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PROPERTIES AND REACTIONS OF SALICYL-COENZYME A

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Abstract—Salicyl-coenzyme A (salicyl-CoA) has been prepared by organic synthesis, and its properties compared to those of benzoyl-CoA. Salicyl-CoA reacts with glycine acyl transferase prepared from both beef and human liver to form salicyluric acid. For both enzymes the rate with salicyl-CoA is approximately 50 per cent of the rate with benzoyl-CoA. In addition, there is a small non-enzymatic acylation of glycine by salicyl-CoA.

EVIDENCE from clinical studies suggests that salicylate congeners such as γ-resorcylic acid (2,6-dihydroxybenzoic acid), gentisic acid (2,5-dihydroxybenzoic acid) and β-resorcylic acid (2,4-dihydroxybenzoic acid) have antirheumatic activity.[1–3] A common feature of these derivatives of benzoic acid is their ortho-hydroxyl group, and this suggests that antirheumatic activity might be related in some way to this molecular configuration which, of course, is characteristic of salicylate itself. This structure–activity correlation receives additional support from observations that benzoic acid derivatives which lack the ortho-hydroxyl group apparently fail to exhibit antirheumatic activity.[3–5] These fragmentary clinical observations suggested that it might be interesting to examine the effect of substituting salicylate for benzoate in certain biochemical reactions of benzoic acid.

A model for this kind of study is the series of reactions leading to the conjugation with glycine of benzoic acid and many of its congeners.[6–9] The most definitive study of these reactions has been made in an enzyme system from beef liver mitochondria,[8,9] and the results indicate that the prototype compound, benzoic acid, is converted to hippuric acid in the following two-step reaction sequence.

$$\text{Benzoate} + \text{CoA} + \text{ATP} \rightarrow \text{benzoyl-CoA} + \text{PP} + \text{AMP (EC 6.2.1.3)}^{10} \quad (1)$$

$$\text{Benzoyl-CoA} + \text{glycine} \rightarrow \text{hippurate} + \text{CoA (EC 2.3.1.13)}^{9} \quad (2)$$

This reaction sequence is responsible for the conjugation with glycine of a number of compounds with structures similar to benzoate,[6,9,11] and it has been assumed to convert salicylate to salicylurate (2-hydroxyhippurate).[12] While this hypothesis is attractive, it takes no recognition of the observation that many ortho-substituted derivatives of benzoate including salicylate are not activated by the enzyme from beef liver mitochondria.[9,10] Specificity studies of this kind raise the question of whether salicyl-CoA, which has not been found in any system, can be formed by reaction (1) or whether some other mechanism must be sought. In addition to this uncertainty it

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is not known whether salicyl-CoA would be capable of reacting with glycine acyl transferase (EC 2.3.1.13) [reaction 2] to form salicyluric acid. Clarification of the biosynthesis of salicylurate seems of particular interest since it would establish a biochemical basis for recent pharmacokinetic studies on salicylate elimination by this pathway.[13,14]

In this paper the synthesis of salicyl-CoA is described and the properties of this compound are compared with those of benzoyl-CoA. In addition, salicyl-CoA is shown to be a substrate for glycine acyl transferase preparations isolated from beef and human livers.

MATERIALS AND METHODS

Materials. Salicylic acid was recrystallized from a mixture of ethanol and water; benzoic and 2-fluorobenzoic acids were recrystallized from benzene. CoA was obtained from P-L Biochemicals, Inc., Milwaukee, Wisc., and glycine-1-^{14}C from New England Nuclear Corp., Boston, Mass.

