*MOLECULAR CHARACTERISTICS OF LIVER ACETYL COA CARBOXYLASE**

By Carlo Gregolin,† Elena Ryder,‡ Albrecht K. Kleinschmidt, Robert C. Warner,§ and M. Daniel Lane**

DEPARTMENT OF BIOCHEMISTRY, NEW YORK UNIVERSITY SCHOOL OF MEDICINE

Communicated by Severo Ochoa, May 26, 1966

Acetyl CoA carboxylase [Acetyl-CoA:CO_2 ligase (ADP), E.C. 6.4.1.2], which catalyzes the carboxylation of acetyl CoA to form malonyl CoA, has been shown[1-5] to be the site of the citrate activation of fatty acid synthesis reported originally by Brady and Gurin.[6] Vagelos *et al.*,[7] Matsuhashi *et al.*,[8] and Numa *et al.*[9] have observed that activation by citrate is accompanied by an increase in the sedimentation velocity of the carboxylase, as measured by the sucrose density-gradient technique. Previous investigations[2, 7, 8-10] into the nature of the citrate activation phenomenon were limited because homogeneous carboxylase preparations, necessary for certain critical physical-chemical experiments, were not available. These enzyme preparations had relatively low specific activities compared to other biotin enzymes. The isolation of liver acetyl CoA carboxylase in pure form in this laboratory has permitted the elucidation of certain physical-chemical properties of the enzyme and their correlation to its catalytic activity.

Experimental Procedure.—Preparation of acetyl CoA carboxylase: Acetyl CoA carboxylase was extracted from chicken liver, using the phosphate-bicarbonate homogenization medium described by Brady and Gurin.[6] The enzyme was purified by $(NH_4)_2SO_4$ and calcium phosphate gel fractionation, DEAE-cellulose and hydroxylapatite chromatography, and preparative sucrose density-gradient centrifugation. Unless otherwise indicated, the carboxylase preparations used in all experiments were dialyzed against 50 mM potassium phosphate, pH 7.0, containing 5 mM 2-mercaptoethanol and 0.1 mM EDTA. Details of the purification procedure will be reported elsewhere.

Acetyl CoA carboxylase assay: The $H^{14}CO_3^-$-fixation carboxylation assay reaction mixture (1.0 ml total vol) contained the following components (in μmoles, unless specified): Tris (Cl^-) buffer, pH 7.5, 60; ATP, 2; $MgCl_2$, 8; $KH^{14}CO_3$ (specific activity, 3×10^5 cpm per μmole), 10; acetyl CoA, 0.2; potassium isocitrate, 20; GSH, 3; bovine serum albumin, 0.6 mg; and carboxylase (diluted with 10 mM potassium phosphate, pH 7.0, 5 mM 2-mercaptoethanol, and 0.1 mM EDTA), 0.01–0.03 ml containing 4×10^{-3} unit. Following a 10-min incubation at 37°, 0.2 ml of 6 *N* HCl was added, an aliquot was taken to dryness in a scintillation counting vial at 85° for 30 min, water and scintillator were added, and acid-stable C^{14}-activity determined using a liquid scintillation spectrometer.

A spectrophotometric assay was frequently used with highly purified carboxylase preparations. This reaction mixture contained in a total volume of 1.0 ml: the components of the $H^{14}CO_3^-$-fixation assay (unlabeled, instead of C^{14}-bicarbonate and up to 0.025 unit of carboxylase rather than 4×10^{-3} unit), 0.5 μmole of PEP, 20 μg of pyruvate kinase, 20 μg of lactate dehydrogenase, and 0.2 μmole of NADH. The rate of NADH oxidation coupled to acetyl CoA-dependent ADP formation was followed spectrophotometrically at 334 mμ and 37°. Both assays were normally initiated by addition of carboxylase; however, no difference in reaction rate could be noted when assays were initiated with any other essential component of the reaction mixture.

One unit of carboxylase is defined as the amount of enzyme capable of catalyzing the carboxylation of 1.0 μmole of acetyl CoA per minute under the conditions described.

