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EFFECT OF SALICYLATES ON ACETYL COENZYME A CARBOXYLASE

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Abstract—The effects of salicylates on rat and chicken liver acetyl-CoA carboxylase were investigated. Acetyl salicylate (2.5 mM) mimicked the activating effect of citrate on rat liver carboxylase if included during the preincubation period. The dissociated inactive form of chicken liver carboxylase could be reconstituted into the partially active form after dialysis against reconstitution buffer containing acetyl salicylate (2.5 mM) instead of citrate (20 mM). However, the inclusion of salicylates (acetyl- or sodium-) in the carboxylase assay medium resulted in inhibition (ranging from 24 to 32 per cent) of both the rat and chicken liver carboxylase activities.

It has been well established that acetyl-CoA carboxylase, the rate-limiting enzyme in fatty acid synthesis [1], requires citrate or isocitrate in order to attain full activity [2]. Subsequently it was shown that citrate or isocitrate transforms the enzyme from a catalytically inactive protomer into an active, fibrous-linear-polymer [3–6]. On the other hand, certain di- and tricarboxylic acids, including malonate, methylmalonate, malate and tricarballylate behave as "pseudo" activators. They appear to activate when the assay is initiated with carboxylase in the active, polymeric, form, but in fact, only retard the rate of transition of active polymer into inactive protomer and are unable to reverse this process [3, 4, 7]. We have shown earlier [8] that the biotin prosthetic group of the carboxylase is involved in the citrate mediated enzyme transition.

Our interest in the effects of salicylates on acetyl-CoA carboxylase arose from conflicting reports on the effect of salicylates on lipid synthesis. Chronic administration of salicylates has been shown to lead to fatty degeneration of the liver in man [9] and rabbits [10, 11]. However, both *in vivo* and *in vitro* inhibitions of fatty acid synthesis in rat liver by salicylates have been reported [12–14].

MATERIALS AND METHODS

ATP, acetyl-CoA, *N*-acetyl cysteine, disodium EDTA, dithiothreitol (DTT), acetyl salicylic acid, *p*-amino salicylic acid, Tris buffer and glutathione (reduced form) were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.); potassium chloride, potassium phosphate (mono- and dibasic) and sodium salicylate were obtained from the Fisher Scientific Co. (NJ, U.S.A.). Analar grades of magnesium sulfate, magnesium chloride and sodium citrate were purchased from the British Drug Houses Ltd., (Toronto, Canada), cystalline bovine serum albumin (fraction V, fatty acid poor) from the Nutritional Biochemical Corp. (Cleveland, OH), and ammonium sulfate (special enzyme grade) from Schwarz/Mann (NY, U.S.A.). $NaH^{14}CO_3$ was purchased from the New England Corp. (Boston, MA, U.S.A.).

Enzyme preparation

All operations were carried out at 4° unless mentioned otherwise.

Rat liver enzyme. The livers were homogenized in 3 vol. of buffer A (0.15 M KCl, pH 7.0. containing 4 mM EDTA, 4 mM $MgSO_4$ and 4 mM *N*-acetyl cysteine). The homogenate was gauze filtered and then centrifuged at 105,000 *g* for 60 min; the enzyme was precipitated by the addition of solid ammonium sulfate (30% saturation). The precipitate was dissolved in buffer B (20 mM potassium phosphate, pH 7.4, containing 0.1 mM EDTA and 0.5 mM DTT) with 20 mM sodium citrate. The enzyme was frozen (−20°) until further use.

Chicken liver enzyme. Acetyl-CoA carboxylase was partially purified from chicken liver, as described by Gregolin *et al.* [3], up to the calcium phosphate gel fractionation and ammonium sulfate precipitation step. The enzyme was dialyzed against buffer B containing 20 mM sodium citrate, divided into 2 or 3 ml batches and stored frozen (−20°) until further use.

Enzyme assay

Acetyl-CoA carboxylase activity was determined by a $H^{14}CO_3^-$ fixation assay procedure [15].