Preparation of acyl-CoA derivatives. Salicyl chloride was prepared by a modification of the method of Adams and Ulich.[15] Salicylic acid (1·3 g) and oxalyl chloride (3 ml) were added to 25 ml of benzene and the mixture allowed to reflux for 1 hr, after which the benzene and excess oxalyl chloride were removed *in vacuo* at room temperature. Salicyl chloride prepared in this way was then added dropwise to a solution containing 100 mg of CoA in 10 ml of 0·5 M $KHCO_3$. During this procedure mixing was provided by a jet of helium gas and the pH of the reaction was maintained at 8–9 by the addition of 0·1 N NaOH. Completion of the reaction was indicated when a spot test on a sample of the reaction mixture showed the disappearance of the free thiol group (which reappeared after alkaline hydrolysis).[16] The solution of salicyl-CoA was then adjusted to pH 3 by the addition of dry Dowex-50 (H^+) which had previously been cycled until clean. The Dowex was removed by filtration and washed with small amount of water. The wash and filtrate were pooled and extracted continuously with ether for 3 hr. The solution of salicyl-CoA, at pH 3, was stable when stored at −196°. A similar procedure was used to prepare 2-fluorobenzoyl-CoA. Benzoyl-CoA was synthesized from benzoic anhydride.[8]

Preparation of glycine acyl transferase. Purification of the enzymes was based on the assay described by Schachter and Taggart which relies on the absorbance change at 280 mμ during the loss of the thiol ester of benzoyl-CoA.[9] For this assay the reaction mixture contained 0·25 μmole of benzoyl-CoA, 60 μmoles of glycine, 50 μmole of potassium phosphate buffer at pH 7·5 and enzyme in a final volume of 1·0 ml maintained at 30°. Insofar as possible, the initial rate of the reaction was determined in a Gilford recording spectrophotometer by the absorbance change at 280 mμ.[9] An enzyme unit is defined as the activity which catalyzes 1 mμmole per min of glycine-dependent loss of benzoyl-CoA under these reaction conditions.

The following operations were carried out at 0–5°: Beef liver mitochondria (16 g),[10] which had been stored at −20° for 3 months, were suspended in 3 vol. of 0·02 M potassium phosphate buffer at pH 7·5 and homogenized in a Potter–Elvehjem homogenizer. This suspension was clarified by centrifugation at 30,000 *g* for 1 hr. Solid ammonium sulfate (19·4 g/100 ml) was added with stirring to the clear supernatant solution. After equilibration for 1 hr the suspension was clarified by centrifugation; to the clear supernatant solution additional ammonium sulfate (8·7 g/100 ml) was

added with stirring. After equilibration the precipitate was collected by centrifugation and dissolved in a minimal volume of 0·02 M potassium phosphate buffer, pH 7·5. The solution was dialyzed for 3 hr against three changes of 0·01 M potassium phosphate buffer at pH 7·5. Three ml of this solution (150 mg of protein) was applied to a Sephadex G-100 column (1 × 50 cm) which had been equilibrated with 0·02 M potassium phosphate buffer at pH 7·5. Elution (at approximately 20 ml/hr) was with the same buffer, and 4-ml fractions were collected. The tubes with the highest specific activity (28 units/mg), which represented a 6-fold purification, were used in the experiments to be described. The enzyme retained full activity when stored at −20° for several months.

For the purification of glycine acyl transferase from human liver the frozen human liver was treated exactly like the frozen beef liver mitochondria except that homogenization was in a Waring blendor, and the homogenizing fluid also contained 0·25 M sucrose. In the ammonium sulfate fractionation the successive additions of ammonium sulfate were 25·8 g/100 ml and 9·0 g/100 ml. The final specific activity of the human preparation was 1·4 units/mg. We thank Dr. Howard Sussman for providing us with the human liver from a patient who died of heart failure approximately 5 hr prior to the autopsy.

Identification of compounds. Compounds were identified by paper chromatography on Whatman 3MM filter paper or silica gel plates (E. Merck A G, Darmstadt). The following solvent systems were used: isopropyl alcohol: concentrated NH_4OH: H_2O (8:1:1) for salicylhydroxamate, salicylurate and hippurate; and *n*-butyl alcohol: acetic acid: water (4:1:5) for salicylhydroxamate.

