

QUANTITATIVE ASPECTS OF THE TRICARBOXYLIC ACID CYCLE IN BAKER'S YEAST¹

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The quantitative significance of the tricarboxylic acid cycle in the oxidation of acetate by yeast has remained in doubt despite many investigations concerning this topic. All of the enzymes of the tricarboxylic acid cycle have been demonstrated in either commercial baker's yeast or in pure cultures of *Saccharomyces cerevisiae*. (Weiland and Sonderhoff, 1932; Lynen and Nعيullah, 1939; Novelli and Lipmann, 1950; Foulkes, 1951; Kornberg and Pricer, 1951; Nossal, 1954; Lynen and Kalb, 1955). The assumption that the individual enzymes can function as a group in effecting this organized sequence of reactions is further supported by isotope experiments. The results of experiments by Sonderhoff and Thomas (1937) and by Lynen (1948) employing deuterio-acetate are in agreement with the concept that succinate arises from acetate in yeast via the currently accepted reactions of the tricarboxylic acid cycle. Similarly, Weinhouse and Millington (1947) found that the isotope distribution in citrate produced by yeast in the presence of magnesium and barium acetate-C¹⁴ was in agreement with the mechanism of the cycle. Such qualitative observations leave little doubt as to the occurrence of the tricarboxylic acid cycle in yeast but provide little information concerning the quantitative significance of the cycle as a terminal oxidative pathway. In experiments designed to evaluate the quantitative role of the tricarboxylic acid cycle in acetate oxidation, Krebs *et al.* (1952) employed yeast preparations which had been made permeable to di- and tricarboxylic acids by treatment with dry ice. The results obtained from studies on the rate of substrate utilization, from isotope carrier experiments, and from additional criteria of the type frequently applied to mammalian systems, were

interpreted as evidence that the tricarboxylic acid cycle is not the main oxidative pathway in yeast. These investigators suggest alternatively that the component reactions of the cycle serve to supply intermediates for synthetic pathways. Studies by Swim and Krampitz (1954a, 1954b) with *Escherichia coli* and by Saz and Krampitz (1954) with *Micrococcus lysodeikticus*, on the other hand, indicate quite conclusively that the rates at which intermediates of the cycle are metabolized and the failure of carriers to become labeled during the oxidation of acetate-C¹⁴ are inadequate criteria for assessing the quantitative role of the cycle. These studies were undertaken to examine the role of the tricarboxylic acid cycle in acetate oxidation by nonproliferating cells of baker's yeast through the use of methods found to be applicable to certain bacteria. The isotope content and distribution found in intermediates of acetate-2-C¹⁴ oxidation, isolated in the absence of added carriers, supports the conclusion that the tricarboxylic acid cycle is a major oxidative pathway under the conditions employed.

METHODS

Baker's yeast. Pressed baker's yeast, purchased from the Red Star Yeast and Products Co., Cleveland, Ohio, was washed 3 times with about 10 vol of distilled water prior to use.

Degradations and isotopic analysis. The methods used to degrade isotopic acetate, citrate, and succinate, and to assay C¹⁴ activity were the same as those described previously by Saz and Krampitz (1954) and by Swim and Krampitz (1954a, 1954b). Amino acids were degraded to carbon dioxide by treatment with chromic acid (Van Slyke and Folch, 1940).

Isolation of intracellular compounds from yeast. Isotope experiments were performed in 2.8-L Fernbach flasks equipped as described previously (Swim and Krampitz, 1954). The flasks and their contents (see protocol beneath table 3) were incubated at 30 C on a reciprocal shaker and the respiratory carbon dioxide was col-

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TABLE 1
Carbon recovery and isotope distribution after oxidation of acetate-2-C¹⁴ in presence of unlabeled acetate and succinate

| Compound* | Quantity, μ Moles | | Specific Activity, cpm per μ Mole | |
|----------------------------------|-----------------------|-------|---------------------------------------|-------|
| | Initial | Final | Initial | Final |
| Acetate-2-C ¹⁴ | 60.0 | 44.3 | 1,500 | 1,520 |
| Succinate..... | 30.0 | 31.0 | | 39 |
| Citrate..... | 30.0 | 29.5 | | 133 |
| Respiratory CO ₂ | | 35.7 | | 755 |

Volume of reactants 4.4 ml, containing 8 mg pressed yeast, 400 μ moles phosphate buffer, pH 6.6, and substrates as indicated; 3 N sodium hydroxide in center well. Temperature 30 C. Reactions terminated after 1 hr by the addition of sulfuric acid.

* Cell residue contained 3,910 cpm total.

