[CONTRIBUTION FROM THE INSTITUTE FOR CANCER RESEARCH AND THE LANKENAU HOSPITAL RESEARCH INSTITUTE, AND THE DEPARTMENT OF CHEMISTRY, TEMPLE UNIVERSITY]

Studies on the Mechanism of Citric Acid Production in *Aspergillus Niger*[1]

BY KATHARINE F. LEWIS[2] AND SIDNEY WEINHOUSE

The metabolism of carboxyl-labeled acetate by *Aspergillus niger* leads to the formation of citric acid labeled in the carboxyl carbons, with approximately 1.5 times more isotope in the primary carboxyls than in the tertiary. Methyl-labeled acetate under the same circumstances yields citrate labeled in both carboxyl and non-carboxyl carbons. The distribution of isotope in the citrates was in accord with the concept that the citric acid cycle is a major pathway for acetate utilization and citrate formation in this organism.

The establishment of the citric acid cycle as a mechanism for oxidative metabolism in animals has naturally led to speculation concerning the extent to which this process occurs in lower organisms.[3]

The fact that various fungi oxidize acetate readily and, under certain conditions, accumulate di- and tricarboxylic acids, which are components of or chemically related to components of the citric acid cycle[4,5] is indicative of its occurrence in these organisms; indeed, long before the citric acid cycle was considered as a metabolic occurrence in animals, a C_2–C_4 condensation between acetate and a dicarboxylic acid such as malate or oxalacetate had been suggested to account for citric acid production by various mold species.[6] The mechanism of citric acid biosynthesis has been obscured, however, by a multiplicity of theories, none of which have stood the test of experimental verification.[4,5] In previous studies some definite evidence was presented in support of the occurrence of the citric acid cycle in the oxidative metabolism of yeast[7,8]; the method consisted in determining the distribution of isotopic carbon in citrate formed during the oxidation of carboxyl-C^{13}-labeled acetate. It was possible to calculate that any citrate formed through the cycle would have in its 2 primary carboxyl carbons an average of 75% of the isotope content of the acetate carboxyl, whereas the corresponding value for the single tertiary carboxyl would be 50%. The close correspondence between the calculated and observed distributions, together with other considerations, including the studies of Lynen[9,10] on acetate oxidation in yeast and the findings of Novelli and Lipmann[11] on the coenzyme A dependence of acetate oxidation led us to conclude that the citric acid cycle represents a major metabolic process in yeast. In the present study similar evidence has been obtained to indicate that the citric acid cycle represents a major pathway of citrate formation in the mold, *Aspergillus niger*.

(1) This work was aided by grants from the National Cancer Institute, U. S. Public Health Service, and by an institutional grant from the American Cancer Society to the Institute for Cancer Research.

(2) This work was carried out by Katharine F. Lewis in partial fulfillment of the requirement for the Ph.D. degree in the Graduate School of Temple University. Presented in part before the Biological Division of the American Chemical Society, Atlantic City, September 22, 1949.

(3) Krebs, *Adv. Enzymol. Related Subjects*, **3**, 191 (1943).

(4) Walker, *ibid.*, **9**, 537 (1949).

(5) Foster, "Chemical Activities of Fungi," Academic Press, N. Y. (1949).

(6) Chrzaszc and Tiukov, *Biochem. Z.*, **218**, 73 (1930).

(7) Weinhouse and Millington, THIS JOURNAL, **69**, 3089 (1947).

(8) Weinhouse, Millington and Lewis, *ibid.*, **70**, 3680 (1948).

(9) Lynen, *Ann.*, **544**, 40 (1943).

(10) Lynen and Neciullah, *ibid.*, **541**, 203 (1939).

(11) Novelli and Lipmann, *J. Biol. Chem.*, **171**, 833 (1948).

Results

The results of an experiment in which carboxyl-labeled acetate was metabolized by a washed mycelial mat of *A. niger* are given in Table I. Owing to wide variations in yields and isotope contents of products no single experiment could be described as typical; however, the experiment cited is fairly representative. The results are similar to those previously carried out with yeast cells except for a generally lower isotope level in the products. There is no doubt that acetate is incorporated in citrate and can also be oxidized completely to carbon dioxide. One of the convenient aspects of the work with yeast was that the cells could be washed and aerated free of endogenous nutrients so that acetate metabolism could be studied without the complications introduced by endogenous substrates. This proved to be impossible in the mold. Despite repeated washing, the removal of endogenous metabolites, or even preformed citrate was incomplete. This is reflected in the specific activities of the products as compared with that of the acetate used as substrate. The citrate, for example, had an over-all activity 11% of that of the acetate carboxyl, whereas the value was 3 times as high in previous experiments with yeast in which the added acetate was the only carbon source for citrate formation. Thus, of the 0.6 mM of citrate recovered, perhaps about $^2/_3$ was either preformed or formed from endogenous carbon. In other experiments the specific activities of citrate ranged from 5 to 18% of the acetate carboxyl. Similarly, the respiratory carbon dioxide had an over-all specific activity 19% of that of the acetate carboxyl, whereas a value of 50% would have been expected if acetate were the only carbon source. As shown by the time course of evolution of the isotopic carbon in Table II, oxidation of acetate was maximal in about 20 hours. Altogether about 85% of the acetate utilized was accounted for by complete oxidation to carbon dioxide. There was oxidized also approximately an equal quantity of endogenous carbon, as shown by the amount and isotope content of the respiratory carbon dioxide. Utilization of acetate was virtually complete in this experiment, though in others it was of the order of 75%. The acetate recovered was diluted approximately eight-fold, indicating the formation of some acetate from endogenous carbon, but in other experiments dilution was only of the order of 10–15%. As shown in the table, oxalate was always formed in substantial quantity, with specific activities similar to those of the citrate.[12]

