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Enzymatic Conversion of Salicylate to Salicylurate

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SUMMARY

The pathway of salicylate conversion to salicylurate has been demonstrated for the first time *in vitro*. A crude preparation of protein from beef liver "mitochondria" catalyzes the following reaction sequence:

$$\text{Salicylate} + \text{ATP} + \text{CoA} \rightleftarrows \text{salicyl-CoA} + \text{PP}_i \quad (1)$$

$$\text{Salicyl-CoA} + \text{glycine} \rightarrow \text{salicylurate} + \text{CoA} \quad (2)$$

In the over-all pathway, salicylurate formation was dependent on the presence of ATP, $MgCl_2$, coenzyme A, glycine, and active enzyme. The crude enzyme catalyzed the conversion of salicylate to a compound identified as salicyl-CoA in a reaction dependent on ATP, $MgCl_2$, and CoA. Salicylurate was also produced enzymatically from glycine and either chemically or enzymatically synthesized salicyl-CoA. The thioester linkages of both chemically and enzymatically synthesized salicyl-CoA were quite stable to alkaline hydrolysis, with half-lives averaging 44 min in 0.25 N KOH at 38°. The first or activating step was rate-limiting in salicylurate biosynthesis from salicylate when both activating and acylating reactions were assayed under approximately optimal conditions. Acyl-CoA:glycine *N*-acyltransferase activity (EC 2.3.1.13) was purified from the crude "mitochondrial" preparation into four protein fractions, each of which could utilize both salicyl-CoA and benzoyl-CoA as substrates.

INTRODUCTION

Salicylurate (salicylglycine) is found in the urine of a variety of mammals after they receive either aspirin or salicylate. In man, over 50% of a given dose of aspirin or salicylate may be converted to salicylurate (1, 2). In contrast, animals such as the rat, rabbit, and dog excrete only small quantities of this metabolite (3–6). The pathway of salicylurate formation from salicylate is not known, but, because of structural similari-

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ties between salicylate and benzoate, salicylurate biosynthesis may resemble that of hippurate. Hippurate is formed from benzoate and glycine as follows:

$$\text{Benzoate} + \text{ATP} + \text{CoA} \rightleftarrows \text{benzoyl-CoA} + \text{AMP} + \text{PP}_i \quad (1)$$

$$\text{Benzoyl-CoA} + \text{glycine} \rightarrow \text{hippurate} + \text{CoA} \quad (2)$$

The glycine-dependent enzymatic conversion of salicyl-CoA to salicylurate recently reported by Davidson and Webster (7) and by Tishler and Goldman (8) is consistent with this hypothesis.

However, two observations raise the possibility that the salicylurate and hippurate pathways might utilize different enzymatic components. Although man and rabbit differ in their capacities on a weight basis to metabolize salicylate to salicylurate, both readily form hippurate from benzoate (9). Furthermore, the partially purified medium-chain fatty acid:CoA ligase (AMP) from beef liver "mitochondria" which catalyzes benzoyl-CoA formation reportedly does not activate *ortho*-substituted benzoates such as salicylate or homogentisate (10). Therefore, an investigation of salicylate conversion to salicylurate *in vitro* was undertaken as a first step toward defining possible differences between salicylurate and hippurate biosynthesis. The findings form the basis of this report.

EXPERIMENTAL PROCEDURE

Enzymatic assays. For the formation of salicylurate from salicylate, complete reaction mixtures contained the following in a final volume of 1 ml: 100 μmoles of Tris-HCl buffer (pH 8.0), 5 μmoles of disodium ATP, 5 μmoles of $MgCl_2$, 0.5 μmole of CoA, 0.5 μmole of sodium salicylate, 100 μmoles of neutralized glycine, and 0.5–5 mg of enzyme protein. Prior to assay, crude preparations of enzyme were freed of endogenous substrates or cofactors by ammonium sulfate precipitation. Incubations were carried out at 38° for up to 10 min, and reactions were terminated with 0.2 ml of 2 M $HClO_4$. After removal of denatured protein by centrifugation, a 1-ml aliquot of the supernatant solution was extracted twice with 10 ml of ether to remove salicylic and salicyluric acids as described by Schachter and Manis (11). The ether phase was evaporated to dryness, and the residue was taken up quantitatively in 3 ml of 0.05 N H_2SO_4. Salicylic acid was then removed from the aqueous phase by extraction twice with 8 ml of water-saturated CCl_4 at 60° and once with 8 ml of water-saturated toluene at 60°. The remaining aqueous phase was adjusted to pH 5 with NaOH, and 0.5-ml aliquots were added to 2.5 ml of 0.5 M Na_2CO_3, pH 11.3, and to 2.5 ml of 0.2 M sodium phosphate buffer, pH 6.0. The fluorescence of these samples was determined along with salicylurate standards and appropriate blanks in an Aminco-Bowman spectrophotofluorometer at an activating wavelength of 340 mμ and emission wavelength of 420 mμ as outlined by Schachter and Manis (11). Recoveries of known amounts of salicylurate ranged from 95 to 102%.

For the enzymatic conversion of salicylate to salicyl-CoA, reaction components and their concentrations were identical with those employed above for salicylurate formation, except that glycine was omitted and 80,000–120,000 cpm of sodium 7-^{14}C-salicylate were added to the incubation mixture (final concentration, 0.5 mM salicylate). After incubation for periods up to 10 min at 38°, the reactions were stopped with 0.2 ml of 2 M $HClO_4$, and protein was removed by centrifugation. Salicylic acid was removed from 1-ml aliquots of the supernatant solutions by extracting them twice with 10 ml of ether. After aeration to remove residual ether, 0.75-ml aliquots of each aqueous phase containing 7-^{14}C-salicyl-CoA were neutralized with concentrated ammonium hydroxide and added to 10 ml of a mixture containing 125 g of naphthalene, 7.5 g of 2,5-diphenyloxazole, and 375 mg of *p*-bis[2-(5-phenyloxazolyl)]benzene per liter of *p*-dioxane. Each mixture was counted in a liquid scintillation counter, and corrections were made for dilution, quenching, and zero-time blanks.

