Succinyl Phosphate

ITS NONENZYMATIC HYDROLYSIS AND REACTION WITH COENZYME A*

(Received for publication, January 26, 1970)

CHRISTOPHER T. WALSH, JR., † § JOHN G. HILDEBRAND, ‡ ¶ AND LEONARD B. SPECTOR||

From The Rockefeller University, New York, New York 10021

SUMMARY

Succinyl phosphate reacts rapidly and nonenzymatically with coenzyme A in the pH range 3 to 8 to yield succinyl coenzyme A. This hitherto unsuspected reaction of an acyl phosphate with a thiol depends critically on the presence of a free carboxyl group, which bears a “succinyl” relationship—and is spatially accessible—to the phosphorylated carboxyl. The free carboxyl group interacts with the anhydride portion of the molecule at neutral pH and below. A variety of kinetic experiments point to the cyclic form of succinyl phosphate as the immediate reactant with coenzyme A in the synthesis of succinyl coenzyme A. The somewhat unorthodox mechanism which is proposed is not to be taken as proved. Succinyl phosphate and its congeners exhibit characteristic pH rate profiles of hydrolysis, which are unmistakably distinct in their contour from those of acyl phosphates which are unreactive with coenzyme A. During hydrolysis at neutral pH and below, succinyl phosphate may also react in a cyclic form, possibly as succinic anhydride following elimination of orthophosphate.

Facts have accumulated in recent years pointing strongly to the probability that the succinate thiokinase reaction proceeds with the intermediary participation of succinyl phosphate (1, 2). Thus, upon reaction of succinate with ATP in the presence of succinate thiokinase (succinyl coenzyme A synthetase, EC 6.2.1.5),

\[
\text{Succinate + ATP + E} \rightarrow \text{E} \cdot \text{succinyl} \sim \text{P} + \text{ADP} \quad (1)
\]

\[
\text{E} \cdot \text{succinyl} \sim \text{P} + \text{CoA} \rightarrow \text{E} + \text{succinyl} \sim \text{CoA} + \text{P}_1 \quad (2)
\]

enzyme-bound succinyl phosphate is formed, and thereafter reacts with coenzyme A to yield succinyl-CoA. While investigating this enzymatic reaction we made the singular observation

* This work was supported by Grant GM-13972 from the United States Public Health Service and GB-8299 from the National Science Foundation.
† Graduate Fellow of The Rockefeller University.
§ Present address, Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02154.
‡ Present address, Department of Neurobiology, Harvard Medical School, Boston, Massachusetts 02115.
¶ To whom inquiries should be addressed.

that synthetic succinyl phosphate reacts rapidly with coenzyme A, and other thiols, in the absence of enzyme (2). This was unexpected in view of the general observation that acetyl phosphate, the prototypic acyl phosphate, fails to react with thiols in the absence of an enzyme or metal catalyst (3, 4). For this reason we were led to examine the nonenzymatic reactivity with coenzyme A of a number of other acyl phosphates. We found that the reactivity of a given acyl phosphate is intimately bound up with its chemical constitution. Only those acyl phosphates are active which stem from dicarboxylic acids, and which possess a free carboxyl function at a distance of 2 carbon atoms from the acyl phosphate portion of the molecule. Moreover, good reactivity depends wholly upon unimpeded interaction between the two functional groups.

MATERIALS AND METHODS

All chemicals and solvents were reagent grade products of Eastman Kodak, Aldrich, or Matheson, Coleman, and Bell. Anhydrous lithium perchlorate was supplied by Alfa Inorganics, Inc. Sigma furnished HEPES (N-2-hydroxyethylpiperezine-N’-2-ethanesulfonic acid) and BICINE (N,N-bis(2-hydroxyethyl)glycine). All solvents and liquid reagents used in synthesis were freshly distilled, with the exception of diethyl ether (Mallinckrodt anhydrous, peroxide-free). H_2^18O was purchased from Miles Laboratories. ^4C-Succinyl acid and ^3H-succinic acid were products of New England Nuclear, and ^14C-glutaric acid was obtained from International Chemical and Nuclear Company. ^4C-Succinic anhydride was made from ^4C-succinic acid by refluxing for 3 hours in acetonitrile with ethoxyacetylene (K and K Laboratories).

