3-Cyanoglutarimide (VII).—Cyanoacetamide (189 g., 2.25 moles) was added to a solution of 46 g. of sodium in 1200 ml. of absolute alcohol at 35° and the suspension was stirred until it was well dispersed. β-Propiolactone was added slowly (30 min.) with good stirring and external cooling while keeping the temperature below 50°. After two additional hours the solution was acidified with 113 g. of concentrated sulfuric acid and filtered to remove the crystalline solid. The solid was washed with water and recrystallized from two liters of hot water to give 34 g. (12.3%) of 3-cyanoglutarimide, m.p. 207–208°.[4]

Anal. Calcd. for $C_6H_6O_2N_2$: N, 20.3. Found: N, 20.4.

The filtrate was concentrated at 15 mm. and the solid which deposited on cooling was filtered, recrystallized from alcohol–water to give 13 g. (4.1%) of 4-cyanoglutaramic acid, m.p. 169–170°. The melting point of a mixture with the 4-cyanoglutaramic acid obtained previously was not depressed.

Attempts to cyclize 4-cyanoglutaramic acid to 3-cyanoglutarimide (VII) by acidifying an alcoholic solution of its sodium salt, by treatment with acetic anhydride containing sulfuric acid or by heating with acids in alcohol gave none of the desired product.

2-Cyanoglutaramic Acid (VIII).—Some of the 3-cyanoglutarimide (6.9 g., 0.05 mole) was dissolved in 50 ml. of water containing 2 g. (0.05 mole) of sodium hydroxide. The solution was heated at 50° for one hour, cooled to 25° and acidified. After standing overnight a crystalline product had separated. This was removed by filtration, the filtrate was evaporated one-half and cooled, a second crop of crystals was obtained. The yield was 4.6 g., 59% of 2-cyanoglutaramic acid, m.p. 142–143° (with gas evolution) after recrystallization from alcohol–water.

Anal. Calcd. for $C_6H_8O_3N_2$: C, 46.15; H, 5.13; N, 17.95; neut. equiv., 156. Found: C, 46.17; H, 5.16; N, 17.97; neut. equiv., 155.

On continued heating of the melted compound it resolidified and then finally remelted at 208–210°, the melting point for 3-cyanoglutarimide, which it was proved to be by the mixture-melting point method.

Diethyl 2-Carbethoxyglutarate (IX) and Diethyl 4,4-Bis-carbethoxypimelate (X).—Diethyl malonate (680 g., 4.25 moles) was added rapidly to a solution of sodium ethylate in alcohol (prepared from 92 g. of sodium and 3 liters of absolute alcohol) with vigorous stirring and the temperature maintained below 40° with external cooling. β-Propiolactone (288 g., 4 moles) was added to this yellow solution over a period of 30 minutes with stirring and controlling of the temperature at 30–35° by use of an ice-bath. After two additional hours during which time some heat was evolved the solution was acidified with 400 ml. of concentrated hydrochloric acid, filtered from salt and the salt washed with alcohol. The combined filtrate and washes were distilled to remove alcohol, some ethyl acrylate and other by-products, until the temperature rose to 100°. The residue was filtered from salt and distilled at 0.5 mm. to give 244 g. (36%) of diethyl malonate, b.p. 48–75°, 588 g. of product, b.p. 75–190° at 0.5 to 2 mm., and 25 g. of tarry residue.

The product fraction was dissolved in an equal volume of ether. The ether solution was washed twice with 800-ml. portions of saturated sodium bicarbonate solutions, twice with 400-ml. portions of water, dried over calcium chloride and distilled at reduced pressure to remove the ether. Distillation of the residue gave two main fractions—(a) diethyl 2-carbethoxyglutarate,[5] b.p. 99–108° (0.5 mm.); 233 g. (21.4%); $n^{25}D$ 1.4319; d^{25}_4 1.0778.

Anal. Calcd. for $C_{12}H_{20}O_6$: C, 55.5; H, 7.7; sapn. equiv., 87; MR_D 62.58. Found: C, 55.3; H, 7.8; sapn. equiv., 85; MR_D 62.59.

(b) Diethyl 4,4-bis-carbethoxypimelate,[9] b.p. 138–149° (0.3 mm.); $n^{25}D$ 1.444; d^{25}_4 1.0983

Anal. Calcd. for $C_{17}H_{28}O_8$: MR_D 87.32. Found: MR_D, 87.21.