For identification of radioactive salicylurate and hippurate, chromatography was performed on Celite 535 (kindly provided by Johns-Manville) by a modification of the method of Swim and Utter.[17] Under these conditions after elution of the column with heptane the products were eluted with chloroform. In these fractions titration values of the free acid standards were seen to be concordant with the radioactivity as determined in a low background Nuclear–Chicago end window gas flow counter. Radioactive salicylurate and hippurate were also identified by radioautograms after paper chromatography.

Other methods. After acidification of the reaction mixture by the addition of 0·1 vol. of 5 N H_2SO_4, hippurate or salicylurate was extracted by shaking with 3 vols. of ether. This extraction was repeated twice and the extracts pooled, and washed with 0·1 N H_2SO_4 to remove any glycine-^{14}C. After standing successively over dry Dowex 50 (H^+) and anhydrous $MgSO_4$, the ether extract was free of glycine-1-^{14}C and could be assayed for radioactivity in an end window gas flow counter.

Salicyl-CoA was converted to salicylhydroxamate with neutral hydroxylamine[16] and a $FeCl_3$ spray reagent was used to locate the hydroxamate after chromatography.

Protein was measured according to Warburg and Christian.[18]

RESULTS

Properties of salicyl-CoA. Spectra of salicyl-CoA before and after hydrolysis (Fig. 1) indicate that the increase in absorbance at 296 mμ ($\Delta\epsilon = 3400$) can be made the basis of a spectral assay of the hydrolysis of this compound. The stability properties of salicyl-CoA in comparison with benzoyl-CoA are summarized in Table 1 which indicates that salicyl-CoA is considerably less stable than benzoyl-CoA at

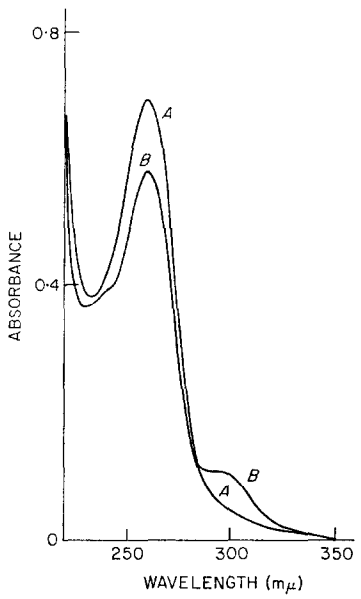

FIG. 1. Spectra of salicyl-CoA before and after hydrolysis. Curve A is the spectrum of salicyl-CoA ($1{\cdot}6 \times 10^{-5}$M) in 0·1 M potassium phosphate buffer, pH 7·5. Curve B is the spectrum of salicyl-CoA ($1{\cdot}6 \times 10^{-5}$ M) which had previously been hydrolyzed in 0·1 N KOH for 10 min and then readjusted to the conditions under which Curve A was recorded.

several different pH values. The inclusion of the stability of 2-fluorobenzoyl-CoA under these conditions makes possible some comparisons about the effects of substituents at the ortho position on the stability of these thiol esters. Apparently the fluorine substituent does not reduce stability as much as the ortho-hydroxyl group.

The increased disappearance of salicyl-CoA in the presence of glycine is shown by the data of Fig. 2. This effect of glycine occurs also with other CoA derivatives, as shown in Table 2. An experiment was then conducted to determine the product of these reactions of the CoA derivatives with glycine. Conditions were identical to those described in Table 2 except that glycine-1-^{14}C of known specific activity was

TABLE 1. STABILITY OF CoA DERIVATIVES*

Conditions	Rate of disappearance (% per min)		
	Sal-CoA	Bz-CoA	2FB-CoA
0·1 N KOH	100	20	40
0·03 M KPO_4 buffer, pH 7·5	0·5	<0·01	<0·01
0·2 M Sodium borate, p 9·0	1·6	<0·01	<0·03

* Reaction mixtures contained 0·1 to 0·3 μmole of the substrate indicated in a 1-ml vol maintained at 30°. The rates of hydrolysis were monitored in a Gilford recording spectrophotometer at 296 mμ for salicyl-CoA and 280 mμ for 2-fluorobenzoyl-CoA and benzoyl-CoA. Rates of disappearance represent initial rates.