H_2^18O Experiments

Hydrolysis of Succinyl Phosphate in H_2^18O—For the experiments of Table I buffers of the desired pH were lyophilized, dried in a vacuum over phosphorus pentoxide overnight, and, just prior to the experiment, dissolved in H_2^18O of the indicated isotope content. The pH (at 37°C) was checked before and after each experiment and was found to be unchanged. Trilithium succinyl phosphate (2) was dissolved in the prepared buffer and hydrolysis proceeded at 37°C for at least five half-lives. Reaction was terminated by freezing at -20°C. Solutions were kept at this temperature for no longer than 2 days before being worked up. The H_2^18O was recovered by lyophilization. Residues were then dissolved in 2 ml of H_2O. Each solution (at 4°C) was placed on a column, 1 x 30 cm, of Dowex 1 X8-formate equilibrated with...
H₂O at 4° (the pH 10.2 solutions were adjusted to pH 7 with 6 N HCl before chromatography). The column was washed with 15 ml of H₂O and then eluted with 2.5 M formic acid. Fractions (3 ml) were collected automatically and analyzed for inorganic phosphates (5). On some columns a low level (10,000 cpm) of 2,3-3H-succinic acid was applied with the sample in order to locate the succinic acid in the eluate. In all cases succinic acid was found in tubes 16 to 24, while phosphate appeared in tubes 34 to 66. A clean separation of succinic acid and phosphate was thus assured. Fractions containing succinic acid were combined, concentrated to 2 to 3 ml by rotary evaporation in a vacuum at temperatures below 40°, and lyophilized. After further desiccation over phosphorus pentoxide in a vacuum, the solid was dissolved, with warming, in 1 ml of acetone. Cooling at 4° for 3 hours gave crystals, which were collected by centrifugation. Yield, 5 to 8 mg of succinic acid, m.p., 185-186° (recorded m.p., 185-187°). The phosphate-containing fractions were combined, concentrated to 12 ml of absolute ethanol (−20°). Crystallization took place overnight at −20°. The crystals were collected by filtration and dried over phosphorus pentoxide in a vacuum. Yield of KH₂PO₄, 8 to 12 mg. Succinic acid and KH₂PO₄ were converted to carbon dioxide for ⁴²⁰⁰ analysis by the procedure of Williams and Hager (6). Mass spectrometric measurements were provided by Gollob Analytical Service, Berkeley Heights, New Jersey. To calculate dilution factors it was essential to know the content of active succinyl in each preparation of succinyl phosphate which was used. This was determined by hydroxamate assay (7). The total succinic acid content in a given weight of hydrolyzed succinyl phosphate was ascertained by adding a known amount of tritiated succinic acid to the hydrolysate and, after isolation on a column as described above, crystallizing the succinic acid to constant specific activity. The succinic acid content of succinyl phosphate, before and after hydrolysis, was calculable from the observed isotope dilution.

Control Experiments—Succinic acid (23.6 mg, 200 μmoles) and 23.2 mg of lithium phosphate (200 μmoles) were dissolved in 2.0 ml of 0.5 M Tris-HCl, pH 6.07, made up in 10.90% H₂O. This solution, in duplicate, was submitted to the same conditions and procedures as were given above. A similar control experiment, in duplicate, was conducted in 6.0 ml of 0.2 M sodium borate, pH 10.2, made up in 10.81% H₂O. In all controls the carbon dioxide derived from succinic acid and KH₂PO₄ was indistinguishable in °⁰¹⁰ content from tank carbon dioxide.

Assay for Succinyl-CoA Synthesis

5,5'-Dithiobis(2-nitrobenzoic acid) Assay (8)—Thiolester synthesis was followed by measuring the amount of unreacted thiol (coenzyme A) remaining in solution after reaction with acyl phosphate. An aliquot (0.10 ml) of test solution was withdrawn at intervals and quickly dispensed into 1.9 ml of assay solution containing 0.05 ml of a solution of 0.0196 g of 5,5'-dithiobis(2-nitrobenzoic acid) (Aldrich) in 5.1 ml of 0.10 M potassium phosphate (pH 7.0), 0.40 ml of potassium phosphate (pH 8.0), and 1.45 ml of H₂O. The mixture was agitated and the absorbance at 420 μm was read exactly 30 sec after mixing in a 1-cm cuvette in a Gilford model 240 spectrophotometer. Under these conditions 0.10 μmole of thiol in the aliquot gave an absorbance of 0.570 OD unit over a blank from which thiol was omitted.

Aldrithiol Assay (9)—To measure thiolester synthesis at neutral pH and below, it was necessary to use the Aldrithiol reagent in place of 5,5'-dithiobis(2-nitrobenzoic acid). An aliquot (0.10 ml) was withdrawn from the test solution and added to 2.9 ml of assay solution containing 10 mg of Aldrithiol (4,4'-dithiopiridine, Aldrich) in 30 ml of 0.5 M potassium phosphate, pH 6.0. The mixture was agitated and the absorbance at 324 μm was read in a 1-cm cuvette in a Gilford model 240 spectrophotometer. The color was stable for at least 1 min. In this procedure 0.10 μmole of thiol in the aliquot gave an absorbance of 0.505 OD unit over a blank from which thiol was omitted.

CHEMICAL SYNTHESSES

Three general synthetic methods were used to prepare the acyl phosphates listed in Table III (10). These methods are described below under separate headings as (a) the aqueous anhydride synthesis, (b) the nonaqueous anhydride synthesis, and (c) the nonaqueous acyl chloride synthesis. Under each heading is given one detailed example of the synthesis. The acyl phosphates thus prepared varied widely in purity, but all were judged adequate for simple chemical experiments, since the contaminants were unlikely to be detrimental. Except for succinyl and citryl phosphates, which were wanted for other investigations, the purity of the product was considered satisfactory.

Aqueous Anhydride Synthesis

Maleyl Phosphate—It proved difficult to obtain this acyl phosphate as the lithium salt. It could, however, be made in solution as the potassium salt. A solution of 68 mg (2.5 mmoles) of KH₂PO₄ in 15 ml of H₂O was magnetically stirred at room temperature and brought to pH 10 with 10 N KOH. Solid maleic anhydride (245 mg, 2.5 mmoles) was added to the stirring solution while the pH was maintained between 8 and 10 by a Radiometer pH meter (type TTT1a) fitted with a motor-driven syringe filled with 10 N KOH. All of the anhydride was dissolved in 5 min, and the final pH was 0.5. The solution of maleyl phosphate was then electrolyzed at 0° and used promptly for the experiments of Table III and Fig. 1. A control, maleic anhydride, was carried through this operation in the absence of phosphate. No hydroxamate-positive material remained in solution at the end.

Phthaloyl Phosphate—Reaction time was 60 min, and the yield of acyl phosphate in solution was 34%.

Itaconyl Phosphate—Reaction time was 5 min, and the yield of acyl phosphate in solution was 69%.

2-Methylsuccinyl Phosphate—Reaction time was 10 min, and the yield of acyl phosphate in solution was 55%.

