THE ACTIVATION OF RAT LIVER ACETYL-COA CARBOXYLASE BY TRYPSIN*

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Acetyl-CoA carboxylase and fatty acid synthetase are the two soluble enzymes required for the conversion of acetyl-CoA into palmitic acid in the presence of appropriate cofactors.[1, 2] In rat liver the synthetase always appears to be present in excess, and the rate of synthesis of saturated long-chain fatty acids is limited by the amount and the activity of acetyl-CoA carboxylase in this organ.[3]

In this report evidence is presented which suggests that nearly all the acetyl-CoA carboxylase is present in an inactive form in freshly prepared 100,000 *g* supernatant fluids from liver homogenates of rats starved for 18 hours; the enzyme is only partly active in homogenates prepared from fed rats. An inactive preparation can be converted to an active form by three different procedures: (*i*) short incubation with trypsin, (*ii*) aging at 0°, and (*iii*) prolonged incubation with citric acid. While activation by citrate[4–6] is reversed by cold storage[7] or by incubation with ATP,[8] the trypsin-treated preparations remain active under these conditions. Activation by aging appears to be due to an enzyme which seems to be present in inactive form in the freshly prepared 100,000 *g* supernatant fluid and which seems to become active itself on standing at 0°.

Results.—Assays: Partially purified fatty acid synthetase was prepared from rat liver.[6] All carboxylase preparations were diluted 20 times for assay and incubated with C^{14}-acetyl-CoA, the other cofactors, and excess synthetase. Under these conditions the enzyme lost its activity nearly instantaneously unless 2×10^{-3} *M* citrate or 1 mg/ml of crystalline bovine serum albumin was present in the incubation mixture. Prolonged incubation with citrate at this dilution of the enzyme did not increase the enzymatic activity;[5] synthesis of labeled fatty acids ceased after 15 minutes of incubation.

100,000 g supernatant fluid: In a series of experiments on fatty acid biosynthesis it was noted that incubation of 100,000 *g* supernatant preparations from rat liver homogenates with trypsin increased the rate at which added C^{14}-acetyl-CoA was converted to fatty acids (Table 1). Incubation was terminated by addition of soybean trypsin inhibitor after various periods of time. Trypsin mixed with soybean trypsin inhibitor before incubation failed to activate C^{14}-acetyl-CoA incorporation (expt. 1, Table 2). Storage of the incubated preparation at 4°C for three days resulted in only a small decrease of activity (expt. 2, Table 2). The kinetics of incorporation of C^{14}-acetyl-CoA after incubation with trypsin are shown in Figure 1. Incubation with chymotrypsin had no such effect (Fig. 2). A larger amount of C^{14}-acetyl-CoA was incorporated by untreated preparations from fed than from fasted rats (Table 1). Enzyme preparations from commercial frozen rat and rabbit liver could similarly be activated by incubation with trypsin, while a preparation from guinea pig liver was not affected. The incorporation of labeled malonyl-CoA was not changed or slightly decreased by incubation with trypsin (Table 3).

TABLE 1

TRYPSIN ACTIVATION OF FATTY ACID SYNTHESIS IN FRESH 100,000 *g* SUPERNATANT PREPARATIONS FROM RAT LIVER

Source*	Incubation† with trypsin (min)	No. of expts.	C^{14}-acetyl-CoA average (picomoles/min/ml *S*‡)	Incorporated range
Fasted rats	0	9	45	20–75
Fasted rats	5	9	2000	1100–2600
Fed rats	0	5	310	120–600
Fed rats	5	5	3300	1900–6000

* Female Holtzman strain rats weighing 90–100 gm were fed water and a fat-free diet *ad libitum* for at least 3 days before being killed by decapitation. The livers were removed immediately and placed in cold 0.25 *M* sucrose (2 ml per gm of liver). The 100,000 × *g* supernatant fluid was then prepared as described.[10] Food was withheld from fasted animals for 18 hr.

