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DEGRADATION OF ISOTOPIC LACTATE AND ACETATE

by

S. Aronoff, V. A. Haas, and B. A. Fries

24 February 1948

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DEGRADATION OF ISOTOPIC LACTATE AND ACETATE

by

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Radiation Laboratory and
Department of Chemistry,
University of California,
Berkeley, California*

24 February 1948

Abstract

A scheme of glucose degradation has been validated by the use of intermediates of known isotopic composition. In this scheme: glucose ⟶ lactic acid ⟶ CO_2 (C-3,4) + acetic acid ⟶ CO_2 (C-2,5) + acetone ⟶ iodoform (C-1,6) + acetate (C-1,6; 2,5), it was found that a) in the oxidation of lactic acid, approximately 4.7% of the acetic acid was oxidized to CO_2; and b) under the conditions prescribed, $BaCO_3$ from the degradation of Ba acetate contained approximately 1.5% of the activity of the methyl group.

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(2) While on leave from California Research Corporation, Richmond, California.

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DEGRADATION OF ISOTOPIC LACTATE AND ACETATE

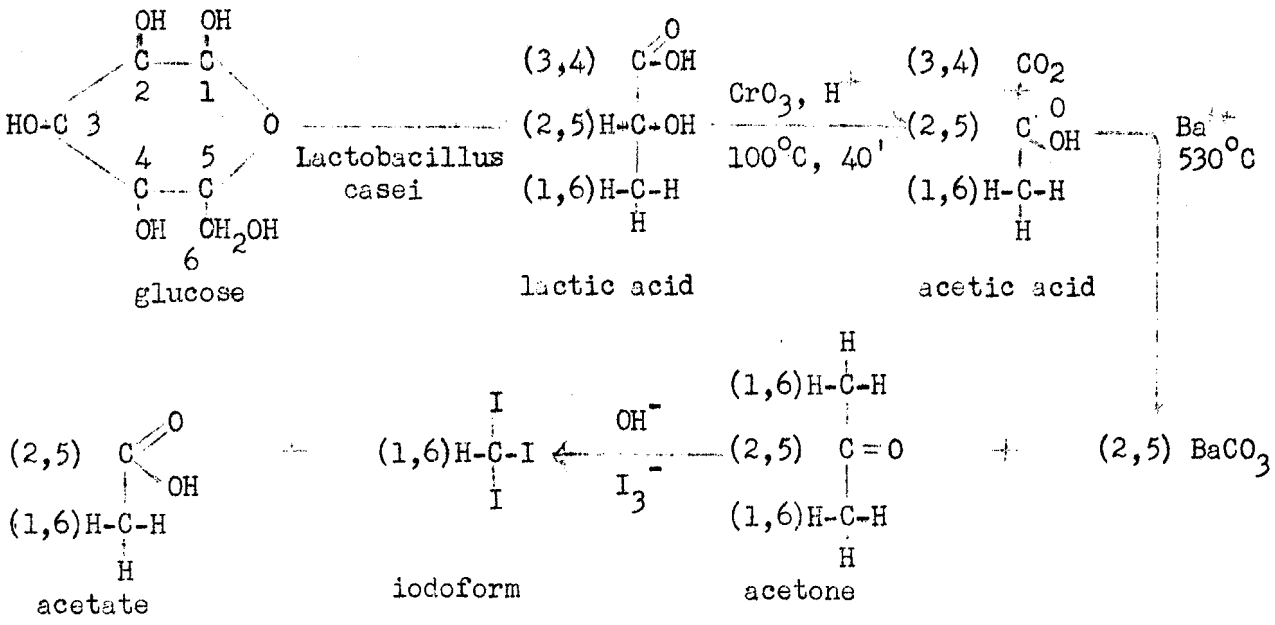
by

S. Aronoff, V. A. Haas, and B. Fries

Radiation Laboratory and
Department of Chemistry of
the University of California,
Berkeley, California.*