2,2-Dimethylsuccinyl and 2,3-Dimethylsuccinyl Phosphates—These were prepared from the corresponding acids, which were converted to the anhydrides with dicyclohexylcarbodiimide in acetonitrile. The anhydrides were then used for acyl phosphate synthesis in the procedure given for maleyl phosphate. Reaction...
tion time was 10 min, and the yields of acyl phosphate in solution were 61 and 45%, respectively, for the 2,2 and 2,3 isomers.

1,2-Cyclobutanedicarboxylic Acid Phosphate—Reaction time was 10 min, and the yield of acyl phosphate in solution was 92%.

With each of the foregoing syntheses a control experiment was carried through in order to be certain that no hydroxamate-positive material remains in solution when the synthetic operations are conducted in the absence of phosphate. It was our experience that, in the absence of phosphate, all anhydrides in alkaline medium are quickly hydrolyzed.

Succinyl Phosphate—The detailed procedure for preparing the solid lithium salt of this compound was given elsewhere (2). It differs from the foregoing syntheses only in the addition of another operation to transform the soluble potassium salt into the solid lithium salt. Yields of succinyl phosphate averaged about 90%, and the purity of routine preparations varied between 70 and 90%.

Nonaqueous Anhydride Synthesis

These syntheses depend upon reaction in acetonitrile of an acid anhydride with triethylammonium phosphate, followed by isolation of the acyl phosphate as the lithium salt.

Glutaryl Phosphate—Glutaric anhydride (342 mg, 3.0 mmoles) was dissolved in 15 ml of acetonitrile, and to the magnetically stirred solution at 0° were added (in rapid succession) 300 mg (2.5 mmoles) of solid triethylammonium phosphate (10) and 0.76 ml 5.0 mmoles) of triethylamine. By the end of a further 30 min of stirring in the cold all of the solid was dissolved. The solution was then added dropwise to a cold, stirring solution of 789 mg (7.5 mmoles) of anhydrous lithium perchlorate in 15 ml of acetonitrile. A voluminous white precipitate formed instantly, and, after 15 min of stirring, was collected by vacuum filtration. The solid was washed with acetonitrile and ether, and dried over phosphorus pentoxide in a vacuum. Yield, 580 mg (96% theory).

Succinyl Phosphate—The synthesis of this compound was detailed earlier (11). It can be made chemically pure and in excellent yields.

Adipyl Phosphate—This was prepared from adipic acid through the anhydride in 91% yield of salt which was 86% pure.

Fumaryl Phosphate—Fumaric acid, as the monoethylammonium salt in acetonitrile, was converted into the intermolecular anhydride with dicyclohexylcarbodiimide. Thereafter, the anhydride in 91% yield of salt which was 86% pure.

Malyl Phosphate—This was made from 2-malic acid through the anhydride. The product contained 22% of malyl phosphate.

Nonaqueous Acyl Chloride Synthesis

Propionyl Phosphate—Triethylamine (2.56 ml, 7.5 mmoles) and 0.16 ml (2.5 mmoles) of phosphoric acid (85%) were dissolved in 15 ml of toluene, and the solvent was removed by rotary evaporation in a vacuum. The residual oil was taken up in 15 ml of toluene, and the solution was again concentrated. The drying cycle was repeated twice more, and the oily triethylammonium phosphate was redissolved in 15 ml of benzene. This solution was added dropwise to a cool, stirring solution of 0.22 ml (2.5 mmoles) of propionyl chloride in 15 ml of benzene. Stirring in the cold was continued for 30 min, after which 0.38 ml (2.5 mmoles) of triethylamine was added. Triethylammonium hydrochloride, which formed instantly and quantitatively, was removed by filtration. The clear, colorless filtrate was concentrated in a vacuum to an oil. This was digested with 15 ml of acetonitrile, and insoluble globules of free triethylamine were separated by decantation of the acetonitrile solution after low speed centrifugation (1000 rpm, 2 min). Addition of the supernatant solution, dropwise, to a cold, stirring solution of 580 mg (5.42 mmoles) of anhydrous lithium perchlorate in 15 ml of ether afforded a precipitate, which was collected, washed with acetonitrile and ether, and dried in a vacuum over phosphorus pentoxide. Yield of off white solid, 277 mg (96% of theory). Content of propionyl phosphate, 88%.

Acetyl Phosphate—Obtained in 79% yield. Purity, 73%.

Butyryl Phosphate—Obtained in 57% yield. Purity, 90%.

Malonyl Phosphate—Malonic acid was converted to malonic acid monochloride by the method of Lynen (13). The acyl phosphate formed in 50% yield. Purity, 40%.

Succinyl Phosphate, Half-Methyl Ester—Methyl hydrogen succinate was converted to β-carbomethoxypropionyl chloride by the method of Cason (14). The acyl phosphate formed in 80% yield. Purity, 84%.

N-Succinyl-Tris—A mixture of succinic anhydride (500 mg, 5 mmoles) and tris(hydroxymethyl)amino methane (free base) (1.21 g, 10 mmoles) in 50 ml of spectroquality acetonitrile was heated to reflux and cooled. The crystalline solid which separated was dissolved in a small volume of water and acetone was added to opalescence. On cooling, an oil separated and subsequently solidified. A second recrystallization yielded white crystals of the Tris salt of N-succinyl-Tris, m.p. 130.5-133°C. The infrared spectrum of the salt (KBr pellet) exhibited strong absorption bands at 3200 cm⁻¹ (OH), 1650 cm⁻¹ (amide I) and 1585 cm⁻¹ (amide II). No ester band was evident. The salt was dissolved in water and applied to a column (2 x 16 cm) of Dowex 1-X8 in the formate form, previously equilibrated with water. After washing with 100 ml of water to remove free Tris, the column was eluted with 0.3 M formic acid. Fractions containing the acid-amide (assayed by heating aliquots in alkaline hydroxylamine followed by ferric chloride reagent) (7) were pooled and evaporated to a colorless oil. The oil was dissolved in absolute ethanol and ethyl acetate was added to opalescence. Crystals separated in the cold. After recrystallization from the same solvent pair, the melting point was 119.5-120°C. The infrared spectrum (KBr pellet) displayed sharp bands at 1650 cm⁻¹ (amide I) and 1585 cm⁻¹ (amide II).