Enzymatic activity was determined using 0.1 ml of the enzyme preparation, 200 μmoles potassium phosphate (pH 7.0), 2 μmoles TPNH, 2 μmoles ATP, 4 μmoles potassium citrate, 20 μmoles $KHCO_3$, 10 μmoles $MgCl_2$, 10 μmoles MgK_2 EDTA, and 20 mμmoles of 1-C^{14}-acetyl-CoA (10^5 cpm) in 2 ml total volume. The pH's of the ATP and EDTA solutions were adjusted to 7.0 before use. Incubation was carried out in 4-ml closed tubes at 38°C usually for 10 min. The reaction was stopped by addition of 0.7 ml of 45% KOH, 1 ml of diethylene glycol was added, and the mixture saponified at 110°C for 45 min. After cooling it was acidified by addition of 1.5 ml of 25% H_2SO_4 (v/v) and extracted with 4 ml of petroleum ether (bp 30–60°C). The ether was washed once with 10% acetic acid and twice with water, dried over sodium sulfate, and evaporated to 1 ml under nitrogen. The samples were counted by a liquid scintillation counter using the 10 ml of a scintillation fluid containing 5 gm of PPO and 0.3 gm POPOP per liter of toluene. C^{14} detected in petroleum ether extracts of the alkaline hydrolysates was negligible.

† Trypsin treatment was carried out as follows: 5μl of the trypsin solution containing 10 mg of crystalline trypsin dissolved in 1 ml of 0.001 *M* HCl was added to 2 ml of prewarmed 100,000 × *g* supernatant fluid and incubated for various periods of time. The reaction was stopped by adding soybean trypsin inhibitor to give a final concentration of 100 μg/ml and the mixture allowed to stand in ice for at least 30 min. Chymotrypsin treatment was carried out in precisely the same way.

‡ *S:* 100,000 × *g* supernatant.

TABLE 2

FATTY ACID SYNTHESIS IN TRYPSIN-TREATED 100,000 *g* SUPERNATANT PREPARATIONS

Expt.	Enzyme preparation treatment	C^{14}-acetyl-CoA incorporation (picomoles/min/ml *S*)
1	Untreated	60
	Incubated with trypsin for 5 min	1100
	Incubated with trypsin plus soybean trypsin inhibitor for 5 min	55
2	Untreated	22
	Incubated with trypsin for 5 min	2400
	Incubated with trypsin for 5 min; stored for 3 days at 0°C	2000
3	Stored for 3 days at 0°; untreated	1600
	Stored for 3 days at 0°; incubated with trypsin for 1 min	5500
	Stored for 3 days at 0°; incubated with trypsin for 2 min	3400
4	Incubated with trypsin for 5 min	2300
	Incubated with trypsin for 5 min; then incubated with 2×10^{-3} *M* ATP for 30 min	2200

See Table 1 for experimental details.

TABLE 3

DISTRIBUTION OF C^{14} IN LONG-CHAIN FATTY ACIDS*

Enzyme treatment	Substrate	Total C^{14} (cpm)	Fraction of C^{14} in carboxyl position†
None	1-C^{14}-acetyl-CoA	320	0.14
Incubated with trypsin for 10 min	"	3900	0.11
None	1,3-C^{14}-malonyl-CoA‡	6500	0.07
Incubated with trypsin for 10 min	"	2700	0.08

See Table 1 for experimental details.

* C^{14}-long-chain fatty acids were identified by gas chromatography:

	Fraction of C^{14} in Fatty Acids					
	$<C_{16}$	C_{16}	C_{17}	C_{18}	C_{19}	$>C_{19}$
Untreated	0.17	0.16	0.06	0.18	0.06	0.37
Incubated with trypsin	0.15	0.22	0.02	0.17	0.02	0.42

† Decarboxylation was carried out with sodium azide and sulfuric acid in Thunberg tubes.[11]

‡ 30 mμmoles; 3×10^3 cpm/mμmoles; also contained 30 mμmoles unlabeled acetyl-CoA.

