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Synthesis of Acetyl Coenzyme A Holocarboxylase *in Vitro* by a Cytosolic Preparation from Chicken Liver[1] (38292)

P. N. ACHUTA MURTHY AND S. P. MISTRY[2]
(Introduced by F. A. Kummerow)

Department of Animal Science, University of Illinois at Urbana–Champaign, Urbana, Illinois 61801

ATP-dependent synthesis of holocarboxylases from their apoproteins and *d*-biotin has been demonstrated by several workers. These studies have been recently reviewed by us (1) and by Moss and Lane (2). The reaction, catalyzed by holocarboxylase synthetases, takes place according to the following scheme:

$$d\text{-Biotin} + \text{ATP} \xrightleftharpoons{Mg^{2+}} d\text{-biotinyl-5'-adenylate} + \text{PPi} \quad (1a)$$

$$d\text{-Biotinyl-5'-adenylate} + \text{apocarboxylase} \rightarrow \text{holocarboxylase} + \text{AMP}. \quad (1b)$$

Both reactions presumably are carried out by the same holoenzyme synthetase, and the biotinyl moiety is covalently linked to an ε-amino group of a lysyl residue of the apoprotein to form the active holocarboxylase.

The synthesis of acetyl CoA holocarboxylase has been demonstrated in yeast (3) and in rat adipose tissue (4–7). Leuthardt and coworkers (8, 9) reported an ATP-dependent binding of ^{14}C-biotin to chicken liver acetyl CoA apocarboxylase. The present study stems from our work on the holocarboxylase synthesizing systems of chicken liver (1). By directly measuring the synthesis of the holoenzyme, we have demonstrated the formation of acetyl CoA holocarboxylase in chicken liver preparations. Some properties of the enzyme system, including the stimulatory effect of citrate, have been investigated. The results of these studies are reported in the present paper.

Materials and Methods. Chemicals. The nucleoside triphosphates and reduced glutathione (GSH) were purchased from the Sigma Chemical Company. Avidin (12–14 U/mg) was supplied by the Worthington Biochemical Corporation. Coenzyme A was a product of P-L Biochemicals Inc. Bovine serum albumin (BSA, fatty acid poor) was obtained from the Mann Research Laboratories. Aquasol was purchased from the New England Nuclear Corporation.

Acetyl coenzyme A was prepared from acetic anhydride and coenzyme A according to the method of Simon and Shemin (10).

Animals. One-day-old female chicks (Columbian female × New Hampshire male) were made biotin-deficient by feeding a 20% egg-white diet (11) for about 6 weeks.

Enzyme preparation. The animals were fasted overnight and killed by cervical dislocation. Livers were quickly removed and placed in an ice-cold medium containing 0.3 *M* sucrose and 0.1 m*M* EDTA. Subsequent operations were carried out at 0–4° in a cold room. The tissue was washed once, minced, and homogenized in a Waring blender with 4 vol of the above medium for 3 min and the homogenate was strained through a layer of cheesecloth. Nuclei and mitochondria were removed by sedimentation at 800*g* for 15 min and 13,000*g* for 30 min, respectively (Sorvall refrigerated centrifuge, model RC2-B). Further centrifugation was carried out at 100,000*g* for 60 min (Beckman preparative ultracentrifuge, model L2-65). Since the clear supernatant had a volume too large for direct lyophilization, solid ammonium sulphate (enzyme grade) was added to 70% saturation; all the enzyme activity was recovered by this treatment. The salt precipitate was dissolved in 0.005 *M* phosphate buffer, pH 7.0, dialyzed against 100 vol of the same buffer overnight, and the dialy-

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[2] To whom requests for reprints should be addressed.

zate was lyophilized (Vortis Unitrap lyophilyzer). The preparation was stored in jars containing anhydrous calcium chloride at −20°. Under these conditions there was little loss in enzyme activity over several months

A 5% extract of the lyophilized preparation in Tris–HCl buffer (0.1 *M*, pH 7.5, extraction time 30 min) was prepared and a 60% saturated ammonium sulfate fraction of this extract was used as the enzyme source.