N-Succinyl-Tris (C₇H₈O₄N)

Calculated: C 49.5, H 6.8, N 6.3

Found: C 49.5, H 6.8, N 6.3

N'-C-Succinyl-Tris—To 0.90 ml of an aqueous solution of Tris-HCl (50 mmoles, pH 7.8) was added 0.10 ml of a dimethoxyethane solution of N'-C-succinimide anhydride (3.0 mmoles, 144,000 cpm). After 15 min at 37°C, the solution was diluted to 5 ml with water and applied to a column (1 x 8 cm) of Dowex 1-X8 in the formate form, previously equilibrated with water. Elution was effected with 0.5 M formic acid. Six 10-ml fractions, comprising 65% of the radioactivity, were collected. The radioactive material in solution was chromatographically identical with authentic N-succinyl-Tris in two thin layer systems: (a) Brink-
**RESULTS**

**Hydrolysis of Succinyl Phosphate and Other Acyl Phosphates**—Set forth in Fig. 1 is the effect of pH on the hydrolysis of succinyl-P at 37°. Compared in the same figure are the pH rate profiles of several additional acyl phosphates of diverse chemical constitution. Most striking is the natural separation of the curves into two general categories: those which display a steep, nearly vertical rise in hydrolytic rate in the neighborhood of neutrality, and those for which the pH must be drastically lowered before a rapid rise in rate becomes manifest. In the former category are succinyl-P, maleyl-P, and phthaloyl-P (15) (not shown). In the latter category are propionyl-P, glutaryl-P, and acetyl-P (16) (not shown). The compounds in the former category possess in common a free carboxyl group which stands in a "succinyl" relationship to the acyl phosphate portion of the molecule. Although glutaryl-P, the next higher homologue of succinyl-P, possesses a free carboxyl group, its hydrolytic behavior conforms to that of the monocarboxylic acyl phosphates.

**Transfer of oxygen from water to succinate and phosphate during hydrolysis of succinyl phosphate**

The hydrolysis of trilithium succinyl phosphate (43.0 mg) in H2O proceeded at pH 10.2 for 70 hours (five half-lives), at pH 6.90 for 5 hours (five half-lives), and at pH 6.07 for 30 min (15 half-lives). The temperature was 37°. The detailed procedure is given under "Materials and Methods."

<table>
<thead>
<tr>
<th>pH</th>
<th>Excess of 18O in CO2 from a</th>
<th>Water-oxygen incorporated b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Succinic acid</td>
<td>KH2PO4</td>
</tr>
<tr>
<td>10.2</td>
<td>0.77 0.08</td>
<td>0.38 0.48</td>
</tr>
<tr>
<td>6.90</td>
<td>2.00 0.06</td>
<td>0.86 0.03</td>
</tr>
<tr>
<td>6.07</td>
<td>1.30 0.03</td>
<td>0.56 0.01</td>
</tr>
<tr>
<td>6.07d</td>
<td>0.60 0.00</td>
<td>0.28 0.00</td>
</tr>
</tbody>
</table>

a Includes the correction of 0.20, the atom per cent excess in the CO₂ from ordinary KH₂PO₄ and succinic acid.

b In these experiments succinyl phosphate (70% active succinyl) was dissolved in 0.9 ml of 0.2 M potassium phosphate buffer (10.81% H₂¹⁸O).

c In these experiments succinyl phosphate (50% active succinyl) was dissolved in 0.9 ml of 0.2 M potassium phosphate buffer (10.90% H₂¹⁸O).

d In these experiments succinyl phosphate (74% active succinyl) was dissolved in 2.0 ml of 0.5 M Tris-HCl buffer (11.49% H₂¹⁸O).

---

**TABLE I**

Transfer of oxygen from water to succinate and phosphate during hydrolysis of succinyl phosphate

---

The hydrolysis of trilithium succinyl phosphate (43.0 mg) in H₂O proceeded at pH 10.2 for 70 hours (five half-lives), at pH 6.90 for 5 hours (five half-lives), and at pH 6.07 for 30 min (15 half-lives). The temperature was 37°. The detailed procedure is given under "Materials and Methods."

1. At pH 10.2, which lies on the pI-independent segment of the pH rate profile (Fig. 1), the hydrolysis of the anhydride function proceeds by a monomolecular reaction in which the carboxylic portion of the molecule is eliminated. This elimination mechanism may well apply to all monoacetyl phosphates. It demands that the P-O bond of the anhydride be split, and that such splitting actually occurs was established earlier by the studies in H₂¹⁸O (18, 19). It becomes a matter of interest to ascertain the point of cleavage of the anhydride linkage in succinyl phosphate as a function of pH. Accordingly, succinyl phosphate was submitted to hydrolysis in H₂¹⁸O at pH 10.2, 6.90, and 6.07, and the data are given in Table I. At pH 10.2, which lies on the pH-independent segment of the pH rate profile (Fig. 1), the hydrolysis of the anhydride function does indeed proceed in large degree by P-O bond rupture. But no P-O bond rupture is evident at pH 6.90 or 6.07. Instead, C-O cleavage of the anhydride predominates at pH 6.90. At this pH nearly 1 atom of ¹⁸O is introduced into succinate from the solvent. Much diminished, however, is the incorporation of oxygen from the solvent into succinate at the top of the steep rise (pH 6.07) in the pH rate profile. Failure to incorporate a whole atom of solvent oxygen at the lower pH is a curious phenomenon, for which no firm explanation is ventured at this time.