TABLE 2
Relative isotope content of fractions isolated from baker's yeast after oxidation of acetate-2-C¹⁴

| Fraction | Per Cent of Total Isotope |
|-----------------------------------|---------------------------|
| Residual acetate..... | 30.2 |
| Respiratory CO ₂ | 1.8 |
| Ether soluble..... | 8.1 |
| Water soluble..... | 47.3 |
| Water insoluble..... | 7.9 |
| Total recovery..... | 95.3 |

lected continuously in 3 N sodium hydroxide by drawing carbon dioxide free air through the apparatus. After incubation, the pH of the reaction mixture was adjusted to about 3 by the addition of a predetermined quantity of sulfuric acid and the mixture was cooled rapidly to 0 C by rotating the flask in a dry ice-methylcellosolve bath. The flasks were then transferred to an ice bath and carbon dioxide free air was drawn through the apparatus for an additional 20 min to collect the carbon dioxide liberated by the addition of sulfuric acid. The cells were collected by centrifugation at 10,000 \times G for 5 min and were then washed twice with 10 vol of distilled water (all operations at 4 C). After washing, the cell-paste was acidified to pH 2 (congo red indicator paper) with 2 N hydrochloric acid and lyophilized.

The lyophilized cells were fractionated as

TABLE 3
Quantity and specific activity of intracellular acids isolated from baker's yeast after oxidation of acetate-2-C¹⁴

| Acid | Quantity | Specific Activity |
|----------------------------------|-------------|-------------------|
| | μ moles | cpm/ μ moles |
| Acetate*..... | 36 | 5,580 |
| Citrate..... | 159 | 4,550 |
| α -Ketoglutarate..... | 0.5 | 4,700 |
| Succinate..... | 208 | 4,260 |
| Fumarate..... | 56 | 4,150 |
| Malate..... | 217 | 4,600 |
| Respiratory CO ₂ | 6,590 | 97 |

Total volume of reactants in 2 vessels 1,000 ml, containing 60 g pressed baker's yeasts, 50 μ moles phosphate buffer, pH 6.8, 5.0 μ moles acetate-2-C¹⁴. (7,000 cpm/ μ mole). Gas phase air. Temperature 30 C. Time of incubation 25 min.

* 1640 μ moles acetate recovered from the medium.

follows: An *ether soluble fraction* was obtained by mixing the dry cells thoroughly with Celite 535 (2 g Celite/g cells). The cell-Celite mixture was then acidified with 0.5 ml 0.2 N sulfuric acid per g of Celite employed and the mixture was extracted with peroxide free ether. The acids were recovered by evaporating the ether (Swim and Utter, 1957). After removing the ether from the cell-Celite mixture it was thoroughly extracted with water. The aqueous extract is referred to as the *water soluble fraction*. This fraction was concentrated by lyophilization prior to analysis. The residue remaining after extraction with water was suspended in 10 per cent hydrochloric acid (2 ml hydrochloric per g cell-Celite mixture) and the suspension was heated at 120 C (15 lb pressure) for 12 hr. The undissolved material was removed by centrifugation and the residue was washed several times with water. The supernatants were combined and evaporated to dryness and this is referred to as the *water insoluble fraction*.

Chromatographic separation and quantitation of acids. The organic acids present in the ether soluble fraction were separated by chromatography on Celite (Swim and Krampitz, 1954a; Swim and Utter, 1957). The α -ketoglutarate isolated in this manner was purified as the 2,4-dinitrophenyl-hydrazone derivative by chromatography on paper prior to degradation

The water soluble fraction was further frac-

TABLE 4
Isotope distribution in citrate, succinate, and acetate isolated from baker's yeast

| Compound | Specific Activity, CPM per μ Moles Carbon | | | | | |
|--|---|----------------|--------------|---------------|------------|------------|
| | 1 | 2 | 3 | 4 | 5 | 6 |
| $\overset{1}{\text{HOOC}}-\overset{2}{\text{CH}_2}-\overset{3}{\text{COH}}-\overset{4}{\text{CH}_2}-\overset{5}{\text{COOH}}-\overset{6}{\text{COOH}}$ | 67 (39)* | 1540 (2210) | 870 (870) | 1540 (870) | 67 (95) | 95 (95) |
| $\overset{1}{\text{HOOC}}-\overset{2}{\text{CH}_2}-\overset{3}{\text{CH}_2}-\overset{4}{\text{COOH}}$ | 435 | 1695 | 1695 | 435 | — | — |
| $\overset{1}{\text{HOOC}}-\overset{2}{\text{CH}_3}$ | 86 | 5700 | — | — | — | — |

