The synthesis of p-aminobenzoate by the insoluble particles of rat liver and kidney homogenates occurs at the expense of phosphate bond energy. Cohen and McGilvery (1) have shown that the necessary energy is provided in an aerobic system by the oxidative reactions of the tricarboxylic acid cycle and in an anaerobic system by ATP. Studies by Kielley and Schneider (2) indicate that the enzymes involved in PAH synthesis are confined to the mitochondria. More recently, Chantrenne (3) has obtained hippurate synthesis in soluble extracts of rat liver acetone powder and has demonstrated a dependence of the over-all process on coenzyme A. The general role of CoA in a variety of condensation reactions involving acetate prompted Chantrenne to suggest the possibility that the energy-rich intermediate in hippurate synthesis is a "coenzyme A compound." The subsequent demonstration by Lynen, Reichert, and Rueff (4) that acetate activation involves the formation of the acetyl mercaptan of CoA provides strong supporting evidence for this hypothesis. However, it has remained uncertain whether benzoic acid or glycine represents the activated member in the condensation reaction. By analogy with the carboxyl activation of acetate, benzoyl CoA has been proposed as the most probable intermediate (5). Benzoyl phosphate (3) and N-phosphoglycine (1) appear to have been excluded as possible intermediates.

The present communication describes the preparation of S-benzoyl CoA by a simple method based on the observation of Simon and Shemin (6) that succinyl CoA can be prepared by the interaction of CoA and succinic anhydride. Evidence is presented that benzoyl CoA is utilized directly in the enzymatic synthesis of hippurate.

Methods and Materials

Preparation of Enzyme—A soluble preparation of pig kidney cortex was used in all of the experiments reported here. An acetone-ether powder of...
pig kidney was prepared at $-5^\circ$. The powder was extracted at room temperature in 10 volumes of 0.02 m potassium phosphate buffer of pH 7.5 and subsequently fractionated with ammonium sulfate at 0°. The fraction obtained between 30 and 40 per cent saturation with ammonium sulfate ($\text{AS}_{30-40}$) was dialyzed overnight against a large volume of 0.07 m KCl-0.02 m KHCO₃ which had been adjusted to pH 8.0 with solid K₂CO₃. When tested for PAH synthesis at 38° in a system containing 0.0025 m PAB, 0.06 m glycine, 0.006 m MgCl₂, 0.006 m ATP, 50 Lipmann units of CoA per ml., and 0.02 m potassium phosphate buffer of pH 7.5, the activity of the $\text{AS}_{30-40}$ fraction was approximately 3 times that of the crude extract and compared favorably with that of the rat liver extracts described by Chantrenne (3). Unlike the rat liver extract, the $\text{AS}_{30-40}$ fraction exhibits a complete CoA dependence without treatment with Dowex 1.

**Analytical Methods**—PAB and PAH were estimated by the method of Cohen and McGilvery (7); hippurate by the method of Chantrenne (3). Sulfhydryl groups were estimated by the procedure of Grunert and Phillips (8), with cysteine·HCl as a standard; for thioglycolate estimations a thioglycolic acid standard was used. Hydroxamic acids were estimated by the Lipmann and Tuttle method (9), with standard acethydroxamic acid prepared from acetamide and standard succinhydroxamic and benzohydroxamic acids prepared from the respective acid anhydrides.

Benzoyl CoA, benzoyl thioglycolate, and benzoyl glutathione have been estimated by two methods: (a) the liberation of $-\text{SH}$ on alkaline hydrolysis, and (b) by reaction with hydroxylamine to form benzohydroxamic acid. The benzoyl thiol esters of CoA and GSH are completely hydrolyzed in 5 minutes in 0.1 m NaOH at 25°, while that of thioglycolate requires 1.0 m NaOH. The results obtained by the alkaline hydrolysis and hydroxylamine methods consistently agreed within ±3 per cent.