Is Succinic Anhydride an Intermediate in the Hydrolysis of Succinyl Phosphate?—A reasonable pathway for the neutral and acidic hydrolysis of succinyl phosphate includes the cyclization of trisuccinyl succinyl phosphate to the cyclic, diionic form (Formulas I → II → III of Scheme 1). Cyclic succinyl phos-
Scheme 1

Comparison of pseudo-first order rate constants for hydrolysis of succinyl-P and succinic anhydride at 37°

Succinyl-P (72% pure by hydroxamate assay) or succinic anhydride (30 μmoles of each) was dissolved in 10 ml of buffer, which finally contained 10% (v/v) of freshly distilled dimethoxyethane. Succinyl-P was added as the solid lithium salt. The anhydride, dissolved in 1.0 ml of dimethoxyethane, was added to 9.0 ml of buffer. Aliquots were withdrawn at intervals and assayed by the Lipmann and Tuttle procedure (7). The buffers (all 0.10 M) were: pH 2.0, HCl-KCl; pH 3.0, potassium phthalate; pH 4.0 and 5.0, potassium succinate; pH 6.0, potassium maleate; pH 6.9 and 7.8, Tris-HCl. Rate constants were taken from linear semilogarithmic plots.

<table>
<thead>
<tr>
<th>pH</th>
<th>(k_{obs}) Succinyl-P</th>
<th>(k_{obs}) Succinic anhydride</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0</td>
<td>0.42</td>
<td>0.69</td>
</tr>
<tr>
<td>3.0</td>
<td>0.33</td>
<td>0.42</td>
</tr>
<tr>
<td>4.0</td>
<td>0.43</td>
<td>0.35</td>
</tr>
<tr>
<td>5.0</td>
<td>0.51</td>
<td>0.40</td>
</tr>
<tr>
<td>6.0</td>
<td>0.36*</td>
<td>0.16</td>
</tr>
<tr>
<td>6.9</td>
<td>0.07*</td>
<td>0.29</td>
</tr>
<tr>
<td>7.8</td>
<td>0.005*</td>
<td>1.73</td>
</tr>
</tbody>
</table>

* Dimethoxyethane was omitted in this experiment. The rate constant is taken from Fig. 1.

not of itself a proof of anhydride participation in the hydrolytic process. On present evidence the possibility is not excluded that cyclic succinyl phosphate itself is the immediate reactant with water (see below). These considerations call to mind the experience of Higuchi, Flynn, and Shah (15) with phthaloyl phosphate, whose hydrolysis between pH 5.5 and 7.1 is said to proceed both by mediation of anhydride and by direct reaction with water.

We interpret as follows the pH rate profile of succinyl phosphate hydrolysis (Fig. 1). In alkaline solution succinyl phosphate exists as the trianion I (Scheme 1). Hydrolysis of the trianion is slow up to pH 12, but thereafter becomes rapid when the concentration of hydroxide ions rises high enough. As the solution of succinyl phosphate is acidified, a proton is ultimately fixed to a phosphate oxygen atom to form the dianion II. Protonation of the phosphate oxygen sufficiently heightens the electrophilic power of the adjoining carbonyl-carbon for the latter to combine with the ionized carboxyl group, and gives the dianionic, cyclic succinyl phosphate III. The monoionized phosphate group of III ought to be an excellent leaving group in either of two conceivable modes of orthophosphate elimination.

At pH 7.8 and above, the anhydride hydrolyzes more rapidly than the acyl phosphate, but, since in alkaline solution succinyl phosphate exists almost certainly as the trianion, there is little possibility that it can furnish anhydride by cyclization.

Table II

Comparison of pseudo-first order rate constants for hydrolysis of succinyl-P and succinic anhydride at 37°

Succinyl-P (72% pure by hydroxamate assay) or succinic anhydride (30 μmoles of each) was dissolved in 10 ml of buffer, which finally contained 10% (v/v) of freshly distilled dimethoxyethane. Succinyl-P was added as the solid lithium salt. The anhydride, dissolved in 1.0 ml of dimethoxyethane, was added to 9.0 ml of buffer. Aliquots were withdrawn at intervals and assayed by the Lipmann and Tuttle procedure (7). The buffers (all 0.10 M) were: pH 2.0, HCl-KCl; pH 3.0, potassium phthalate; pH 4.0 and 5.0, potassium succinate; pH 6.0, potassium maleate; pH 6.9 and 7.8, Tris-HCl. Rate constants were taken from linear semilogarithmic plots.

