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Evidence for a Medium-Chain Fatty Acid:Coenzyme A Ligase (Adenosine Monophosphate) That Activates Salicylate

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SUMMARY

A medium-chain fatty acid:CoA ligase (AMP) which activates salicylate has been purified approximately 30-fold from bovine liver "mitochondria." The new enzyme is not completely free of a medium-chain fatty acid:CoA ligase (AMP) (EC 6.2.1.2) of the type first described by Mahler, Wakil, and Bock [*J. Biol. Chem.* **204,** 453 (1953)], but the two enzymes can be distinguished readily from each other. The enzyme with salicyl-CoA synthetase activity is more easily inactivated, migrates more slowly toward the anode during disc gel electrophoresis, and utilizes a wider spectrum of aromatic acid substrates than the enzyme described earlier. Evidence was obtained that both enzymes could catalyze the activation of hexanoate, benzoate, *o*-methoxybenzoate, and anthranilate, but only the "salicylate" enzyme displayed activity with salicylate and *p*-aminosalicylate.

INTRODUCTION

In mammalian liver and kidney, the formation of thioesters of coenzyme A with benzoate and salts of several other aromatic carboxylic acids is reportedly catalyzed by a single enzyme with broad acyl substrate specificity, i.e., the mitochondrial medium-chain (C_4–C_{11}) fatty acid:CoA ligase (AMP) (EC 6.2.1.2) of Mahler, Wakil, and Bock (1). It has been shown in a companion paper (2) that salicylurate biosynthesis from salicylate in bovine liver mitochondria proceeds by formation of salicyl-CoA, which then acylates glycine in the second and final reaction of the pathway. However, salicylate has been reported not to serve as a substrate for preparations of partially purified medium-chain fatty acid:CoA ligase (AMP) from the same tissue source (3). This raised the possibility that another acyl-CoA synthetase activates salicylate. The heterogeneity of aromatic acid-activating enzymes might also explain the differences in ratios of salicylurate to hippurate formation observed in various species *in vivo*, especially if these enzymes catalyze relatively slow steps in each pathway and if there is little discrimination between salicyl-CoA and benzoyl-CoA as substrates for the *N*-acylation of glycine (2).

Therefore, the enzymatic activation of

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several acids by a "mitochondrial" fraction of bovine liver was examined on the supposition that more than one enzyme with aromatic acid:CoA ligase (AMP) activity is present. The experimental results supporting this concept are presented in this report.

EXPERIMENTAL PROCEDURE

Assays for enzymatic activity and protein. Complete reaction mixtures for the formation of CoA thioesters of hexanoate, benzoate, salicylate, and *p*-aminosalicylate contained the following in a final volume of 1 ml: 100 μmoles of Tris-HCl buffer (pH 8.2), 5 μmoles of disodium ATP, 5 μmoles of $MgCl_2$, 0.5 μmole of CoA, 0.5–2.0 μmoles of acyl substrate containing 80,000–120,000 cpm of carboxyl-^{14}C radioactive label, 1 μg of crystalline inorganic pyrophosphatase, and 20–800 μg of enzyme protein. After incubation periods of up to 15 min, the reactions were terminated with 0.2 ml of 2 M $HClO_4$, and denatured protein was removed by centrifugation. The contaminating radioactive acid was removed from the acid supernatant solution by ether extraction, and the remaining ^{14}C-acyl-CoA was determined in a neutralized aliquot as outlined previously (2). Maximum backgrounds were 60 cpm for the salicylate, benzoate, and hexanoate assays and 200 cpm in the *p*-aminosalicylate assay.

The concentrations of reaction components for conversion of anthranilate and *o*-methoxybenzoate to the corresponding thioesters of CoA were the same as above except that the acyl substrate was nonradioactive. Reaction volumes were 0.5 ml, and enzymatic catalysis was terminated with 70 μl of 20% trichloroacetic acid, after which protein was removed by centrifugation. Aliquots of each supernatant solution were employed for determination of pyrophosphate (equivalent to 2 moles of P_i) by the method of Fiske and SubbaRow (4). The acyl substrate or CoA was omitted from the control, which was processed in parallel with the complete reaction mixtures. For balance studies, residual CoA (—SH) was determined by the method of Ellman (5).