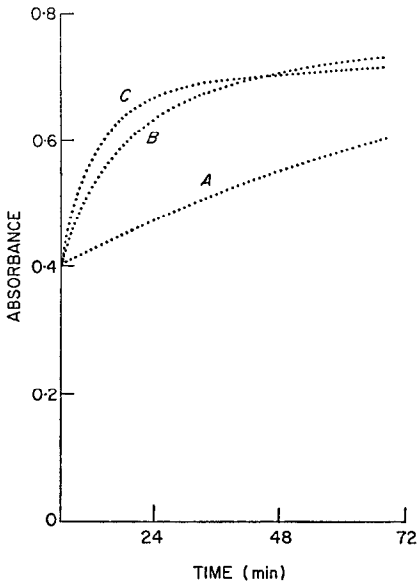

FIG. 2. The effect of glycine on salicyl-CoA hydrolysis. Each cuvette contained 0·3 μmole of salicyl-CoA and sodium borate–boric acid buffer at pH 9·0 in a total volume of 3·0 ml. In addition, Curve B contained 50 μmoles of glycine and Curve C contained 100 μmoles of glycine. Spectral changes at 296 mμ were recorded with time on a Gilford recording spectrophotometer.

TABLE 2. STABILITY OF CoA DERIVATIVES IN THE PRESENCE OF GLYCINE*

Conditions	Rate of disappearance (% per min)		
	Sal-CoA	2FB-CoA	Bz-CoA
0·03 M KPO_4 buffer, pH 8·0	7	<0·01	<0·01
0·2 M Glycine-NaOH, pH 9·0	100	0·1	<0·005

* Conditions were as in Table 1 except that each cuvette contained 0·2 M glycine. Rates of disappearance represent initial rates.

used. After 24 hr the glycine which had been conjugated was extracted by ether, as described in Methods. Under these conditions approximately 4% of salicylate and less than 1% of the other acyl groups had been transferred to glycine.

Enzymatic reactions. Studies with partially purified extracts containing glycine-acyl transferase(s) derived from beef and human livers indicate that both are capable of catalyzing the conversion of salicyl-CoA to salicylurate and benzoyl-CoA to hippurate (Table 3). The experiments of Table 3 indicate that under these conditions benzoyl-CoA is a better substrate. A more meaningful comparison of enzyme reactivity with the two CoA derivatives on the basis of initial rates was not possible for several reasons. The complicated kinetics and the difficulty in obtaining initial rates with the beef liver enzyme have already been discussed by Schachter and Taggart.[19] In addition, because of spontaneous hydrolysis and nonenzymatic reactions, it was not possible

to obtain a spectral assay with salicyl-CoA that was comparable to that for benzoyl-CoA. A time-course of the reaction with the two CoA derivatives (Fig. 3) shows that benzoyl- CoA seems to react approximately twice as rapidly as salicyl-CoA during the early part of the reaction. Since end-product inhibition by hippurate has been offered to explain the kinetics of the beef liver enzyme,[19] experiments were performed to see if the presence of salicylurate inhibited the formation of salicylurate by the human enzyme. Conditions for these experiments were as in Fig. 3 except that various concentrations of salicylurate were added to the reaction vessels. It was found that salicylurate concentrations as high as 3×10^{-4} M (10-fold higher than the concentration reached in Fig. 3) did not diminish the salicylurate formed from glycine-