A sample of diethyl 4,4-bis-carbethoxypimelate was saponified with alcoholic potassium hydroxide to give, after acidification and decarboxylation, 4-carboxypimelic acid,[9] m.p. 115–116°.

Acknowledgment.—The authors are indebted to J. R. Kubik for microanalyses and to W. P. Tyler and A. K. Kuder for the other analyses.

(9) Bottomley and Perkins, *J. Chem. Soc.*, **77**, 299 (1900).

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[CONTRIBUTION NO. 92 FROM THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF TENNESSEE]

The Action of Formaldehyde on *m*-Hydroxybenzoic Acid. III. Stepwise Degradation of the Lactone of 6-Hydroxymethyl-1,3-benzodioxan-5-carboxylic Acid

BY C. A. BUEHLER, ARCHIE V. SLACK, D. A. SHIRLEY, PHILIP A. SANGUINETTI AND SHELDON H. FREY

The lactone of 6-hydroxymethyl-1,3-benzodioxan-5-carboxylic acid has been degraded stepwise by two routes to 4-hydroxybenzene-1,2,3-tricarboxylic acid or a simple derivative of this acid. The results confirm the structure originally assigned to the lactone.

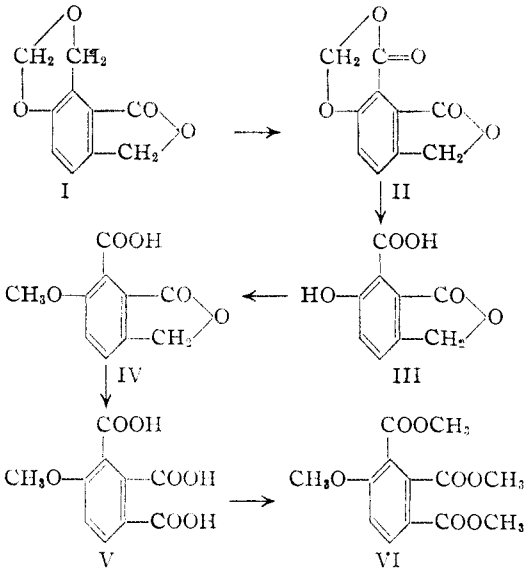
In a previous article[1] the structure of 6-hydroxymethyl-1,3-benzodioxan-5-carboxylic acid lactone was established by its synthesis and the conversion of its permanganate oxidation product into 4-hydroxyphthalide-7-carboxylic acid. Original attempts at structural proof through oxidation in one operation to the hydroxytricarboxylic acid were unsuccessful. However, degradation stepwise by two routes which involved the cleavage of the two non-benzenoid rings in opposite order and which led to 4-hydroxybenene-1,2,3-tricarboxylic acid or suitable derivatives of this acid were successful. These results, which support the structure originally assigned, are reported in the present paper.

The dioxanyl phthalide I was oxidized with chromium trioxide in acetic acid[2] to give the dioxanone II, which on hydrolysis produced the hydroxycarboxylic acid III. Methylation of the latter gave the methoxy acid IV which on oxidation with permanganate yielded the known 4-methoxybenzene-1,2,3-tricarboxylic acid (V). Methylation of the latter produced the known trimethyl-4-methoxybenzene-1,2,3-tricarboxylate (VI).

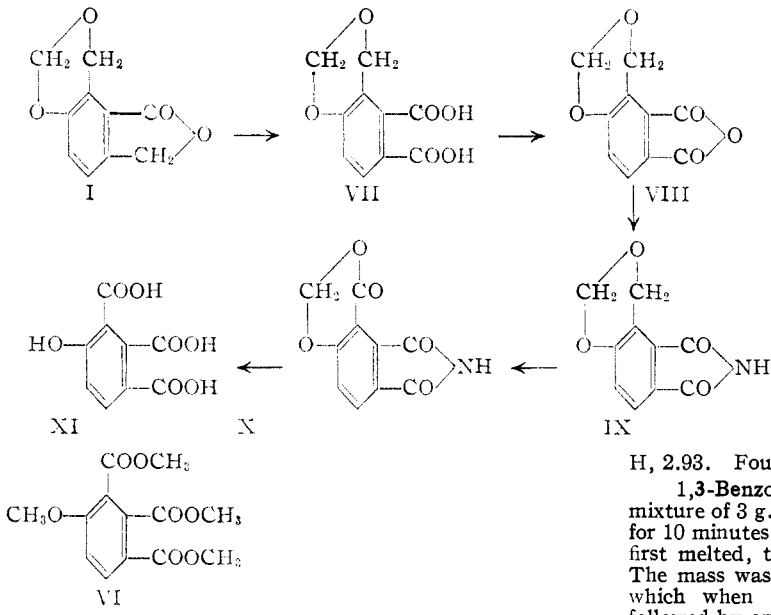
The oxidation of I to the dicarboxylic acid VII was accomplished with alkaline permanganate followed by careful acidification.[1] To protect the two ortho carboxyl groups in VII, the compound was converted first into the anhydride VIII and then into the imide IX. Oxidation of the latter with chromium trioxide in acetic acid gave the dioxanone X which was converted first into the known 4-hydroxybenzene-1,2,3-tricarboxylic acid

