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RADIOACTIVE CARBON AS AN INDICATOR OF CARBON DIOXIDE REDUCTION. IV. THE SYNTHESIS OF ACETIC ACID FROM CARBON DIOXIDE BY CLOSTRIDIUM ACIDI-URICI

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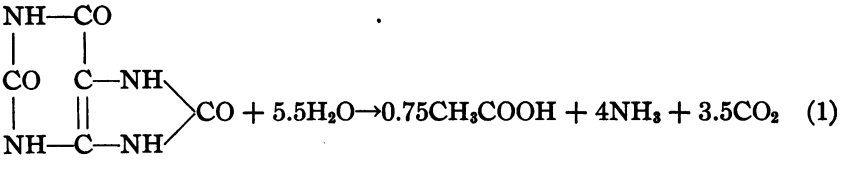
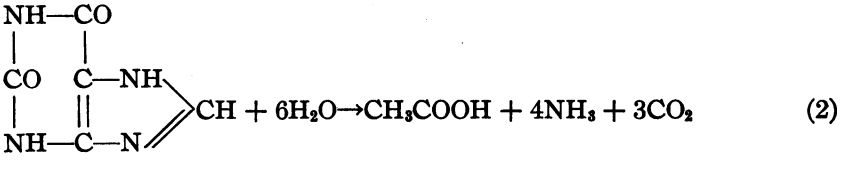
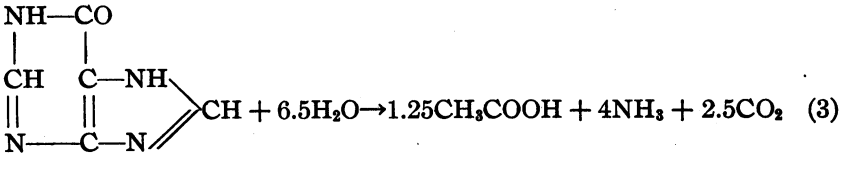
Clostridium acidi-urici is a little-known organism* capable of rapidly fermenting uric acid, xanthine, hypoxanthine and other purines under strictly anaerobic conditions. Besides cell materials, only three products are formed in considerable quantities in these fermentations, namely, ammonia, carbon dioxide and acetic acid. These substances account for about 98% of the nitrogen and 95% of the carbon of the purines decomposed.

The relative quantities of the fermentation products depend, of course, upon the particular compound used. Uric acid, xanthine and hypoxanthine, for example, are fermented approximately according to the following equations:







Actually the observed yields of acetic acid are a little lower than indicated by these equations. With uric acid the observed yields in moles of acetic acid per mole of purine fermented have been 0.6–0.7, with xanthine 0.9–1.0 and with hypoxanthine 1.1–1.25.

Now the yields of acetic acid obtained from uric acid and xanthine are in no way remarkable since these quantities might well be derived from the central C_3 chain of these purines. With hypoxanthine, however, the quantity of acetic acid is greater than could be obtained directly from this source. To explain the formation of more than one mole of acetic acid per mole of hypoxanthine it must be postulated that either (1) an intra- or intermolecular condensation involving the C_3 chains occurs followed by a splitting into the required number of C_2 fragments, or (2) part or all of the acetic acid is built up from the carbon dioxide or other C_1 fragments of the purine.

Using radioactive carbon (C^{11}) as an indicator it has been possible to test the latter alternative and to show that acetic acid is formed by the reduction of carbon dioxide.

For the experiments reported below, suspensions of *Cl. acidi-urici*, strain 9*a*, were used. The organisms were grown in a medium containing 0.3–0.4% uric acid, 0.1% yeast autolyzate, mineral salts and sodium sulfide or thioglycollate as a reducing agent. The reaction was adjusted to pH 7.4. After 18–24 hours' incubation at 35°C. under strictly anaerobic conditions, the cells were centrifuged, washed and resuspended in $M/10$ phosphate buffer pH 7 containing 0.015% $Na_2S.9H_2O$. Cells derived from 250–1000 cc. of medium were used in each experimental vessel. A brief description of the mode of preparation and use of C^{11} (21 minutes half-life) is given elsewhere.[5]

Experiment 1.—The object of the first experiment was to see whether radioactive acetic acid is formed during fermentations of uric acid, guanine and hypoxanthine carried out in the presence of radioactive carbon dioxide. Three vessels were used, each containing 5 cc. of cell suspension plus the substances indicated in column 2 of table 1. Glycine was added to vessels 1 and 2 because it has been found to eliminate an induction period in the decomposition of these compounds. The cells were mixed with their substrates just before adding the radioactive carbon dioxide (C^*O_2). The suspensions were then shaken in a N_2-C^*O_2 atmosphere at 37°C.

Following the incubation period the contents of each vessel were treated with 0.5 g. $NaHCO_3$, 0.5 cc. 50% acetic acid and sufficient sulfuric acid to bring the reaction to pH 1; the mixture was then boiled briefly to remove all residual radioactive carbon dioxide. The volatile acids were then removed by steam distillation, neutralized and tested for radioactivity with a Geiger counter.