The activity of acyl-CoA:glycine *N*-acyltransferase (EC 2.3.1.13) was assayed with benzoyl-CoA as substrate by monitoring the glycine-dependent decrease in absorption at 280 mμ or by coupling coenzyme A released

from the acyl-CoA substrate to 2,6-dichlorobenzenoneindophenol and phenazine ethosulfate in a glycine-dependent reaction (12, 13). Reaction mixtures for the latter assay contained the following in a volume of 0.89 ml: 25 μmoles of potassium *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate buffer (pH 8.0), 64 mμmoles of either salicyl- or benzoyl-CoA, 50 mμmoles of 2,6-dichlorobenzenoneindophenol, and 100 mμmoles of phenazine ethosulfate. Components were placed in 1.2-ml cuvettes with 10-mm light paths and allowed to equilibrate at 38° while being continuously monitored in a Gilford recording spectrophotometer at a wavelength of 600 mμ. In a volume of 0.1 ml, 100 μmoles of glycine, adjusted to pH 8.0, were pipetted into each cuvette, and the rate of color disappearance was recorded for 2 min after mixing. Finally, 10 μl of enzyme solution were added to complete each reaction mixture, and the rate was monitored for an additional 7 min. Maximal rates were usually achieved within 15 sec after adding the enzyme, and recorder sensitivity and enzyme concentrations were adjusted so that no more than 15 μM product was formed during this phase. Acyl-CoA deacylase activity was determined separately by omitting glycine from an otherwise complete reaction mixture. Both the deacylase rate and the glycine-dependent rate of nonenzymatic reduction of dye were subtracted from the maximal initial rate obtained with the complete system. The net rate in absorbance change per minute was divided by the molar extinction coefficient of the dye (21×10^3 M^{-1} cm^{-1} and multiplied by a factor of 1.75 to extrapolate it to the maximal rate at infinite concentration of dye.

For studies of the stoichiometry of the acyltransferase reaction, reaction mixtures contained 25 mM potassium *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate buffer (pH 8.0), 64 μM salicyl-CoA or benzoyl-CoA, and 100 mM glycine, adjusted to pH 8.0. After 3 min of incubation at 38°, 10 μl of enzyme solution were added per milliliter of each reaction mixture. Reactions were terminated 1.5 min and 6.5 min after enzyme addition with 0.1 ml of 20% trichloracetic acid added per milliliter. Controls from which glycine or enzyme was omitted were run in parallel. After denatured protein was removed by centrifugation, aliquots of the supernatant solutions were analyzed for several components. Salicylurate was removed by extraction with ether and determined by the fluorometric method of Schachter and Manis (11). Sulfhydryl release (CoA) was assessed by the method of Ellman (14). Other aliquots of the trichloracetic acid supernatant solution were extracted with ether and then used to determine residual acyl-CoA substrates. Salicyl-CoA was completely converted to salicylurate at pH 8.0 in a glycine-dependent reaction, and tde salicylurate formed was measured by fluorometry (11). The benzoyl-CoA content was estimated at pH 8.0 by monitoring the glycine-dependent enzymatic disruption of its thioester bond at 280 mμ (12).

Enzyme units for all assays are expressed as nanomoles or micromoles of product formation (or substrate disappearance) per minute at 38°; specific activities are given in units per milligram of protein. Enzyme for the acyltransferase assay was routinely diluted in 10 mM potassium *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate buffer, pH 8.0, containing 10 μg of bovine serum albumin per milliliter. Protein concentrations were determined by the biuret reaction with crystalline bovine plasma albumin used as the standard (15).

Preparation of enzyme that catalyzes formation of salicylurate from salicylate. For studies of the over-all reaction, a crude fraction of beef liver "mitochondrial" protein was obtained as follows. Whole fresh beef liver was sliced and chilled in ice immediately after removal from the animal. Subsequent operations were performed at 0–4°. After the capsule and major vessels were removed, 800 g of the diced organ were homogenized for 45 sec at high speed in a 1-gallon Waring Blendor with 2400 ml of 0.13 M KCl adjusted to pH 7.8 with 1.5 M NH_4OH. Following homogenization, the pH was adjusted to 7.8 with concentrated NH_4OH, and cell wall debris and nuclei were removed by centrifugation at $500 \times g$ for 10 min. The supernatant solution was filtered through cheesecloth and centrifuged in a Lourdes LRA re-

frigerated continuous-flow centrifuge at 28,000 × *g* at a flow rate of 40–55 ml/min. Particles obtained from 2400 g of liver were suspended in approximately 1500 ml of 0.13 M KCl–4 mM 2-mercaptoethanol–1 mM EDTA adjusted to pH 7.8 with NH_4OH, and frozen at −70°. After 24 hr or more, the preparation was thawed rapidly into glycerol, which was added to reach a final concentration of 10% (v/v); then the suspension was centrifuged at 78,000 × *g* for 60 min at 4°. The clear supernatant solution was decanted, and its volume and protein concentration were determined. When stored at −70°, this crude fraction lost no enzymatic activity in 6 months, and the average yield of soluble protein approximated 0.2–0.5% of the net weight of the liver.