Protein determination: Protein was determined by the method of Lowry *et al.*[11] using crystalline bovine serum albumin as standard. Protein concentration of pure carboxylase preparations was also determined by the Warburg and Christian method,[12] in which case values were multiplied by 0.64 to convert to a protein concentration equivalent to that determined by the Lowry method.

Electron microscopy: Enzyme preparations were mounted on carbonized Formvar support films and stained with uranyl acetate using the droplet technique as described by Van Bruggen *et al.*[13] Stained and unstained preparations were photographed using a Siemens-Elmiskop I electron microscope (original magnification, 40,000×, 80,000×).

Results.—Sedimentation velocity and molecular weight of acetyl CoA carboxylase: Examination of the purified acetyl CoA carboxylase in the analytical ultracentrifuge (Fig. 1*A* and *B*) revealed a single, sharp symmetrical peak with no apparent contamination. The hypersharp character of the sedimenting boundary indicates artificial sharpening resulting from a high concentration dependence of the sedimentation coefficient. This suggests that the enzyme molecule has a high axial ratio. The asymmetric character of the carboxylase was subsequently confirmed by electron microscopy. Relatively low protein concentrations (0.5–2.0 mg per ml) were employed in sedimentation velocity experiments due to the tendency of the protein to aggregate at higher concentrations. In view of the asymmetric character of the enzyme and results to be described in the following sections, the sedimentation characteristics were investigated under a variety of conditions. In all cases a single sedimenting peak was observed on schlieren patterns with $S_{20,w}$ values falling within the range 53–58*S*. The conditions tested included: 50 mM potassium phosphate, pH 7.0; 50 mM potassium phosphate plus 20 mM DL-isocitrate, pH 7.0; 50 mM Tris (Cl^-), pH 7.5; 50 mM Tris (Cl^-) plus 20 mM DL-isocitrate, pH 7.5; and the carboxylation assay reaction mixture (albumin omitted) in the presence and absence of 20 mM DL-isocitrate. With the exception of the reaction mixture, all solutions contained 5 mM 2-mercaptoethanol and 0.1 mM EDTA, and were dialyzed to equilibrium against the solution indicated. A