Enzyme assay. Acetyl CoA holocarboxylase synthesis was measured as follows. The crude enzyme preparation was preincubated with avidin (1 U/5 mg protein) for 10 min at 37° in order to suppress endogenous holocarboxylase activity and increase the sensitivity of the assay. Holocarboxylase synthesis was carried out using an assay system similar to that described by Kosow *et al.* (12) for propionyl CoA holocarboxylase synthesis. The reaction mixture contained 20 μmoles Tris–HCl buffer (pH 7.5), 0.75 μmole $MgCl_2$, 0.75 μmole ATP, 0.625 μmole GSH, 0.25 μmole *d*-biotin, 2.5 μmoles sodium citrate, and avidin-treated enzyme in a final volume of 0.5 ml. ATP was omitted from the blanks. A large excess of biotin is present in the reaction mixture in order to neutralize the avidin, which was used to inactivate the residual endogenous enzyme. The reason for adding citrate is discussed later. Incubation was carried out at 37° for 60 min. Acetyl CoA carboxylase activity was then determined by directly adding a cocktail (13) containing 10 μmoles of Tris–HCl buffer (pH 7.5), 10 μmoles $MgCl_2$, 10 μmoles sodium citrate, 2 μmoles ATP, 2 μmoles GSH, 0.2 μmole acetyl CoA, 20 μmoles $NaH^{14}CO_3$ (sp. act. 0.4 μCi/μmole), and 0.6 mg BSA in a final volume of 0.5 ml. At the end of 6 min, reaction was stopped by adding 0.2 ml of 6 *N* HCl. The precipitated protein was removed by centrifugation, and the supernates were treated with pieces of dry ice to remove unreacted ^{14}C-bicarbonate (14). Aliquots were then mixed with 15 ml of Aquasol, and radioactivity was determined in a Packard liquid scintillation spectrophotometer.

Protein was estimated by the procedure of Lowry *et al.* (15).

Results and Discussion. Citrate was found to have a stimulatory effect on acetyl CoA holocarboxylase synthesis, as shown in Table I. At a concentration of 5 m*M* (2.5 μmoles/0.5 ml and with 2.4 mg protein per tube), citrate increased the amount of holocarboxylase formed by 66%. Higher concentrations did not result in any appreciable further increases in enzyme activity. With twice the amount of protein (4.3 mg), citrate at 10 m*M* concentration doubled the enzyme activity. However, at higher concentrations, the beneficial effect of citrate appeared to be reduced. Therefore, citrate at 5 m*M* concentration was included in all enzyme assays. Whether the observed effect of citrate is on the synthetase *per se* or on the apoprotein is not clear. It is possible that citrate activates the apocarboxylase and makes it a more receptive substrate for the synthetase reaction. Such effects have been observed for the stimulation of pyruvate holocarboxylase synthesis by acetyl CoA in a bacterial system (16).

The time course of the formation of holocarboxylase is shown in Fig. 1. At a protein concentration of approximately 2 mg, routinely used in all the experiments, holoenzyme synthesis

TABLE I. Effect of Citrate on Acetyl CoA Holocarboxylase (ACCHC) Synthesis.

	ACCHC synthesized[a]			
	With 2.4 mg protein		With 4.3 mg protein	
μmoles citrate added	mU/60 min	% of control	mU/60 min	% of control
None (control)	0.184	100.0	0.285	100.0
2.5	0.306	166.3	—	—
5.0	0.306	166.3	0.594	208.0
10.0	0.313	170.1	0.579	203.2
15.0	0.315	171.2	0.405	142.1
20.0	0.296	160.9	0.481	168.8

[a] Assay conditions are as described in the text except for the indicated amounts of citrate used. Values given are averages of duplicate determinations.

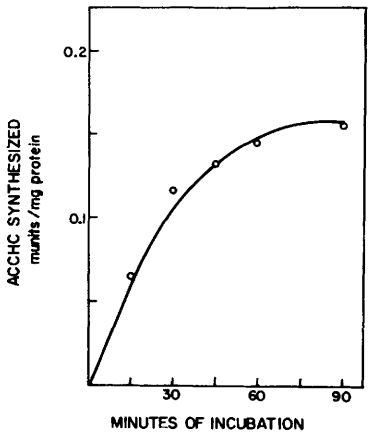

FIG 1. Kinetics of acetyl CoA holocarboxylase (ACCHC) synthesis. Assay conditions are described in the text. Values shown are averages of duplicate assays.

was fairly linear up to 60 min. A nonlinear relationship was obtained between enzyme activity and protein concentration, as shown in Fig. 2. This is to be expected since the crude enzyme preparation contains both apoenzyme and the holoenzyme synthetase.

