the electron microscope. The purified enzyme was adsorbed on carbon films prepared on cleaved mica and negatively stained with sodium silicotungstate (5% w/v) at pH 7.0 for 2-4 min at room temperature. The films were mounted on copper grids and studied in a Siemens Elmiskop 102. Pictures were taken at a magnification of 50 000.

2.6 Chemicals

Avidin, ATP, Coenzyme A and dithiothreitol were obtained from Sigma Chemical Company St. Louis, Mo., U.S.A. Sodium ^{14}C bicarbonate was obtained from Radio Chemical Centre, Amersham, U. K. Acetyl CoA was prepared from acetic anhydride and coenzyme A according to SIMON and SHEMIN (22). Biotin carboxylase and transcarboxylase were obtained from *Escherichia coli* according to the method of NERVI *et al.* (19).

3. RESULTS AND DISCUSSION

The purity of barley embryo acetyl CoA carboxylase obtained by the two methods employed is illustrated in Tables I and II. Both procedures resulted in protein fractions with closely similar specific enzymatic activities (7 to 7.4 μmoles of Co_2 fixed per min per mg protein). However, the enzyme could be purified faster by repeated ultracentrifugation than by the procedure described by HEINSTEIN and STUMPF (10). With both methods the yield of purified enzyme varied considerably from one experiment to another. This variability was in part due to the condition of the embryos at the initial step of extraction. Thus, homogenates made rom fresh embryos contained enzyme at a specific activity of 0.0025 to 0.0030 μmoles CO_2 fixed/min/mg protein, whereas extracts made from embryos which had been frozen and stored for 7 days gave specific activities of approximately 0.001 μmoles CO_2 fixed/min/mg protein. Subjecting the enzyme preparation to DEAE cellulose chromatography, sephadex G 200 and sepharose 6B gel filtration produced no change in the specific activity of the preparation. The evidence presented below suggests a high purity of the enzyme in these preparations. Polyacrylamide disc gel electrophoresis of purified enzyme in the asparagine system of HEDRICK and SMITH (9) caused a complete loss of enzyme activity and five protein bands

Table I

Purification of acetyl CoA carboxylase from barley according to the method described by HEINSTEIN and STUMPF (10). The enzyme was extracted from embroys that had been stored frozen at -20°C. The activation was performed by preliminary incubation of the protein for 60 min at 30°C with 0.01 M dithiothreitol and 0.25 M phosphate buffer pH 8.0.

Fraction	Total protein mg	Specific activity μmoles CO_2 fixed/min/mg protein	Recovery %
1. Extract	70 890	0.001	100
2. $MnCl_2$ supernatant	15 614	0.004	88.1
3. $(NH_4)_2SO_4$ (0.28-0.38 saturation)	5 246	0.01	74.0
4. DEAE cellulose	19.0	0.8	21.4
5. DEAE sephadex A 50	10.4	1.4	20.6
6. Sucrose gradient centrifugation	1.06	6.9	10.3
7. Activation	1.06	7.1	10.6

appeared in the gels in agreement with the situation encountered by HEINSTEIN and STUMPF with an over 1000 fold purified preparation of wheat germ acetyl CoA carboxylase. The latter authors showed by studying the individual protein bands for biotin content, ATP-^{32}Pi exchange and malonyl CoA-acetyl CoA exchange that all five protein bands had activities ascribable to wheat germ acetyl CoA carboxylase. The polyacrylamide disc gel electrophoretic pattern of barley embryo acetyl CoA carboxylase in the phenol: acetic acid: urea system is shown in Fig. 2. Three polypeptide bands with relative mobilities of 0.44, 0.53 and 0.66 were observed in the gels. In order of their increasing relative mobility these bands corresponded to apparent molecular weights of 41 000, 32 000 and 21 000 daltons respectively. Proteins corresponding to the individual bands were eluted from unstained gels and analysed for their biotin content and enzyme activities. None of the protein fractions obtained showed any acetyl CoA carboxylase, biotin carboxylase or transcarboxylase activities. Attempts to reconstitute these enzyme activities by combining different protein fractions were also unseccessful. The protein corresponding to a relative mobility of 0.66 contained 10.1 ± 2.2 μg biotin/mg protein and was able to function as carboxyl carrier in the *E. coli* acetyl CoA

Table II

Purificaton of barley embryo acetyl CoA carboxylase by repeated ultracentrifugation.
The enzyme was extracted from freshly obtained embryos.