For determinations of acyl-CoA synthetase activity in analytical disc gels, the gels were sliced transversely into 1.5-mm-wide segments with the marker dye positioned in the middle of one segment. Individual segments were then crushed with a glass rod in 1.15 ml of an ice-cold mixture of 120 μmoles of Tris-HCl buffer (pH 8.0), 6 μmoles of disodium ATP, 6 μmoles of $MgCl_2$, 0.5–2.0 μmoles of acyl substrate (hexanoate, benzoate, or salicylate) containing 400,000–1,200,000 cpm of carboxyl-^{14}C, and 1 μg of crystalline pyrophosphatase. The pH of each mixture was adjusted to 8.2 at 4° prior to addition of the gel. Tubes were first incubated for 15 min at 4°, followed by 10 min at room temperature. They were brought to 38°, and reactions were started by addition of 0.6 μmole of CoA in 50 μl. After 17 min, reactions were terminated with 0.2 ml of 20% $HClO_4$. Mixtures were centrifuged, and ^{14}C-acyl-CoA was determined in an aliquot of each supernatant solution after ether extraction (2). Background ranged from 100 to 200 cpm, depending on the acyl substrate.

Units of enzymatic activity are expressed as nanomoles of product formation (or substrate disappearance) per minute at 38°. Specific activities are presented as units of activity per milligram of protein. Protein concentrations were determined by the biuret procedure and related to a standard of crystalline bovine serum albumin (6).

Purification of an enzyme with salicyl-CoA synthetase activity from bovine liver "mitochondria." The ratio of hexanoyl-CoA to benzoyl-CoA to salicyl-CoA synthetase activities differed among various crude preparations of beef liver "mitochondria." Because this phenomenon might be expected if more than one protein catalyzed these reactions, fractions of "salicylate" enzyme were selected during the purification for low ratios of hexanoyl-CoA to salicyl-CoA synthetase activity as well as for increasing specific activities in the salicyl-CoA synthetase assay. During certain steps of the purification, use of these criteria resulted in only modest increases in specific activity and/or recoveries of units for the "salicylate" enzyme (Table 1).

An aliquot of frozen bovine liver "mitochondria" was thawed into glycerol (10%, v/v), and the suspension was clarified by centrifugation at 78,000 × *g* for 45 min as described previously (2). The supernatant solution, which contained 4.4 g of protein, was diluted to a protein concentration of 7 mg/ml with 20 mM $KHCO_3$–130 mM KCl–2 mM $MgCl_2$–2 mM ATP–0.5 mM sodium salicylate–4 mM 2-mercaptoethanol (solution A). All subsequent procedures were performed at 0–4°. Solid ammonium sulfate was added (23 g/100 ml of solution), and the pH was kept at 8 with M NH_4OH. After stirring for 15 min, the suspension was centrifuged at 24,000 × *g* for 10 min and the precipitate was discarded. Ammonium sulfate was added to the supernatant solution (8 g/100 ml of original solution) while the pH was maintained at 8. The mixture was stirred for 15 min and centrifuged for 10 min at 24,000 × *g*. The resulting supernatant solution was stored at −70°, and aliquots were used later for purification of a medium-chain fatty acid: CoA ligase (AMP) with minimal salicyl-CoA synthetase activity (Table 3). The precipitate was packed by a second centrifugation at 24,000 × *g* to remove excess liquid $(NH_4)_2SO_4$.

A reverse ammonium sulfate fractionation was performed by first suspending the precipitate in 15 ml of 2.12 M $(NH_4)_2SO_4$ in solution A. The mixture was stirred for 15 min and then centrifuged at 24,000 × *g* for 10 min. The resulting precipitate was suspended in 80 ml of 1.81 M $(NH_4)_2SO_4$ in solution A, stirred for 15 min, and centrifuged again. The new precipitate was subjected to the same procedure, except that 160 ml of 1.81 M $(NH_4)_2SO_4$ in solution A were used. The final precipitate was dissolved in solution A to a protein concentration of 35 mg/ml. The volume at this stage was between 16 and 17 ml. The solution was divided into three aliquots and stored at −70°.