(1) C. A. Buehler, J. O. Harris, C. Shacklett and B. P. Block, THIS JOURNAL, **68**, 574 (1946).

(2) Borsche and Berkhout, *Ann.*, **330**, 92 (1904).



(XI) and finally into the known trimethyl 4-methoxybenzene-1,2,3-tricarboxylate (VI).



Experimental[3]

Lactone of 6-Hydroxymethyl-1,3-benzodioxan-5-carboxylic Acid (I).—This lactone was prepared by a method described previously.[1] Since there is some evidence that both the 5- and 7-carboxylic acid lactones are produced in the reaction, great care was taken in purification. The samples used in the degradation processes melted at 175.0–176.0° or better.

Lactone of 6-Hydroxymethyl-1,3-benzodioxan-4-one-5-carboxylic Acid (II).—The benzodioxane-lactone, 10 g., was dissolved by heating in 250 ml. of glacial acetic acid. To the solution cooled to 60–70° was added 35 g. of chromium trioxide[2] at such a rate so as to prevent the acid from boiling out of the flask. The cooled solution was diluted with water to 2 liters and on standing overnight in the refrigerator needles formed. These were washed free from the green color and crystallized from water to give 3 g. of white needles, m.p. 241–243°.

Anal. Calcd. for $C_{10}H_6O_5$: C, 58.26; H, 2.93. Found: C, 58.29, 58.49; H, 2.82, 2.80.

(3) Melting points in the first sequence were determined in an oil-bath; in the second sequence by use of an aluminum block.

6-Hydroxy-7-carboxyphthalide (III).—The benzodioxanone-lactone, 0.39 g., was saponified in 10% sodium hydroxide solution to give 0.27 g. of a white solid. Crystallization from water produced white crystals, m.p. 212–213°.

Anal. Calcd. for $C_9H_6O_5$: C, 55.68; H, 3.12. Found C, 55.72, 55.83; H, 2.96, 2.89.

6-Methoxy-7-carboxyphthalide (IV).—The hydroxycarboxyphthalide, 7.5 g., in 65 cc. of 10% aqueous sodium hydroxide and 12 cc. of dimethyl sulfate were stirred for 1 hour at room temperature. Base, 11 cc. more, and 12 cc. more of dimethyl sulfate were then added and stirring was continued for another hour. After the addition of 40 cc. more of base the mixture was heated on a steam cone for 2 hours and acidified to yield 7 g. of crude product. One crystallization from water gave 6.5 g. of white needles, m.p. 261–263°.

Anal. Calcd. for $C_{10}H_8O_5$: C, 57.70; H, 3.87. Found: C, 57.34, 57.64; H, 3.94, 3.88.

4-Methoxybenzene-1,2,3-tricarboxylic Acid (V).—The methoxycarboxyphthalide, 3 g., in 200 cc. of 3.5% potassium hydroxide solution was heated and agitated while 105 cc. of 0.27 *M* potassium permanganate solution was added over a period of 1.5 hours. After removing the excess permanganate with sodium bisulfite and filtering off the solid manganese dioxide, the filtrate was acidified with sulfuric acid and extracted twice with 50-cc. portions of ether to remove impurities. The water layer was saturated with sodium sulfate and extracted seven times with ethyl acetate. Evaporation of the combined acetate extracts gave 2 g. of crude product which after crystallization first from acetone-benzene (2:1) and then from acetic acid yielded white crystals, m.p. 214.5–215.5° (Buehler, Spees and Sanguinetti[4] give 215.0–216.0°). No depression in melting point was produced when mixed with an authentic sample.