Chicken liver enzyme was assayed without prior incubation with citrate because the enzyme, as prepared, was in the active polymeric form [6, 16]. The rat liver enzyme had to be converted into the active form by preincubation with citrate before it could be assayed.

Rat liver enzyme (0.15 ml) was preincubated at 37° for 15 min with either aqueous salicylate solution (pH 7.0, 0.05 ml) or buffer, as indicated in individual experiments (see legends to figure and tables) and then incubated for 30 min (37°) with 0.05 ml of the regular preincubation medium (RPIM). The RPIM had the following composition: Tris–Cl^-, 0.3 M; glutathione (reduced form), 15 mM; $MgCl_2$, 40 mM; bovine serum albumin, 150 μg; and sodium citrate, 0.1 M, pH 7.6.

Assay procedure

The assay mixture (0.5 ml total volume, final pH 7.5 at 25°) contained the following components (in

μmoles): Tris–Cl^-, 60; ATP, 3; $MgCl_2$, 8; $NaH^{14}CO_3$ (sp. act. 0.6 μCi/μmole), 10; acetyl-CoA, 0.2; sodium citrate, 20; glutathione (reduced form), 3; and bovine serum albumin, 0.6 mg. The reaction was initiated by the addition of 0.05 ml of the enzyme and was carried out at 37° for 2 min; the reaction was then terminated by adding 0.1 ml of 6 N HCl. An aliquot (0.2 ml) was taken to near dryness in a scintillation vial followed by the addition of 15 ml of scintillation fluid [15]. ^{14}C-activity was determined with the use of a liquid scintillation spectrometer. The reaction was linear with respect to the amount of carboxylase added, up to a reaction period of 5 min (at 37°).

Dissociation and association of chicken liver acetyl-CoA carboxylase

The polymeric enzyme obtained from chicken liver, as described above, was dissociated into its protomeric form according to the method described by Gregolin *et al.* [16]. The enzyme (3 to 3.2 mg protein/ml) was dialyzed overnight against buffer D (50 mM Tris–Cl^-, and 0.5 M NaCl, pH 8.0, containing 0.1 mM EDTA and 0.5 mM DTT). Reconstitution of the dissociated enzyme back into the active, polymeric form was achieved by overnight dialysis against buffer T (50 mM Tris–Cl^-, pH 7.5, containing 0.1 mM EDTA and 0.5 mM DTT) with 20 mM sodium citrate. In the experiment where the effect of salicylate was tested on the association of the enzyme, 20 mM sodium citrate (in buffer T) was replaced by 2.5 mM acetyl salicylate.

Protein was determined by a BIO-RAD protein determination procedure (BIO-RAD Protein-Assay, Technical Bulletin 1051). One milliunit of carboxylase is defined as that amount of enzyme which catalyzes the carboxylation of 1 nmole acetyl-CoA/min of reaction at 37°.

RESULTS

As shown in Fig. 1, more than 85 per cent of the activity of rat liver acetyl-CoA carboxylase was lost when the enzyme was incubated at 37° for 15 min in the absence of citrate. However, when salicylate was included during this incubation, the loss in enzymatic activity was prevented, proportionate to the concentration of salicylate added. Acetyl salicylate was found to be more effective than either sodium- or *p*-amino salicylate. At 2.5 mM, acetyl salicylate completely prevented the inactivation of the carboxylase during 15 min of preincubation, thus mimicking the effect of citrate.

In contrast to this, an entirely opposite effect of the salicylates was observed if the drug was included in the acetyl-CoA carboxylase assay mixture instead of being added during the preincubation period. In the experiments where salicylate mimicked the effect of citrate, the concentration of salicylate in the assay medium was one order of magnitude lower (i.e. 0.01 mM to 0.25 mM), since 0.05 ml of preincubated enzyme (including salicylate ranging from 0.1 to 2.5 mM) was added to a total assay mixture of 0.5 ml.