After thawing, each of the three aliquots was chromatographed on a Sephadex G-100 column (36 × 2.5 cm) equilibrated with solution A. Each column was eluted with solution A at a flow rate of 0.3–0.4 ml/min, and 1.8-ml fractions were collected. Salicyl-CoA synthetase activity appeared after the main protein peak, and those fractions having specific activities greater than the starting material were pooled. The active fractions from each column were combined and stored at −70°.

The pooled active fractions were thawed, and the protein was precipitated by adding 40 g of $(NH_4)_2SO_4$ per 100 ml while the pH was maintained at 8 with NH_4OH. The precipitate was washed successively with 7 ml and 10 ml of 2.12 M $(NH_4)_2SO_4$ in solution A, and centrifuged after each wash. The resulting precipitate was dissolved in solution A, and the protein concentration was adjusted to 32 mg/ml. This material was placed on a Sephadex G-100 column (40 × 2 cm) equilibrated with solution A. The column was eluted with the same solution at a flow rate of 0.3 ml/min, and 1.0-ml fractions were collected. Active fractions were pooled and subjected to a second reverse $(NH_4)_2SO_4$ fractionation after concentration against 20% polyethylene glycol.

The protein was precipitated by adding 40 g of $(NH_4)_2SO_4$ per 100 ml while the pH was maintained at 8. After 15 min, the suspension was centrifuged at 24,000 × *g* for 10 min. The precipitate was then equilibrated at a protein concentration estimated at 5 mg/ml in successively decreasing concentrations of $(NH_4)_2SO_4$ in solution A (2.12, 1.78, 1.48, and 1.15 M). After stirring for 10 min, each suspension was centrifuged for 10 min at 24,000 × *g*, and the resulting precipitate was resuspended and stirred for 10 min in the next lower concentration of $(NH_4)_2SO_4$. Protein was precipitated from each supernatant solution by addition of sufficient crystalline $(NH_4)_2SO_4$ to bring the final concentration to 2.6 M. The precipitates were collected by centrifugation, dissolved in 0.3–0.5 ml of solution A, and stored at −70°.

Preparation of a medium-chain fatty acid:CoA ligase (AMP) with minimal salicyl-CoA synthetase activity. A side fraction obtained during the purification of the "salicylate" enzyme, which also had a low hexanoyl-CoA to salicyl-CoA activity ratio, was used to purify hexanoyl-CoA synthe-

tase activity. However, substrates and cofactors which protected salicyl-CoA synthetase activity (solution A) were not used during the purification. The final enzyme preparation had only a trace of salicyl-CoA synthetase activity, and its properties were then compared with those of the "salicylate" enzyme.

The supernatant solution remaining after the first $(NH_4)_2SO_4$ precipitation of the "salicylate" enzyme (23–31 g of $(NH_4)_2SO_4$ added per 100 ml of solution) was thawed, and 19 g of $(NH_4)_2SO_4$ were added per 100 ml of solution while the pH was kept at 8. After 15 min of stirring, the suspension was centrifuged for 10 min at 24,000 × *g*. The precipitate was dissolved in 20 mM $KHCO_3$–4 mM 2-mercaptoethanol, and the protein concentration was adjusted to about 32 mg/ml. Six milliliters of this solution were placed on a Sephadex G-100 column (36 × 2.5 cm) equilibrated with 20 mM $KHCO_3$–4 mM 2-mercaptoethanol. The protein was eluted with the equilibrating solution in 2.4-ml fractions at a flow rate of 0.7 ml/min. Two major peaks of protein were identified, and fractions having the highest hexanoyl-CoA synthetase activity were centered in the trailing peak. The gel filtration step was repeated with a second aliquot of the dissolved $(NH_4)_2SO_4$ precipitate, and active fractions from both columns were pooled.