Trimethyl-4-methoxybenzene-1,2,3-tricarboxylate (VI).—The methoxy tricarboxylic acid, 1 g., was methylated in the usual manner with diazomethane to give 1 g. of white needles, m.p. 91.8–92.0° (Buehler, Spees and Sanguinetti[4] give 91.0–92.0°).

1,3-Benzodioxan-5,6-phthalic Acid (VII).—This acid was prepared as described previously.[1]

1,3-Benzodioxan-5,6-phthalic Anhydride (VIII).—The acid, 2.4 g., heated with acetic anhydride gave 2.0 g. of a yellow product, m.p. 126.0–127.0°.

Anal. Calcd. for $C_{10}H_6O_5$: C, 58.26; H, 2.93. Found: C, 58.52, 58.30; H, 3.29, 3.26.

1,3-Benzodioxan-5,6-phthalimide (IX).—An intimate mixture of 3 g. of the anhydride and 3 g. of urea was heated for 10 minutes at about 150° during which time the mixture first melted, then evolved ammonia and finally solidified. The mass was dissolved in 2000–2400 cc. of boiling water which when cooled produced fluffy needles. Washing followed by crystallization from water gave 1.1 to 2.0 g. of white crystals, m.p. 231.5–232.0°.

Anal. Calcd. for $C_{10}H_7NO_4$: C, 58.54; H, 3.44; N, 6.83; neut. equiv., 205. Found: C, 58.17, 58.38; H, 3.34, 3.37; N, 7.02; neut. equiv. (indirect), 198, 197.

1,3-Benzodioxan-4-one-5,6-phthalimide (X).—The dioxan-imide, 2.2 g., was oxidized with chromium trioxide and glacial acetic acid to give 1.6 g., m.p. 326.5–328.0° (dec.). Ethyl benzoate appeared to be the preferred solvent for crystallization.

Anal. Calcd. for $C_{10}H_5NO_5$: C, 54.80; H, 2.30; N, 6.39. Found: C, 54.76, 54.76; H, 2.48, 2.46; N, 6.31, 6.37.

4-Hydroxybenzene-1,2,3-tricarboxylic Acid (XI).—The dioxanone-imide, 0.5 g., was saponified in 25% aqueous sodium hydroxide solution and the acidified solution was evaporated to dryness. Extraction with absolute alcohol followed by evaporation to dryness gave a second residue which was taken up in water, from which on evaporation and cooling 0.25 g. of a white solid, m.p. 203–205° with efferves-

(4) C. A. Buehler, R. B. Spees and P. A. Sanguinetti, THIS JOURNAL, 71, 13 (1949).

cence[5] (Buehler, Spees and Sanguinetti[4] give 205–206° with effervescence).

Anal. Calcd. for $C_9H_6O_7$: C, 47.80; H, 2.68. Found: C, 47.87, 47.87; H, 2.74, 2.83.

(5) The melting point of this compound was found to vary with the initial temperature of the block. The above value was obtained when the tube containing the sample was placed in the block at a temperature of 200°.

Trimethyl-4-methoxybenzene-1,2,3-tricarboxylate (VI). —The acid, 0.4 g., was methylated with diazomethane in the usual manner to give 0.3 g. of crude ester which when purified melted at 91.5–92.5°. A mixed melting point with an authentic sample showed no depression.

KNOXVILLE, TENN. RECEIVED NOVEMBER 20, 1950

NOTES

A Simple and Rapid Biosynthesis of Isotopically Labeled Succinic Acid

BY SAMUEL J. AJL AND MARTIN D. KAMEN

Cell suspensions of *Escherichia coli* (Strain E26) can be adapted to oxidize acetate.[1] If such suspensions oxidize acetate and succinate simultaneously most of the acetate carbon metabolized is trapped in succinate.[2] Based on this observation, a simple and rapid method for the synthesis of isotopically labeled succinate from labeled acetate has been developed and will be described in this report.

Procedure

Preparation of Bacteria.—*Escherichia coli* (Strain E26) is adapted to oxidize acetate readily by preliminary inoculation into 10 ml. of a medium consisting of 1.5% anhydrous sodium acetate, 0.4% ammonium sulfate, 0.8% KH_2PO_4, 0.07% peptone and 2% tap water (for inorganic ions), at an initial *p*H of 7.0. After 18 hours of incubation at 30°, the suspension is added to 80 ml. of the same medium which is incubated at 30° for another 18-hour period. The resultant suspension is added to 800 ml. of the original medium and again incubated in the same fashion. A final transfer is made to 8 liters of medium in a 12-liter florence flask. The bulk medium is aerated at 30° for 48 hours with compressed air using a carborundum aerating ball. The cells are then harvested, resuspended in a minimal volume of phosphate buffer and aerated several hours to deplete endogenous reserves.