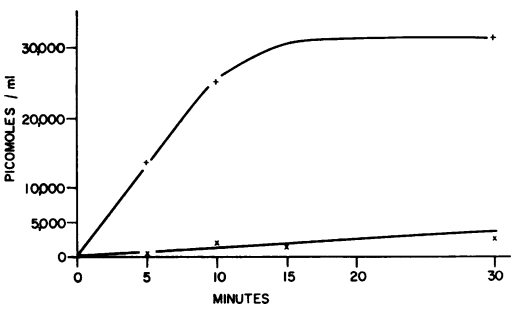

FIG. 1.—Rate of incorporation of C^{14}-acetyl-CoA by a fresh 100,000 *g* supernatant preparation: (×) untreated; (+) after incubation for 10 min with trypsin.

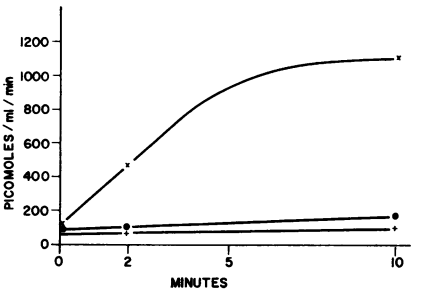

FIG. 2.—C^{14}-acetyl-CoA incorporated in 10 min versus time of incubation of a fresh 100,000 *g* supernatant preparation: (+) without additions; (×) with trypsin; (●) with chymotrypsin.

If an aliquot of the enzyme preparations incubated with trypsin was mixed with a nonincubated aliquot, incorporation of C^{14}-acetyl-CoA was the same or greater than the sum of the individually assayed aliquots (Table 4). It therefore appears that trypsin does not destroy either a competitive inhibitor of the carboxylation step or a malonyl-CoA decarboxylase[11] since the rate of incorporation of C^{14}-acetyl-CoA in the mixture was not depressed. The products of the reactions with and without incubation with trypsin were identical mixtures of long-chain fatty acids.[12] The products were identified by gas chromatography (Table 3). The distribution of labeled carbon with C^{14}-acetyl-CoA as substrate between the carboxyl group and the other carbon atoms of the products was not much affected by incubation of the 100,000 *g* supernatant preparation with trypsin (Table 3).

Experiments were carried out to determine the rate of activation of fatty acid synthesis by incubation with trypsin. It was found that the rate was dependent upon whether the 100,000 *g* supernatant preparation used was fresh or aged. In fresh preparations the rate increased with increase in time of incubation with trypsin. A maximum was usually reached after five to ten minutes with 25 μg of trypsin per ml (Fig. 2). In contrast, aged preparations were fully activated by trypsin in one minute or less (expt. 3, Table 2). Activation of fatty acid synthesis by trypsin could not be reversed by standing at 0° for several days or by incubation with ATP (expt. 4, Table 2).

Aging: The rate of incorporation of C^{14}-acetyl-CoA into fatty acids by the 100,000 *g* supernatant preparations increased on storage at 0° for some nine days (Fig. 3). However, after 15 days a precipitate appeared and the enzymatic activity was lost.

TABLE 4

FATTY ACID SYNTHESIS IN MIXED TRYPSIN-TREATED AND UNTREATED 100,000 *g* SUPERNATANT PREPARATIONS

Prep. no.	Preparation treatment	C^{14}-acetyl-CoA incorporated (cpm)
1	0.1 ml nonincubated	540
2	0.1 ml incubated with trypsin for 2 min	5,300
3	0.1 ml incubated with trypsin for 10 min	8,600
	Mixture of 0.1 ml of #1 and 0.1 ml of #2	8,100
	Mixture of 0.1 ml of #1 and 0.1 ml of #3	15,900

See Table 1 for experimental details.