Protein in the active fractions was precipitated by adding $(NH_4)_2SO_4$ to a final molarity of 2.8 while the pH was kept at 8. After 15 min of stirring, the mixture was centrifuged at 24,000 × *g* for 10 min, and the precipitate was subjected to a series of washes at decreasing concentrations of $(NH_4)_2SO_4$ dissolved in 20 mM $KHCO_3$–50 mM KCl–4 mM 2-mercaptoethanol, adjusted to pH 8 with NH_4OH. The precipitate was stirred in each wash for 10 min and then centrifuged for 10 min at 24,000 × *g*. The volumes and concentrations of $(NH_4)_2SO_4$ used in successive washes were 14 ml of 2.15 M, 10 ml of 2.0 M, and 10 ml of 1.85 M. The 2.0 and 1.85 M $(NH_4)_2SO_4$ washes, which contained most of the activity and protein, were combined, and the protein was precipitated by adding 3.7 g of $(NH_4)_2SO_4$ while the pH was kept at 8 with NH_4OH. After 15 min of equilibration, the precipitate was collected by centrifugation, taken up in a small volume of 2 mM ATP–2 mM $MgCl_2$–50 mM KCl, and stored at −70°.

After thawing, the preparation was dialyzed for 2 hr in a rocking dialysis chamber against 100 volumes of 20 mM $KHCO_4$–4 mM 2-mercaptoethanol. The dialyzed sample (protein concentration, approximately 25 mg/ml) was applied to a 10 x 0.9 cm column of Sephadex G-100 equilibrated with 20 mM $KHCO_3$–4 mM 2-mercaptoethanol. The column was eluted with the same solution, and 0.5-ml fractions were collected. Those with the highest hexanoyl-CoA synthetase activity were combined and stored at −70°.

Other methods. Analytical disc gel electrophoresis was performed as described previously in 7.5% polyacrylamide gels at 4° with 0.04 M Tris–0.2 M β-alanine–4 mM 2-mercaptoethanol adjusted to pH 8.9 in the electrode compartments (2). Bromphenol blue was used for the marker dye, and its distance from the interface of the stacking and separatory gels was measured for each gel immediately after the electrophoresis. Two of the gels were stained with Amido black for protein visualization, and the remaining gels were measured and transected into 1.5-mm segments for enzymatic assays as indicated above. Gels stained with Amido black were scanned at a rate of 1 cm/min in a Gilford recording spectrophotometer at a wavelength of 600 mμ.

Materials. Carboxyl-^{14}C-labeled acids were purchased from New England Nuclear Corporation. The sources of other materials were the same as those specified previously (2).

RESULTS

Salicyl-CoA synthetase activity was purified in the presence of salicylate, ATP, $MgCl_2$,[3] and 2-mercaptoethanol to a specific activity of 24.7 units/mg of protein. This represented about a 30-fold purification from the crude mitochondrial protein (Table 1). With added inorganic pyrophosphatase, salicyl-CoA formation up to 60 mμmoles/ml was linear with enzyme concentration and

TABLE 1

Purification of medium-chain fatty acid:CoA ligase (AMP) with salicyl-CoA synthetase activity

Assay mixtures contained 0.5 μmole of salicylate or 2 μmoles of hexanoate per milliliter. The concentrations of other reaction components are given in the text.

Step	Total protein	Enzyme activity				Activity ratio, hexanoate: salicylate
		Salicylate		Hexanoate		
		Specific activity	Total units	Specific activity	Total units	
	mg					
Crude mitochondrial fraction	4,350	0.8	3,480	65	282,800	81
Ammonium sulfate (23–31%)	1,239	1.8	2,230	84	104,100	47
1st reverse ammonium sulfate	573	2.9	1,660	104	59,590	36
1st Sephadex G-100	95	12.5	1,190	398	37,800	32
2nd Sephadex G-100	27	19.5	530	698	18,850	36
2nd reverse ammonium sulfate						
1.78 M	2.4	7.9	19	272	650	34
1.48 M	8.5	24.7	210	810	6,890	33
1.15 M	7.3	22.9	167	794	5,800	35

with time during the first 15 min. In the presence of the pyrophosphatase, twice as much phosphate appeared as CoA disappeared or salicyl-CoA was formed, which is consistent with an AMP-pyrophosphate cleavage in this reaction (Table 2). The "salicylate" enzyme lost appreciable activity during the purification if not kept in the presence of salicylate, ATP, $MgCl_2$, and 2-mercaptoethanol.

