FURTHER EVIDENCE FOR POLYMERIC STRUCTURE OF LIVER ACETYL CoA CARBOXYLASE

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Acetyl CoA carboxylase (E.C. 6.4.1.2.) appears to play a critical role in the regulation of fatty acid synthesis (Numa et al., 1965a). This enzyme is known to be activated by tri- and dicarboxylic acids, especially by citrate (Waite, 1962; Matsuhashi et al., 1962; Martin et al., 1962), and inhibited by long-chain acyl CoA derivatives (Bortz et al., 1963; Numa et al., 1965b). A correlation between activation of the carboxylase and an increase in its sedimentation coefficient has been shown by Vagelos et al. (1963) with the rat adipose tissue enzyme and in this laboratory with the rat liver enzyme (Numa et al., 1965b and c). In these studies, the sucrose density gradient centrifugation method was mainly employed, and sedimentation of the enzyme was followed by its catalytic activity alone, owing to the relatively low purity of the carboxylase preparations used. The isolation of a highly purified liver enzyme preparation in this laboratory has allowed us to demonstrate the citrate-effect on the sedimentation coefficient of the carboxylase as protein in the analytical ultracentrifuge. Furthermore, the sedimentation pattern of the enzyme has been found to depend largely upon centrifugation conditions including protein concentration and the composition of medium.

Enzyme preparation - Acetyl CoA carboxylase was prepared from chicken liver. The purification procedure reported previously for the rat liver carboxylase (Matsuhashi et al., 1964; Numa et al., 1964) was employed with minor modifications up to the DEAE-cellulose chromatography step. Further purification was achieved by preparative sucrose density gradient centrifugation.

Specific activity and biotin content - The enzyme thus obtained catalyzed carboxylation of approximately 3 μmoles of

acetyl CoA per minute per mg of protein* under the standard assay conditions at 25° described previously (Numa et al., 1965b). The enzyme contained 0.72 µg (+)-biotin per mg of protein (for biotin determination, see Lynen et al., 1961). This value corresponds to 1 mole of bound biotin per 340,000 g of protein. Accordingly, approximately 1,000 molecules of acetyl CoA are carboxylated per minute per molecule of bound biotin at 25°.

Sedimentation velocity experiments in the analytical ultracentrifuge - Fig. 1 shows the effects of citrate and protein concentration on the sedimentation pattern of acetyl CoA carboxylase in the pyrophosphate media, pH 8.1. The sedimentation coefficient was increased largely by the addition of citrate. The $s_{20,w}$ value** of the major component in the absence of citrate was 14.1 S (A), 14.5 S (B), 14.1 S (C), 14.3 S (D) and 14.0 S (E), whereas that in the presence of citrate 42.5 S (A), 44.3 S (B), 48.3 S (C), 46.3 S (D) and 43.4 S (E), respectively.

In the presence of citrate at lower protein concentrations, a minor slowly sedimenting component appeared in addition to the "48 S" form and possessed a $s_{20,w}$ value of 14.4 S (B), 13.6 S (C) and 13.9 S (D). In Experiment E, where the centrifugation media were slightly different from those for Experiments A-D, the minor component sedimenting ahead of the "14 S" form in the absence of citrate was resolved more distinctly from the major component, and its $s_{20,w}$ value was 32.4 S. Thus, 3 forms with different sedimentation coefficients were visible in the pyrophosphate media, pH 8.1. The formation of the faster sedimenting components appeared to be favored by higher protein concentrations, due presumably to aggregation (see Fig. 1). Furthermore, sedimentation runs in pyrophosphate media of pH 7.0 and 9.1

*Protein concentrations, which were determined routinely by the method of Lowry et al. (1951) with bovine serum albumin as standard or by the method of Warburg et al. (1942), were corrected to dry weight basis.

**In all runs, the sedimentation coefficient was calculated from displacement of the position of the peak on the schlieren patterns; the asymmetry of the boundary observed at higher protein concentrations was not taken into consideration. The partial specific volume of the protein was assumed to be 0.735 cm^3/g on the basis of a preliminary amino acid analysis (Schachman, 1957).