The effect of omission of reaction components is shown in Table II. No holocarboxylase was formed in the absence of ATP. Although biotin is obviously essential for the reaction, its exclusion from the assay system did not result in complete loss of activity. Lack of $MgCl_2$ or GSH caused a partial decrease in the amount of holoenzyme formed: 32 and 35% of the control, respectively. Presumably the crude enzyme preparation contained traces of biotin as well as some Mg^{2+} ions which were responsible for the partial activity observed when omitted from the complete system.

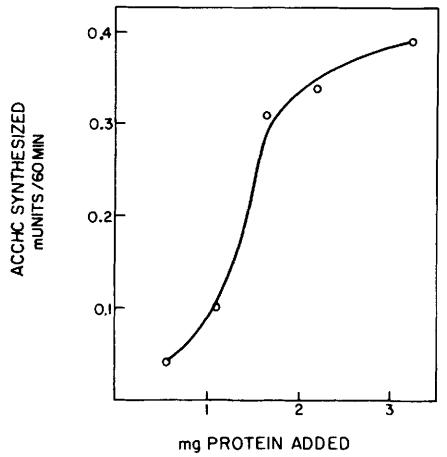

FIG. 2. Synthesis of acetyl CoA holocarboxylase *in vitro* by a cytosolic preparation from chicken liver by P. N. Achuta Murthy and S. P. Mistry.

TABLE II. Requirements for Acetyl CoA Holocarboxylase (ACCHC) Synthesis.

Component omitted from the complete system[a]	ACCHC synthesized	
	mU/60 min/mg protein	% of control
None (control)	0.142	100.0
ATP	0	0
d-Biotin	0.047	33.1
$MgCl_2$	0.097	68.3
GSH	0.093	65.5

[a] Complete system and assay procedure are given in the text. Each value is the mean of duplicate assays.

As seen from Table III, the enzyme preparation can effectively utilize other nucleoside triphosphates in place of ATP. ITP and UTP were as active as ATP in promoting holocarboxylase formation, while GTP appeared more effective than ATP. CTP gave about 80% activity as compared to the control. It is interesting to point out that lack of a strict specificity towards ATP is also found to be true for pyruvate (17) and propionyl CoA holocarboxylase (18–20) synthesizing systems of chicken liver. In contrast to these observations, holocarboxylase synthesizing systems derived from other sources are quite specific for ATP (1). Generation of ATP from other triphosphates via the kinases that could be present in the crude enzyme preparation is not ruled out. However, it has been

TABLE III. Nucleoside Triphosphate Requirement for Acetyl CoA Holocarboxylase (ACCHC) Synthesis.

Nucleoside triphosphate added[a]	ACCHC synthesized[b]	
	mU/mg protein/ 60 min	% of control
ATP (control)	0.142	100.0
CTP	0.113	79.6
GTP	0.167	117.6
ITP	0.140	98.6
UTP	0.141	99.3

[a] Where indicated ATP was replaced by an equimolar concentration of the other nucleoside triphosphate.

[b] Assay procedure is described in the text. The values are means of duplicate determinations.

observed (17) that UTP and GTP gave much higher activities than ATP for chicken liver pyruvate holocarboxylase synthesis, although all the nucleoside triphosphates were used at equimolar concentrations. This suggests that the above possibility is probably unlikely.

It is implied that the crude enzyme preparation used in these studies contained the apocarboxylase and the synthetase which catalyzes the activation. The values for the acetyl CoA holocarboxylase synthesis reported here are somewhat low when compared with those obtained for pyruvate and propionyl CoA holocarboxylase synthesis. Very likely this is because hepatic acetyl CoA carboxylase activity is not reduced as markedly as in the case of other biotin-dependent carboxylases in the rat and also in the chicken (11). We showed that in the chick, even prolonged biotin deprivation results in a loss of only about 35% in hepatic acetyl CoA carboxylase activity. This would mean that the apoprotein level is low in our crude enzyme preparation. Indeed Jacobs *et al.* (5) have shown that in biotin deficiency, very small amounts of acetyl CoA apocarboxylase accumulate in rat liver.

Summary. Using 0–60% ammonium sulfate fraction of a cytosolic preparation from biotin-deficient chicken liver as the enzyme source, the ATP-dependent formation of acetyl CoA holocarboxylase was demonstrated. Citrate significantly stimulated the holoenzyme formation. Apart from ATP, other nucleoside triphosphates proved to be about equally effective in promoting synthesis of the holoenzyme.

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