When the purified "salicylate" enzyme (specific activity, 24.7 units/mg) was subjected to analytical disc gel electrophoresis, multiple bands of protein were visualized after staining with Amido black, indicating that the preparation was not homogeneous (Fig. 1, upper panel). Salicyl-CoA synthetase activity, which, depending on the preparation, was recovered in 1.5–2.5% yield, formed a peak at an R_F of about 0.69 of the marker dye, as did the bulk of the hexanoyl-CoA and benzoyl-CoA synthetase activity (Fig. 1, lower panel). The major band of protein was also found in the same location, at an R_F of 0.685, and this comprised roughly 28% of the total protein added to the gel (Fig. 1, upper panel).

The remainder of the recoverable hexanoyl-CoA and benzoyl-CoA synthetase activity in the "salicylate" preparation migrated in a smaller peak at an R_F of about 0.82–0.84 (Fig. 1, lower panel).

TABLE 2

Balance study of salicyl-CoA synthetase reaction

A 2-ml reaction mixture was used with concentrations of components, conditions of assay, and analytical techniques as given in the text. The enzyme had a specific activity of 12.6 units/mg in the salicyl-CoA synthetase assay, and 0.92 μg of this protein was added; incubation was conducted for 10 min.

Salicyl-CoA formed	CoA (—SH) disappearance	PP_i appearance
mμmoles	*mμmoles*	*mμmoles*
114.8	108.2	118.2

Less than 1.6% of the added hexanoyl-CoA and benzoyl-CoA synthetase activity was recovered in this region. A protein band containing less than 4% of the total stained material was visualized at an R_F of 0.83. This corresponded, within experimental error, to the smaller peak of hexanoyl-CoA and benzoyl-CoA synthetase activity described (Fig. 1).

To investigate whether the hexanoyl- and benzoyl-CoA synthetase activity found in the region of the gel showing salicyl-CoA synthetase activity was due to the "salicylate" enzyme or to some other protein, a preparation capable of utilizing hexanoate and benzoate as substrates, but possessing minimal salicyl-CoA synthetase activity,

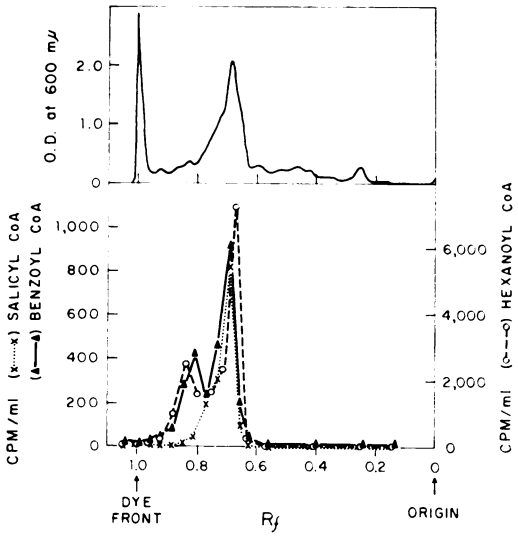

FIG. 1. *Analytical disc gel electrophoresis of a medium-chain fatty acid:CoA ligase (AMP) with salicyl-CoA synthetase activity*