**Preparation of S-Benzoyl CoA**—Benzoyl CoA was prepared by a modification of the acid anhydride method used by Simon and Shemin (6) for the synthesis of succinyl CoA (Equation 1).

$$\text{COA-SH} + \text{R-CO-}O\text{-CO-R} \rightarrow \text{COA-S-CO-R} + \text{RCOOH}$$ (1)

The following summarizes a typical preparative run. To a standard Warburg vessel were added 50 mg. of Pabst CoA (50.4 μm of free $-\text{SH}$), 3.0 ml. of water, solid NaHCO₃ to pH 7.0, and 75 μm of finely ground benzoic anhydride. After thorough flushing with nitrogen, the vessel was shaken in a 38° water bath. After 3 hours the reaction mixture contained 2.4 μm of free $-\text{SH}$ and 37.0 μm of alkali-hydrolyzable acyl mercaptan. The reaction mixture was adjusted to pH 3.5 with HCl and extracted four times with ether to remove any remaining benzoic anhydride. The material was then extracted successively into phenol-benzyl alcohol (3:1) and
water, according to the procedure utilized by Littlefield and Sanadi (10) in the purification of acetyl CoA. The final aqueous extract contained 19.6 \( \mu M \) of acyl mercaptan. Lyophilization yielded a fine white powder with an estimated purity of 27 per cent, as based on a molecular weight of 871 for benzoyl CoA. This material was dissolved in water and streaked for an ascending chromatogram on washed Whatman No. 3 paper with a 1:1 mixture of absolute ethanol and 0.1 M sodium acetate buffer of pH 4.5 as the solvent (11). Subsequent examination of the chromatogram with the nitroprusside Reagent I of Toennies and Kolb (12) failed to reveal any free sulfhydryl compound. However, treatment of the paper with methanolic KOH (11) prior to the application of Reagent I led to the appearance of a sharply defined band with an \( R_F \) of 0.73. The corresponding \( R_F \) of CoA

TABLE I
Hydrolysis Characteristics of Benzoyl CoA

<table>
<thead>
<tr>
<th>Condition</th>
<th>Time</th>
<th>Temperature</th>
<th>Per cent hydrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 1.0</td>
<td>20 min.</td>
<td>100 °C.</td>
<td>0</td>
</tr>
<tr>
<td>&quot; 3.5</td>
<td>20</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>&quot; 6.5</td>
<td>20</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>&quot; 8.5</td>
<td>20</td>
<td>25</td>
<td>39</td>
</tr>
<tr>
<td>&quot; 10.5</td>
<td>20</td>
<td>25</td>
<td>80</td>
</tr>
<tr>
<td>0.1 N NaOH</td>
<td>2</td>
<td>25</td>
<td>63</td>
</tr>
<tr>
<td>0.1 &quot; &quot;</td>
<td>5</td>
<td>25</td>
<td>100</td>
</tr>
</tbody>
</table>

Following incubation, all samples were readjusted to pH 6.5 prior to the estimation of free \(-\text{SH} \) (8). Stability in the acid range was confirmed by the hydroxylamine reaction (9).

in this solvent system was 0.55 to 0.59, as previously reported by Stadtman (11). Elution of the \( R_F \) 0.73 band in water and subsequent lyophilization yielded a white powder containing 44 per cent benzoyl CoA, as estimated by \(-\text{SH} \) liberation on alkaline hydrolysis and by the hydroxamic test.

A portion of the product was treated with an excess of hydroxylamine and applied to an ascending chromatogram on Whatman No. 1 paper with water-saturated butanol as the solvent (13). Subsequent color development with FeCl₃-HCl revealed a single spot with an \( R_F \) of 0.80. The observed \( R_F \) of a standard benzohydroxamic acid was 0.80, while that of succinhydroxamic acid was 0.35 and of acethydroxamic acid 0.43.

Table I summarizes data on the hydrolysis of benzoyl CoA over a wide
range of pH and at various temperatures. The stability of the thiol ester in acid solutions at 100° resembles that reported for acetyl CoA (11). In alkaline solutions, the rate of hydrolysis increases with increasing pH.

**Benzylothyioglycolic Acid and Benzyol Glutathione**—Benzylothyioglycolic acid was prepared from thioglycolate and benzoic anhydride by a procedure similar to that described for benzyol CoA. After recrystallization from water, the colorless plates which were obtained melted at 104—106°, as compared with a melting point of 107° reported by Holmberg (14) for benzylothyioglycolic acid prepared by another method. Alkaline hydrolysis liberated the theoretical amount of —SH and reaction with hydroxylamine gave the expected amount of benzo hydroxamic acid.