It can be seen in table 1 that the volatile acids derived from all three

compounds were highly radioactive. The non-volatile fractions were also active. The non-volatile material derived from the hypoxanthine cells contained only about 5% of the total activity; a considerable part of this can be attributed to an incomplete removal of volatile acids. The non-

TABLE 1

DISTRIBUTION OF RADIOACTIVE CARBON IN PRODUCTS OF PURINE FERMENTATION. (ACTIVITIES ARE EXPRESSED IN ARBITRARY UNITS; ALL VALUES ARE CORRECTED FOR DECAY AND ARE DIRECTLY COMPARABLE)

VESSEL	SUBSTRATE	INCUBATION TIME, MINUTES	PURINE DECOMPOSED MG.	RADIOACTIVITY VOLATILE ACIDS	RADIOACTIVITY NON-VOLATILE FRACTIONS
1	30 Mg. Na urate 1 Mg. glycine	15	23	2.3	1.97
2	20 Mg. guanine 1 Mg. glycine	39	9.6	1.35	...
3	10 Mg. hypoxanthine	79	ca3	7.9	0.45

volatile activity from the uric acid cells was much larger, being sufficient to account for 46% of the total reduced C*. Not more than about 10% of this 46%—5% of the total—can be attributed to residual volatile acids. It must be concluded that a considerable quantity of some truly non-volatile material has been formed by a reduction of carbon dioxide.

In order to positively identify the volatile acid fraction, it was subjected to Duclaux distillation; the volatile acid from the uric acid culture was used. Five 20-cc. fractions of distillate were collected from a total initial volume of 110 cc. In table 1 the activities of each fraction, expressed in per cent of the activity in 100 cc. of distillate, are compared with similar data obtained by distillation and titration of pure acetic acid solution. The agreement is sufficiently close to leave no doubt that the volatile acid produced from uric acid is very largely if not entirely acetic acid.

TABLE 2

IDENTIFICATION OF VOLATILE ACID FRACTION BY DUCLAUX DISTILLATION

VOLUME OF DISTILLATE	UNKNOWN % OF ACTIVITY	ACETIC ACID % OF TITRATION
20	14.3	15.7
40	30.9	32.7
60	50.8	51.3
80	73.8	72.7
100	100	100

Experiment 2.—A second experiment was undertaken to find out whether (1) the large quantity of radioactive non-volatile material formed from uric acid is dependent upon the presence of glycine; (2) the non-volatile fraction is soluble or is associated with the insoluble cell materials; (3) the radioactive carbon from carbon dioxide is in the methyl group of the acetic acid.

The procedure was essentially the same as in the previous experiment. Two vessels were used, each containing 5 cc. of cell suspension. In addition, one contained about 30 mg. of sodium urate and 1 mg. glycine; the other vessel contained urate only. Incubation was at 40°C. for 17 minutes and 65 minutes, respectively. In both vessels 22.8 mg. of uric acid were decomposed.

The distribution of radioactive carbon in various fractions of the fermented media is given in table 3. The fractionation of the contents of vessel 1 (containing glycine) was most complete. Immediately after stopping the incubation the cell suspension was centrifuged sharply to separate the cells from the soluble constituents. Volatile acid and non-volatile fractions were then prepared from both cells and supernatant solution. The suspension of vessel 2 (without glycine) was not centrifuged prior to separating the volatile and non-volatile fractions.

TABLE 3

DISTRIBUTION OF RADIOACTIVE CARBON IN PRODUCTS OF URIC ACID FERMENTATION. (ACTIVITIES ARE EXPRESSED IN ARBITRARY UNITS; ALL VALUES ARE CORRECTED FOR DECAY AND ARE DIRECTLY COMPARABLE)

FRACTION	VOLATILE	NON-VOLATILE	TOTAL	NON-VOLATILE / TOTAL
Vessel 1 (glycine)				
Supernatant solution	17.4	8.5	25.9	0.33
Cells	1.9	4.7	6.6	0.71
Total	19.3	13.2	32.5	0.41
Vessel 2 (no glycine)				
Total	22.0	11.5	33.5	0.34

Table 3 shows that glycine does not markedly affect the total quantities or ratios of volatile and non-volatile activities. Non-volatile activity is present in both the cell fraction and the supernatant liquid. But although the total non-volatile activity is about twice as great in the liquid (9 cc.) as in the cell fraction (1 cc.), the concentration is much higher in the cells. This suggests that at least a considerable part of the radioactive non-volatile material is a cellular constituent. Further experiments would be required to elucidate the nature of this material.