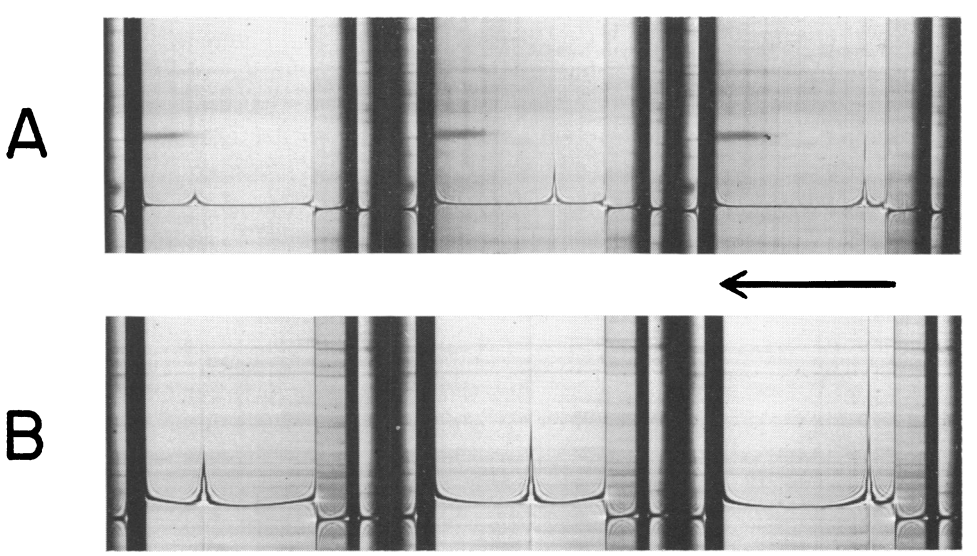

FIG. 1.—Sedimentation patterns of acetyl CoA carboxylase. (*A*) Enzyme in 0.05 *M* potassium phosphate, pH 7.0, 0.1 mM EDTA, 5 mM 2-mercaptoethanol; protein concentration, 1.08 mg/ml; centrifugation at 25,980 rpm and 22°C; photographs taken at 9, 23, 51 min after reaching speed; direction of centrifugation, right to left. (*B*) Enzyme in 0.05 *M* potassium phosphate, pH 7.0, 0.1 *M* ammonium sulfate, 0.1 mM EDTA, 5 mM 2-mercaptoethanol; protein concentration, 1.70 mg/ml; centrifugation at 25,980 rpm and 20°C; photographs taken 20, 48, 68 min after reaching speed; direction of centrifugation, right to left.

significant decrease in sedimentation velocity was noted, however, when 0.1 *M* $(NH_4)_2SO_4$ was present in the phosphate-containing medium. In this case, the $S_{20,w}$ was found to be 40–42*S*.

The molecular weight of acetyl CoA carboxylase in phosphate was determined by sedimentation equilibrium according to the method of Yphantis.[14] Five experiments were conducted at protein concentrations from 0.08 to 0.6 gm per liter in 50 mM potassium phosphate, pH 7.0, 5 mM 2-mercaptoethanol, and 0.1 mM EDTA. The molecular weight was found to be $7.8 \pm 0.6 \times 10^6$. The linearity of plots of log Rayleigh fringe displacement versus $\times$ coordinate position indicated that the carboxylase preparation was monodisperse.

Molecular activity and biotin content: The pure carboxylase catalyzes the carboxylation of 8 μmoles of acetyl CoA per minute per mg of protein under the standard assay conditions. The (+)-biotin content (determined microbiologically with *Lactobacillus arabinosus*[15]) of the enzyme is 0.63 μg per mg of protein, which corresponds to 1 mole of biotin per 390,000 gm of protein or 20 moles of biotin per mole of enzyme. The catalytic center activity calculated from these data is 3,100 moles of acetyl CoA carboxylated per minute per mole of biotin, and the molecular activity 62,000 moles per minute per mole of enzyme. The pure enzyme catalyzes the carboxylation of propionyl CoA at a maximum velocity 80 per cent that of acetyl CoA. The products of acetyl CoA and propionyl CoA carboxylation were identified as malonyl CoA and methylmalonyl CoA, respectively, by methods previously described.[16]

Activation by tricarboxylic acids: Acetyl CoA carboxylase exhibits an essentially absolute requirement for tricarboxylic acid and dicarboxylic acid anions, the most active being isocitrate, citrate, and malonate. This requirement is apparent when either acetyl CoA or propionyl CoA is used as substrate. Figure 2*A* shows that a linear carboxylation rate is achieved instantly upon addition of enzyme to the reaction mixture containing isocitrate. Figure 2*B* reveals that the enzyme, which is active upon addition to the reaction mixture without isocitrate, is short-lived and decays to nearly zero within 1 min under the influence of the components of the reaction mixture. Addition of isocitrate instantly reactivates the enzyme to its maximal level of activity. The pure chicken liver carboxylase differs from the rat liver,[8] and adipose tissue[5] carboxylase preparations in that prolonged preincubation with isocitrate (or citrate) is not required for its activation. It appears that the method of preparation outlined for the chicken liver carboxylase leads to an "active" form of the enzyme which undergoes inactivation under the assay conditions (in the absence of isocitrate) and reactivation in the presence of isocitrate. This behavior is identical at 25°; furthermore, this enzyme does not appear to be cold-labile under the conditions used to demonstrate the cold-lability of the rat liver carboxylase.[10]

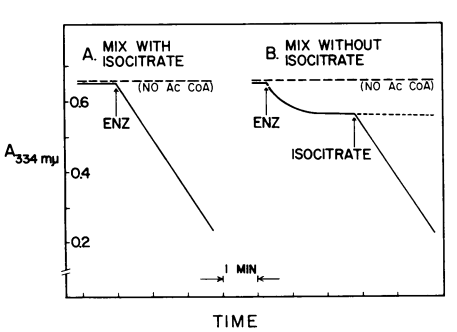

FIG. 2.—Kinetics of the acetyl CoA carboxylase-catalyzed reaction. Enzyme was assayed by the spectrophotometric test described in the text; 3.3 μg of enzyme were used.