Fraction	Total protein mg	Specific activity μmoles CO_2 fixed/min/mg protein	Recovery %
1. Extract	7 080	0.003	100
2. $MnCl_2$ supernatant	1 493	0.01	70.3
3. 1st ultracentrifugation	13.8	0.9	58.5
4. 2nd ultracentrifugation	1.6	5.0	37.7
5. 3rd ultracentrifugation	0.6	7.3	20.6
6. 4th ultracentrifugation	0.54	7.4	18.8
7. Sepharose 6B	0.49	7.4	17.1

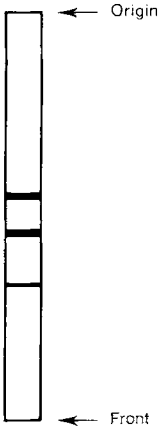

Figure 2. *Disc gel electrophoretic pattern of barley embryo acetyl CoA carboxylase.*
The purified enzyme was electrophoresed for 6 hours at room temperature in a polyacrylamide gel using the phenol: acetid acid: 8 M urea (2:1:1 by vol) system as described by NIELSEN (20). The gels were stained with 1% amido black in 7% acetid acid.

carboxylase reaction (Table III) in the same way as the corresponding polypeptide from the chloroplast acetyl CoA carboxylase (cf. 13). In the other two protein fractions obtained from the gels biotin could not be detected. Acetyl CoA carboxylase (400 µg) purified from barley embryos gave an unstable carboxylated enzyme when incubated with all the cofactors except CoA (Table IV). Addition of acetyl CoA to carboxylated enzyme resulted in a rapid formation of labelled malonyl CoA. Therefore we concluded that in addition to biotin carboxyl carrier protein both biotin carboxylase and transcarboxylase activities are associated with the purified barley embryo acetyl CoA carboxylase. Phenol: acetid acid: urea allowed only biotin carboxyl carrier protein to be obtained in an active form. The biotin containing polypeptide isolated from the soluble embryo acetyl CoA carboxylase shows similarities to barley chloroplast biotinyl protein both in function and in behaviour during disc gel electrophoresis. The behaviour of purified barley embryo acetyl CoA carboxylase in sepharose 6B gel filtration chromatography as illustrated in Figs. 3 and 4 reveals molecular aggregation. The enzyme was excluded as a single protein peak from sepharose 6B when eluted with 55 mM imidazole buffer pH 8.0

Table III

Evidence for the participation of the biotinyl peptide isolated from barley embryo acetyl CoA carboxylase in the Escherichia coli acetyl CoA carboxylase reaction.
The complete reaction mixture contained in a total volume of 1.0 ml the following: 55 mM imidazole HCl buffer pH 8.0, mM dithiothreitol, 0.5 mM $MnCl_2$, 0.5 mM $MgCl_2$, 2mM ATP, 0.17 mM $NaH^{14}CO_3$ (10 µCi), 0.4 mM acetyl CoA, 0.9 mg *E.coli* transcarboxylase, 0.4 mg *E.coli* biotin carboxylase and 14 µg of biotinyl peptide.
Incubations were carried out at 30°C for 30 min. and terminated by the addition of 200 µl of concentrated HCl. Aliquots were placed on filter paper discs dried and acid stable radioactivity determined.