Preparation of purified acyl-CoA:glycine N-acyltransferase. Acyl-CoA:glycine *N*-acyltransferase activity was purified from the frozen crude mitochondrial fraction (see above). An aliquot containing about 1.5 g of protein was thawed, and the protein concentration was adjusted to 10 mg/ml with 20 mM $KHCO_3$–4 mM 2-mercaptoethanol. Twenty-four grams of $(NH_4)_2SO_4$ were added per 100 ml of this solution, which was maintained at pH 8 by addition of NH_4OH. After 15 min of stirring, the suspension was centrifuged at 24,000 × *g* for 10 min, and 21 g of $(NH_4)SO_4$ were added to each 100 ml of supernatant solution. The pH was kept at 8, and the resulting suspension was stirred for 15 min. The precipitate was collected by centrifugation at 24,000 × *g* for 10 min, packed by an additional centrifugation at the same speed to remove excess $(NH_4)_2SO_4$, and dissolved in a minimal volume of 20 mM $KHCO_3$–0.1 M KCl–4 mM 2-mercaptoethanol. Approximately 40 ml, at a protein concentration adjusted to 30 mg/ml, were placed on a 58 × 5 cm column of Bio-Gel P-150 (100–200 mesh) equilibrated with 20 mM $KHCO_3$–0.1 M KCl–4 mM 2-mercaptoethanol. The column was eluted with the same solvent at a flow rate of 0.5 ml/min. The acyltransferase activity was recovered in 3-ml fractions after the bulk of the protein and a reddish pigment had been eluted. Fractions having specific activities at least 6 times greater than the material put on the column were pooled, and 50 g of $(NH_4)_2SO_4$ were added per 100 ml of this solution. The pH was kept at 8, stirring was carried out for 60 min, and the mixture was centrifuged for 30 min at 78,500 × *g*. The precipitate was dissolved in a minimal volume of 20 mM $KHCO_3$–0.1 M KCl–4 mM 2-mercaptoethanol and stored in two equal portions at −70°.

One fraction of precipitate (30–35 mg of protein) was thawed and dialyzed against 200 volumes of 20 mM $KHCO_3$–50 mM KCl–4 mM 2-mercaptoethanol in a rocking chamber for 1 hr. The sample, containing about 30 mg of protein, was subjected to preparative disc gel electrophoresis in a Canalco apparatus. A PD-2/150 upper column was used under experimental conditions specified by the manufacturer, except that a solution of 40 mM Tris–20 mM β-alanine, pH 8.9, was substituted for the usual Tris-glycine or imidazole buffer in the electrode compartments. The combination of a 1.5-cm stacking gel (3.5% acrylamide) and a 3-cm separatory gel (10% acrylamide) gave good separation of protein and enzymatic activity. Stacking of the protein was usually obtained within 3–4 hr after the sample was applied, and the activity was usually eluted within 8 hr.

Chemical syntheses and purification. Salicyl-CoA was synthesized from acetylsalicylic acid anhydride and CoA by a method analogous to that used by Schachter and Taggart for the synthesis of benzoyl-CoA (12). One hundred micromoles of lithium CoA and 300 μmoles of acetylsalicylic acid anhydride were added to 6 ml of water, and the pH was adjusted to 7.8 with solid $NaHCO_3$. The reaction was carried out in a Thunberg tube flushed with N_2, and constant agitation was maintained for 3 hr at 38°, during which period the disappearance of CoA (—SH) was monitored by the nitroprusside reaction (16). The mixture was then acidified to pH 3.0 with HCl, and the remaining anhydride and acetic and salicylic acids were removed by four extractions with 8 volumes of anhydrous ether. A stream of nitrogen was used to evaporate ether from the residual aqueous phase containing the salicyl-CoA product.

Salicyl-CoA was then freed of acetyl-CoA and oxidized and reduced CoA by passage over a Sephadex LH-20 column (30 × 2.5 cm) equilibrated and eluted with ethanol–H_2O–acetic acid (40:60:1 by volume). Components, in 2.5-ml fractions of effluent, were assayed qualitatively by thin-layer chromatography (see below). A compound appeared in the Sephadex fractions after the bulk of the oxidized and reduced CoA had been eluted. Fractions containing this substance were pooled, evaporated to a small volume several times after small additions of H_2O and ether, and stored at −70°. The material, identified as salicyl-CoA by criteria listed below, was quite stable, and the final yield ranged from 5 to 20% of the CoA initially employed.

Benzoyl-CoA was synthesized from benzoic acid anhydride and CoA as described by Schachter and Taggart (12). The product, purified by gel filtration on Sephadex LH-20 as described above for salicyl-CoA, migrated like salicyl-CoA in both the Sephadex and thin-layer systems. Fractions containing benzoyl-CoA free of other reaction components were identified by thin-layer chromatography, concentrated, and stored at −70° as was done for salicyl-CoA. The yield was 20–65% of the CoA used in the initial reaction.

Identification procedures. For isolation of salicyluric acid from enzymatic reaction mixtures, samples were processed through the extractions with warm CCl_4 and toluene as described for the enzymatic assay for salicylurate formation. Salicyluric and contaminating salicylic acids left in the acidic aqueous phase were then extracted into ether. The ether was evaporated to dryness, and the residue was taken up in a minimal volume of methanol. Salicyluric acid was then identified as a product of the over-all and acylation reactions by ascending thin-layer chromatography on Eastman silica gel 6060 plates under conditions that provided ready separation from salicylic acid. Approximate R_F values for salicyluric acid and salicylic acid standards in the several solvent systems were: benzene–acetic acid (30:3 by volume), 0.05 and 0.39; chloroform–acetic acid (30:3 by volume), 0.11 and 0.52; benzene–ether–acetic acid–methanol (120:60:18:1 by volume), 0.29 and 0.69. In these systems salicyluric acid was readily identified by its characteristic blue fluorescence. When chromatography was done on aluminum-backed silica gel plates (Silica F-254, Merck) in benzene–1,4-dioxane–acetic acid (2:1:1 by volume), salicyluric acid had an R_F of 0.55 and salicylic acid an R_F of 0.71. The salicyluric acid spot did not react with ninhydrin until concentrated HCl was added and the chromatogram was heated to 110° between two glass plates. This is consistent with disruption of the protected peptide linkage.

