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THE ACTIVATION OF RAT LIVER ACETYL-CoA CARBOXYLASE BY INCUBATION

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SUMMARY

1. Partially purified acetyl-CoA carboxylase (acetyl-CoA:CO_2 ligase (ADP), EC 6.4.1.2) from the livers of rats starved for 12 h is activated by incubation at 37° for 3–4 h; in contrast to previous reports the presence of citrate is not required for activation. The rate of [^{14}C]acetyl-CoA incorporation into fatty acids by 100 000 × *g* supernatant preparations from rat liver is also increased by incubation without citrate.

2. Acetyl-CoA carboxylase activities of partially purified and 100 000 × *g* supernatant preparations activated by treatment with trypsin were found in the low-density portions of sucrose gradients in contrast to preparations activated by incubation with citrate.

INTRODUCTION

From experiments of VAGELOS *et al.*[1,2] and LYNEN *et al.*[3,4] it appeared that the specific enzymatic activity of acetyl-CoA carboxylase (acetyl-CoA:CO_2 ligase (ADP), EC 6.4.1.2) depended on its state of aggregation since the molecular weight and the specific activity of the enzyme simultaneously increased on incubation with citrate. Citrate was the most effective among a number of polycarboxylic acids and was thought to act as an allosteric effector[5]. The experiments reported here show that partially purified acetyl-CoA carboxylase becomes activated by incubation even in the absence of citrate; acetyl-CoA incorporation by a 100 000 × *g* supernatant preparation from rat liver behaves similarly upon incubation without citrate.

Partially purified acetyl-CoA carboxylase was previously shown to be activated by incubation with trypsin[6]. The experiments reported here demonstrate that the molecular weight of acetyl-CoA carboxylase does not change on incubation with trypsin and that the trypsin-treated enzyme does not aggregate if it is incubated with citrate.

EXPERIMENTAL

Holtzman rats weighing about 150 g were fasted for 12 h before killing. Incu-

bations and assays were carried out as previously described[6]. Care was taken to insure that all assays contained an excess of fatty acid synthetase. Azide (10^{-3} M) was added to prevent bacterial growth; it was without effect on fatty acid synthesis. The enzyme preparations were incubated with trypsin* as previously described[6].

Sucrose gradient centrifugations were carried out by the method of MARTIN AND AMES[8]. 100 000 × *g* supernatant or partially purified enzyme preparations were layered on sucrose gradients (5–20%) containing 0.01 M Tris buffer at pH 7.5 and centrifuged for 3 h at 39 000 rev./min in a Spinco SW-39 rotor at 5°. The gradient tubes were punctured at the bottom and 0.1-ml fractions collected. All fractions from the 100 000 × *g* supernatant preparations were supplemented with excess synthetase and assayed for [^{14}C]acetyl-CoA incorporation into fatty acids. The fractions obtained from the partially purified enzyme preparations were analyzed for acetyl-CoA carboxylase activity by estimating the rate of $^{14}CO_2$ incorporation into malonyl-CoA[2].

RESULTS

Activation of partially purified acetyl-CoA carboxylase by incubation in the absence of citrate

Acetyl-CoA carboxylase was partially purified as previously described[6] and aliquots incubated at 5° and 26° at pH 7.5 (Fig. 1). At lower or higher H^+ concentrations the increase of the specific activity was smaller at both temperatures. Addition

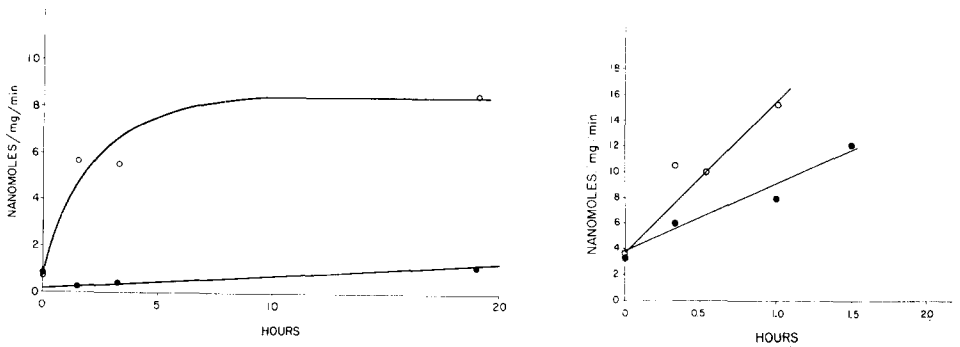

Fig. 1. Specific activity of partially purified acetyl-CoA carboxylase as a function of time of incubation at 5° (●) and 26° (○).