Reaction mixture	Acid stable radioactivity (cpm fixed into malonyl CoA)
Complete	2 984
- biotinyl peptide	484
- *E.coli* biotin carboxylase	16
- *E.coli* transcarboxylase	12

Table IV

Formation enzyme-$^{14}CO_2$complex and transfer of $^{14}CO_2$ to malonyl CoA.
The complete reaction mixture contained in 1.0 ml the following: 55 mM imidazole HCl buffer pH 8.0, 0.5 mM $MgCl_2$, 0.5 mM $MnCl_2$, 2 mM ATP, 0.17 mM $NaH^{14}CO_3$ (10 µCi) and 400 µg acetyl CoA carboxylase. The miture was incubated for 15 min. at 30°C and applied on to a sephadex G-50 (2 x 30 cm) column equilibrated with 55 mM imidazole HCl buffer pH 8.0. Aliquots from the fractions containing protein were counted for radioactivity. The protein fractions (1.5 ml) containing bound $^{14}CO_2$(48 300 cpm) were incubated with and without 0.4 mM acetyl CoA for 30 min. at 30°C. Reactions were terminated by the addition of 200 µl of concentrated HCl and acid stable radioactivity was determined after drying aliquots on filter paper discs.

Enzyme-$^{14}CO_2$ complex formation	$^{14}CO_2$ (cpm) bound to protein
Complete	62 260
- Enzyme	0
- ATP	0
Transfer of $^{14}CO_2$ to malonyl CoA	**Acid stable radioactivity (cpm)**
+ Acetyl CoA	30 900
- Acetyl CoA	27

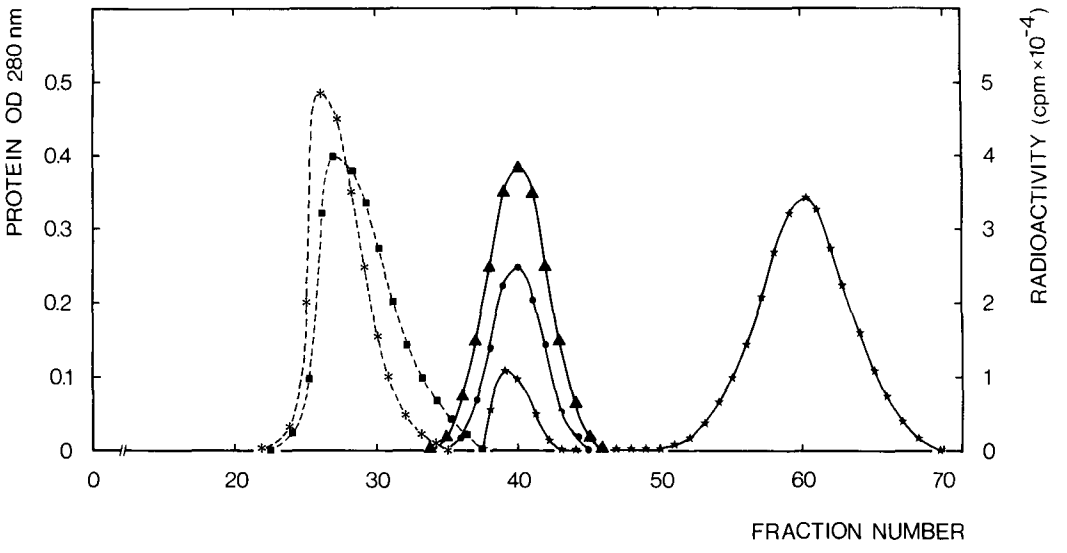

Figure 3. *The elution profiles of avidin treated and untreated barley embryo acetyl CoA carboxylase in a sepharose 6B column (40 × 2.5 cm).*
Eluant contained 55 mM imidazole buffer pH 8.0, 1 mM dithiothreitol with and without addition of 1% (w/v) NaCl. The dotted lines show the profiles of protein (---■---) and carboxylase activity (---✳---) in the absence of 1% NaCl. The solid lines refer to protein (—●—), carboxylase activity (—▲—) and avidin treated protein (—★—) profiles observed in the presence of 1% NaCl. The avidin treated enzyme (—★—) gave two uv light absorbing peaks. The first peak corresponds to the enzyme-avidin complex while the second larger peak represents free avidin.