TABLE 3. STUDIES WITH GLYCINE-*N*-ACYLASE*

Enzyme source	Starting compound	(mμmoles)	Glycine conjugation at completion (mμmoles)	
			No enzyme	With enzyme
Beef liver	Bz-CoA	240	0	80
	Sal-CoA	70	0	10
	Sal-CoA	200	10	30
	Sal-CoA	400	10	50
Human liver	Bz-CoA	240	0	80
	Sal-CoA	200	10	40
	Sal-CoA	400	20	60

* Reaction mixtures contained 100 μmoles of potassium phosphate buffer, pH 7·5; 60 μmoles of glycine-1-^{14}C; the amount of acyl-CoA derivative indicated and either 1·4 units of beef liver enzyme or 0·14 units of human liver enzyme in a final volume of 1·0 ml maintained at 30°. Incubation was for 2 hr with the beef enzyme and for 17 hr with the human enzyme. Glycine conjugates were extracted and assayed as described in Methods.

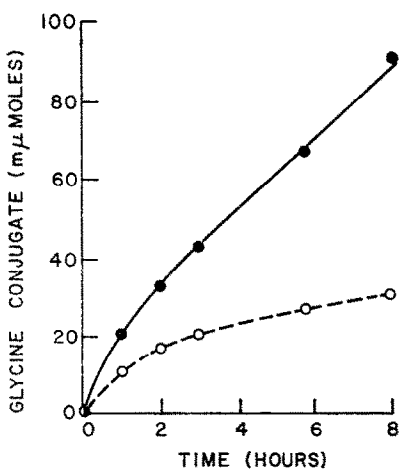

FIG. 3. Time-course of glycine conjugation. Reaction conditions were as in Table 3 for the experiments with the human enzyme except that each tube contained 240 mμmoles of either benzoyl-CoA or salicyl-CoA. Incubation was for the time intervals shown. The curve of hippurate formation is shown with closed circles and of salicylurate formation in open circles.

1-^{14}C. Presumably the slowing in the relative rate in salicylurate formation at later times is due to the greater loss of salicyl-CoA through spontaneous hydrolysis. Although the enzymes use in these studies were partially purified, it has not been established whether the glycine acyl transferase reactions with salicyl-CoA and benzoyl-CoA are catalyzed by the same enzyme.

DISCUSSION

Steric hindrance between the ortho-hydroxyl group of salicyl-CoA and the sulfur atom of the thiol ester can be demonstrated in studies with Cory–Pauling–Koltun models.[20] Since steric hindrance of this kind is only slight in models of 2-fluorobenzoyl-CoA and is not observed at all in models of benzoyl-CoA, one can invoke steric factors to explain the stability difference which increases in the order: salicyl-CoA, 2-fluorobenzoyl-CoA, benzoyl-CoA. It should be pointed out, however, that this correlation of thiol ester stability and steric factors ignores the effects of relative pK of the carboxyl groups and a consideration of hydrogen bonding of the ortho-hydroxyl group. These factors also would affect the stability of the thiol esters under consideration.

Evidence suggesting that salicyl-CoA is a substrate for glycine acyl transferase is in accord with a number of pharmaco-kinetic studies on salicylate elimination in man. It has been shown, for example, that salicylate retention is increased and salicylurate formation is reduced when *p*-aminobenzoate is given with salicylate.[21] These observations would be explained by the demonstration that salicyl-CoA, like *p*-aminobenzoyl-CoA,[9] is a substrate for glycine acyl transferase. Competition of the two CoA derivatives for glycine acyl transferase is a valid explanation for these observations even if, as appears possible, salicyl-CoA arises in a manner different from benzoyl-CoA or *p*-aminobenzoyl-CoA.