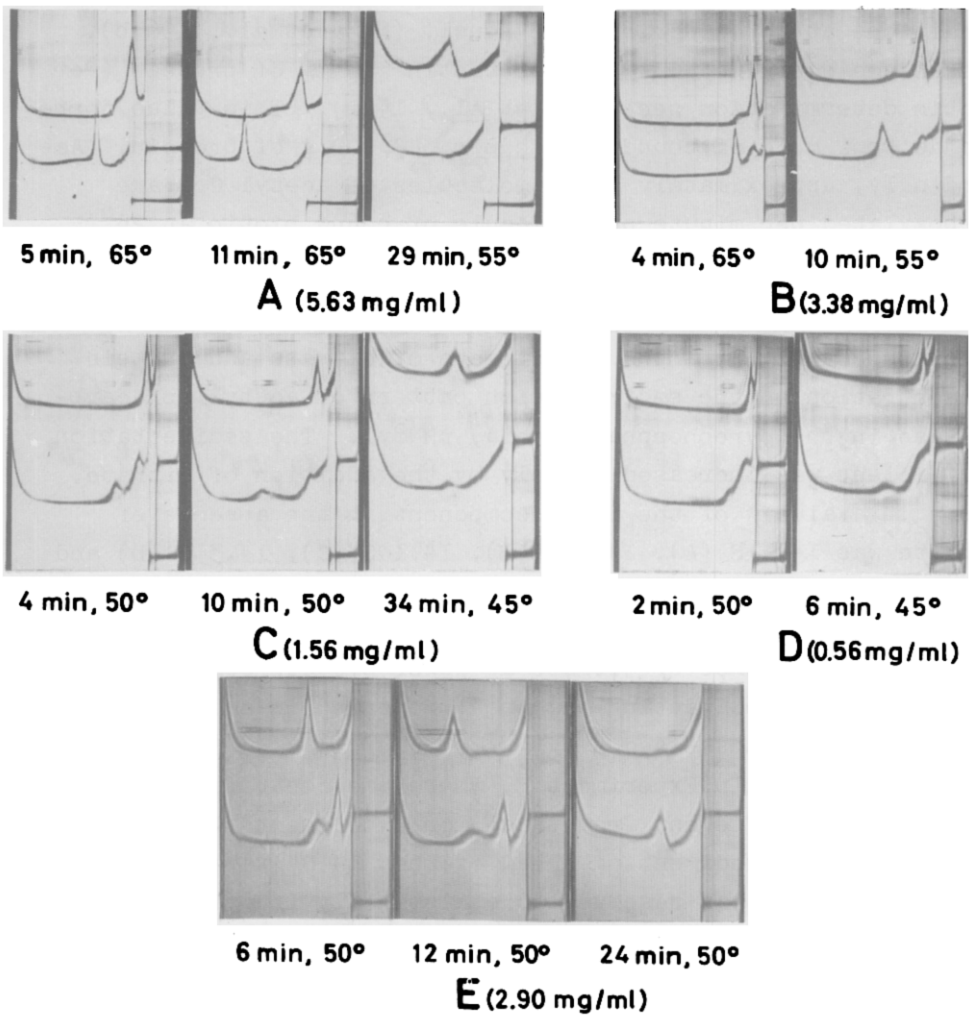

Fig. 1. Sedimentation patterns of acetyl CoA carboxylase in the presence and absence of citrate at varying protein concentrations.

Experiments A-D; lower patterns with citrate (normal window), upper patterns without citrate (wedge window); centrifugation in the Spinco model E ultracentrifuge at 59,780 r.p.m. and 5.1°; solvent: 50 mM $Na_4P_2O_7$-HCl, pH 8.1, NaCl (final Cl^- concentration, 100 mM), 2 mM 2-mercaptoethanol and 1 mM EDTA with and without 10 mM potassium citrate; enzyme dialyzed against the corresponding solvent; protein concentration (mg/ml), 5.63 (A), 3.38 (B), 1.56 (C) and 0.56 (D); photographs taken at indicated times after reaching speed and at indicated phase plate angle; direction of sedimentation, right to left.