* The degradation procedures employed for citrate do not permit the separation of carbons 1 and 5 and carbons 2 and 4. If it is assumed that citrate is formed by the condensation of a C_2 with a C_4 derived from succinate in which the isotope is distributed equally in each half of the molecule, the distribution indicated by the figures in parentheses can be calculated from the data ($\text{C}_5 = \text{C}_6 = 95$; $\text{C}_3 = \text{C}_4 = 870$; $\text{C}_1 = 67 + 67 - 95$; $\text{C}_2 = 1540 - 870 + 1540$).

tionated on a Dowex 2-X4 column by a modification of the procedure of Stein (1953). A mixture of amino acids obtained in this manner were separated according to the method of Moore and Stein (1951). The individual amino acids were quantitated by the Moore and Stein (1954) modification of the ninhydrin method.

RESULTS

Isotope carrier experiments. The results of an experiment, in which nonproliferating yeast was permitted to oxidize acetate-2- C^{14} in the presence of unlabeled citrate and succinate, are presented in table 1. It will be noted that the baker's yeast used in these studies behaves in a typical fashion in that acetate is oxidized to carbon dioxide whereas citrate and succinate are not metabolized. Furthermore, the carrier citrate and succinate did not become labeled significantly in spite of the fact that about $\frac{1}{3}$ of the acetate was utilized. The significance of the C^{14} activity in the cell residue is discussed in a subsequent section.

Isolation of intermediates of acetate-c- C^{14} oxidation in the absence of added carriers. Subsequent experiments were designed to examine the possibility that more than one terminal oxidative pathway might be operative in nonproliferating baker's yeast and to evaluate the quantitative importance of the tricarboxylic acid cycle in the oxidation of acetate by such preparations. It appeared reasonable to assume, on the basis of studies with bacteria (Saz and Krampitz, 1954; Swim and Krampitz, 1954a), that intermediates of acetate oxidation could also be isolated from

yeast in experiments employing large quantities of cells. The use of acetate- C^{14} makes it possible to distinguish intermediates, as well as metabolically related compounds from other intracellular constituents. The isotope distribution in the intracellular compounds and their relative C^{14} -content permits certain conclusions regarding their origin and the extent to which they participate in the metabolism of acetate.

A suspension of baker's yeast was allowed to oxidize acetate-2- C^{14} (see protocol beneath table 3) and then the cells were fractionated as described under Methods. The individual fractions were assayed for C^{14} and their relative isotope content is indicated in table 2. The quantity of each of the compounds isolated from the ether soluble fraction by chromatography on Celite and their specific activities are presented in table 3. It will be noted that the citrate, α -ketoglutarate, succinate, malate and fumarate are not only highly labeled but are in isotopic equilibrium with each other. Of particular interest is the fact that only intermediates of tricarboxylic acid cycle were isolated and that these account for all of the isotope in the ether soluble fraction. The distribution of isotope found in the intracellular citrate, succinate, and acetate is presented in table 4. These results attest to the reliability of the degradation procedures since the sum of the specific activities of the individual carbons in each compound agrees with the values obtained on total combustion (compare tables 3 and 4).

An interpretation of the quantitative significance of the data presented in tables 3 and 4 was

TABLE 5
Quantity and specific activity of amino acids
isolated from baker's yeast

| Amino Acid | Quantity* | Specific Activity |
|---------------|-------------------|------------------------------|
| | μmoles | $\text{cpm}/\mu\text{moles}$ |
| Glutamic..... | 2,900 | 4,770 |
| Aspartic..... | 165 | 960 |
| Alanine..... | 770 | 715 |
| Serine..... | 165 | 590 |
| Glycine..... | 297 | <300 |

* Quantity isolated from 60 g pressed baker's yeast; see protocol beneath table 3.

complicated by the fact that a large proportion of the isotope was not recovered in the residual acetate, respiratory carbon dioxide and intermediates of the tricarboxylic acid cycle but was located in the *water soluble* and *water insoluble* fractions (table 2). To ascertain whether other potential intermediates of acetate oxidation were present in the cells and to account for the isotope contained in ether-insoluble compounds, the cell residue (after ether extraction) was fractionated according to the procedures described under Methods. Essentially all of the isotope in the water soluble fraction was located in 5 amino acids. The quantity and specific activity of each of the labeled amino acids which were isolated are indicated in table 5. About 2 per cent of the total isotope in the water soluble fraction was not trapped in the Dowex 2-X4 column, indicating that basic amino acids or neutral compounds containing isotope were present. No attempt was made, however, to resolve this mixture into its individual components. Within the limits of experimental error all of the isotope in the water insoluble fraction was located in the glutamic acid isolated after hydrolysis of this fraction. The over-all analytical data demonstrate that, with the one exception already noted, all the intracellular C^{14} derived from acetate-2- C^{14} is accounted for by intermediates of the tricarboxylic acid cycle, glutamate, aspartate, alanine, serine, and glycine. A comparison of tables 3 and 5 reveals the degree to which the size of the intracellular pools of intermediates of the cycle and amino acids differ under the conditions employed. The values for 60 g of pressed yeast vary from 0.5 μmole of α -ketoglutarate to 2900 μmoles of glutamate although most of the compounds fall into the range of 50 to 300 μmoles . In view of