Each column received 0.15 mg of protein [1.48 M $(NH_4)_2SO_4$ fraction from the second reverse $(NH_4)_2SO_4$ step, Table 1] with 810 units of hexanoyl-CoA synthetase specific activity per milligram of protein and a hexanoyl-CoA to benzoyl-CoA to salicyl-CoA synthetase activity ratio of 33:11:1. A densitometry scan of one typical gel stained with Amido black is shown in the upper panel. Assays of acyl-CoA synthetase activity are depicted in the lower panel as counts per minute of acyl-CoA formed per milliliter of reaction mixture. Recoveries of added enzymatic activity were 4.2, 2.5, and 1.5% for hexanoyl-CoA, benzoyl-CoA, and salicyl-CoA synthetase, respectively. Specific assay methods are given in the text.

was prepared (Table 3). When examined by analytical disc gel electrophoresis, this material exhibited only one major peak of hexanoyl-CoA and benzoyl-CoA synthetase activity (approximately 30% of the added units), which was positioned at about 84% of the mobility of the marker dye. A heavily stained band was located at the 83% region, which by densitometry contained approximately 12% of the total protein added to the gel (Fig. 2, upper panel). No protein peak was seen in the region of the gel where salicyl-CoA synthetase activity characteristically migrates (Fig. 1), although both this and the "salicylate" preparation displayed an inactive protein peak at an R_F of 0.715.

The purified "salicylate" preparation catalyzed acyl-CoA formation with hexanoate, benzoate, anthranilate, *o*-methoxybenzoate, and *p*-aminosalicylate (Table 4). The preparation of fatty acyl-CoA synthetase with minimal salicyl-CoA synthetase activity (Table 3) showed a different substrate spectrum. This material readily utilized hexanoate, benzoate, and anthranilate as acyl donors; less activity was seen with *o*-methoxybenzoate, and only minimal activity was noted with salicylate and *p*-aminosalicylate (Table 4).

DISCUSSION

The experimental data support the existence of a previously unrecognized acid:CoA ligase (AMP) which activates salicylate.

TABLE 3

Purification of medium-chain fatty acid:CoA ligase (AMP) with negligible salicyl-CoA synthetase activity

Assay mixtures contained 0.5 μmole of salicylate or 2 μmoles of hexanoate per milliliter. The concentrations of other reaction components are given in the text.

Step	Total protein	Enzyme activity				Activity ratio, hexanoate: salicylate
		Salicylate		Hexanoate		
		Specific activity	Total units	Specific activity	Total units	
	mg					
Ammonium sulfate precipitation	384	1.7	653	59	22,620	35
1st Sephadex	105	0.58	61	87	9,190	150
Reverse ammonium sulfate	47	0.35	16	195	9,160	557
Dialysis		0.30		186		620
2nd Sephadex	22	0.16	4	144	3,170	900

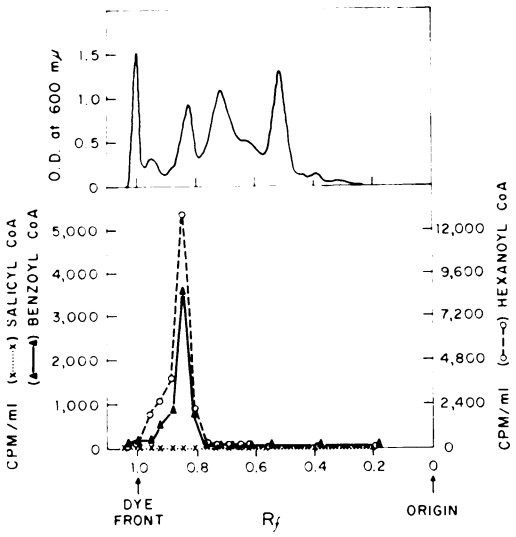

FIG. 2. *Analytical disc gel electrophoresis of a medium-chain fatty acid:CoA ligase (AMP) with little salicyl-CoA synthetase activity*

Each column received 0.16 mg of protein [reverse $(NH_4)_2SO_4$ step, Table 3] with 238 units of hexanoyl-CoA synthetase specific activity per milligram of protein and a hexanoyl-CoA synthetase to salicyl-CoA synthetase activity ratio of 595:1. A densitometry scan of one such gel stained with Amido black is shown in the upper panel. Assays of acyl-CoA synthetase activity in gel segments are depicted in the lower panel as counts per minute of acyl-CoA formed per milliliter of reaction mixture. Recoveries of added enzymatic activity were 32.6 and 29.5% for hexanoyl-CoA and benzoyl-CoA synthetase, respectively. No significant salicyl-CoA synthetase activity was detectable in any gel segment. Specific assay methods are given in the text.