The reaction of neutral, reduced glutathione (General Biochemicals, Inc.) with benzoic anhydride yields products which are very sparingly soluble in acid solutions. Recrystallization from dilute HCl gave dense clusters of thin needles which melted with decomposition at 244°. However, elementary analysis of this product indicates a mixture of the mono- and dibenzyol derivatives of glutathione. Further purification procedures are in progress.

### Results

**Formation of Hippurate**—In the synthesis of hippurate by the soluble AS$_{38.40}$ fraction of pig kidney, benzyol CoA completely replaces the usual additions of benzoate + CoA + ATP + Mg$^{++}$ and GSH (or cysteine). In a system containing only the enzyme, benzyol CoA, glycine, and potassium phosphate buffer of pH 7.5, the formation of hippurate is essentially complete in 10 minutes at 38° (Fig. 1). The yield of hippurate decreases after 20 minutes incubation owing to the action of a hippuricase present in the AS$_{38.40}$ fraction. When the incubation period was limited to 20 minutes, the amount of hippurate formed was found to be directly proportional to either the quantity of benzyol CoA added (up to 0.6 $\mu$M per ml.) or to the concentration of enzyme (up to 2.0 mg. of protein per ml.). In a large series of experiments, the conversion of added benzyol CoA to hippurate averaged 75 per cent.

In several experiments, the liberation of —SH groups and the disappearance of hydroxylamine-reactive material were balanced against hippurate synthesis. In each experiment comparison was made between the complete system and one lacking glycine. As shown in Table II, the three estimations are in good agreement and are consistent with Equation 2.

$$\text{CoA-S-benzoyl + glycine} \rightarrow \text{hippurate + CoA-SH}$$

In the absence of added glycine there is no demonstrable formation of hippurate. However, it should be noted that benzyol CoA is slowly hy-
drolyzed, even in the absence of glycine. This may be due to the presence in the AS_{30-40} fraction of a benzoyl CoA deacylase analogous to those described by Gergely et al. (15) for acetyl CoA and succinyl CoA.

The possibility that benzoyl CoA is not utilized directly in hippurate synthesis appears to have been excluded by the following experiment.

![Graph showing time curve for synthesis of hippurate from benzoyl CoA.](image)

**Fig. 1.** Time curve for synthesis of hippurate from benzoyl CoA. The complete system contained 0.25 μM of benzoyl CoA, 60 μM of glycine, 20 μM of potassium phosphate buffer of pH 7.5, and 6 mg. of protein of the pig kidney AS_{30-40} fraction; final volume 1.0 ml. Temperature, 38°C.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Δ -SH (μM)</th>
<th>Δ hydroxamic acid (μM)</th>
<th>Δ hippurate (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+0.37</td>
<td>-0.59</td>
<td>+0.57</td>
</tr>
<tr>
<td>2</td>
<td>+0.62</td>
<td>-0.60</td>
<td>+0.60</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table II**

*Balance Studies for Hippurate Synthesis from Benzoyl CoA*

Hippurate was synthesized from benzoyl CoA and glycine in the presence of a 10-fold excess of PAB. An exchange of the S-benzoyl group with either PAB or glycine prior to condensation should result in the appearance of PAH among the reaction products. The actual experimental data showed that 0.25 μM of benzoyl CoA gave rise to 0.18 μM of hippurate, but no detectable quantity of PAH.

It has not been possible to establish a requirement for any additional cofactors in the condensation reaction. Although a potassium phosphate buffer of pH 7.5 was used in all of the experiments reported, it could be
replaced by a Tris-HCl buffer with only a 10 per cent reduction in the rate of hippurate synthesis. Chantrenne (3) has previously shown 0.003 M MgCl₂ to be essential for a maximal rate of synthesis in the ATP-dependent system. However, the addition of Mg²⁺ has no demonstrable effect when benzoyl CoA serves as the activated intermediate.

Hippurate synthesis was inhibited 73 per cent by 0.02 M cysteine; the basis for this inhibition will be discussed in a later section. No inhibition was observed with either 0.001 M 2,4-dinitrophenol, 4'-carboxyphenylmethanesulfonanilide (carinamide), or dipropylsulfamyl benzoate (benemid). The latter two compounds have been reported by Beyer and Wielbelhaus (16, 17) to be potent inhibitors of PAH synthesis in respiring kidney slices and washed particle systems.