<table>
<thead>
<tr>
<th>pH</th>
<th>(k_{obs}) Succinyl-P</th>
<th>(k_{obs}) Succinic anhydride</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0</td>
<td>0.42</td>
<td>0.69</td>
</tr>
<tr>
<td>3.0</td>
<td>0.33</td>
<td>0.42</td>
</tr>
<tr>
<td>4.0</td>
<td>0.43</td>
<td>0.35</td>
</tr>
<tr>
<td>5.0</td>
<td>0.51</td>
<td>0.40</td>
</tr>
<tr>
<td>6.0</td>
<td>0.36*</td>
<td>0.16</td>
</tr>
<tr>
<td>6.9</td>
<td>0.07*</td>
<td>0.29</td>
</tr>
<tr>
<td>7.8</td>
<td>0.005*</td>
<td>1.73</td>
</tr>
</tbody>
</table>

* Dimethoxyethane was omitted in this experiment. The rate constant is taken from Fig. 1.
since the acyl phosphate is very acid-labile (Fig. 1), whereas the theory of succinic monohydroxamate, which was unambiguously identified by paper chromatography. These conditions, when applied to succinyl phosphate, afforded no hydroxamate, indicating that the addition of two methyl groups (as in 2,2-dimethyl succinyl phosphate) trebles the reactivity, and the addition of the ethylene bridge (in 1,2-cyclobutane-dicarboxylate monophosphate) doubles it. Maximal reactivity is attained when the two polar groups of succinyl phosphate are locked into the cis configuration by inserting a double bond between them to give maleyl phosphate. Signally different is the corresponding trans isomer (fumaryl phosphate), which is inert. Also inert are malyl and citryl phosphates and the half-methyl ester of succinyl phosphate. Of the larger homologues (glutaryl, cis-1,2-cyclobutane-dicarboxylate) only malonyl phosphate possesses any reactivity, and this is a slight one. The larger homologues (glutaryl and adipyl phosphates) are inactive, as are the monocarboxyl phosphates, acetly, propionyl, and butyryl phosphates. The kinetic course of the reaction for several of the acyl phosphates of coenzyme A and succinyl phosphate is represented in Fig. 3.

The product which formed in the reaction of succinyl phosphate with coenzyme A was identified as succinyl-CoA, after spraying the succinyl-CoA zone with sodium acetate (pH 4.6)-absolute ethanol, 1:1) revealed that all of the ultra-violet-absorbing material co-chromatographed with authentic succinyl-CoA. After spraying the succinyl-CoA zone with alkali, nitroprusside-reactive thiol appeared (20).

**Acyl-CoA Synthesis with Acyl Phosphates**—In Table III are set out the initial velocities of acyl-CoA synthesis with 18 different acyl phosphates. Itaconyl, 2-methyl, and phthaloyl phosphates exhibit reactivities comparable to that of succinyl phosphate itself. Reactivities are enhanced when still other modifications are made in the skeletal structure of succinyl phosphate. Thus, the addition of two methyl groups (as in 2,2-dimethyl succinyl phosphate and 2,3-dimethyl succinyl phosphate) trebles the activity, and the addition of the ethylene bridge (in 1,2-cyclobutane-dicarboxylate monophosphate) doubles it. Maximal reactivity is attained when the two polar groups of succinyl phosphate are locked into the cis configuration by inserting a double bond between them to give maleyl phosphate. Signally different is the corresponding trans isomer (fumaryl phosphate), which is inert. Also inert are malyl and citryl phosphates and the half-methyl ester of succinyl phosphate. Of the homologues of succinyl phosphate, only malonyl phosphate possesses any reactivity, and this is a slight one. The larger homologues (glutaryl and adipyl phosphates) are inactive, as are the monocarboxyl phosphates, acetly, propionyl, and butyryl phosphates. The kinetic course of the reaction for several of the acyl phosphates of Table III is shown in Fig. 3.

These data make it plain that the reaction between coenzyme A and succinyl phosphate hinges on the availability of a free carboxyl group at the precise distance of 2 carbon atoms from the phosphorylated carboxyl. If the free carboxyl group is blocked through esterification, as with the half-methyl ester of succinyl

**Table III**

Nonenzymatic acyl-CoA synthesis with various acyl phosphates

In each experiment, 1.0 μmole each of acyl phosphate and coenzyme A and 50 μmoles of Tris-HCl (pH 7.8) were incubated in 1.0 ml at 37° for 30 min. Acyl-CoA synthesis was assayed indirectly by measuring unreacted coenzyme A (see "Materials and Methods").

<table>
<thead>
<tr>
<th>Acyl phosphate</th>
<th>Initial velocity of CoA disappearance (μmole/min × 10&lt;sup&gt;3&lt;/sup&gt;)</th>
<th>Acyl phosphate</th>
<th>Initial velocity of CoA disappearance (μmole/min × 10&lt;sup&gt;3&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Succinyl</td>
<td>6.0</td>
<td>Fumaryl</td>
<td>0</td>
</tr>
<tr>
<td>Itaconyl</td>
<td>6.0</td>
<td>Malyl</td>
<td>0</td>
</tr>
<tr>
<td>2-Methylsuccinyl</td>
<td>7.9</td>
<td>Citryl</td>
<td>0</td>
</tr>
<tr>
<td>Phthaloyl</td>
<td>5.5</td>
<td>Succinyl, half-methyl</td>
<td>0</td>
</tr>
<tr>
<td>2,2-Dimethylsuccinyl</td>
<td>18.5</td>
<td>Ester</td>
<td>0</td>
</tr>
<tr>
<td>2,3-Dimethylsuccinyl</td>
<td>18.5</td>
<td>Glutaryl</td>
<td>0</td>
</tr>
<tr>
<td>cis-1,2-cyclobutane-dicarboxyl</td>
<td>12.0</td>
<td>Adipyl</td>
<td>0</td>
</tr>
<tr>
<td>Maleyl&lt;sup&gt;a&lt;/sup&gt;</td>
<td>44.0</td>
<td>Acetly</td>
<td>0</td>
</tr>
<tr>
<td>Malonyl</td>
<td>0.3</td>
<td>Propionyl</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup> The possibility is discounted that coenzyme A adds to the double bond of maleyl phosphate, since no such addition is evident with fumarate phosphate. The high reactivity of maleyl phosphate with coenzyme A parallels its high reactivity with water (Fig. 1).
sonable pathway for succinyl-CoA synthesis requires the prior reaction with coenzyme A. Subsequent combination of anhydride with coenzyme A involves cyclization of succinyl phosphate to succinic anhydride (Reaction 3). Indeed, any activity of the phosphates of monocarboxylic acids. The inactivity of succinic anhydride. Moreover, the kinetic superiority of succinyl-CoA synthesis from succinyl phosphate. If succinic anhydride is the actual intermediate in the synthesis, its reactivity with coenzyme A ought to equal, or exceed, that of succinyl phosphate. Curves 1 and 2 of Fig. 4 disclose that succinyl phosphate reacts with coenzyme A more rapidly than does succinic anhydride. Moreover, the kinetic superiority of succinyl phosphate over anhydride is maintained across the entire range of pH from 2 to 7. It seems reasonable to infer from this that, at pH 7 and below, succinic anhydride does not participate in the synthesis of succinyl-CoA from succinyl phosphate.