these results and the fact that acetate was added as substrate, it is surprising that only 36 μmoles of this compound were isolated from the cells.

DISCUSSION

According to the mechanism of the tricarboxylic acid cycle the following occurs during the oxidation of acetate. The citrate formed by the condensation of a C_4 with a C_2 derived from acetate is converted to succinate by the loss of carbons 5 and 6 as carbon dioxide (table 4). Since succinate is a symmetrical molecule, the isotope distribution in the 1—4 and 2—3 carbons represents an average value of the 1—4 and 2—3 carbons of citrate respectively (table 4). As a result of repeated turns of the cycle, isotope from acetate-2- C^{14} is distributed into the carboxyl carbons of citrate (carbons 5 and 6) and succinate. The specific activity of the carboxyl carbons in the respective molecules should therefore be considerably less than that of the noncarboxyl carbons. The specific activity carbons 1 and 2 of citrate should reflect the isotope content in the carboxyl and methyl carbons of acetate, respectively, since citrate is metabolized in an asymmetric fashion (see Krebs, 1949 for review). All of the respiratory carbon dioxide, on the other hand, is derived from carbons 5 and 6 of citrate. The data presented in tables 3 and 4 are in excellent agreement with each of these requirements. If a pathway not involving intermediates of the tricarboxylic acid cycle is operating to an appreciable extent, the respiratory carbon dioxide would be expected to be higher than that of the carboxyl carbons of citrate. It is also evident that isocitratase (Smith and Gunsalus, 1954) does not play a significant role in either the breakdown or formation of citrate. Alternatively, carbon dioxide with a low specific activity could be derived from another pathway if the cells contained large pools of the corresponding intermediates. The analytical data indicate that such compounds are not present in baker's yeast under the conditions employed. It is considered that the foregoing data justify the conclusion that the tricarboxylic acid cycle is a major pathway of acetate oxidation by nonproliferating suspensions of baker's yeast. It is to be noted however, that the data in no way exclude the possibility that acetate may be metabolized to a limited extent by oxidative condensation to succinate (Thunberg condensation) and the other reactions of the dicar-

boxylic acid cycle or that other oxidative pathways may be operative during the growth of cells. It is of interest that recent experiments by Eaton and Kline (1957) employing glucose- C^{14} indicate that the tricarboxylic acid cycle is an important terminal respiratory pathway in *S. cerevisiae*.

The foregoing interpretations are not in accord with the conclusion of Krebs *et al.* (1952) that the tricarboxylic acid cycle plays a minor role in the oxidation of acetate by baker's yeast. This conclusion was based largely on the following results obtained in experiments with baker's yeast which had been rendered permeable to di- and tricarboxylic acids by treatment with dry ice. (1) When acetate- C^{14} is oxidized in the presence of unlabeled members (carriers) of the tricarboxylic acid cycle, the latter fail to become labeled to an appreciable extent. (2) Acetate is oxidized much more rapidly than other intermediates in the cycle as judged by the rate of oxygen utilization. (3) Acetate is converted to carbon dioxide whereas, succinate is oxidized only as far as fumarate. Such observations, however, do not permit the conclusion that the tricarboxylic acid cycle is of little quantitative importance since studies with *M. lysodeikticus* (Saz and Krampitz, 1954) and with *E. coli* (Swim and Krampitz, 1954a, 1954b) demonstrate quite conclusively that such factors as the ability of an organism to utilize members of the cycle, the rate at which they are metabolized, and the degree to which they become labeled in isotope carrier experiments employing acetate- C^{14} are invalid criteria for evaluating the role of this pathway in terminal oxidation. The data obtained with *M. lysodeikticus* are of particular significance in relation to yeast treated with dry ice since the former was made permeable by treatment with lysozyme. The fact that yeast treated with dry ice does not oxidize succinate beyond fumarate although acetate is converted to carbon dioxide may indicate that such preparations contain 2 metabolically distinct types of cells as suggested by Weinhouse (cited in Krebs *et al.*, 1952). On the other hand, evidence has not been presented which excludes the possibility that treatment with dry ice merely renders a limited number of enzymes (including succinoxidase) accessible to exogenous substrate and that a complete set of enzymes of the tricarboxylic acid cycle are located within a physiological compartment in the

damaged cells which does not permit the free exchange of compounds (pools) contained therein with the corresponding compounds of exogenous origin. It is of particular interest that such a concept is also consistent with all the data concerning the bacterial systems.