Chemically synthesized and purified salicyl-CoA gave a single ultraviolet-absorbing spot which migrated with an R_F of ~0.60 on thin-layer Eastman 6064 cellulose plates developed with butanol–acetic acid–H_2O (10:3:5 by volume). Approximate R_F values of other compounds in this system were: oxidized CoA, 0.11; reduced CoA, 0.31; acetyl-CoA, 0.39; free salicylic acid migrated near the solvent front. Salicyl-CoA also separated from the above reaction components with an R_F of approximately 0.68 when it was chromatographed on the same cellulose plates in *tert*-amyl alcohol–formic acid–H_2O (3:1:1 by volume). In addition to absorbing ultraviolet light, the salicyl-CoA spot on either chromatogram exhibited a bluish fluorescence. It also displayed a positive $FeCl_3$ test (free phenolic hydroxyl groups) and gave a delayed positive nitroprusside reaction only after alkaline hydrolysis (release of —SH groups). Chemically or enzymatically synthesized salicyl-CoA purified by gel filtration was determined quantitatively by sulfhydryl release (CoA) and by salicylurate production upon its reaction with glycine in the presence of excess acyl-CoA:glycine *N*-acyltransferase; these methods were also used to estimate the molar extinction coefficient at 260 mμ. The salicylurate formed was measured fluorometrically and also identified by thin-layer chromatography as described above; CoA release was monitored with Ellman's reagent (14). A coupled enzymatic reaction with limiting concentrations of acetate or acetyl-CoA was used to monitor acetate or acetyl-CoA content in the chemi-

cally synthesized acyl-CoA substrate before and after these compounds were subjected to alkaline hydrolysis (17, 18).

Benzoyl-CoA migrated as a single spot which absorbed ultraviolet light on the thin-layer chromatogram. Its R_F on Eastman cellulose plates developed with either butanol–acetic acid–H_2O (10:3:5) or *tert*-amyl alcohol–formic acid–H_2O (3:1:1) was the same as that recorded for salicyl-CoA. Benzoyl-CoA was measured by means of the disappearance of its absorption at 280 mμ upon complete enzymatic conversion to hippurate in a glycine-dependent reaction (12) or by sulfhydryl appearance during the same reaction (14).

Materials and general methods. Chemically synthesized salicyluric acid was a kind gift of the A. H. Robbins Company. Reagent kits and equipment for analytical and preparative disc gel electrophoresis were obtained from Canalco. Other products were purchased from the following sources: coenzyme A, acetyl-CoA, and nucleotides, from P-L Biochemicals; phenazine ethosulfate, malate dehydrogenase, citrate-condensing enzyme, and pyridine nucleotides, from Sigma Chemical Company; acetylsalicylic acid anhydride, from Eastman Organic Chemicals; sodium 7-^{14}C-salicylate, from New England Nuclear Corporation; Sephadex LH-20 powder, from Pharmacia; Bio-Gel P-150 (100–200 mesh), from Bio-Rad Laboratories; ammonium sulfate, ultrapure, from Mann Research Laboratories; and yeast inorganic pyrophosphatase, from Nutritional Biochemicals. Remaining chemicals were the commercially available reagent grades.

Dialysis tubing was boiled in 0.1 N KOH–1 mM EDTA for 10 min and later washed prior to use. Glass-distilled water was used to make up all reagents employed for enzyme purification and assay.

RESULTS

Salicylurate biosynthesis from salicylate, ATP, $MgCl_2$, CoA, and glycine. The crude preparation of protein from beef liver particles catalyzed the formation of salicylurate from salicylate, ATP, $MgCl_2$, CoA, and glycine (Table 1). The formation of up to 60 mμmoles of salicylurate per milliliter of reaction mixture was linear with protein concentration and with time over a 15-min period. Little or no salicylurate was produced from salicylate if ATP, $MgCl_2$, CoA, or glycine was omitted from the incubation mixture or if heat-inactivated enzyme was used. Neither cysteine or glutathione substituted for CoA in the reaction. Salicylurate was isolated and identified as a product of the reaction by methods outlined in EXPERIMENTAL PROCEDURE. There was also an approximate stoichiometry between salicylate disappearance and salicylurate formation.

TABLE 1

Requirements for formation of salicylurate from salicylate in the over-all pathway

Reaction mixtures of 1 ml contained 4 mg of crude mitochondrial protein, and incubations proceeded for 10 min. The concentrations of other components and the assay for salicylurate are given in the text.

System	Salicylurate formed
	mμmoles
Complete (salicylate, ATP, $MgCl_2$, CoA, glycine, enzyme)	31.0
−Salicylate	<0.04
−ATP	0.52
−$MgCl_2$	<0.12
−CoA	<0.12
−Glycine	<0.12
+Heat-treated enzyme[a]	<0.12

[a] Enzyme was aged for 1 hr at 60°.

Evidence for both glycine-acylating and salicylate-activating reactions in the pathway of salicylurate formation from salicylate. The requirement for CoA in the biosynthesis of salicylurate from salicylate suggested that salicyl-CoA was an intermediate in this pathway. To substantiate this hypothesis, salicyl-CoA was synthesized chemically from acetylsalicylic anhydride and CoA, and the product was purified both by ether extraction at acid pH and by gel filtration on Sephadex LH-20. The final substance was found by thin-layer chromatography to be free of anhydride, acetylsalicylic acid, salicylic acid, reduced CoA, and oxidized CoA. The gel filtration and concentration steps also eliminated acetyl-CoA, which was readily detectable in

TABLE 2

Acetate content of chemically synthesized salicyl-CoA before and after alkaline hydrolysis

A stock solution of 3.74 mM salicyl-CoA was used for the unhydrolyzed control. Reaction tubes for alkaline hydrolysis contained 0.2 ml of 3.74 mM salicyl-CoA stock solution, 40 μl of 12.5 N KOH, and either 10 μl of water or 10 μl of 30 mM potassium acetate. Incubation was conducted for 22 hr at 40°, after which 40 μl of 12.4 N HCl were added to each mixture. Aliquots of 50 μl were used for analysis (17, 18). Results are expressed as nanomoles of acetate per milliliter of 3.74 mM salicyl-CoA stock solution and are corrected for recoveries of acetate added to the assay mixtures.