Experiment E; lower patterns without citrate (normal window), upper patterns with citrate (wedge window); deviations of the experimental conditions were as follows: temperature, 3.7°; solvent, without further addition of NaCl and with 5 mM EDTA; citrate added after dialysis of enzyme; protein concentration, 2.90 mg/ml.

suggested that aggregation occurrs more readily at lower pH.

In 50 mM potassium phosphate buffer, pH 7.4, containing 2 mM 2-mercaptoethanol and 5 mM EDTA, the protein (3.6 mg/ml) showed very extensive aggregation, which was further accentuated by the presence of 10 mM citrate. In both cases, however, a relatively small peak was visible behind a very broad boundary formed by aggregated material and sedimented with nearly equal $s_{20,w}$ values (53 to 55 S). The addition of 50 mM NaCl to the phosphate medium without citrate reduced the tendency of aggregation and resulted in the formation of a larger peak with a $s_{20,w}$ value of 46.5 S (protein concentration, 5.0 mg/ml).

Sucrose density gradient centrifugation studies . Investigations with sucrose density gradients revealed that the sedimentation patterns of the protein observed in the analytical

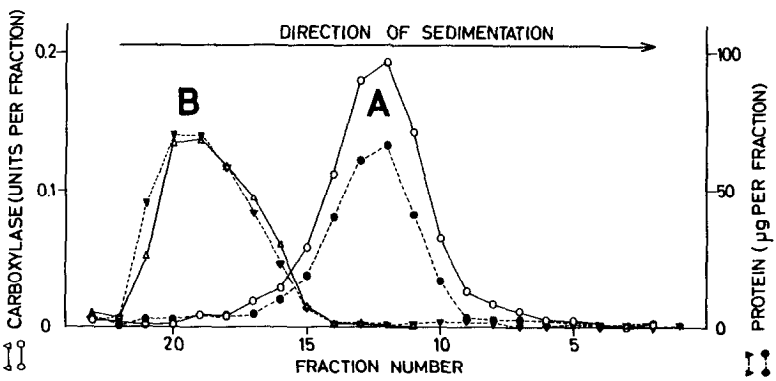

Fig. 2. Concomitant sedimentation of protein and carboxylase in sucrose density gradients in the presence (A) and absence (B) of citrate. The method of Martin and Ames (1961) was employed with a gradient from 5 to 20 per cent (w/v) sucrose. The gradients contained 50 mM $Na_4P_2O_7$-HCl, pH 8.1, 2 mM 2-mercaptoethanol, 5 mM EDTA and, when present, 10 mM potassium citrate. Enzyme (specific activity, approximately 3) was dialyzed against the corresponding solution containing no sucrose. One tenth ml of dialyzed enzyme (3.67 mg/ml; specific activity, 2.9 (A) and 2.1 (B)) was applied on the respective gradient. Liquid paraffin was layered on it to prevent a possible disturbance of the meniscus. The gradient tubes were centrifuged at 38,000 r.p.m. and about 5° in a SW 39 rotor in the Spinco model L ultracentrifuge for 126 minutes. Twenty-two (A) or 23 (B) fractions were collected, and aliquots assayed for carboxylase activity (solid lines) and for protein (dashed lines).

ultracentrifuge actually represent those of acetyl CoA carboxylase. In these studies, sedimentation of the carboxylase was followed by determinations of both protein and enzymatic activity. An experiment of this kind in the pyrophosphate media, pH 8.1, which is represented in Fig. 2, showed good coincidence of protein and carboxylase activity. With yeast fatty acid synthetase ($s_{20,w}$ = 43 S) as standard, the $s_{20,w}$ value of the carboxylase in the presence and absence of citrate was estimated to be 47 S and 14 S, respectively, being in close agreement with the values found in the analytical ultracentrifuge. Analogous experiments were carried out in sucrose gradients containing other buffers as listed in Table I. Despite various sedimentation patterns obtained in different media, concomitant sedimentation of protein and activity was always observed.* This supports the homogeneity of the carboxylase preparations employed in the present studies.