Fig. 2. Specific activity of partially purified acetyl-CoA carboxylase as a function of time of incubation at 26° without (●) and with (○) $5 \cdot 10^{-3}$ M citrate.

of $5 \cdot 10^{-3}$ M citrate increased the initial rate nearly twofold (Fig. 2); no lag period was seen.

Partially purified acetyl-CoA carboxylase was also prepared from a 100 000 × *g* supernatant preparation which had been incubated with trypsin. About a third of the

* It has also been possible to increase [^{14}C]acetyl-CoA incorporation by 100 000 × *g* supernatant preparations to some extent by incubation with chymotrypsin or cocoonase[7], however both proteases completely destroy the fatty acid synthetase in the 100 000 × *g* supernatant fluids. The authors are indebted to J. H. LAW for the cocoonase.

TABLE I

RECOVERY OF ACETYL-CoA CARBOXYLASE ACTIVITY ON PARTIAL PURIFICATION OF 100 000 × *g* SUPERNATANT PREPARATIONS AFTER INCUBATION WITH AND WITHOUT TRYPSIN

Preparation	*[^{14}C]Acetyl-CoA incorporated (nmoles/ml per min)*	
	Not incubated with citrate	*Incubated with citrate for 30 min*
100 000 × *g* supernatant*	3	32
Partially purified carboxylase** prepared from above	2	8
100 000 × *g* supernatant* incubated with trypsin for 5 min at 37°	57	
Partially purified carboxylase** prepared from above	20	

* Total protein: 33 mg/ml.

** Assayed in volumes equal to the 100 000 × *g* supernatants from which they were prepared; protein concentration: 1.5 mg/ml.

carboxylase originally present in the trypsin-treated supernatant and a quarter present in the untreated control were recovered in the final $(NH_4)_2SO_4$ precipitates (Table I). The quantities of protein were about the same in both precipitates. If a 100 000 × *g* supernatant preparation and the partially purified acetyl-CoA carboxylase prepared from it were each incubated with trypsin, activation occurred to nearly the same extent (Expt. 1; Table II), the capacity of the enzyme to become activated was therefore not changed by the purification procedure. For comparison, activation of [^{14}C]acetyl-CoA incorporation by incubation of the 100 000 × *g* supernatant preparation with and without citrate are also presented (Table II).

TABLE II

EFFECT OF PROLONGED INCUBATION AND TRYPSIN TREATMENT ON PARTIALLY PURIFIED ACETYL-CoA CARBOXYLASE PREPARATIONS AND ON THE 100 000 × *g* SUPERNATANTS OF RAT LIVER FROM WHICH THEY WERE PREPARED

Preparation	*Incubation (min)*	*Addition*	*[^{14}C]Acetyl-CoA incorporated*	
			*Expt. 1**	*Expt. 2***
			(nmoles/ml per min)	
100 000 × *g* supernatant	—	—	2	0.6
100 000 × *g* supernatant	5	trypsin	26	96
100 000 × *g* supernatant	180	citrate	12	120
100 000 × *g* supernatant	180	—	8	
100 000 × *g* supernatant	240	citrate		132
100 000 × *g* supernatant	240	—		132
			(nmoles/mg per min)	
Partially purified carboxylase	—	—	0.5	
Partially purified carboxylase	5	trypsin	17	

* Total protein: 30 mg/ml.

** Total protein: 26 mg/ml.

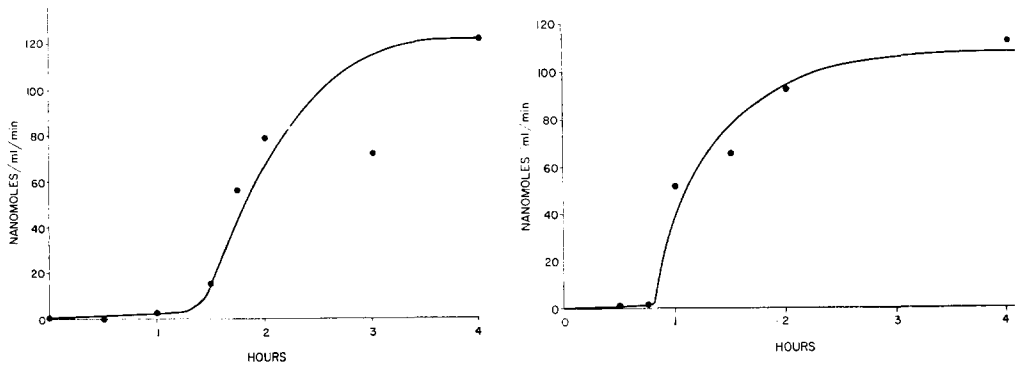

Fig. 3. Rate of [^{14}C]acetyl-CoA incorporation by a 100 000 × *g* supernatant preparation from rat liver as a function of time of incubation at 37°. Homogenates were prepared in 2 ml of isotonic sucrose per g of liver.