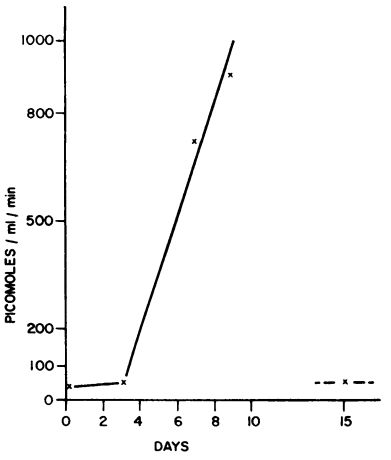

FIG. 3.—C^{14}-acetyl-CoA incorporated in 10 min: 100,000 *g* supernatant preparation stored at 0°C. After 15 days a precipitate formed in the stored solution with simultaneous loss of the enzymatic activity.

Citrate activation: Incubation with citrate brought about an increase of the rate of C^{14}-acetyl-CoA incorporation in fresh 100,000 *g* supernatant preparations from fasted rats (Fig. 4). A lag period was generally seen; the length of the lag decreased as the preparation was aged by storage at 0° or as the concentration of citrate was increased. Little or no activation was seen if a fresh preparation was incubated without citrate for one hour.

Partially purified carboxylase: Acetyl-CoA carboxylase was partially purified as previously reported[6] by precipitation with ammonium sulfate at 0.3 saturation, adsorption on and elution from calcium phosphate gel, and reprecipitation with ammonium sulfate. This partially purified preparation could be activated by incubation with citrate.[4-6] Incubation with trypsin resulted in greater activation than incubation with citrate and the effects of both seemed to be additive (expt. 1, Table 5). Incubation in the presence of Mg^{++} did not result in further activation. Carboxylase was similarly prepared from trypsin-treated 100,000 *g* supernatant fluid (expt. 2, Table 5). The kinetics of acetyl-CoA incorporation are given in Figure 5.

Discussion.—The enzyme system involved in the synthesis of long-chain fatty acids from C^{14}-acetyl-CoA exists largely in an inactive state in homogenates of livers from fasted rats, and it is present in partly activated form in liver homogenates prepared from animals fed a fat-free diet. It was found that such preparations can be activated by incubation with trypsin. The activation depends on the specific enzymic activity of trypsin, since prior treatment of trypsin with soybean trypsin inhibitor prevented the activation. Since the rate of incorporation of

TABLE 5

ACTIVATION OF PARTIALLY PURIFIED ACETYL-CoA CARBOXYLASE* BY TRYPSIN

Expt.	Additions	Incubation (min)	2×10^{-3} citrate	C^{14}-acetyl-CoA incorporated (picomoles/min/mg A†)
1	None	15	−	90
	None	15	+	270
	Trypsin‡	15	−	570
	Trypsin	15	+	880
2	None	—	−	2.5
	Trypsin	5	−	650
	Prepared from trypsin-treated 100,000 *g* supernatant	—	−	40

* The 100,000 *g* supernatant fluid was brought to 0.3 saturation with ammonium sulfate; the precipitate was dissolved in 0.005 *M* phosphate buffer (pH 7.5) and 1 mg of calcium phosphate gel per mg of protein was added. Fatty acid synthetase was eluted with 0.033 *M* phosphate buffer and acetyl-CoA-carboxylase with 0.2 *M* phosphate buffer. Each enzyme was reprecipitated with ammonium sulfate to 0.25 saturation and the precipitates were dissolved in 0.1 *M* phosphate buffer.[6]

Enzymatic activity was determined using 0.01 ml of the acetyl-CoA carboxylase solution and 0.02 ml of the synthetase as described in Table 1.

† A: acetyl-CoA carboxylase.

‡ Acetyl-CoA carboxylase was incubated with trypsin as described in Table 1. After inactivation of the trypsin by soybean trypsin inhibitor, the cofactors and synthetase were added for assay.

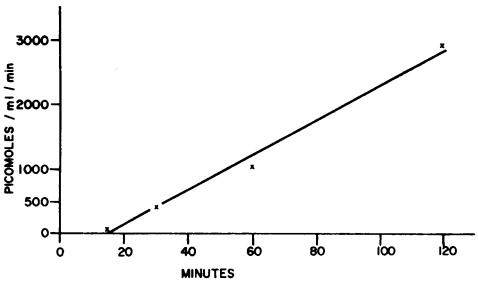

FIG. 4.—C^{14}-acetyl-CoA incorporated in 10 min: fresh 100,000 *g* supernatant preparation incubated with 2×10^{-3} *M* citrate.