Absorption Spectrum—The ultraviolet absorption spectrum of a sample of benzoyl CoA (44 per cent pure)³ is shown in Fig. 2. Of particular interest are the changes in light absorption which accompany the reaction of benzoyl CoA and glycine to form hippurate. Similar changes occur on the alkaline hydrolysis of benzoyl CoA. The increased light absorption associated with the formation of a thiol ester linkage has been reported to be maximal at 232 mp for acetyl CoA (18) and succinyl CoA (6) and at 235 mp for lactyl glutathione (19). In the case of benzoyl CoA, the difference spectrum has an apparent maximum at 272 mp. This observation, together with the balance data presented in Table II, appears to provide the basis for a sensitive spectrophotometric method for following the conversion of benzoyl CoA to hippurate.⁴ At 280 mp the light absorption contributed by the thiol ester linkage represents approximately 55 per cent of the total for benzoyl CoA. With a 1 cm. light path, a decrease in optical density of 0.730 corresponds to the cleavage of 0.1 μM of benzoyl CoA per ml. In estimating hippurate synthesis, comparison must be made with a glycine-free control, as is shown in Fig. 3.

Benzoyl Transfer Reactions—Stadtman has previously reported the non-enzymatic transfer of acetyl groups from acetyl CoA to various sulfhydryl compounds and from acetyl thioglycolate to glutathione (11). Very similar transfer reactions have now been shown to occur with benzoyl CoA. When benzoyl CoA is incubated at 38° in the presence of an excess of either cysteine or BAL at pH 7.5, there is a rapid and almost complete disappearance of hydroxylamine-reactive material. In contrast, incubation with

³ This sample was obtained by paper chromatography, as described under "Methods and materials," to minimize contamination with oxidized CoA. The bulk of the residual contaminant is presumed to be sodium acetate from the solvent system.

⁴ Stadtman has described a similar spectrophotometric method for following the arsenolysis of acetyl CoA (18).
either thioglycolate or glutathione does not decrease the yield of benzo-
hydroxamic acid. The S-benzoyl derivatives of cysteine and BAL are
apparently unstable and undergo spontaneous cleavage or rearrangement
to give products which do not react with hydroxylamine, as is the case
with the corresponding S-acetyl derivatives (11). Evidence for the fol-

![ absorption spectrum of benzoyl CoA during the formation of hippurate. ]

FIG. 2

Fig. 2. Changes in the absorption spectrum of benzoyl CoA during the formation of hippurate. The complete system contained 0.15 µM per ml. of benzoyl CoA and other additions as in Fig. 1. Neutralized perchloric acid filtrates (1:10) were pre-
pared of the initial reaction mixture (●) and after 25 minutes incubation at 37° (○). Absorption measurements were made in a Beckman DU spectrophotometer in silica
cells of 1 cm. light path; a perchloric acid filtrate containing all the components of
the complete system, except benzoyl CoA, served as the blank. The difference spec-
trum is indicated by the dash line.

Fig. 3. Spectrophotometric method for following hippurate synthesis from ben-
zoyl CoA. The complete system contained 0.09 µM of benzoyl CoA, 60 µM of glycine,
100 µM of potassium phosphate buffer of pH 7.3, and 0.6 mg. of protein of AS20-46;
total volume 3.0 ml. Readings were made in 1 cm. silica cells in a Beckman DU
spectrophotometer at 280 μm. The initial optical density of the complete system
was 0.472 when read against a blank containing all the components except benzoyl
CoA.

The reaction products were identified by the appearance on the chro-
matograms of spots with RF values corresponding to those listed in Table
III. The samples of benzoyl thioglycolate, whether prepared with benzoic
anhydride or by the exchange reaction with benzoyl CoA, migrated at

(3) CoA-S-benzoyl + GSH → GS-benzoyl + CoA-SH
(4) CoA-S-benzoyl + thioglycolate → benzoyl thioglycolate + CoA-SH
identical rates. In contrast, the benzoyl derivative of glutathione prepared with benzoic anhydride migrated a little more rapidly in both solvent systems ($R_F$ in ethanol-acetic acid 0.24, in phenol-water 0.67) than did the corresponding product of the exchange reaction. The latter is presumed to be the monobenzyol thiol ester of glutathione. Glutathione and its derivatives were detected on the chromatograms by reaction with ninhydrin as well as with the nitroprusside reagent.