Modification of sedimentation velocity in sucrose density gradients: On the basis of previous reports[7-9] that activation of acetyl CoA carboxylase is accompanied by changes in sedimentation velocity in sucrose density gradients, the chicken liver acetyl CoA carboxylase was subjected to similar analysis using the conditions found to influence its state of activation. Figure 3 shows that in sucrose density gradients in the presence of potassium phosphate buffer, pH 7.0, the carboxylase sediments as a "large" structure with an apparent sedimentation coefficient of 54*S* (30*S* and 50*S* *E. coli* ribosomes used as external markers). This value is in agreement with the values determined in the analytical ultracentrifuge. In the presence

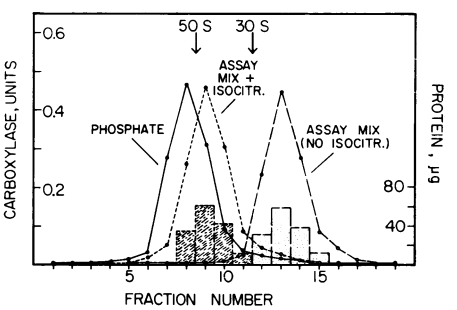

FIG. 3.—Sedimentation of acetyl CoA carboxylase in sucrose density gradients. The method of Martin and Ames[17] was employed using a 5–20 per cent (w/v) sucrose gradient, total volume 4.5 ml. Sucrose gradients contained: (*a*) phosphate—0.05 *M* potassium phosphate, pH 7.0, 0.1 mM EDTA; (*b*) assay mix + isocitrate—0.06 *M* Tris (Cl^-), pH 7.5, 0.02 *M* DL-isocitrate, 2 mM ATP, 8 mM $MgCl_2$, 0.01 *M* potassium bicarbonate, 0.2 mM acetyl CoA, 0.1 mM EDTA; (*c*) assay mix (no isocitrate)—as in (*b*) *minus* isocitrate. On the gradients were applied 200 µg of acetyl CoA carboxylase, specific activity 7.2, dissolved in 0.2 ml of 0.05 *M* potassium phosphate, pH 7.0, 0.1 mM EDTA. Gradient tubes were centrifuged at 39,000 rpm (SW 39 rotor; Spinco model L ultracentrifuge) for 60 min, at 25°C. Eighteen fractions were collected, and aliquots assayed for activity (*curves*) and for protein (*bars;* cross-hatched bars refer to gradient *b*, dotted bars to gradient *c*).

of the components of the assay reaction mixture (minus albumin) without isocitrate, a condition shown earlier (Fig. 2) to cause reversible inactivation, the enzyme sediments (Fig. 3) as a "small" structure of about 20*S*. In the presence of isocitrate, a condition shown earlier (Fig. 2) to give rise to the active form, the enzyme sediments as a "large" structure of 46*S*. The correlation between the sedimentation characteristics of the carboxylase and its structural features as revealed by electron microscopy is illustrated in the next section. It should be noted (Fig. 3) that the change in sedimentation velocity of carboxylase activity from 20*S* to 46*S* on the density gradients exactly corresponds to the change in *S* of the bulk of the protein in the gradient. It is clear, therefore, that the activation phenomenon is associated with a corresponding change in the sedimentation velocity of the protein component of the enzyme preparation. It should be emphasized that subjecting the carboxylase to the components of the assay reaction mixture does not constitute the minimal conditions giving rise to the 20*S* form. Indeed, this form is also accessible from Tris (Cl^-) buffer, pH 7.5–8.0, containing 5 mM 2-mercaptoethanol and 0.1 mM EDTA. The 46*S* form can be restored with isocitrate.