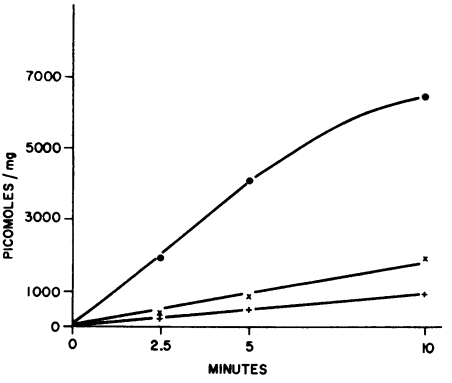

FIG. 5.—Rate of incorporation of C^{14}-acetyl-CoA by a partially purified acetyl-CoA carboxylase preparation: (+) untreated; (×) incubated with trypsin for 0 min; (●) for 3 min.

C^{14}-malonyl-CoA into long-chain fatty acids was slightly decreased by incubation with trypsin, it can be assumed that activation by trypsin is due only to action on acetyl-CoA carboxylase. The activity of partially purified acetyl-CoA carboxylase was similarly increased by incubation with trypsin. Partially purified carboxylase prepared from a trypsin-treated aliquot of the 100,000 *g* supernatant fluid was 15 times more active than carboxylase purified from an untreated aliquot. It appears that the purification procedure used[6] gives a poor yield of the trypsin-activated carboxylase, since incubation with trypsin of the partially purified carboxylase from the untreated aliquot yields considerably more active enzyme.

It seems reasonable to assume that an "activating" enzyme can account for the observation that C^{14}-acetyl-CoA incorporation in the 100,000 *g* supernatant fluid becomes partially activated on prolonged aging (7 days or more) at 0°. The lag observed in the appearance of carboxylase activity during storage and the fact that freshly prepared 100,000 *g* supernatant fluid is not activated upon incubation without citrate make it likely that the "activating" enzyme itself is present in an inactive form in fresh liver homogenates of fasted rats. It is uncertain whether an active form of the "activating" enzyme is present in livers of fed animals.

It has been shown that acetyl-CoA carboxylase can be activated by incubation with citrate[4–6] and that the rate of this reaction is accelerated by Mg^{++}.[8] In contrast to activation by trypsin treatment, activation of the100,000 *g* supernatant fluid from fasted rats by citrate requires more than one hour and is reversed in part by storage at 0° and completely by incubation with ATP.[8] It is likely, therefore, that the active forms of acetyl-CoA carboxylase obtained by trypsin treatment and by incubation with citrate are not identical. It is known that activation by citrate is accompanied by polymerization of subunits and is reversible.[7, 13] The mechanism of action of trypsin is at present unknown. It is possible that this enzyme removes a tightly bound inhibitor, possibly palmitoyl-CoA, from the carboxylase.[14, 15] It is more reasonable to assume that trypsin splits one or more peptide bonds of the inactive form of the carboxylase molecule. The formation of active carboxylase catalyzed by trypsin is probably irreversible since

the activated enzyme is resistant to inactivation by low temperatures and by ATP. Presumably, the configurational change which follows the splitting of the peptide bond(s) is not associated with a polymerization process, since activation by trypsin is a very rapid reaction.

Summary.—Preparations of 100,000 *g* supernatant fluids and partially purified acetyl-CoA carboxylase from rat liver were incubated with trypsin. A many-fold stimulation of C^{14}-acetyl-CoA incorporation by such preparations was observed. The presence of citrate was not required for this reaction. C^{14}-malonyl-CoA incorporation remained unchanged.

Abbreviations used: CoA, Coenzyme A; ATP, adenosine triphosphate; EDTA, ethylenediaminetetraacetate; PPO, 2,5-diphenyloxazole; and POPOP, 1,4-bis[2-(4-methyl-5-phenoxazolyl)] benzene.

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