**Table III**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Solvent system</th>
<th>$R_F$</th>
<th>$R_F$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ethanol-acetic acid-H$_2$O (80:1:10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CoA</td>
<td></td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>Glutathione</td>
<td></td>
<td>0.12</td>
<td>0.45</td>
</tr>
<tr>
<td>Thioglycolate</td>
<td></td>
<td>0.70</td>
<td>0.51</td>
</tr>
<tr>
<td>BENZOYL CoA</td>
<td></td>
<td>0.07</td>
<td>0.43</td>
</tr>
<tr>
<td>&quot; glutathione</td>
<td></td>
<td>0.19</td>
<td>0.60</td>
</tr>
<tr>
<td>&quot; thioglycolate</td>
<td></td>
<td>0.84</td>
<td>0.78</td>
</tr>
</tbody>
</table>

For the demonstration of non-enzymatic benzoyl transfer, 3 µM of benzoyl CoA were incubated with either 30 µM of glutathione or 60 µM of thioglycolate in 0.6 ml. at pH 7.5, temperature 37°, time 60 minutes. The final reaction mixtures were chromatographed on Whatman No. 1 paper with the indicated solvent system. CoA, thioglycolate, and their benzoyl derivatives were detected by the nitroprusside reaction (11), either directly or after hydrolysis with methanolic KOH. Glutathione and benzoyl glutathione were detected by the nitroprusside test and by reaction with ninhydrin.

* No free —SH spot for CoA could be detected with the phenol-water solvent system.

**DISCUSSION**

In the experimental section it has been assumed that the product arising from the interaction of CoA and benzoic anhydride is a monobenzyol derivative of the coenzyme. Lacking a sample of sufficient purity to permit a proof of structure, we cannot exclude the possibility that groups other than the sulfhydryl are benzoylated as well by the procedure described. Consequently, the absorption spectrum in Fig. 2 and estimates of the purity of benzoyl CoA preparations must be regarded as tentative. However, evidence that the procedure yields the benzoyl mercaptan of CoA has been presented. In addition, data from the balance studies indicate that the $S$-benzoyl group is utilized directly in hippurate synthesis.
The availability of benzoyl CoA has made possible a definition of the essential factors in the final condensation reaction (Equation 2). In contrast to the system studied by Chantrenne (3), in which ATP served as the energy donor, benzoyl CoA replaces the usual requirements for benzoate + CoA + ATP and, therefore, is considered to represent the activated intermediate in hippurate synthesis. In accordance with the proposed mechanism for acetate activation (4, 20), the phosphate bond energy of ATP may be utilized in the preliminary formation of an appropriate phosphoryl CoA. A subsequent exchange of benzoyl for phosphate on the coenzyme would yield the activated intermediate. Since neither Mg++ nor a reduced sulfhydryl compound is required in the final condensation reaction, these factors must be essential for the reactions leading to the formation of benzoyl CoA. Similarly, the inhibition of hippurate synthesis by carinamide and benemid in respiring or ATP-driven systems (16, 17) must be due to an interference with benzoate activation rather than with the condensation reaction. In support of this view, unpublished observations in this laboratory have shown that carinamide and benemid likewise interfere with the activation of acetate by ATP.

SUMMARY

A method for the S-benzoylation of coenzyme A by benzoic anhydride has been described. Evidence was presented that S-benzoyl CoA represents the activated intermediate in the enzymatic synthesis of hippurate by a soluble pig kidney preparation. The essential factors in the condensation reaction were found to be limited to benzoyl CoA, glycine, and the enzyme. A spectrophotometric method for following the synthesis of hippurate from benzoyl CoA and glycine was described. The benzoyl group of benzoyl CoA can be transferred non-enzymatically to cysteine, BAL, glutathione, or thioglycolate. While such transfer reactions can interfere with the synthesis of hippurate from benzoyl CoA in vitro, their physiological significance is not known at the present time.

BIBLIOGRAPHY

BENZOYL COENZYME A AND HIPPURATE SYNTHESIS
David Schachter and John V. Taggart


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