The chicken liver acetyl CoA carboxylase, prepared as described, apparently differs from the rat liver[8, 9] and adipose tissue[5, 7] carboxylases in that the former is isolated in a "large" form (53–58*S*) and the latter enzymes in the "small" form. Furthermore, once the catalytically inactive 20*S* form of the chicken liver enzyme is produced, *preincubation* (with isocitrate) is unnecessary for its transition into the active 46*S* form.

The inability to detect the 20*S* form in the analytical ultracentrifuge under conditions similar to those which give rise to this form in sucrose density gradients might be due to differences inherent in the two centrifugation techniques, differences in enzyme concentration, or the effect of sucrose.

Electron microscopy: Preliminary electron microscopy has revealed a striking correlation between the state of activation and sedimentation velocity (sucrose density-gradient technique) of acetyl CoA carboxylase and its structural characteristics. Electron micrographs (Fig. 4*A*) of carboxylase subjected to the assay reaction mixture (without isocitrate), which gives rise to its catalytically inactive 20*S* form, reveal small particles having a minimum dimension of 70–150 Å and a maximum dimension of 100–300 Å. Some of these stained structures are elongated and many appear to have open ends connected by a central channel. In the presence of isocitrate, which activates the enzyme and gives rise to its 46*S* ("large") form, it assumes the filamentous structure shown in Figure 4*B*. These filaments are 70–100 Å in width and up to 4,000 Å in length, which constitutes an axial ratio of up to 40–60. Figure 5*A* shows an electron micrograph prepared from the 54*S* form of the enzyme in phosphate buffer, pH 7.0. At a somewhat higher protein concentration than those already described, this form of the enzyme appears as a netlike arrangement of filaments of relatively uniform width (70–100 Å). The inset shows a single filament from a more dilute preparation which has approximate dimensions of 70–100 Å by 4,000 Å. It appears to be a twisted structure and shows regular, well-defined indentations. While the results described above have dealt with uranyl acetate-stained enzyme, the electron micrograph shown in Figure 5*B* represents an exceptional unstained preparation of carboxylase from the assay reaction mixture plus isocitrate on a particularly thin support film. It is apparent that the unstained preparation has similar structural characteristics to those of the stained enzyme shown in Figure 4*B*.

Discussion.—Acetyl CoA carboxylase, which has been isolated in pure form from chicken liver, has a biotin content, specific activity, catalytic center activity, and molecular activity similar to those of other biotin enzymes.[18] The carboxylase has been found to be an unusually large ($S_{20,w}$ = 53–58*S* and molecular weight, 7.8 million in phosphate, pH 7.0) and asymmetric protein structure. Its characteristic filamentous structure, shown by electron microscopy, is easily dissociated into constituent subunits with concomitant loss of enzymatic activity. The active filaments (70–100× up to 4,000 Å) can be rapidly reconstituted by isocitrate and other tri- and dicarboxylic acid anions. The present investigation demonstrates a striking correlation between enzyme structure and activity. It is suggested that the subunit conformation in the presence of phosphate or isocitrate permits a linear organization of subunits and expression of catalytic activity. It has not yet been established what minimal filament length is necessary for catalytic activity.

The present findings shed further light on the process by which Krebs cycle tri- and dicarboxylic acids activate acetyl CoA carboxylase and thereby regulate fatty acid biosynthesis. It is tempting to speculate that in addition to its catalytic role, acetyl CoA carboxylase has a structural role in the fatty acid synthesizing system. Acetyl CoA carboxylase filaments could provide a basic structure to which fatty acid synthetase molecules are complexed, thereby bringing into close proximity the malonyl CoA-synthesizing and -utilizing systems.