Fig. 4. $5 \cdot 10^{-3}$ M Mg^{2+} added at zero time; conditions as in Fig. 3.

Activation of 100 000 × g supernatant by incubation without citrate

The 100 000 × *g* supernatant fluid from livers of rats fasted for 12 h was prepared as previously described[6] and assayed for capacity to incorporate [^{14}C]acetyl-CoA into fatty acids after incubation for various periods of time (Fig. 3). The rate of [^{14}C]acetyl-CoA incorporation was small in fresh preparations and remained unchanged during 70–80 min of incubation at 37°. It then increased with time for the next 100–150 min after which it remained constant. After about 350 min of incubation the rate of incorporation slowly declined. Continued incubation after addition of $5 \cdot 10^{-3}$ M citrate or trypsin did not further increase the rate of [^{14}C]acetyl-CoA incorporation of preparations incubated for 4 h.

Since addition of Mg^{2+} had been reported to increase the enzymic activity of acetyl-CoA carboxylase[9] an experiment was carried out in which $5 \cdot 10^{-3}$ M Mg^{2+} had been added to the 100 000 × *g* supernatant preparations before incubation: a similar lag period of 50–60 min was observed but the maximal rate of acetyl-CoA incorporation was reached after only 70 min of further incubation (Fig. 4). Citrate had been regarded as a specific activator of acetyl-CoA carboxylase. If the 100 000 × *g* supernatant

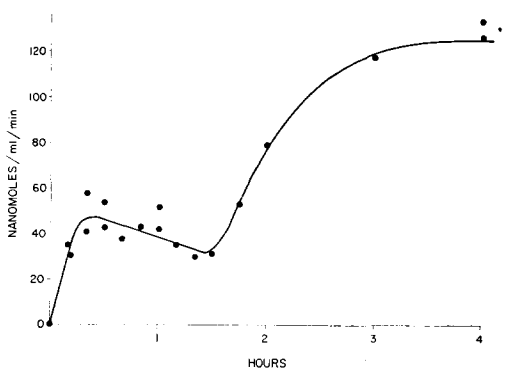

Fig. 5. $5 \cdot 10^{-3}$ M citrate added at zero time; conditions as in Fig. 3.

preparation was incubated with $5 \cdot 10^{-3}$ M citrate the rate of [^{14}C]acetyl-CoA incorporation started to increase immediately and leveled off after some 20–30 min of incubation. The level reached at this time corresponded to about 1/3 of the maximal rate observed in the experiment with the unsupplemented preparation; on continued incubation for another 70 min the rate of [^{14}C]acetyl-CoA incorporation began to increase again and reached the same maximum level as the unsupplemented aliquot after about the same total time of incubation (Fig. 5). If the concentration of citrate was increased to $2 \cdot 10^{-2}$ M a larger initial rate of activation was obtained; the time course of activation showed a lag period if a citrate concentration of less than $2 \cdot 10^{-3}$ M was used.

The dependence of the rate of activation of the 100 000 × *g* supernatant preparation on its acetyl-CoA carboxylase concentration was not examined. A 100 000 × *g* preparation aged for 2 days at 0°, was fully activated after 1 h of incubation with or without the addition of either citrate or Mg^{2+}. If the 100 000 × *g* preparation was incubated with trypsin for 5 min (Table III) the rate of [^{14}C]acetyl-CoA incorporation

TABLE III

RATES OF INCORPORATION OF [^{14}C]ACETYL-CoA BY 100 000 × *g* SUPERNATANT PREPARATIONS* FROM RAT LIVER AFTER INCUBATION WITH TRYPSIN

Trypsin (mg/ml)	*Incubation (min)*	*[^{14}C]acetyl-CoA incorporation (nmoles/ml per min)*
—	—	0.5
0.25	5	16
0.25	10	32
0.5	2.5	40
0.5	5	96
1.0	2.5	44

* Total protein: 26 mg/ml.

was approximately the same as that seen after 4 h of incubation without additions (Table II). The 100 000 × *g* supernatants prepared from chicken liver or from yeast could not be activated by incubation with trypsin.