24 February 1948

The biological preparation of glucose containing isotopic carbon has made necessary the development of degradation procedures for the determination of the distribution of the isotope within the molecule. Two such procedures have been given by Wood, Lifson, and Lorber (1), one being entirely chemical, the other partially microbiological. The results of a modified version of the latter were recently published by Aronoff, Barker, and Calvin (2). Their scheme can be depicted as:



(1). H. G. Wood, N. Lifson, and V. Lorber, J. Biol. Chem., 159, 475 (1945)

(2). S. Aronoff, H. A. Barker, and M. Calvin, J. Biol. Chem., 169, 459 (1947)

It was of interest to determine the reliability of the above procedure by the degradation of synthetic radio-lactic and acetic acid of known isotopic distribution. Synthetic radio-glucose is not yet available.

Fermentation

Inasmuch as our fermentation procedure has not been published elsewhere, the following details are given. The homofermentative bacterium L. casei ϵ was used to convert glucose to lactic acid. Sucrose may be fermented also, but at a rate approximately one-tenth that of glucose. Sixteen-hour cultures, grown in Stokes and Gunness medium (3), were used. The activity of cultures older than 24 hours decreased appreciably with time, so that 96 hour cultures were almost inactive. With sixteen hour cultures the rate of fermentation was 1.0 g. glucose/cmm wet bacteria/min.

The bacterial culture was centrifuged and the organisms washed twice with distilled water. Five cc. of a 20% (vol.) aqueous suspension were then mixed in a manometric reaction vessel with an equal volume of a solution consisting of $\frac{M}{10}$ bicarbonate and $\frac{M}{200}$ phosphate (pH = 6.8) and fifteen to thrity mg. of glucose added. It is evident that the amount of glucose added as substrate should be sufficient to prevent excessive glucose incorporation into the internal bacterial metabolism and yet not be so excessive as to require an undue fermentation time. In our experiments less than 1% of the original activity is found within the cells. The fermentation was allowed to proceed at 37°C., its progress being noted by the evolution of CO_2 as lactic acid was formed. It was found convenient to use mercury as the manometer fluid because of the large amount of gas evolved. Upon completion of the reaction the suspension was removed quantitatively from the reaction vessel with two washes

(3). J. L. Stokes and M. Gunness, J. Biol. Chem., 157, 651 (1945); 160, 35 (1945); 163, 159 (1946).

and centrifuged. The supernatant was combined with two subsequent washings and after acidification with dilute sulfuric acid was steam distilled. The residual solution was made alkaline to thymol blue, and ether-extracted for 12 hours. Following acidification with dilute sulfuric acid, the aqueous solution was again extracted with ethyl ether. The latter ethereal solution, containing the lactic acid, was evaporated and water added to make a volume of approximately 5 cc. The solution was then quantitatively transferred to an oxidation vessel.

Lactic Acid Degradation.

2-C^{14}-lactic acid was prepared by the reduction of 2-C^{14}-pyruvic acid with Pt and H_2. The lactate was isolated and purified as the zinc salt. The pyruvic acid in turn had been prepared from carboxyl-labeled acetic acid (4). The oxidative degradation of lactic acid to acetic acid and CO_2 by means of CrO_3 in dilute acid solution has been used by agricultural chemists (5). Determinations are generally made of the acetic acid which is formed in 95-97% yield. Acids such as succinic are not affected by the oxidation mixture; others, as tartaric and malic, are completely oxidized. Oxidation of the 2-C^{14}-lactic acid by the modified procedures given below resulted in $BaCO_3$ with 4.3% of the original activity. Assuming that the activity appearing in the $BaCO_3$ arises from oxidation of some of the acetic acid, the results indicate 4.7% oxidation of the acetic acid to CO_2 and H_2O.