containing 1 mM dithiothreitol. Carboxylase activity was associated with the leading edge of the protein peak. The approximate exclusion limit for sepharose 6B is a protein molecular weight 4×10^6 daltons. However, sepharose 6B gel filtration of purified enzyme in the presence of 1% sodium chloride caused the protein molecules to penetrate the agarose beads. The enzyme was then eluted at a position corresponding to a molecular weight of 610 000 daltons (Figs. 3 and 4). Carboxylase activity was associated uniformly throughout the peak. These data reveal that 1% sodium chloride under conditions used prevent aggregation of barley embryo acetyl CoA carboxylase. The purified enzyme contained 0.42 ± 0.06 µg biotin per mg protein which amounts to 1.05 moles of biotin per 610 000 g of carboxylase. Thus it is concluded that the barley embryo enzyme has 1 mole of biotin per mole of enzyme. Since the biotin is covalently bound to the 21 000 dalton peptide, each enzyme molecule contains a single biotinyl polypeptide. The egg white protein avidin (M. W. 63 000) has the property of strongly and specifically binding to biotin. Avidin has four biotin binding sites localized in two fold symmetry (5). The purified enzyme (4 mg) was treated with excess avidin (6 mg) and subjected to sepharose 6B gel filtration in the presence of 1% NaCl. This avidin treatment resulted in a 100% inhibition of the carboxylase activity of the enzyme. Both avidin treated and untreated barley embryo acetyl CoA carboxylase eluted very close to each other from the sepharose 6B column (Fig. 3). This reveals that under the conditions employed, excess of avidin and presence of NaCl, a single avidin molecule did not bind more than a single carboxylase molecule. This result further supports the finding that only one biotin molecule is present per enzyme molecule as binding of several avidin molecules would have shifted the peak position measurably.

Acetyl CoA carboxylase purified from barley embryos was unstable. It lost over 50% of its activity during a week stored frozen in the presence of 50% saturated ammonium sulphate and 1.0 mM dithiothreitol. The loss of enzyme activity was associated with an increase in turbidity of the preparation indicating protein aggregation.