Reaction mixture	Acetate
	mμmoles/ml
Unhydrolyzed salicyl-CoA stock	197
Hydrolyzed salicyl-CoA	451
Hydrolyzed salicyl-CoA with 1.5 μmoles of potassium acetate added per milliliter of salicyl-CoA stock	1920
Acetate released by hydrolysis	223–254

less purified material (17, 18). However, the final preparation of salicyl-CoA probably contained about 6.4% of acetylsalicyl-CoA, as revealed by liberation of this amount of acetate during prolonged hydrolysis in 2 N KOH for 22 hr at 40° (Table 2). The extinction coefficients of four preparations of purified salicyl-CoA ranged from 11.1 to 15.7 × 10^3 M^{-1} cm^{-1} (average, 13.1 × 10^3 M^{-1} cm^{-1}) in 0.05 M sodium phosphate buffer, pH 7.0. The thioester linkage of purified salicyl-CoA was found to be quite resistant to alkaline hydrolysis in 0.25 N KOH at 38°. The half-life, calculated from data obtained over the first 30 min, averaged approximately 40 min according to the experiments depicted in Fig. 1; after 60 min of hydrolysis the values were less reproducible but indicated that the reaction was proceeding at a comparable rate.

When chemically synthesized salicyl-CoA was substituted for salicylate, ATP, $MgCl_2$, and CoA in a glycine-dependent reaction, salicylurate production ranging up to 7 mμmoles/ml of reaction mixture was proportional to protein concentration during the linear phase of dye reduction. Salicylurate formation was not detected in the absence of glycine or when heat-inactivated enzyme was used. The salicylurate isolated from such reaction mixtures was identified by its characteristic fluorescence and migration on thin-layer chromatograms.

The crude mitochondrial enzyme also catalyzed the formation of salicyl-CoA from salicylate, ATP, $MgCl_2$, CoA, and active enzyme. In the presence of added inorganic

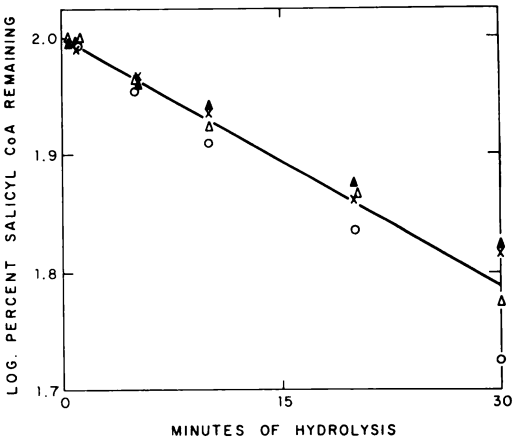

FIG. 1. *Alkaline hydrolysis of salicyl-CoA in 0.25 N KOH at 38°.*

Three separate experiments were performed. (a) Hydrolysis of 787 μM salicyl-CoA prepared by chemical synthesis (○): at the intervals shown, 0.1-ml aliquots were withdrawn and added to 0.1 ml of 30% metaphosphoric acid; thiol release was measured by reaction with Ellman's reagent (14). The calculated half-life is 37.5 min. (b) Hydrolysis of 20 μM 7-^{14}C-salicyl-CoA (160 cpm/mμmole) formed by enzymatic synthesis from 7-^{14}C-salicylic acid, ATP + Mg^{++}, and CoA-SH (×): at the intervals shown, 0.4-ml aliquots were withdrawn, added to 0.1 ml of 20% perchloric acid, and extracted with 5.0 ml of anhydrous ether to remove 7-^{14}C-salicylic acid; residual ^{14}C-salicyl-CoA was then determined as outlined in the text. The calculated half-life is 46.8 min. (c) Hydrolysis of a mixture of 1.257 mM chemically synthesized salicyl-CoA (△) and 49 μM enzymatically synthesized 7-^{14}C-salicyl-CoA (▲) containing 175 cpm/mμmole: at the intervals shown, aliquots were removed and assayed for thiol release and residual 7-^{14}C-salicyl-CoA as outlined above. The half-life is 42 min for thiol release and 48.7 min for ^{14}C-salicyl-CoA disappearance.

pyrophosphatase, product formation up to 60 mμmoles/ml was linear with time up to 15 min, as well as with enzyme concentration. ^{14}C-Salicyl-CoA was isolated, purified over Sephadex LH-20, and identified as a product of this reaction by thin-layer chromatography. The purified, isotopically labeled salicyl-CoA disappeared in alkali with a half-life of approximately 48 min; this is nearly the same rate at which thiol was released from chemically synthesized salicyl-CoA (Fig. 1). Salicyl-CoA also was enzymatically converted to salicylurate in a glycine-dependent reaction, and the radioactive salicylurate was isolated and identified as outlined under EXPERIMENTAL PROCEDURE.

Properties of purified acyl-CoA:glycine N-acyltransferase. To test whether glycine acylation with salicylate and benzoate is catalyzed by the same or separate enzymes, acyl-CoA:glycine *N*-acyltransferase activity was fractionated with ammonium sulfate and by gel filtration. After these procedures the ratio of specific activities with benzoyl-CoA compared to salicyl-CoA as the acyl donor varied from 2.0 to 3.2, depending on the enzyme preparation, but was reasonably constant for each preparation over the 15-fold purification achieved (Table 3). There was no detectable salicyl-CoA synthetase activity when 3 mg of this protein were added to the activating assay. Balance studies revealed that the amount of either salicyl-CoA or benzoyl-CoA disappearing approximated the amount of —SH groups (free CoA) formed, and that utilization of salicyl-CoA approached the formation of salicylurate (Table 4). The apparent Michaelis constants for glycine were approximately 20 mM at 64 μM salicyl-CoA and 10 mM at 64 μM benzoyl-CoA (Fig. 2). At 150 mM glycine the apparent K_m for salicyl-CoA was 8 μM, whereas the comparable figure for benzoyl-CoA at 80 mM glycine was about 15 μM (Fig. 3). At 64 μM salicyl- or benzoyl-CoA and 100 mM glycine, acyl-CoA:glycine *N*-acyltransferase activity was inhibited somewhat more by salicylurate than by hippurate at equimolar concentrations of inhibitor (Table 5).