Weinhouse and Millington (1947) demonstrated that the isotope distribution in the citrate formed by yeast in the presence of C^{14} labeled barium acetate is in accord with the mechanism of the tricarboxylic acid cycle. This raised the question as to the nature of the C_4 which must be formed from endogenous sources to account for the data. It is of interest to consider the results of the present studies in this connection. Carbon 2 of citrate (table 4) represents the methyl carbon of acetate and carbons 3, 4, 5 and 6 are derived from succinate via oxalacetate. The specific activity of these carbons is considerably less than that of their corresponding precursors indicating that dilution from endogenous sources occurs at both the C_2 and C_4 level prior to condensation. In the case of the C_4 it appears that dilution occurs at oxalacetate since fumarate and malate are in isotopic equilibrium with the succinate (table 3). This is further suggested by the fact that the isotope content of the intracellular aspartic acid is also less than that of succinate. The isotope distribution in citrate also reveals that the carboxyl carbons of oxalacetate are diluted more than the α, β carbons by a factor of 2. This cannot be explained by the exchange of isotope between the respiratory carbon dioxide and the carboxyl carbons of oxalacetate since the latter is not a symmetrical molecule. The data are more consistent with the possibility that some oxalacetate is produced via the condensation of a C_2 (containing C^{14} from acetate) with two C_1 units or a single C_2 unit of endogenous origin.² It is of interest in this regard that considerable C^{14} is located in the intracellular pools of glycine, serine and alanine although the significance of these observations remains to be established.

It is noteworthy that most of the C^{14} removed from the acetate pool which is not accounted for

² Examples of how oxalacetate could be produced are illustrated in the following equations: (a) $C^{14}H_3-COOH + C_1 \rightarrow (C-C^{14}-COOH) + C_1 \rightarrow HOOC-CH_2-C^{14}O-COOH$; (b) $C^{14}H_3-COOH \rightarrow (C^{14}-C) + (C-C) \rightarrow HOOC-CH_2-C^{14}O-COOH$ or $HOOC-C^{14}H_2-CO-COOH$.

by intermediates of the cycle and respiratory carbon dioxide is located in amino acids. The glutamate is in isotopic equilibrium with α -ketoglutarate (compare tables 3 and 5). Although the aspartate is not as highly labeled as glutamate it appears that the degree of equilibration with oxalacetate is reasonably complete for reasons already discussed. These findings are in accord with the concepts of Krebs *et al.* (1952) that component reactions of the cycle can function in supplying intermediates for the synthesis of amino acids. Similarly these data confirm earlier observations by Wang *et al.* (1953) that the isotope distribution in glutamate isolated from yeast after oxidation of acetate- C^{14} conforms to the mechanism of the tricarboxylic acid cycle. These investigators also suggested that glutamic acid may be an important assimilatory product of acetate metabolism in resting cells of baker's yeast. Winzler (1940) on the other hand, presented evidence that the acetate which is assimilated by *S. cerevisiae* during the oxidation of acetate is located in the polysaccharide fraction. In the present experiments, isotope was not found in either the glycogen isolated by a modification of the procedure of Somogyi (1934) or in the free reducing sugars contained in the *water soluble fraction*. These results may be a reflection of intracellular nitrogen pools, strain of cells and their over-all physiological state and thus, do not necessarily contradict the conclusions of Winzler.

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SUMMARY

A suspension of baker's yeast was permitted to oxidize acetate-2- C^{14} in the absence of additional substrates. The cells were separated into several fractions and the compounds containing C^{14} were separated from each fraction by chromatographic procedures. All of the isotope was located in acetate, citrate, α -ketoglutarate, succinate, fumarate, malate, glutamate, aspartate, alanine and glycine. The citrate, α -ketoglutarate, succinate, fumarate and malate were highly labeled and in isotopic equilibrium with each other. Similarly the glutamate was in isotopic equilibrium with the α -ketoglutarate. The specific activities of the remaining amino acids were

variable and consistently less than those of the intermediates of the tricarboxylic acid cycle. The isotope distribution found in citrate and succinate, coupled with the other isotope data are presented as evidence that the tricarboxylic acid cycle is a major pathway of acetate oxidation by nonproliferating suspensions of baker's yeast.

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