The changing ratios of hexanoyl-CoA to salicyl-CoA synthetase activity observed during various fractionation procedures suggested this possibility, but the most definitive evidence that the "salicylate"-activating enzyme differs from an intermediate-chain fatty acyl-CoA synthetase of the type first described by Mahler, Wakil, and Bock (1) is derived from the separation of these two proteins by analytical disc gel electrophoresis (Figs. 1 and 2). The "salicylate" enzyme migrated more slowly and was far less stable in this system than the enzyme described by Mahler *et al.* Furthermore, preparations of medium-chain

TABLE 4

Substrate specificity of two medium-chain fatty acid:CoA ligases (AMP), one with high and the other with minimal salicyl-CoA synthetase activity

A 1.78 M fraction from the second reverse ammonium sulfate step in Table 1 was used to investigate the substrate specificity of the "salicylate" enzyme. The second Sephadex fraction from Table 2 was employed as the acid:CoA ligase with minimal salicyl-CoA synthetase activity. Assay conditions are given in the text.

Substrate	Concentration	Low activity with salicylate	High activity with salicylate
	mM	*units/mg*	*units/mg*
Hexanoate	2.0	145	272
Benzoate	2.0	42.8	88.1
Salicylate	0.5	0.2	7.9
Anthranilate	1.0	14.1	7.9
p-Aminosalicylate	1.0	0.2	13.2
o-Methoxybenzoate	1.0	2.2	12.1

fatty acid:CoA ligase (AMP) with minimal salicyl-CoA synthetase activity lacked a detectable protein peak in the "salicylate" region of the gel (Fig. 2).

The previous finding that partially purified preparations of medium-chain fatty acyl-CoA synthetase displayed no salicyl-CoA synthetase activity is readily explained not only by the insensitivity of the former assay methods (3), but also by inactivation of the "salicylate" enzyme during its purification in the absence of protecting substrates. The assay problem was eliminated in the present study by the use of a more sensitive procedure which can detect accurately the formation of less than 1 mμmole of salicyl-CoA (2).

The densitometry tracings suggested that the best preparation of "salicylate" enzyme (specific activity, about 25 units/mg) was approximately 28% pure and contained less than 4% of the protein previously described by Mahler *et al.* (1). Thus, a detailed appraisal of the substrate specificity of the "salicylate" enzyme must await its further purification and demonstrated freedom from closely related acyl-CoA synthetases such as the one assumed but not proven here to be the enzyme described by Mahler *et al.* However, the evidence strongly suggests

that the "salicylate" enzyme has intrinsic hexanoyl-CoA and benzoyl-CoA synthetase activity. Not only did the last two activities form a peak in the "salicylate" locus on the disc gels (R_F 0.69), but activity with hexanoate and benzoate also disappeared from this region when the "salicylate" protein was missing (Fig. 2). In further experiments, not shown, there was a good inverse correlation between the hexanoyl-CoA to salicyl-CoA synthetase ratio of a given preparation and the proportion of total hexanoyl-CoA and benzoyl-CoA synthetase activity found in the "salicylate" region of the gel.