When the above protein was subjected to preparative disc gel electrophoresis, enzymatic activity was separated into four peaks, and roughly 85% of the activity was recovered (Fig. 4). Analytical disc gel electrophoresis of each of the four peak fractions (fractions 52, 67, 80, and 103) revealed a major protein band which migrated differently from the major protein component in each of the other peak fractions, and most of the detectable enzymatic activity in each gel was found in the location occupied by its major protein band. Each of the four peak fractions was found to catalyze the acylation of glycine with either benzoyl-CoA or salicyl-CoA; ratios of acylating rates with benzoyl-CoA as compared to salicyl-CoA varied from 1.5 to 2.6, which was a greater variation than usually obtained with less highly purified material (Fig. 4).

Rate-limiting step in pathway of salicylurate formation from salicylate. When each

TABLE 3

Purification of acyl-CoA:glycine N-acyltransferase activity

Incubation mixtures contained 64 mμmoles of the acyl-CoA substrate and 100 μmoles of glycine per milliliter, as stated in the text. Specific activities are expressed as micromoles of sulfhydryl groups released per minute per milligram of protein at 38°.

Fraction	Total protein	Enzyme activity				Activity ratio, benzoyl-CoA: salicyl-CoA
		Salicyl-CoA		Benzoyl-CoA		
		Specific activity	Total units	Specific activity	Total units	
	mg					
Crude mitochondrial fraction	1650	0.9	1490	1.8	2980	2.0
Ammonium sulfate	1200	1.2	1440	2.9	2480	2.4
Bio-Gel P-150	77.5	13.8	1070	29.0	2250	2.1

TABLE 4

Balance study of the acyl-CoA : glycine N-acyltransferase reaction

The composition of the reaction mixtures, conditions of assay, and analytical techniques for estimating individual components are given in the text. Each milliliter of reaction mixture contained 186 mμg of protein (specific activity, 24 μmoles of benzoyl-CoA disappearance per minute per milligram of protein).

Acyl-CoA substrate	Thiol formed	Salicylurate formed	Salicyl-CoA disappearance	Benzoyl-CoA disappearance
	μM	μM	μM	μM
Salicyl-CoA	9.5[a]	10.2	9.4	
	8.4[b]			
Benzoyl-CoA	21.6[a]			22.5
	22.6[b]			

[a] Determined by assay with 2,6-dichlorobenzenoneindophenol and phenazine ethosulfate.

[b] Determined by assay with Ellman's reagent.

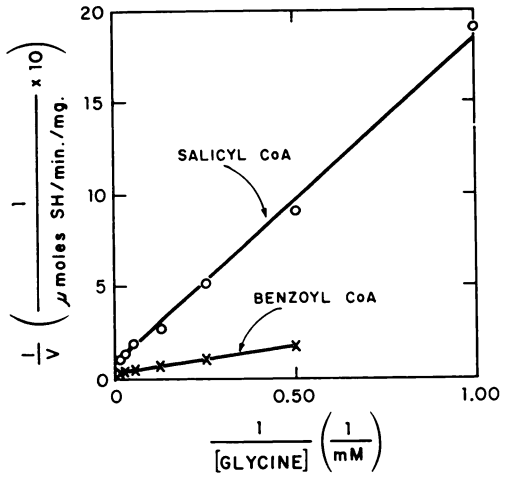

FIG. 2. *Effect of glycine concentration on acyl-CoA : glycine N-acyltransferase activity*

The concentrations of salicyl-CoA and benzoyl-CoA were 64 μM each. The quantities of purified enzyme added (Bio-Gel step, Table 3) were adjusted so that there was no more than 10% conversion of the limiting substrate to product. Other assay conditions are given in EXPERIMENTAL PROCEDURE.

was determined at nearly optimal substrate concentrations, more than a 1000-fold excess of acylating as compared to activating ac-

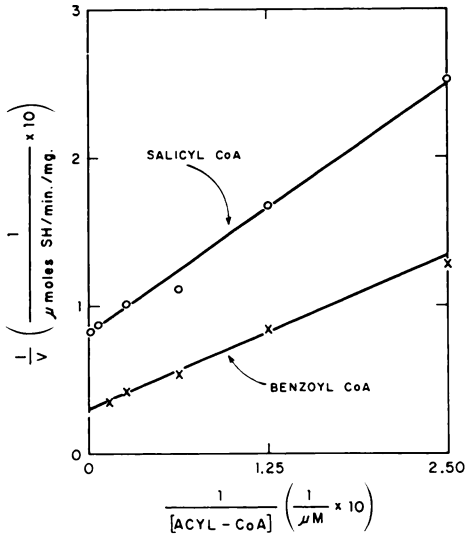

FIG. 3. *Effect of acyl-CoA concentration on acyl-CoA : glycine N-acyltransferase activity*

The concentration of glycine in the salicyl-CoA system was 150 mM (approximately 7 times the apparent K_m for glycine), and in the benzoyl-CoA system, 80 mM (approximately 8 times the apparent K_m for glycine). The quantities of purified enzyme added (Bio-Gel step, Table 3) were adjusted so that no more than 50% of the limiting substrate was converted to product. Other assay conditions are given in EXPERIMENTAL PROCEDURE.

tivity was present in the crude "mitochondrial" preparation of protein from bovine liver (Table 6). The rate of the activating step matched that for the over-all pathway, and the latter was not increased by addition of highly purified acylating enzyme.