Conclusions drawn from a comparison of Curves 1 and 2 of Fig. 4 may, however, be faulted on grounds of the "mixing" problem inherent in the execution of these experiments. In the experiments of Curve 1, an aqueous solution of lithium succinyl phosphate is added to buffer solutions of coenzyme A which contain dimethoxyethane. In the experiments of Curve 2, a dimethoxyethane solution of anhydride is added to buffer solutions of coenzyme A which are totally aqueous. It is not certain that the "mixing" problem does in fact account for the large differences in initial velocity portrayed in Curves 1 and 2. But, the difficulty is circumvented in the comparison of Curves 2 and 3 of Fig. 4, where all of the reactions were initiated in the same way. Compared are the initial velocities of reaction of succinic anhydride with coenzyme A in a series of different buffers (Curve 2) and in phosphate buffers throughout (Curve 3). The experiments of Curve 3 present a remarkable contrast to those of Curve 2, as well as to those of Curve 1. It is plain that at pH 6 to 7 succinic anhydride is vastly more reactive with coenzyme A in phosphate buffer than in Tris buffer. The difference resides in the capacity of phosphate to combine with anhydride. The rising portion of Curve 3, between pH 5 and 7, matches the rising proportion of dianionic orthophosphate, which combines with succinic anhydride in reversal of Reaction 3. This accords with established procedures for the chemical synthesis of succinyl phosphate (2, 22, 23). Thus, the enhanced reactivity with coenzyme A, expressed in Curve 3, coincides precisely with the synthesis of succinyl phosphate from succinic anhydride and dianionic orthophosphate. It follows that coenzyme A reacts more rapidly with succinyl phosphate than with succinic anhydride. Above pH 7, the reactivity with coenzyme A (Curve 3) declines as the newly synthesized succinyl phosphate ionizes increasingly to the trianion which is slow to hydrolyze (Fig. 1) and is inactive with coenzyme A as well (Fig. 7 of Reference 2).

Remarkable is the finding that, between pH 6.5 and 8, succinyl phosphate made in situ (Curve 3) is more reactive with coenzyme A than exogenous succinyl phosphate (Curve 1), which is permitted added as the trianionic (open chain) lithium salt. When orthophosphate combines with anhydride (Curve 3), the first event is the addition of phosphate to a carbonyl group of the anhydride. The product is cyclic succinyl phosphate (Scheme 3).

The same species is of course derived from the open chain, tri-
Succinyl Phosphate

Vol. 245, No. 21

Effect of various buffers on velocity of reaction between succinic anhydride and coenzyme A at pH 6.5

Coenzyme A (1.0 mmole) was dissolved in 0.90 ml of 0.05 M buffer. Reaction was initiated by the addition of succinic anhydride (3.0 mmoles) dissolved in 0.10 ml of freshly distilled dimethoxyethane. The temperature was 37°. The disappearance of coenzyme A was measured with the Aldrithiol reagent (see "Materials and Methods").

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Initial velocity of COA disappearance (umoles/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate</td>
<td>0.24</td>
</tr>
<tr>
<td>Tri-HCl</td>
<td>0.28</td>
</tr>
<tr>
<td>Fumarate</td>
<td>0.48</td>
</tr>
<tr>
<td>Malonate</td>
<td>2.28</td>
</tr>
<tr>
<td>Glutarate</td>
<td>2.44</td>
</tr>
<tr>
<td>Succinate</td>
<td>2.52</td>
</tr>
<tr>
<td>Malate</td>
<td>2.64</td>
</tr>
<tr>
<td>Phosphate</td>
<td>2.80</td>
</tr>
<tr>
<td>Citrate</td>
<td>2.84</td>
</tr>
</tbody>
</table>

In some experiments of Table IV, the possibility is present that succinyl-CoA may be accompanied by a second acyl-CoA. Since an anhydride and a carboxylate ion in solution are likely to react and give a mixed anhydride (24, 25), a mixture of thioesters may, in principle, form in presence of a thiol. In glutarate buffer, for instance, glutaryl-CoA is also a conceivable product. Our test of this possibility reveals that glutaryl-CoA is not a coproduct. In one experiment, 14C-succinyl anhydride reacted with coenzyme A in unlabeled glutarate buffer. In the converse experiment, unlabeled anhydride and 14C-glutarate were the reactants. After 3-min incubations and thin layer chromatography, careful scanning for radioactivity made it clear that no glutaryl-CoA appears.