Biosynthesis from Labeled Acetate.—Two ml. of a 10% bacterial suspension is incubated with 10–20 μM 2-C^{14}-acetate and 125 μM. unlabeled succinate buffered with 1 ml. 0.2 *M* phosphate solution (*p*H, 7.0) in a 30-ml. Warburg–Barcroft vessel at 30° with air as the gas phase and with constant shaking. The center well contains 0.3 ml. of 10% KOH absorbed on a filter paper in the usual way to catch evolved CO_2. The reaction is allowed to continue until the uptake of oxygen indicates that approximately half of the succinate has disappeared. The flask then is quickly removed from the manometer and the contents acidified rapidly with 1–2 ml. of 4 *N* H_2SO_4. The flask is quickly corked to prevent atmospheric contamination by the $C^{14}O_2$ released suddenly in this operation. After 5–10 minutes, the flask is opened and the suspension is separated into supernatant and cells by centrifugation. The supernatant is steam distilled to remove residual labeled acetate. A second distillation is made after addition of approximately 10 μM. unlabeled acetate. This procedure is repeated until no appreciable radioactivity is found in the distillate.

Separation of Succinic Acid.—The residual solution from the above procedure is made ~ 2 *M* in manganous sulfate and oxidized by heating with 0.03 *M* permanganate. This treatment eliminates all oxidizable contaminants such as fumarate, malate and pyruvate. Practically all labeled contaminants formed are convertible to volatile formate, acetate or CO_2. To remove all such volatile contaminants, the oxidized mixture is again steam distilled. α-Ketoglutaric acid, if it were present, would yield succinate and carbon dioxide. However, it has been shown that labeled α-ketoglutarate is not formed under the experimental conditions.[3]

The residue from this distillation is adjusted to *p*H 2–3 and extracted with ether for 24 hours. The ether extract is separated and contains the pure labeled succinate. This can be extracted as the sodium salt with alkaline water or precipitated as the silver salt.

Determination of Purity of Labeled Succinate.—An aliquot of the final ether extract has been subjected to analysis by strip-paper chromatography[3] and only one distinct band identical to the one obtained with known pure succinate is found. In the presence of inactive carriers, *e.g.*, fumarate, malate, α-ketoglutarate and tartrate, corresponding bands appear but contain no radioactivity.

Degradation of Labeled Succinate.—Total concentration of succinate is determined using a succinoxidase preparation obtained from fresh beef-heart. Distribution of C^{14} in the C_4 acid may be determined by the usual degradation procedures involving permanganate oxidation of the fumarate–malate mixture (resulting from the succinoxidase reaction) to acetaldehyde, formate and carbon dioxide.[4]

Experimental Results and Discussion.—The isotopic distribution found in the succinate varies from preparation to preparation. This is a consequence of the cycling mechanism of the acetate oxidation whereby methyl carbon of acetate rapidly equilibrates with methylene carbons of so-called "Krebs cycle acids" and more slowly with carboxyl carbons. Practically, the oxidation of small quantities of labeled acetate in the presence of quantities of unlabeled succinate tenfold greater leads to succinate containing label mainly in the methylene carbons. Short oxidation periods using minimal amounts of labeled acetate should be best for obtaining methylene-labeled succinate. For uniform labeling it is best to begin by using uniformly-labeled acetate.

A typical synthesis yielded the following results. Beginning with ~10 μM. acetate containing ~2 $\times$ 10^6 c./min. in the methyl carbon and 125 μM. unlabeled succinate, 60 μM. succinate were isolated containing a total of 730,000 c./min., a yield relative to originally labeled acetate of some 37%. Approximately uniform distribution of labeled carbon was observed.

The usual time required for the biosynthesis is

(1) S. J. Ajl, *J. Bact.*, **59**, 499 (1950).

(2) S. J. Ajl and M. D. Kamen, *J. Biol. Chem.*, in press.

(3) J. W. H. Lugg and B. T. Overell, *Austral. J. Sci. Res.*, **1**, 98 (1948).

(4) H. G. Wood, C. H. Werkman, A. Hemingway and A. O. Nier, *J. Biol. Chem.*, **139**, 377 (1941).