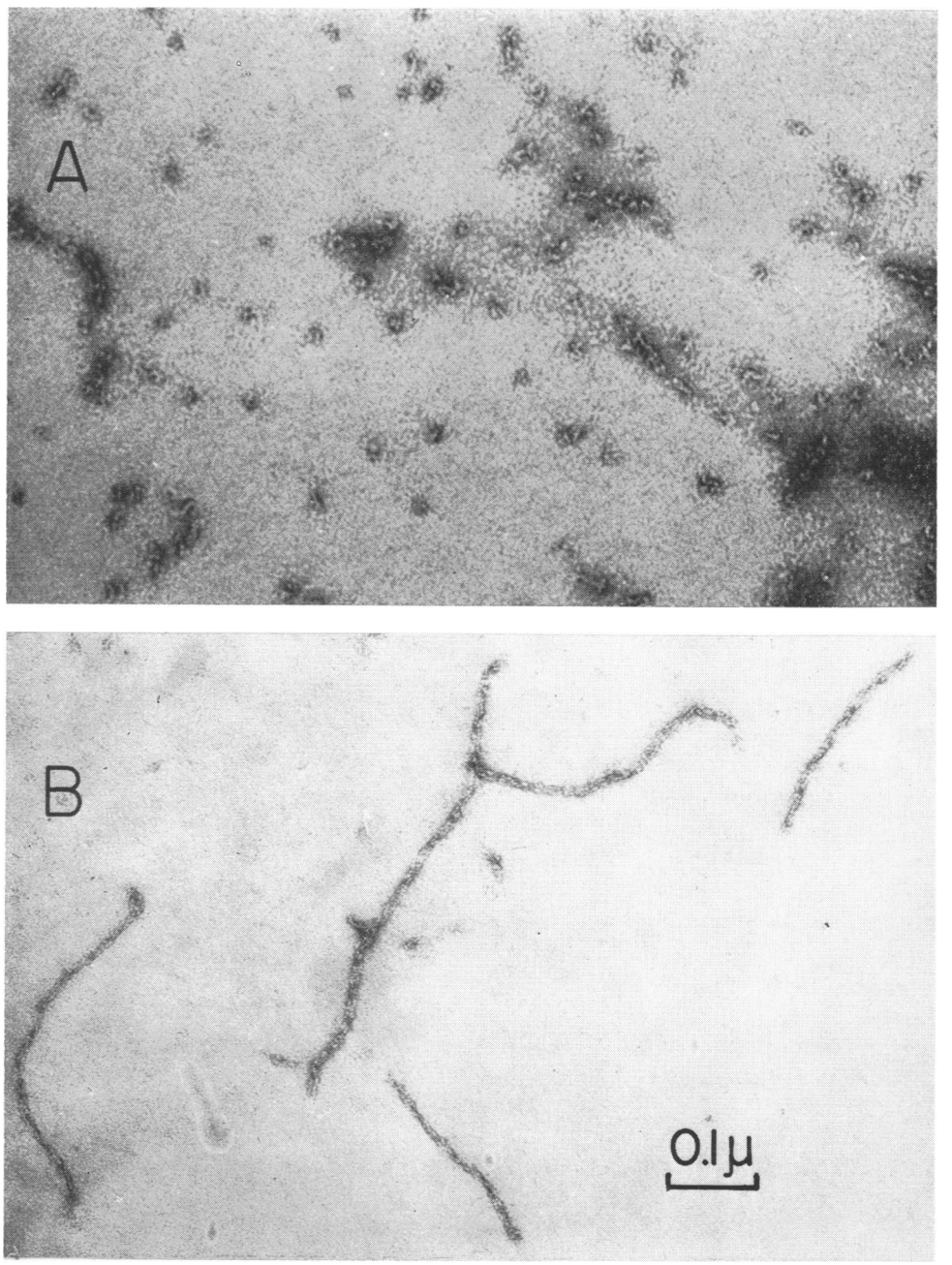

FIG. 4.—Electron micrographs of acetyl CoA carboxylase in assay reaction mixture (*A*) without, and (*B*) with isocitrate. Acetyl CoA carboxylase, specific activity 7.5, was dissolved in: (*A*) 0.06 *M* Tris (Cl^-), pH 7.5, 2 mM ATP, 8 mM $MgCl_2$, 0.01 *M* potassium bicarbonate, 0.2 mM acetyl CoA, 3 mM GSH; enzyme, 50 μg/ml. (*B*) as in (*A*) *plus* 0.02 *M* DL-isocitrate. After a 10-min incubation, mixtures were diluted 5-fold with water. Uranyl acetate stained ×120,000.