The acetic acid was decarboxylated in order to find out if carbon from carbon dioxide was present in the methyl group. The volatile acid from vessel 1 was neutralized with $Ba(OH)_2$, evaporated to dryness, finely powdered and heated at 450–500°C. for 20 minutes in a stream of oxygen-free nitrogen. The procedure used by Ardagh, *et al.*,[1] was followed. The resulting acetone was trapped in ice-cold 1*N* KOH and converted into iodoform by oxidation with iodine. The equations for the decarboxylation and oxidation reactions are:

$$Ba(CH_3COO)_2 \rightarrow BaCO_3 + CH_3COCH_3 \quad (4)$$

$$CH_3COCH_3 + 3I_2 + 4OH^- \rightarrow CHI_3 + CH_3COO^- + 3I^- + 3H_2O \quad (5)$$

The iodoform, separated by centrifugation and ether extraction, was found to be radioactive, the activity being sufficient to account for approximately 18% of the total activity of the initial acetic acid. Since only one molecule of iodoform is produced from two molecules of acetic acid, the observed activity is sufficient to account for 70% of the theoretical yield on the assumption that the C* is distributed equally between the methyl and carboxyl groups. This calculated yield is a lower limit since the experiment was not carried out in such a way as to give a quantitative recovery of iodoform. Nevertheless the data constitute proof that an appreciable part of the reduced carbon is present in the methyl group.

Experiment 3.—This experiment was designed to give further information on the distribution of radioactive carbon between the methyl and carboxyl groups of acetic acid. The procedure was essentially the same as employed in the latter part of experiment 2 except that more care was exercised in the manipulations and, following decarboxylation, the activity of the residual $BaCO_3$ as well as of the CHI_3 was measured.

In this experiment the decarboxylation was allowed to proceed at 450–500°C. for 50 minutes. The yield of acetone was determined, following the iodoform reaction, by back titration with thiosulfate. There was obtained 68.5% of the theoretical yield. The iodoform was separated by centrifugation and exhaustive ether extraction, care being taken to have the aqueous phase alkaline to prevent contamination with acetic acid. The carbon dioxide resulting from decarboxylation was obtained by acidifying the residual $BaCO_3$, heating and collecting the evolved gas in alkali. The radioactivity of an aliquot of the alkali solution was determined.

The result of the experiment was the recovery of 23% of the activity of the acetic acid as iodoform derived from the methyl group and 11.3% as carbon dioxide derived from the carboxyl group. When these figures are corrected for the yield of acetone, they become 33.6 and 16.5%, respectively. From equations 4 and 5 it is evident that one of the two acetate radicals in the decomposed barium acetate is regenerated intact so that only 50% of the C* can be recovered in carbon dioxide and iodoform. The observed recovery ($33.6 + 16.5 = 50.1\%$) is in remarkably good agreement with the theoretical maximum.

As for the distribution of C* it appears that considerably more is in the methyl than in the carboxyl group. Since a preferential reduction of carbon dioxide to a methyl group seems unlikely, the observed distribution suggests the existence of reversible exchange reactions between the carboxyl group and carbon dioxide. If such a reaction occurs, the radioactive

carbon in the carboxyl group will be diluted with carbon dioxide produced by the oxidation of uric acid. This point requires further investigation.

Discussion.—Although the above experiments show that carbon dioxide is reduced by *Cl. acidi-urici* to acetic acid in fermentations of purines, they do not prove that all the acetic acid is formed in this way. However, it is not improbable that this is the case. If acetic acid is derived entirely from carbon dioxide these "fermentations" would have to be regarded as *complete oxidations of purines with carbon dioxide acting as the ultimate oxidizing agent.* They would then be entirely analogous to the methane "fermentation" with acetic acid replacing methane as the reduction product. The generalized reaction would be

$$4H_2A + 2CO_2 \rightarrow 4A + CH_3COOH + 2H_2O$$

where H_2A represents the reducing agent, A its oxidation product. This interpretation is not without supporting evidence for it has been shown by Wieringa[2] that another Clostridium species is capable of oxidizing molecular hydrogen by means of carbon dioxide, the latter being reduced to acetic acid. In this connection it will be of interest to study the reduction of radioactive carbon dioxide by other acetic acid producing anaerobes.

In conclusion, it may be pointed out that these experiments furnish another example of the already large group of non-photosynthetic, heterotrophic organisms[6] that are able to reduce carbon dioxide. They also illustrate again the use of bacteria for the preparation of organic compounds containing radioactive carbon.[3, 4]

We are indebted to Dr. M. D. Kamen and Professor E. O. Lawrence for the radioactive carbon.

* An extensive study of this organism will be published in the near future.

[1] Ardagh, E. G. R., Barbour, A. D., McClellan, G. E., and McBride, E. W., *Ind. Eng. Chem.*, **16**, 1133–1139 (1924).

[2] Wieringa, K. T., *Antonie van Leeuwenhoek*, **3**, 1–11 (1936).

[3] Carson, S. F., and Ruben, S., *Proc. Nat. Acad. Sci.*, **26**, 422–426 (1940).

[4] Barker, H. A., Ruben, S., and Kamen, M. D., *Ibid.*, **26**, 426–430 (1940).

[5] Ruben, S., Hassid, W. Z., and Kamen, M. D., *Jour. Am. Chem. Soc.*, **61** 661 (1939).

[6] Ruben, S., and Kamen, M. D., *Proc. Nat. Acad. Sci.*, **26**, 418–422 (1940).