The oxidation was performed in the reaction vessel shown in Figure 1a. An inert carrier gas (e.g. N_2) was used to carry the CO_2 evolved in the reaction to the absorption vessel (Figure 1b.) The latter consisted of a

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(4). R. M. Lemmon and M. Calvin, J. Am. Chem. Soc., 169, 1232 (1947).

(5). E. Semichon and M. P. P. Flanzy, Ann. Fals. 25, 414 (1932); 24, 516 (1931).

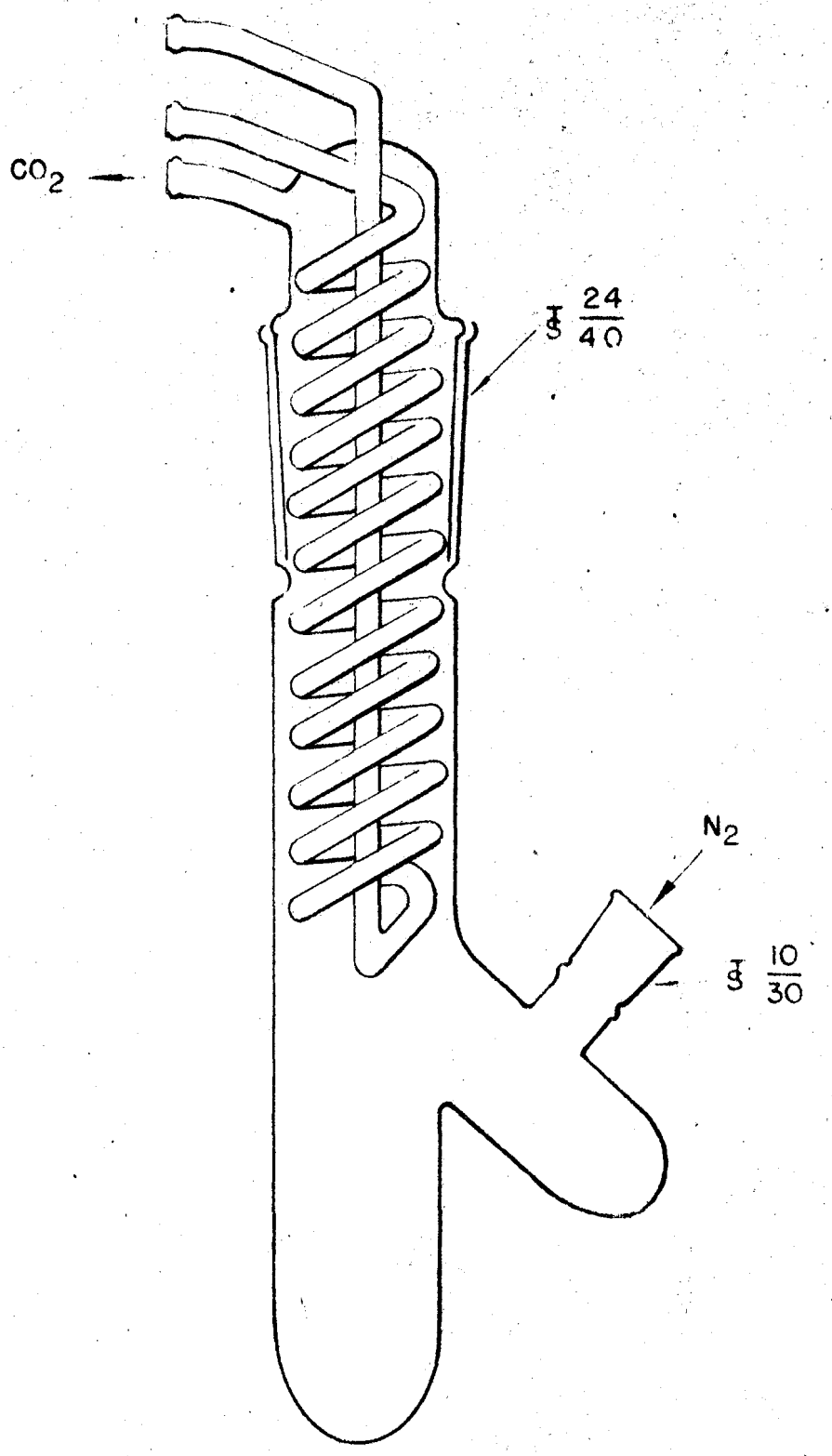

Figure 1a
Reaction vessel

sintered glass bubbler within a weighed tube containing $\frac{N}{2}$ CO_2-free NaOH. The reaction vessel contained 10-50 mg. of the lactic acid in 5-8 cc. of 1 N H_2SO_4. Two cc. of CrO_3 -H_2O (1:1, wt.) were pipetted into the side arm. The atmosphere of the reaction vessel was swept free of CO_2, connected to the absorption vessel and the CrO_3 solution tipped into the lactic acid solution. The reaction proceeded at 100°C for 40 minutes. When the reaction was complete, the residual solution on the surface of the sintered glass bubbler was carefully washed with distilled water into the alkali while the carrier gas was still flowing. A few drops of saturated $BaCl_2$ were added to the NaOH to precipitate $BaCO_3$. The precipitate was centrifuged (protected from air by a rubber stopper), washed once with water and twice with methanol. After oven drying (or, more rapidly, in a continuously evacuated Abderhalden drying pistol), the precipitate was weighed and plated for C^{14} determination. The acetic acid was quantitatively steam distilled from the residual oxidation mixture and the distillate titrated with $Ba(OH)_2$. Recoveries of acetic acid as the barium salt were 97% $\pm$ 1%, based on the initial lactic acid.

Acetate Degradation

Two methods of acetate degradation have been employed: 1) pyrolysis of the barium salt to $BaCO_3$ and acetone; and 2) electrolysis of the sodium salt to CO_2 and ethane. In the first method both methyl- and carboxyl-labeled acetate were used; in the second, only methyl-labeled acetate. In the pyrolysis method finally adopted, the dried barium salt was heated at 530°C for 10 minutes (Figure 2) in an atmosphere of nitrogen or argon. The inert gas was used to sweep the acetone fromed into a gas washing bottle. Satisfactory results could be obtained only at sufficiently high temperatures. At 450°C as much as 15% of the activity of the methyl group could be found in the residual $BaCO_3$. The residual $BaCO_3$, which was dark gray, was purified by the