As seen in Table I, the sedimentation pattern of the carboxylase depended largely upon centrifugation medium, concentration of the enzyme applied on the gradient, as well as addition of citrate. The concentration dependence was especially marked in the pyrophosphate, pH 7.0, and Tris media without citrate; at higher concentrations the enzyme sedimented as very broad or nearly biphasic band. The presence of citrate caused an increase in the sedimentation coefficient of the enzyme in the pyrophosphate and Tris media. However, the citrate-effect was essentially not noticeable in the phosphate medium, in which the enzyme sedimented fast even in the absence of citrate. The addition of 50 mM NaCl to the phosphate medium without citrate decreased to some extent the $s_{20,w}$ value of the enzyme, whereas no such effect of NaCl was seen in the presence of citrate. Recovery of enzyme activity from gradient centrifugations was generally lower, when more dilute enzyme solutions were applied on gradients.

*Occasionally, there was no precise coincidence of protein and activity peaks, the former showing a slightly larger trailing than the latter (see Fig. 2). This occurred in some media without citrate and might be due to the relative instability of the enzyme in more slowly sedimenting forms.

Table I. Effects of buffer, protein concentration and citrate on the sedimentation pattern of acetyl CoA carboxylase in sucrose density gradients.

Experimental details, see the legend to Fig. 2. Each centrifugation medium contained 50 mM of the respective buffer and the same additions as described in the legend. At lower protein concentrations, sedimentation was followed by enzymatic activity alone. When the peak was very broad, the $s_{20,w}$ value given in the Table represents that of the fraction located in the middle of the peak. Standard enzyme, see text.

Medium	Without citrate		With 10 mM citrate	
	Protein concentration* (mg/ml)	$s_{20,w}$ (S)	Protein concentration* (mg/ml)	$s_{20,w}$ (S)
$Na_4P_2O_7$-HCl, pH 8.1	0.23	12	0.23	33
	0.92	12	0.92	40
	3.67	14	3.67	47
$Na_4P_2O_7$-HCl, pH 7.0	0.24	15		
	0.97	22		
	2.66	29	2.66	45
	3.88	23;33**		
Tris (Cl^-), pH 7.8	0.29	16	0.26	48
	1.16	19;34**	1.05	57
	4.62	37	4.21	65
Potassium phosphate, pH 7.4	0.32	49	0.27	46
	1.29	53	1.08	53
	5.16	55	4.31	55

* Concentration of protein applied on the gradient.
** Biphasic.

Discussion - The present studies demonstrate that the sedimentation pattern of chicken liver acetyl CoA carboxylase varies greatly with centrifugation conditions. This is consistent with the hypothesis that the enzyme is in an association-dissociation equilibrium which is influenced by various factors; addition of citrate, higher enzyme concentrations and certain media favor

association. The smallest component so far obtained without irreversible inactivation is the "14 S" form, which might represent the unit containing 1 molecule of biotin, assuming that the frictional ratio of the protein is relatively low. Further investigations are in progress to characterize the molecular properties of the different components.

It should be noted that the citrate-effect on the sedimentation coefficient of the chicken liver carboxylase was seen even at approximately 5°; all centrifugation studies were carried out at this temperature, and the enzyme had not been preincubated with citrate at a higher temperature before centrifugation. This is in accordance with the fact that the chicken liver carboxylase, in contrast to the rat adipose tissue and liver enzymes (Vagelos et al., 1963; Matsuhashi et al., 1964; Numa et al., 1965c), showed no noticeable induction period, when the reaction was started by the addition of enzyme (without preincubation in the presence of citrate at a higher temperature). Reversible inactivation of citrate-treated enzyme by cold was also not observed with the chicken liver carboxylase under the identical conditions used to demonstrate the inactivation of the rat liver enzyme (Numa et al., 1965c).

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