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.

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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 $\sim$ 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.[2]

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 $\sim$10 μM. acetate containing $\sim$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).

3–5 hours. The purification procedure requires ~30 hours.

The simplicity and rapidity of this method appear to surpass any purely chemical method so far reported for the preparation of labeled succinate. It compares favorably with the biosynthesis of carboxyl-labeled succinate using cell suspensions of *Tetrahymena gelii*[5] while possessing the advantage that predominantly methylene-labeled succinate as well as uniformly-labeled succinate can be prepared.

(5) C. B. Van Niel, J. O. Thomas, S. Ruben and M. D. Kamen, *Proc. Natl. Acad. Sci. (U. S.)*, **28**, 157 (1942).

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The Electrophoretic Migration of Cellulose in Cupriethylenediamine Solution

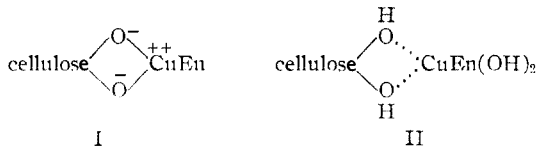
BY MABELLE E. ADAMS, MELVIN L. KARON AND RICHARD E. REEVES

By substituting copper plates for the silver–silver chloride electrodes and filling the entire system with dilute cupriethylenediamine solution the migration of cellulose can be observed conveniently in a Tiselius electrophoresis apparatus[1] equipped with a Longsworth scanning device. In agreement with previous observations[2] cellulosic material was found to migrate slowly toward the anode under the influence of an electric current.

In eleven experiments employing cupriethylenediamine solutions of various celluloses the ascending boundaries invariably remained sharp and unresolved, while the descending boundaries usually separated into two distinguishable peaks or bands. The patterns of the descending boundaries appeared to be characteristic of the dissolved cellulose.

In view of the known interaction between cellulose and cupriethylenediamine it is not surprising that the ascending and descending boundaries are not mirror images. Such disymmetry has often been attributed to interaction among components. Smith and Briggs[3] cite many such examples, and they have obtained patterns showing a single ascending peak and multiple descending peaks from interaction between serum albumin and methyl orange.

Cupriethylenediamine exists in solution largely in the dissociated form, $CuEn_2^{++}$. In order to account for the electronegative character of the cellulose-cupriethylenediamine complex the copper-containing component must lose its strong positive charge upon combination with cellulose. This could occur through ionic linkage with cellulose as in I; or the complexing valences could be non-ionic, but of such a nature that the copper is no longer strongly basic. The complex would then exist largely in the undissociated form, II.



I II

The *p*H of the solvent is approximately 12.7, hence a small amount of dissociation of cellulosic hydroxyl would account for the observed anodic migration.

Figure 1 shows initial and final boundaries for a low molecular weight methanolyzed cellulose derived from cotton linters. The ascending boundary moved as a single component, while the descending boundary split into two well-defined peaks having mobilities of 0.72 and 0.85 × 10^{-5} sq. cm./volt/sec. To obtain this result, a sample containing 250 mg. of methanolyzed cotton cellulose[4] was dissolved in 5.0 ml. of stock cupriethylenediamine solution (one molar in copper, 2.01 molar in ethylenediamine) and diluted to exactly 50 ml. with water. This solution was placed in the bottom and descending leg of the 92-mm. electrophoresis cell and the remainder of the cell and electrode chambers were filled with about 1500 ml. of cupriethylenediamine solution of the same concentration. The assembly was brought to temperature equilibrium at 0° and the boundaries were formed and moved into position for observation by the usual procedures. The boundaries were photographed by the schlieren technique employing a mercury vapor light source. A current of 30 ma. was then passed through the cell for 1300 minutes and the final position of the boundaries was recorded.

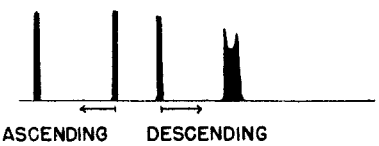


Fig. 1.—The initial and 1300-minute boundaries for methanolyzed mercerized cotton cellulose; 0.5% concentration in 0.1 *N* cupriethylenediamine solution: current, 30 ma.; field strength, 2.98 v./cm.

There was no evolution of gases at either electrode during the experiment. The solutions in the anode and cathode chambers were analyzed separately for copper and nitrogen at the end of the run. The results of these analyses are compared in Table I with the composition of the initial diluted cupriethylenediamine solution. It is apparent that some change in composition of the solutions in the electrode chambers occurred during the run. There was a migration of ethylenediamine toward the cathode and an observable plating out of copper on this electrode. Some of the migration of cellulose toward the anode might have arisen from displacement due to the transport of ethylenediamine and copper to the closed cathode compartment; however, it is difficult to explain the splitting of the descending boundary on this basis.

(1) L. G. Longsworth, *Chem. Revs.*, **30**, 323 (1942).

(2) W. A. Sisson, *Contrib. Boyce Thompson Institute*, **10**, 113 (1938).

(3) R. F. Smith and D. R. Briggs, *J. Phys. and Colloid Chem.*, **54**, 33 (1950).

(4) This material was prepared by autoclaving purified, mercerized cotton linters for one hour at 120° with methanol containing, initially, 3.6 per cent. anhydrous hydrogen chloride. Its intrinsic viscosity was 0.35 in A. C. S. cuprammonium.