However, when acetyl salicylate was added to the carboxylase assay medium at a concentration of 2.5 mM, it inhibited both rat and chicken liver carboxylase activities by 27 and 24 per cent respectively. At the same concentration (2.5 mM), sodium- and *p*-amino salicylate also inhibited rat liver carboxylase by 32 and 19 per cent respectively (Table 1).

From the data presented in Fig. 1 one cannot distinguish between the two possible modes of action of salicylate on the carboxylase, viz. prevention of enzyme inactivation or promotion of enzyme activation. To investigate this, we studied the effect of salicylate on chicken liver acetyl-CoA carboxylase, which is more

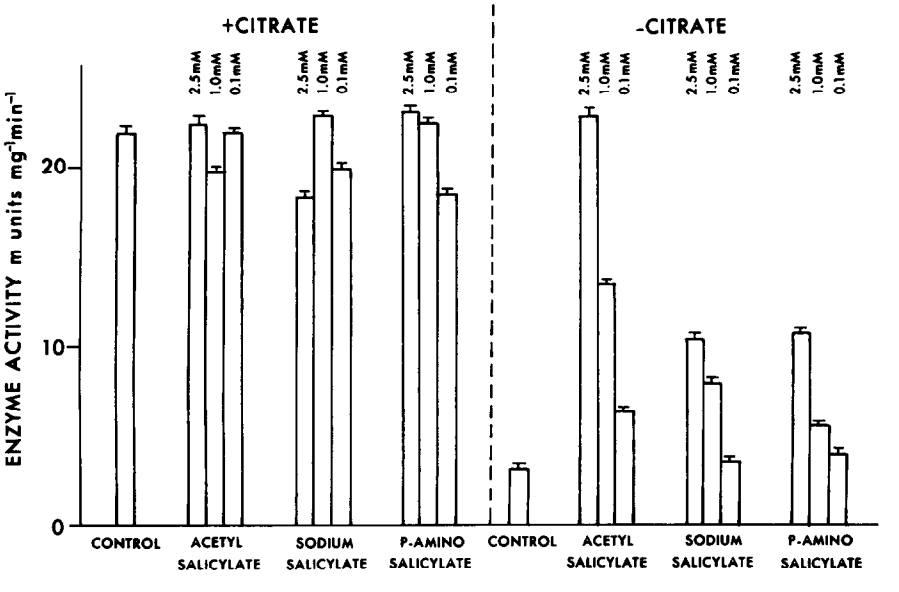

Fig. 1. Effect of salicylates on rat liver acetyl-CoA carboxylase preincubated (37°, 15 min) in the presence or absence or citrate. The rat liver enzyme (~4 mg protein/ml) was dialyzed overnight against buffer B. The dialyzed enzyme (0.15 ml) was incubated at 37° for 15 min with 0.05 ml of buffer B (control) or salicylate solution (aqueous, pH 7.2). RPIM (0.05 ml) was then added to all the tubes and incubation (37°) was carried for another 30 min. The reaction was initiated by the addition of 0.05 ml of the preincubated enzyme and the assay was carried out as described under Materials and Methods.

Table 1. Effects of salicylates, when added to the reaction mixture during assay, on the activity of rat and chicken liver acetyl-CoA carboxylase *

Salicylate concn × 10^{-3} M	Rat liver enzyme		Chicken liver enzyme	
	Sp. act. (m-units/ mg protein)	% Inhibition	Sp. act. (m-units/ mg protein)	% Inhibition
None (control)	4.51		19.41	
Acetyl salicylate				
2.5	3.29	27.1	14.73	24.1
1.0	3.22	28.6	16.75	13.7
0.1	3.97	12.0	17.01	12.9
Sodium salicylate				
2.5	3.06	32.15		
1.0	4.06	10.0		
0.1	3.82	15.3		
p-Amino salicylate				
2.5	3.66	18.9		
1.0	3.77	19.4		
0.1	4.40	2.4		

* Rat liver enzyme was dialyzed against buffer B and preincubated with RPIM at 37° for 45 min. Chicken liver enzyme was dialyzed against buffer B (containing 20 mM sodium citrate) and assayed without preincubation. Enzyme (0.05 ml) was assayed (at 37° for 2 min), in the presence of the drug added to the reaction mixture, as described under Materials and Methods.

stable than the rat liver enzyme. The chicken liver enzyme was disaggregated and the effect of salicylate on enzyme activation studied.