Although the ability of the "salicylate" enzyme to activate two *o*-hydroxybenzoates (salicylate and *p*-aminosalicylate)—which the enzyme of Mahler *et al.* did not—seems apparent (Table 4), the results of substrate studies with anthranilate and *o*-methoxybenzoate are more difficult to interpret because neither enzyme preparation was completely devoid of the other. However, inspection of the anthranilyl- and *o*-methoxybenzoyl-CoA synthetase to salicyl-CoA synthetase activity ratios of both preparations in Table 4 reveals that the enzyme described by Mahler *et al.* must be able to activate both substrates. This not only is contrary to previous findings (3) but also shows that the protein found by Mahler *et al.* is active with certain *ortho*-substituted benzoates, including one (anthranilate) which, like salicylate, may display appreciable hydrogen bonding between the *ortho* substituent and the carboxylate anion. Obviously, the differential substrate specificity between the enzyme of Mahler *et al.* and the "salicylate" enzyme has a more subtle explanation.

The possibility that activation of anthranilate and/or *o*-methoxybenzoate by the "salicylate" enzyme is due to contamination with the enzyme described by Mahler *et al.* is more difficult to exclude, but this seems unlikely if certain assumptions are valid. Thus, after electrophoresis of the "salicylate" enzyme, the over-all yield of hexanoyl-CoA synthetase activity in the region of the gel in which the enzyme of Mahler *et al.* appeared (R_F ~0.83) was less than 1.6% of the activity added. If this 1.6% represents roughly 30% of the latter hexanoyl-CoA synthetase activity added (Fig. 2), then a maximum of 5.3% of the hexanoyl-CoA synthetase activity in the "salicylate" preparation was provided by the enzyme of Mahler *et al.* This conclusion seems valid for the "salicylate" preparation used for substrate studies (Table 4), which had about the same hexanoyl- to salicyl-CoA synthetase activity ratio as the more highly purified material used in the electrophoresis experiment (Fig. 1). From the ratios of hexanoate to anthranilate or *o*-methoxybenzoate activation shown in the fourth column of Table 4, it is calculated further that the presence of the enzyme described by Mahler *et al.* in the "salicylate" preparation would yield specific activities of less than 1.5 units/mg for anthranilate and 0.22 unit/mg for *o*-methoxybenzoate. The actual specific activities recorded for these two substrates were 5.3 and 12.1 units/mg of "salicylate" preparation, or 3.5 and 55 times the specific activities estimated on the basis of contamination with the enzyme of Mahler *et al.* Therefore, it seems likely that the "salicylate" enzyme can utilize both anthranilate and *o*-methoxybenzoate as substrates, although the case for *o*-methoxybenzoate is stronger.

Depending on their tissue and species of origin, mammalian mitochondria can contain more than one medium-chain fatty acid:CoA ligase (AMP). For example, this report shows that bovine liver mitochondria have at least two medium-chain fatty acyl-CoA synthetases with different substrate specificities. Beef heart mitochondria contain a medium-chain fatty acyl-CoA synthetase with a narrower substrate specificity than the enzyme described by Mahler *et al.*; the former enzyme activates fewer fatty acids and cannot utilize aromatic acids as substrates (7). Dog kidney mitochondria contain a fatty acyl-CoA synthetase which activates butyrate but not benzoate; this enzyme is derived from a preparation that also displays benzoyl-CoA synthetase activity, which indicates the presence of at least one other re-

lated enzyme.[4] To date, no acyl-CoA ligases (AMP) have been isolated from mammalian mitochondria which activate aromatic carboxylic acids but not aliphatic acid substrates.

The heterogeneity of the medium-chain fatty acid:CoA ligases complicates the understanding of pathways in which these enzymes participate. For example, cattle lacking the synthetase described by Mahler *et al.* could still metabolize benzoate to hippurate, because the "salicylate" enzyme would provide an alternative pathway for benzoate activation. In man, in whom benzoate conversion to hippurate has been used as a test of hepatic function, a similar situation may exist (8). It is probable that the "salicylate" enzyme is also responsible for activation of *p*-aminosalicylate and 3-methylsalicylate, both of which are excreted as glycine conjugates by man (9, 10). Because this enzyme may well be rate-limiting in the conjugation of certain pharmacologically important compounds with glycine (2, 8), the study of its properties and metabolic regulation should provide a fruitful area for future investigation.

[4] Z. R. Vlahcevic and L. T. Webster, Jr., unpublished observations.

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