Recently, Levy[14] has calculated from his own data and that of Quick,[22] Schachter and Manis[23, 24] and Salassa *et al.*[21] figures for the maximum excretion of salicyluric acid in man. These figures range from 250 to 400 μmoles per hr; the maximum excretion of hippurate, on the other hand, is at least 20-fold greater.[23, 25] Since our studies with human glycine acyl transferase show that benzoyl-CoA reacts only approximately twice as fast as salicyl-CoA, this property of the enzyme(s) cannot explain the difference in excretion of the two glycine conjugates. Presumably it is the synthesis of salicyl-CoA which is rate limiting for salicylurate formation. Physiological studies have shown that the availability of glycine is the limiting factor in hippurate synthesis,[25, 26] but not of salicylurate synthesis.[13, 22, 27]

The small nonenzymatic acylation of glycine that is seen with salicyl-CoA but not with benzoyl-CoA is a phenomenon that can excite some speculation. One wonders if acylation by salicylate which occurs by such a mechanism might alter biologically important molecules and thus explain some of the effects of salicylate *in vivo*.

REFERENCES

1. K. Meyer and C. Ragan, *Science, N.Y.* **108**, 281 (1948).
2. J. Reid, R. D. Watfon, J. B. Cochran and D. H. Sproull, *Br. med. J.* **2**, 321 (1951).
3. N. E. Clarke, C. N. Clarke and R. E. Mosher, *Am. J. med. Sci.* **235**, 7 (1958).
4. A. K. Done, *Clin. Pharmac. Ther.* **1**, 141 (1960).
5. R. Stockman, *Rheumatism and Arthritis*, p. 39. Green, Edinburgh (1921).
6. R. K. Kielley and W. C. Schneider, *J. biol. Chem.* **185**, 869 (1950).

7. H. CHANTRENNE, *J. biol. Chem.* **189**, 227 (1951).
8. D. SCHACHTER and J. V. TAGGART, *J. biol. Chem.* **203**, 925 (1953).
9. D. SCHACHTER and J. V. TAGGART, *J. biol. Chem.* **208**, 263 (1954).
10. H. R. MAHLER, S. J. WAKIL and R. M. BOCK, *J. biol. Chem.* **204**, 453 (1953).
11. K. M. JONES, *Biochem. J.* **73**, 714 (1959).
12. G. LEVY, *The Salicylates* (Eds. M. J. H. SMITH and P. K. SMITH), p. 32. Interscience, New York (1966).
13. C. BEDFORD, A. J. CUMMINGS and B. K. MARTIN, *Br. J. Pharmac. Chemother.* **24**, 418 (1965).
14. G. LEVY, *J. Pharmac. Sci.* **54**, 959 (1965).
15. R. ADAMS and L. H. ULICH, *J. Am. chem. Soc.* **42**, 599 (1920).
16. E. R. STADTMAN, *Methods in Enzymology* (Eds. S. P. COLOWICK and N. O. KAPLAN), vol. 3, p. 931. Academic Press, New York (1957).
17. P. GOLDMAN, *J. biol. Chem.* **240**, 3434 (1965).
18. O. WARBURG and W. CHRISTIAN, *Biochem. Z.* **310**, 384 (1941).
19. D. SCHACHTER and J. V. TAGGART, *J. biol. Chem.* **211**, 271 (1954).
20. W. I. KOLTUN, *Biopolymers* 3, 665 (1965).
21. R. M. SALASSA, J. L. BALLMAN and T. J. DRY, *J. Lab. clin. Med.* **33**, 1393 (1948).
22. A. J. QUICK, *J. biol. Chem.* **101**, 475 (1933).
23. D. SCHACHTER, *J. clin. Invest.* **36**, 297 (1947).
24. D. SCHACHTER and J. G. MANIS, *J. clin. Invest.* **37**, 800 (1958).
25. A. J. QUICK, *J. biol. Chem.* **92**, 65 (1931).
26. A. DE VRIES and B. ALEXANDER, *J. clin. Invest.* **27**, 665 (1948).
27. E. NELSON, M. HANANO and G. LEVY, *J. Pharmac. exp. Ther.* **153**, 159 (1969).