Sucrose gradient analysis

Partially purified acetyl-CoA carboxylase preparations were incubated with citrate and trypsin for 45 and 5 min, respectively. Acetyl-CoA carboxylase activities as a function of position in the gradient were determined (Fig. 6). With the untreated enzyme, activity was found in both high and low density portions of the gradient*. Acetyl-CoA carboxylase activity in the aliquot incubated with citrate was found in the denser part of the gradient in agreement with previous reports[2]. In contrast, acetyl-CoA carboxylase activity in an aliquot incubated with trypsin was found to be in-

* It should be noted that the partially purified acetyl-CoA carboxylase used here does not disaggregate in the cold to the same extent that has been previously observed[4].

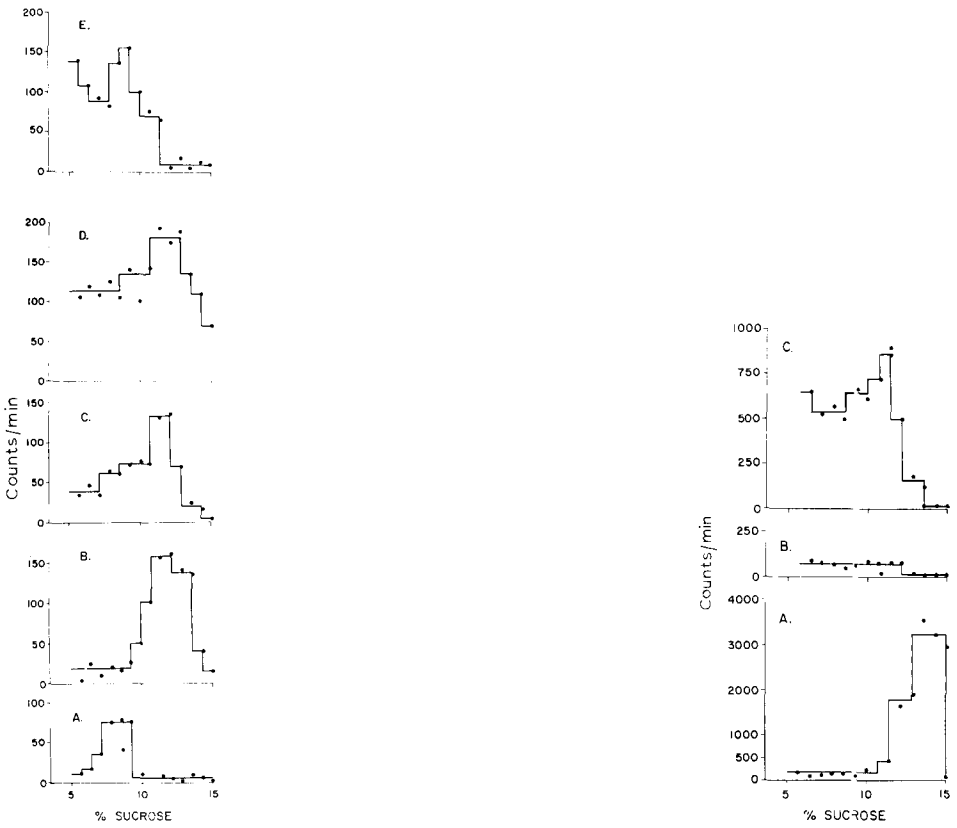

Fig. 6. Specific acetyl-CoA carboxylase activity of a partially purified preparation as a function of position after centrifugation in a 5–20% sucrose gradient: (A) 1 mg incubated with 10^{-3} M pyrophosphate for 100 min at 32° (1400 counts/min per mg) (B) 1.5 mg incubated with $5 \cdot 10^{-3}$ M citrate for 45 min (4000 counts/min per mg) (C) 1.5 mg not incubated (550 counts/min per mg) (D) 1.5 mg incubated with trypsin for 6 min at 32° (2300 counts/min per mg) (E) incubated with trypsin for 5 min at 32° and then with $5 \cdot 10^{-3}$ M citrate for 45 min more (2800 counts/min per mg).

Fig. 7. Rate of [^{14}C]acetyl-CoA incorporation by a 100 000 × *g* supernatant preparation as a function of position after centrifugation in a 5–20% sucrose gradient. (A) incubated with $5 \cdot 10^{-3}$ M citrate for 45 min at 33° (0.1 ml; 46 000 counts/min per ml) (B) not incubated (0.1 ml; 2000 counts/min per ml) (C) incubated with trypsin for 10 min at 33° (0.1 ml; 18 000 counts/min per ml).

creased mainly in the lighter portion of the gradient. On further incubation of a trypsin-treated aliquot with citrate, acetyl-CoA carboxylase activity was only found in the lighter part of the gradient (Fig. 6E). Incubation of an aliquot with 10^{-3} M pyrophosphate resulted in a small increase of the specific enzymatic activity which on gradient centrifugation appeared in the low density region (Fig. 6A). Some 100 000 × *g* preparations were also centrifuged after layering on sucrose gradients (Fig. 7).