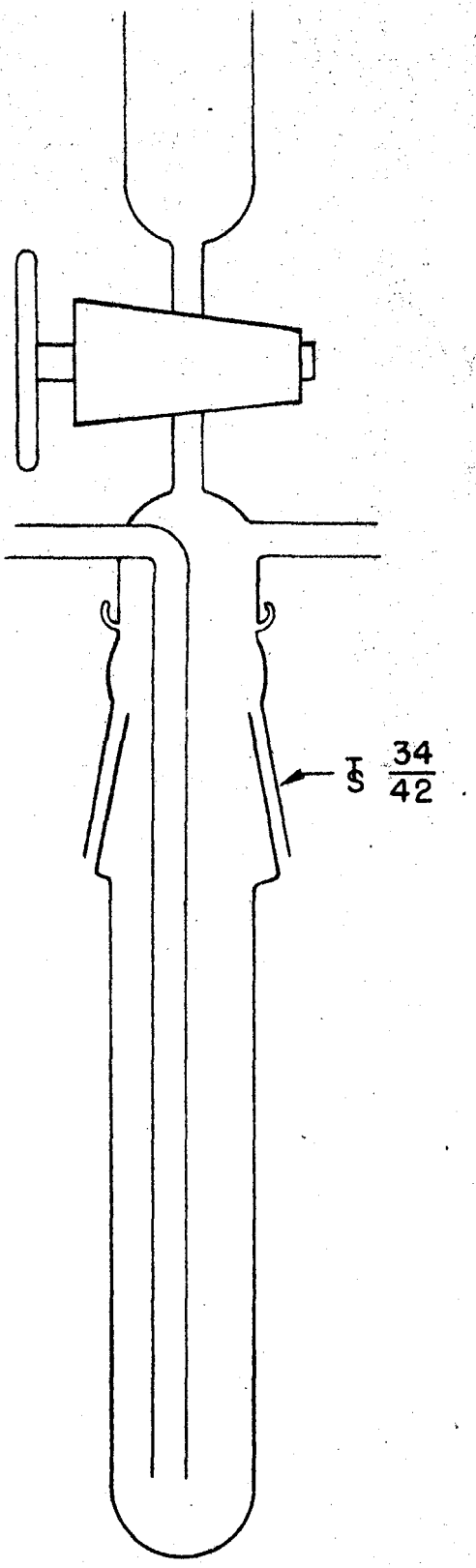

Figure 2
Reaction vessel for pyrolysis

liberation of CO_2 with lactic acid and subsequent reprecipitation. The yield of iodoform from acetone is 95% (6); under our experimental conditions, the yield of dry iodoform from acetone was approximately 90% and that from the barium acetate pyrolysis was of the order of 70%.

Table I enumerates the results under diverse conditions:

Table I

Pyrolysis of Methyl-labeled Barium Acetate

Experiment	Temperature (time = 10 min.)	% of Activity in carboxyl
1. High vacuum (closed system)	530°C	2.3
2. Low vacuum (vapor pressure of water)	450°C	4.0 5.7 6.5
3. a) Flow (argon)	450°C	8.3
b) Flow (liberation of CO_2 from residue with 50% H_2SO_4)	530°C	3.0
c) Flow (argon) (Liberation of CO_2 from residue with 50% lactic)	530°C	1.6 1.1 0.86

Pyrolysis of carboxyl-labeled acetate under conditions of experiment 3a yielded iodoform with an activity of less than 0.1% of the carboxyl carbon.

The work of Höleman and Clusius (7) indicated that electrolysis was an additional and possibly useful method of degradation of acetate. It was conceivable that with this type of bond cleavage, fewer side reactions might occur. Under the conditions prescribed by these authors the ratio of CO_2

(6). W. H. Hatcher and W. H. Mueller, Trans. Royal Soc. of Canada, 23, Series 3 part 1, Sect. III, (1929).

(7). P. Höleman and K. Clusius, Zeit. fur Physiol. Chem., 35B, 261 (1937).

yield to ethane produced would indicate almost complete conversion of methyl free radicals to ethane. A simple electrolysis vessel with bright platinum electrodes (2.5 cm x 1 cm.) was filled with 4 cc. of 1.6 M sodium acetate (methyl-labeled). A current of 100 μamps. was passed through for ½ hour. Using N_2 as carrier gas, the CO_2 was collected in a gas washing bottle containing NaOH. The unabsorbed gases were passed through a CuO-filled combustion furnace and the resulting CO_2 also collected in NaOH. The yield of $BaCO_3$ from the ethane fraction was only ¼ of that from the carboxyl group. 1% of the activity was found in the carbon dioxide produced by the electrolysis. It is not known whether the same results would be obtained from electrolysis of a more dilute solution.

Summary

A scheme of glucose degradation has been validated by the use of intermediates of known isotopic composition. In this scheme: glucose $\longrightarrow$ lactic acid $\longrightarrow$ CO_2 (C-3,4) + acetic acid $\longrightarrow$ CO_2 (C-2,5) + acetone $\longrightarrow$ iodoform (C-1,6) + acetate (C-1,6; 2,5), it was found that a) in the oxidation of lactic acid, approximately 4.7% of the acetic acid was oxidized to CO_2; and b) under the conditions prescribed, $BaCO_3$ from the degradation of Ba acetate contained approximately 1.5% of the activity of the methyl group.