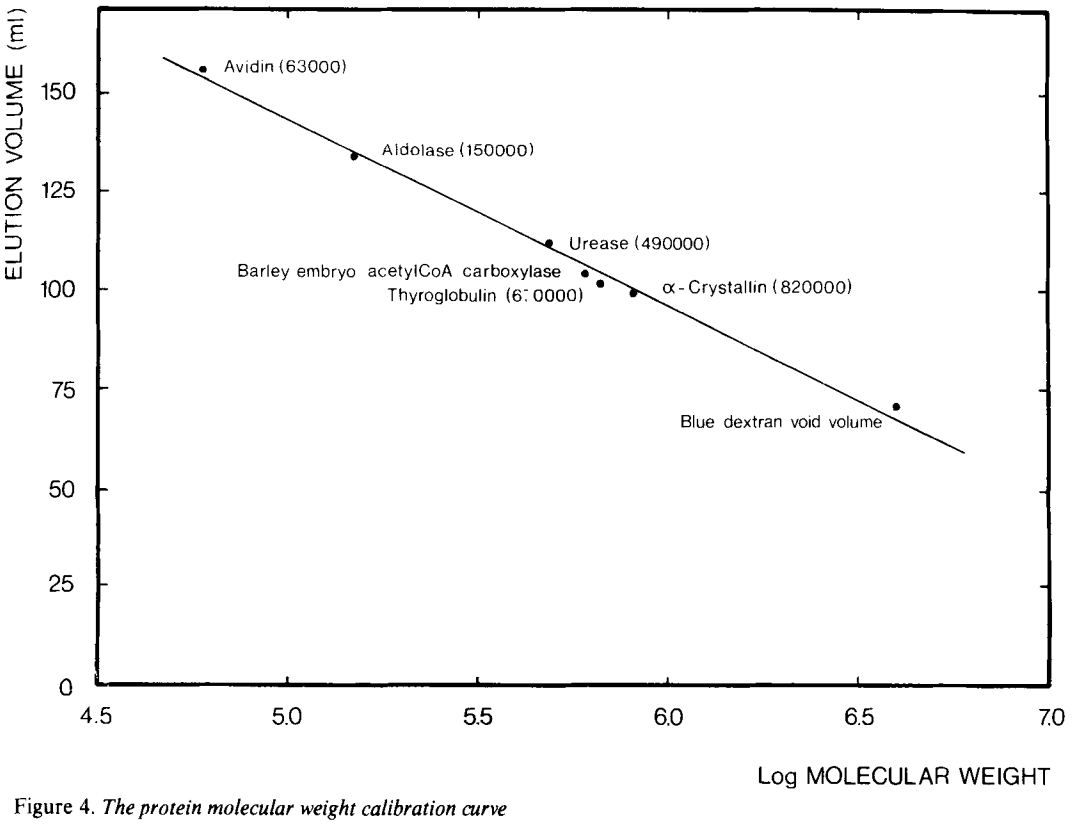

Figure 4. *The protein molecular weight calibration curve for the sepharose 6B column (40 x 2.5 cm).*
The standard proteins were eluted with 55 mM imidazole buffer pH 8.2 containing 1 mM dithiothreitol and 1% (w/v) NaCl at 4°C. The eluate was monitored for uv absorption at 254 nm using a LKB UVICORD II. The numbers within brackets refer to the molecular weights of the proteins in daltons. Barley embryo acetyl CoA carboxylase was eluted at a position corresponding to a molecular weight of 610 000 daltons.

In electron micrographs of negative contrast preparations, the purified enzyme appeared as a globular protein. Measuring 90 particles on electron micrographs at a magnification of 400 000 gave 275 ± 8 Å for the longest visible diameter and 192 ± 45 Å for the shortest visible diameter (Fig. 5). A substructure is recognizable in the molecules. The stain penetrates into three cavities of the molecules which have an average diameter og 50-60 Å. In cases where the protein material was recognizable between two adjacent cavities, it measured approximate-

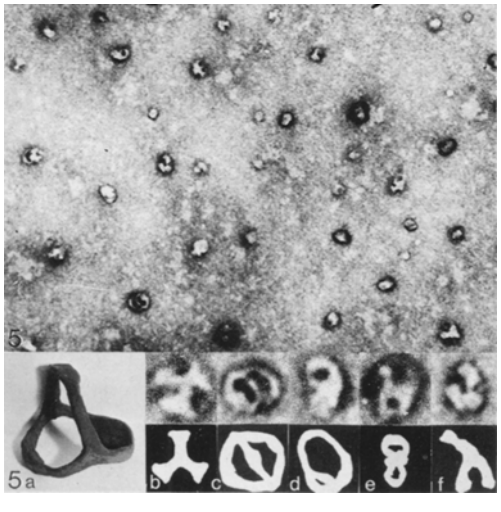

Figure 5. *Electron micrograph of purified barley embryo acetyl CoA carboxylase.*
A carbon film freshly stripped from mica was floated on a solution of protein (50μg/ml) as described by VALENTINE *et al.* (23). The molecules adsorbed on the film were negatively stained in 5% (w/v) sodium silicotungstate pH 7.0 for 4 min. The preparation was studied in a Siemens Elmiskop 102 at a magnification of 50 000. The final magnification is 80 000. *Fig. 5a* shows the proposed model for the molecule. *Figs. 5b to 5f* present five characteristic images of the carboxylase magnified 175 000 fold together with a corresponding shadowgraph of the model in Fig. 5a.

ly 55 Å in thickness. A model that would fit the observed shapes of the molecule in the micrographs is depicted in Fig. 5a. In Figs. 5b to 5f higher magnifications of some characteristic images of the carboxylase are presented together with the corresponding shadowgraphs of the model in Fig. 5a. Filament formation as is characteristic for the active liver enzyme has not been observed for barley embryo acetyl CoA carboxylase. The morphology of the barley embryo enzyme is in agreement with the biochemical characteristics of its active state as reported above.

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