DISCUSSION

Partially purified acetyl-CoA carboxylase is activated by incubation even in the absence of citrate, provided the time of incubation is sufficiently prolonged (Fig. 1).

It had previously been shown that incubation of acetyl-CoA carboxylase with citrate results in an increase of the molecular weight and it had therefore been suggested that the enzymatically active form of the enzyme is an aggregate of inactive or less active protomers[2,5]. This effect of citrate could either be due to binding of citrate to the enzyme, with citrate participating in holding the protomeric units together in the aggregate, or to a catalytic action of citrate upon the process of aggregation. Since activation of partially purified acetyl-CoA carboxylase is observed on incubation without citrate, it is likely that citrate serves only as a catalyst of the aggregation process and that the forces holding the protomeric units together in the aggregate do not require the permanent attachment of citrate. The notion of citrate having only a catalytic effect is supported by the observations that on incubation with citrate the rate of activation of the carboxylase increases (Fig. 2) and aggregates remaining after trypsin treatment disappear (Fig. 6D and E).

If a 100 000 $\times$ *g* supernatant preparation of rat liver is incubated at 37°, the rate of [^{14}C]acetyl-CoA incorporation into the fatty acids is increased; this increase is presumably also due to activation of acetyl-CoA carboxylase, since this enzyme was shown to be rate limiting in such preparations[10]. The largest rate of [^{14}C]acetyl-CoA incorporation is obtained after 3–4 h of incubation. The presence of citrate does not alter the final level of activity, but does bring about an immediate if partial increase and abolishes the lag period (Fig. 5). The mechanism of this biphasic increase of enzyme activity during incubation is unclear. The lag period observed for the activation of fatty acid biosynthesis in 100 000 $\times$ *g* supernatant preparations cannot be ascribed to some property of the fatty acid synthetase, since incorporation of [^{14}C]malonyl-CoA into fatty acids is catalyzed by 100 000 $\times$ *g* preparations at a maximal rate without any treatments. The lag period may be due to an energy barrier in the initial aggregation step, since it was not seen on incubation of partially purified preparations which are in part aggregated (Fig. 2). On the other hand the lag may be due to an agent present in the homogenate and removed on partial purification.

A large number of aggregation states appear to be present in partially purified acetyl-CoA carboxylase preparations[2]; these presumably were formed during the purification procedure since acetyl-CoA carboxylase activity, which was found only in the light portion of the gradient on centrifugation of the 100 000 $\times$ *g* supernatant (Fig. 7), was distributed along the major portion of the gradient after purification (Fig. 6C). It may be assumed that the different aggregation states are in equilibrium with each other since their number appears to decrease on incubation with pyrophosphate or with trypsin followed by incubation with citrate (Fig. 6E); aggregates of different sizes may have different specific activities.

Activation of acetyl-CoA carboxylase by incubation with trypsin can not be due to aggregation since sucrose gradient centrifugation showed the increased activity predominantly in the less dense section of the gradient. This distribution was even more clearly seen if incubation with trypsin was followed by further incubation with citrate. It appears that under these circumstances all aggregates were broken up. It may be concluded that trypsin-treated protomeric units differ from untreated ones, since they are incapable of aggregating under the conditions used here.

It may be noted that the final level of activity reached by incubation of a 100 000 $\times$ *g* supernatant preparation with or without citrate for 4 h is of the same order of magnitude as that obtained by incubation with trypsin for 5 min (Expt. 2; Table II),

even though the mechanisms of activation appear to be different in the two cases. For this reason the maximal rate of [^{14}C]acetyl-CoA incorporation which was obtained irrespective of the activation procedure used may be regarded as representing the total quantity of acetyl-CoA carboxylase present in livers from rats fasted for about 12 h.

It is doubtful that the increase of the specific activity of acetyl-CoA carboxylase seen on prolonged incubation is relevant to the control of the rate of fatty acid synthesis in fed and fasted rats; it is possible however, that the rate of activation on refeeding *in vivo* is greater than the rate observed on simple incubation *in vitro*. LOWENSTEIN had concluded that the change of concentration of citrate in the liver is not related to the control of fatty acid biosynthesis[11,12]. In agreement with this notion is the observation that acetyl-CoA carboxylase can be activated by incubation without citrate.

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