* Our notions respecting the mechanism of this reaction include the addition of coenzyme A to the lactone carboxyl group to form a tetrahedral intermediate, which is not formulated in the scheme. As indicated by the arrows, the intermediate collapses with ring opening and elimination of orthophosphoric acid.
Table IV.

buffer to yield glutaryl-CoA in the experimental conditions of the experiments reported here, we know now that succinyl-CoA possesses no precedent (3, 4, 11). But, in the presence of thiol, exhibit a like tendency toward thiolester synthesis. Such acyl phosphates, moreover, display a distinctive hydrolytic behavior, as exemplified in the pH rate profiles of hydrolysis (Fig. 1). The sharp, nearly vertical rise in velocity of hydrolysis at about neutral pH is typical only for acid catalysis, and is thought to interact with the ring oxygen and promote rupture of the lactone linkage during reaction with coenzyme A. Alone among the dicarboxylate ions, fumarate is a poor catalyst. Molecular models reveal that steric constraints prevent easy access of the second carboxyl of fumarate to the ring oxygen of Structure I (Scheme 5). Citrate, possessing three carboxyl groups, is fittingly the most active oxyanion of Table IV. Acetate ion, wanting a second carboxyl group altogether, is no more active than Tris.

DISCUSSION

The easy reaction of succinyl phosphate with coenzyme A to give succinyl-CoA is a singular phenomenon. Earlier experience with acyl phosphates affords no precedent (3, 4, 11). But, in light of the experiments reported here, we know now that succinyl phosphate is not unique in its high reactivity. Any acyl phosphate possessing a free carboxyl group in a “succinyl” relationship will, in the presence of thiol, exhibit a like tendency toward thiolester synthesis. Such acyl phosphates, moreover, display a distinctive hydrolytic behavior, as exemplified in the pH rate profiles of hydrolysis (Fig. 1). The sharp, nearly vertical rise in velocity of hydrolysis at about neutral pH is typical only of acyl phosphates with the designated molecular specifications. These two manifestations—rapid hydrolysis and rapid reaction with thiols at neutral pH and below—are traceable, we feel, to the same root cause.

Clearly, the free carboxylate group of succinyl phosphate must interact in some way with the phosphorylated carboxyl. The rapid reaction of maleyl phosphate with coenzyme A and the failure of fumaryl phosphate to react in the same circumstances (Table III) are best explained on the same basis. In all probability the free carboxylate group shares in the reactions of succinyl phosphate by making a covalent bond with the carbonyl carbon of the mixed anhydride function. The product is cyclic succinyl phosphate—a y-disubstituted butyrolactone (I of Scheme 6). Thus the carboxylate group is thought to act here as a nucleophilic catalyst. This is a reasonable expectation, since pKₐ (~8) and pKₐ (~12) of the potential leaving group (orthophosphate) are both below 13.5, which marks the borderline between nucleophilic and general base catalysis by a free carboxyl group (26-28).

The contention that coenzyme A reacts with the cyclic form of succinyl phosphate finds fresh force in the data of Table IV. Dicarboxylic acids, like orthophosphate, can catalyze the synthesis of succinyl-CoA from succinic anhydride. It is difficult to devise a mechanism for this catalysis more plausible than the one of Scheme 5. This assumes that succinyl anhydride and the dicarboxylic acid of the buffer will want to join to form the corresponding mixed acyl anhydride. The y-disubstituted-y-butyro lactone (II of Scheme 6) must be the first stage of the synthesis. The acyclic anhydride, which would form on opening of the lactone, ought to react with coenzyme A to yield a mixture of succinyl- and glutaryl-CoA, since there is no obvious reason for coenzyme A to react solely with the succinyl portion of the acyclic succinyl-glutaryl mixed anhydride. Yet careful experiment reveals that only succinyl-CoA appears. We are driven to conclude that the acyclic mixed anhydride has no existence, and that the thiol in its reaction with Structure II acts only on the carbonyl group of the lactone ring. It is pertinent to recall that nucleophilic phenylhydrazine, in its reaction with y-acetoxy-y-valerolactone, also acts only on the lactone carbonyl (29).

It may occasion some wonder that lactones with Structures I and II (Scheme 6) should exhibit reactivity with nucleophiles commensurate with that of anhydrides. But, we note in this connection that y-lactones, bearing in the y-position a single electronegative substituent, possess carbonyl groups with C=O stretching frequencies as high as 1815 cm⁻¹ (30). This matches the frequencies found in anhydrides. Cyclic succinyl phosphate and the putative intermediate in dicarboxylic acid catalysis are y-lactones substituted on the y-carbon with two electronegative groups. The ring carbonyls of these molecules ought to possess even higher stretching frequencies. The reactivity of these carbonyls may reasonably be of the same order as that of anhydrides, and the more so if the lactone ring opening is abetted by intramolecular hydrogen bonding to the ring oxygen. The experi-

...
ments of Table IV support the notion that such hydrogen bonding is indeed real.

We have no information on which species of succinyl phosphate participates in the enzyme action of succinic thiokinase. Expectation is that the enzymatic synthesis of succinyl-CoA also proceeds via the cyclic species. But, whether enzymatic ATP synthesis proceeds from the cyclic or acyclic succinyl phosphate is at this time a moot question.

Acknowledgment—To Professor William P. Jencks we are grateful for keen criticism of some of our experiments, which forced revision in some of our earlier conceptions.

REFERENCES
Succinyl Phosphate: ITS NONENZYMATIC HYDROLYSIS AND REACTION WITH COENZYME A
Christopher T. Walsh, Jr., John G. Hildebrand and Leonard B. Spector


Access the most updated version of this article at http://www.jbc.org/content/245/21/5699

Alerts:
  • When this article is cited
  • When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/245/21/5699.full.html#ref-list-1