As seen in Table 2, almost 86 per cent of the original activity was lost when the chicken liver carboxylase was dialyzed against the dissociation buffer (buffer D). Overnight dialysis in Tris–Cl^- buffer (50 mM, pH 7.5) containing 20 mM citrate almost completely restored the enzymatic activity. Approximately 50 per cent of the activity (of the control in citrate buffer) could be restored when citrate was replaced by 2.5 mM acetyl salicylate in the reconstitution buffer, suggesting that acetyl salicylate (2.5 mM) could promote the aggregation of the carboxylase into the active polymeric form.

DISCUSSION

The results presented suggest that salicylates, when included during the preincubation of acetyl-CoA carboxylase, can replace the citrate requirement for maximal catalytic activity. Also, it has been demonstrated that acetyl salicylate prevents the inactivation caused by the irreversible disaggregation of the rat liver carboxylase during a 15-min exposure of the enzyme to 37° in the absence of citrate. The observation that, in the presence of citrate, salicylate had no effect on enzyme activity indicated that salicylates act on the protomeric form of the enzyme, and once the enzyme is in the polymeric state, salicylates can not activate it any further. It is possible that, like citrate, salicylates bind only to the protomeric form of the enzyme. The effect of salicylate is different from the effects of the "pseudo" activators.

Gregolin *et al.* [16] have demonstrated that chicken liver carboxylase can be dissociated by dialysis in a high salt-buffer at alkaline pH (8.0) in which the enzyme is converted to the catalytically inactive protomeric form (13–15 S). Dialysis overnight against Tris–

Table 2. Effect of acetyl salicylate on the reconstitution of the depolymerized acetyl-CoA carboxylase from chicken liver

Enzyme * dialyzed in	Sp. act. (m-units/mg protein/min reaction)	% Control
Buffer B + 20 mM sodium citrate followed by buffer T	19.43	100
Buffer D followed by buffer T	2.79	14
Buffer D followed by buffer T + 20 mM sodium citrate	18.25	94
Buffer D followed by buffer T + 2.5 mM acetyl salicylate	9.08	47

* Protein concentration, 3 to 3.5 mg/ml; all buffers contained 0.1 mM EDTA and 0.5 mM DTT. Buffer B: 20 mM potassium phosphate, pH 7.3; Buffer D: 50 mM Tris–Cl^- + 0.5 M NaCl, pH 8.0; and Buffer T: 50 mM Tris–Cl^-, pH 7.4. Enzyme assay was carried out as described under Materials and Methods.

Cl^- (50 mM, pH 7.5) buffer containing citrate (20 mM) restored the enzyme activity, with the concomitant formation of the polymeric filamentous enzyme structure as observed under the electron microscope [16]. In our study we have demonstrated that the requirement of citrate in the reconstitution dialysis buffer can be partially replaced by acetyl salicylate (2.5 mM).

The relatively mild inhibition of the carboxylase activity by salicylates when added to the carboxylase assay medium is in contrast to the activation of the enzyme when the drug is present in the preincubation medium of the rat liver acetyl-CoA carboxylase. However, this inhibition could be attributed to the 10-fold higher concentration of the drug in these assays.

Thus, the *in vivo* effect of salicylate on fatty acid synthesis is quite complex depending, perhaps, on the actual concentration of the drug at the site of the enzyme. This could explain the contradictory data in the literature suggesting either increased [9–11] or decreased [12–14] lipogenesis following administration of salicylates to animals and man.

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