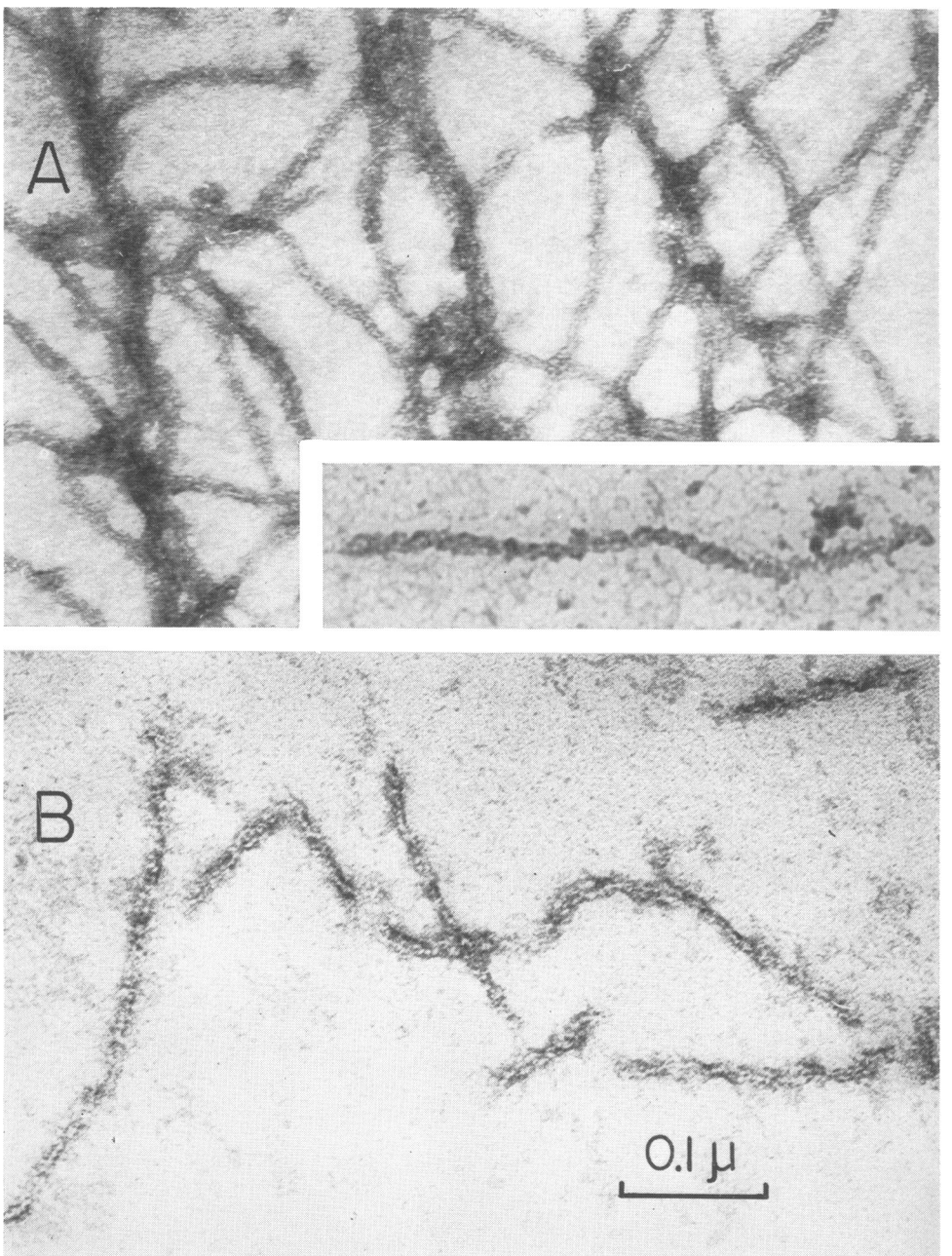

FIG. 5.—Electron micrographs of acetyl CoA carboxylase in (*A*) phosphate, and (*B*) assay reaction mixture with isocitrate. Acetyl CoA carboxylase, specific activity 7.5, was dissolved in: (*A*) 0.05 *M* potassium phosphate, pH 7.0, 0.1 mM EDTA, 5 mM 2-mercaptoethanol; enzyme, 100 μg/ml. *Inset*, the same, enzyme 50 μg/ml. (*B*) 0.06 *M* Tris (Cl^-), pH 7.5, 0.02 *M* DL-isocitrate, 2 mM ATP, 8 mM $MgCl_2$, 0.01 *M* potassium bicarbonate, 0.2 mM acetyl CoA, 3 mM GSH; enzyme, 50 μg/ml. After a 10-min incubation, mixtures were diluted 5-fold with water. (*A*) Uranyl acetate-stained, (*B*) unstained ×200,000.

The authors wish to express their appreciation to Dr. Severo Ochoa for his encouragement during the course of this investigation, and to Miss Inge Grumm and Miss Wilhelmine Hellmann for their skillful technical assistance. The assistance of Mr. Frank Zaboretsky with the ultracentrifuge experiments is gratefully acknowledged. We express our gratitude to Dr. M. Ross, Department of Anatomy, for the use of the electron microscope.

* This investigation was supported by research grants AM-09116 and GM-06967 from the National Institutes of Health, U.S. Public Health Service, and by a grant from the John A. Hartford Foundation.

† Recipient of a NATO fellowship (1965), on leave of absence from the University of Padua, Padua, Italy.

‡ Postdoctoral fellow of Instituto Venezolano de Investigaciones Cientificas, Caracas, Venezuela.

§ Career Development Awardee (USPHS Research Career Program Award 2-K3-14899).