DISCUSSION

Aside from its theoretical significance, biochemical examination of the components, reaction sequence, and control of the pathway for converting salicylate to salicylurate should prove of practical value because salicylurate is the major end metabolite of two of the most commonly used therapeutic agents in man, aspirin and salicylate. To our knowledge, the present study provides the first convincing evidence of enzymatic formation of salicylurate from salicylate *in vitro*. No positive experiments have been reported for cell-free systems, and only equivocal results have been obtained with rat liver

TABLE 5

Inhibition of acyl-CoA:glycine N-acyltransferase activity by salicylurate or hippurate

Experiments were performed as outlined in the text, except that the stated concentrations of inhibitors (salicylurate or hippurate) were added to the initial reaction mixtures. The specific activity of the enzyme was 22.2 μmoles of benzoyl-CoA disappearance per minute per milligram of protein.

Inhibitor	Concentration	Velocity observed without inhibitor	
		Salicyl-CoA as substrate	Benzoyl-CoA as substrate
	μM	%	%
Salicylurate	20	100	100
Hippurate	20	100	100
Salicylurate	40	62	96
Hippurate	40	100	
Salicylurate	100	59	68
Hippurate	100	87	78
Salicylurate	300	49	54
Hippurate	300	65	60
Salicylurate	500	20	16
Hippurate	500	45	45
Hippurate	1000	27	27

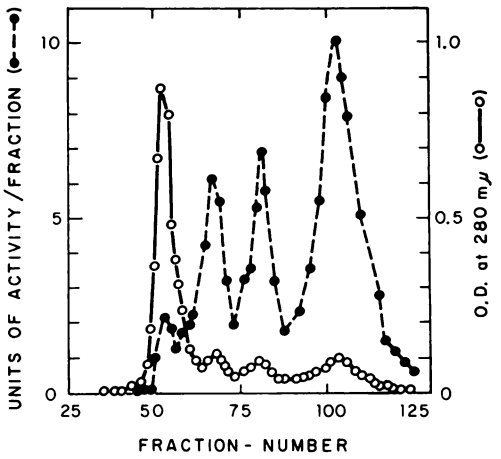

FIG. 4. *Preparative disc gel electrophoresis of purified acyl-CoA:glycine N-acyltransferase*

Thirty milligrams of a Bio-Gel P-150 fraction (Table 3) containing 290 units of benzoyl-CoA: glycine N-acyltransferase activity were applied to the column. Details are given in EXPERIMENTAL PROCEDURE. Units of enzyme activity are expressed as micromoles of 2,6-dichlorobenzenoneindophenol reduced per minute per each 3.5-ml fraction with benzoyl-CoA as substrate. The ratios of acylating rates with benzoyl-CoA compared to salicyl-CoA as acyl substrate were 1.5 in fraction 52, 2.5 in fraction 67, 1.9 in fraction 80, and 2.6 in fraction 103. These fractions corresponded to the four peaks of enzymatic activity observed.

and kidney tissue slices (19). Failure to demonstrate salicylurate biosynthesis from salicylate *in vitro* is not surprising in view of the possible uncoupling effects of salicylate on oxidative phosphorylation, the small quantity of salicylurate formed by the liver and kidney of most species, and the high sensitivity of methods required for the detection of salicylurate in biological materials. Fortunately, the sensitive fluorometric procedure of Schachter and Manis (11) was readily adapted in the present study to the determination of salicylurate in enzymatic reaction mixtures, and the predominant localization of enzymatic activity in this pathway to the "mitochondrial" fraction permitted the addition of protein of relatively high specific activity to the crude assay system.

Salicylurate formation from salicylate is apparently accomplished sequentially by salicylate activation and *N*-acylation of glycine by salicyl-CoA, as judged from the deletion and balance experiments described above, the participation of chemically synthesized salicyl-CoA, and the identification of the products of each individual reaction. The over-all pathway is similar to that described for hippurate biosynthesis from benzoate:

$$\text{Salicylate} + \text{ATP} + \text{CoA} \rightleftarrows \text{salicyl-CoA} + \text{AMP} + \text{PP}_i$$

$$\text{Salicyl-CoA} + \text{glycine} \rightarrow \text{salicylurate} + \text{CoA}$$

Acylating activity was purified from the beef liver particulate fraction in order to determine whether conjugation of glycine with salicyl-CoA and benzoyl-CoA is catalyzed by the same or different enzymes. Schachter and Taggart (10) have already reported a broad acyl-CoA substrate specificity for their partially purified preparation

TABLE 6

Rate-limiting step in salicylurate biosynthesis from salicylate under optimal assay conditions

The crude mitochondrial protein fraction was assayed under conditions for each reaction given in the text.

System	Specific activity
	mμmoles/min/mg protein
Over-all reaction:	
Salicylate, ATP, $MgCl_2$, CoA, glycine	0.75
+ 1 mg of acyltransferase[a]	0.78
Activating reaction (salicylate, ATP, $MgCl_2$, CoA)	0.75
Acyltransferase reaction (salicyl-CoA, glycine)	900

[a] The purified acyl-CoA:glycine *N*-acyltransferase was free of detectable acyl-CoA synthetase activity and had a specific activity of 9 μmoles of salicylurate formed per minute per milligram of protein.

of acyl-CoA:glycine *N*-acyltransferase from beef liver, but did not test it with salicyl-CoA. The present purified enzyme displays characteristics different from Schachter's preparation, as evidenced by a roughly 20-fold greater specific activity and a lower apparent molecular weight (approximately 32,000 by gel filtration on Sephadex G-100).[4] Subjecting the crude particulate material to a freezing and thawing step rather than to acetone precipitation probably was the major factor responsible for the improved yield and specific activity displayed by the present enzyme.