** Career Development Awardee (USPHS Research Career Program Award, K3-AM-18487).

[1] Waite, M., *Federation Proc.*, **21**, 287 (1962).

[2] Waite, M., and S. J. Wakil, *J. Biol. Chem.*, **237**, 2750 (1962).

[3] Matsuhashi, M., S. Matsuhashi, S. Numa, and F. Lynen, *Federation Proc.*, **21**, 288 (1962).

[4] Martin, D. B., and P. R. Vagelos, *Federation Proc.*, **21**, 289 (1962).

[5] Martin, D. B., and P. R. Vagelos, *J. Biol. Chem.*, **237**, 1787 (1962).

[6] Brady, R. O., and S. J. Gurin, *J. Biol. Chem.*, **199**, 421 (1952).

[7] Vagelos, P. R., A. W. Alberts, and D. B. Martin, *J. Biol. Chem.*, **238**, 533 (1963).

[8] Matsuhashi, M., S. Matsuhashi, and F. Lynen, *Biochem. Z.*, **340**, 263 (1964).

[9] Numa, S., W. M. Bortz, and F. Lynen, in *Advances in Enzyme Regulation*, ed. G. Weber (New York: Pergamon Press, 1965), vol. 3, p. 407.

[10] Numa, S., and E. Ringelmann, *Biochem. Z.*, **343**, 258 (1965).

[11] Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

[12] Warburg, O., and W. Christian, *Biochem. Z.*, **310**, 384 (1942).

[13] Van Bruggen, E. F. J., E. H. Wiebenga, and M. Gruber, *Biochim. Biophys. Acta*, **42**, 171 (1960).

[14] Yphantis, D. A., *Biochemistry*, **3**, 297 (1964).

[15] Wright, L. D., and H. R. Skeggs, *Proc. Soc. Exptl. Biol. Med.*, **56**, 95 (1944).

[16] Lane, M. D., D. R. Halenz, D. P. Kosow, and C. S. Hegre, *J. Biol. Chem.*, **235**, 3082 (1960).

[17] Martin, R. G., and B. N. Ames, *J. Biol. Chem.*, **236**, 1372 (1961).

[18] Kaziro, Y., and S. Ochoa, in *Advances in Enzymology*, ed. F. F. Nord (New York: Interscience Publ., 1964), vol. 26, p. 283.