Although Tishler and Goldman (8) also have described enzymatic preparations which catalyze the *N*-acylation of glycine by salicyl-CoA, major differences exist between their results and ours. For example, when assayed under their conditions, the Bio-Gel P-150 fraction (Table 3) had 340 times the specific activity published for their partially purified beef liver enzyme. Discrepancies were also found between the chemical behavior of the present preparation of salicyl-CoA and that of Tishler and Goldman. After hydrolysis of the present material in 0.25 N KOH for 17 min at 38°, 28 mμmoles of sulfhydryl groups were released from 116 mμmoles of substrate, and a decrease in absorbance of 0.063 at 296 mμ was recorded after readjustment of the pH to 7.5. Tishler and Goldman noted an increase in extinction at 296 mμ after alkaline hydrolysis of their salicyl-CoA preparation and concluded on the basis of this extinction change that hydrolysis of salicyl-CoA by 0.1 N KOH was complete in 1 min at 30° (conditions under which little —SH was released from the present material).

Stability of the thioester bond of salicyl-CoA to alkali would be rather unexpected in view of its *o*-hydroxyl group and the known lability of salicylester glucuronide (20). However, despite the use of acetylsalicylic acid anhydride in its synthesis, the resistance of the present preparation to alkaline hydrolysis cannot be explained by supposing that a major fraction of its thioester linkages are in the form of acetylsalicyl-CoA. Thus, acetate released from the purified acyl-CoA during extensive alkaline hydrolysis amounted to less than 7% of the thioester linkages present (Table 2). Also, acetyl-CoA was a major by-product of the chemical synthesis, indicating that the acetyl group was cleaved from acetylsalicylic acid anhydride, acetylsalicylic acid, or both, during the reaction. Furthermore, the "salicyl-CoA" spot on the thin-layer chromatograms reacted with ferric chloride, which would not have been expected if the phenolic hydroxyl group had remained acetylated. Finally, the thioester linkages of both chemically and enzymatically synthesized "salicyl-CoA" were hydrolyzed by alkali at about the same rate (Fig. 1); because the enzymatically synthesized CoA ester was derived from a mixture of salicylate, MgATP, and CoA, it obviously could not have been acetylsalicyl-CoA.

A more plausible explanation for the differences obtained in the two laboratories is that the chemically synthesized salicyl-CoA studied by Tishler and Goldman contained impurities which absorb in the ultraviolet region. Indeed, these investigators did not report a purification procedure which might be expected to remove either oxidized or reduced CoA from the product of their

[4] W. B. Forman, unpublished observations.

chemical synthesis, and their product was not examined directly (identification was made by conversion to salicylhydroxamate). Contamination of the salicyl-CoA by ultraviolet-absorbing material is consistent with the high extinction coefficient at 260 mμ (approximately 42×10^3 M^{-1} cm^{-1} in 0.1 M potassium phosphate buffer, pH 7.5) calculated from their data. This might make spectral differences such as those used to assess the rate of alkaline hydrolysis difficult to interpret; moreover, an impure acyl-CoA substrate may also have contributed to the barely detectable rates of enzymatic acylation which these investigators reported. The present preparations of salicyl-CoA appeared freer of extraneous ultraviolet-absorbing compounds, as judged by thin-layer chromatography and the much lower extinction coefficient obtained at 260 mμ.

The fact that the present transferase could utilize both benzoyl-CoA and salicyl-CoA as acyl-CoA substrates throughout purification to high specific activity, and particularly after resolution of the enzymatic activity in good yield into four peaks by preparative disc gel electrophoresis, argues strongly against the concept of separate acyl-CoA:glycine *N*-acyltransferases for benzoyl-CoA and salicyl-CoA in beef liver. Whether the four peaks of enzymatic activity obtained after the last procedure represent isolation artifacts, isoenzymes, degradative products, aggregates, or different complexes is now under investigation.

The rate-limiting step under favorable assay conditions for both activating and acylating enzymes in the salicylurate pathway was localized to the activating level, as shown by the results of Table 6. However, the rates of both activation and acylation must be compared at more physiological concentrations of pathway components in order to gain insight into significant mechanisms for metabolic control. Because of the localization of the enzymes in this pathway to the inner aspects of mitochondria,[5] it is quite possible that the two reactions are closely coupled *in vivo*. Therefore, an understanding of substrate and product compartmentation and transport within these organelles is required before findings such as product inhibition of the acylating reaction can be appraised in a physiological context (Table 5). Species and organs (liver and kidney) must also be evaluated independently for metabolic control of salicylurate biosynthesis, because the ratios of glycine acylation to salicylate activation vary in different species and in different organs within the same species (21).

The salicylurate model may offer advantages over the more commonly used benzoate-hippurate system to study peptide conjugations between aromatic acids and amino acids. For example, the maximal rate of salicylurate elimination after therapeutic doses of salicylate has already been equated with the ability of a species to form this metabolite, but whether the metabolite is formed by zero- or first-order kinetics is still under debate (22–25). Because the maximal rate of salicylurate formation is relatively slow, its assessment under normal physiological circumstances is less likely to be influenced by such factors as the size of the glycine pool or the renal excretory capacity, both of which variables are known to complicate evaluation of the maximal rate of hippurate biosynthesis *in vivo* (2, 9, 11, 23). In several species tested, the rates of salicylate activation are much slower than benzoate activation, whereas the rates of acylation of glycine with salicyl-CoA and benzoyl-CoA are more nearly comparable (21). Thus, other factors being equal, activation would be more likely to be rate-limiting in the salicylurate pathway than in the benzoate-hippurate system, and this should be kept in mind when considering mechanisms involved in the inhibition of salicylurate formation by benzoate or *p*-aminobenzoate (6, 25, 26). Finally, the evaluation of factors controlling hippurate biosynthesis is made more complex by the recent findings that more than one enzyme can activate benzoate (27). For the above reasons it appears that a species-specific correlative biochemical and pharmacokinetic approach to salicylurate biosynthesis

[5] E. D. Davidson and C. L. Hoppel, unpublished observations.

from salicylate will provide fruitful information as to the control of this and analogous pathways *in vivo*.

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