(12) The formation of oxalate by *A. niger* will be taken up in a separate publication.

TABLE I

DISTRIBUTION OF C^{14} IN PRODUCTS OF COOH-LABELED ACETATE METABOLISM IN *A. niger*

Specific activities are expressed as per cent. of acetate carboxyl; *i.e.*, rel. sp. act. = spec. act. of compound as $BaCO_3$ × 100/sp. act. of acetate COOH as $BaCO_3$.

	Quantity, mM.	Relative specific activity	Per cent. of total activity
Acetate, start	10.0	100	100
Acetate, recovered	1.36	12.5	1.7
Citrate, over-all	0.62	11.4	4.2
Oxalate	2.69	6.0	3.2
Formate	0.94	4.5	0.4
Ethanol	0.17	0	0
Cell lipides	280[a]	4.3	7.5
Cell non-lipides	2941[a]	0.6	7.2
Resp. CO_2	43.8	19.4[b]	85

[a] Milligrams. [b] Weighted mean of fractions shown in Table II.

TABLE II

TIME COURSE OF CARBON DIOXIDE EVOLUTION. SAME EXPERIMENT AS TABLE I

	Time, hours 8	20	30	48	54	Bound[a]	Total
Millimoles	4.93	9.47	7.99	13.2	7.22	0.93	43.76
Rel. sp. act.	11.5	27.6	27.4	17.7	9.9	4.6	19.4
Total activity, %	5.7	26.1	21.9	23.3	7.1	4.3	85

[a] Residual CO_2 liberated by addition of acid.

Some formate was always found, but its activity was extremely low. In the experiment of Table I, the formate activity was unaccountably high; in all of the other experiments the values were less than one-tenth of that given in the table and we are therefore inclined to attribute this to some unknown contamination. Appreciable activity was found in the cell components and, as expected, incorporation of acetate carbon in the lipides was high.

Distribution of Activity among Citrate Carbons.—The presence of acetate carbon in citrate formed by *A. niger* having been established, a study of its distribution was made. Results on citrates obtained from six separate experiments are given in Table III.

TABLE III

ISOTOPE DISTRIBUTION IN CARBONS OF CITRATE FORMED BY *A. niger* IN PRESENCE OF COOH-TAGGED ACETATE

Specific activities are in per cent. of the acetate carboxyl

	Experiment number[a] 1	2	3	4	5	6
Citrate, over-all	18.1	4.8	11.4	16.6	16.4	11.7
Citrate, primary COOH's	38.4	10.4	23.5	37.5	38.3	25.4
Citrate, tertiary COOH	23.5	7.0	20.6	23.2	20.1	13.3
Primary/tertiary	1.6	1.5	1.1	1.6	1.9	1.8

[a] Expts. 4–6 also contained glucose as substrate.

Despite the wide range in over-all activity, which can be reasonably attributed to varying amounts of preformed and endogenously formed citrate, the relative proportions of activity in the primary and tertiary carboxyls are rather constant; the ratio of primary to tertiary activity ranged between 1.1 and 1.9 and a mean of 1.6. No activity was present in the three central, non-carboxyl carbons. It was noted previously that in the citrate formed by way of the citric acid cycle from carboxyl-labeled acetate as the sole substrate, the primary carboxyls should have 1.5 times the activity of the tertiary carboxyl, and the close correspondence of the observed ratios to this calculated value was taken as strong evidence for the occurrence of the cycle in yeast.[7] In the present experiments the good correspondence between the observed average and the calculated values indicates that the processes of citrate formation in *A. niger* are essentially similar to those in yeast, though the deviations from the calculated value, observed in some experiments, suggest that other processes are occurring simultaneously to yield citrate or precursors thereof. It is clear that during citrate accumulation this acid cannot supply the 4-carbon acids necessary to carry on the cycle, and hence other mechanisms must be available for the maintenance of their supply. To this extent, therefore, deviations from an "ideal" value are not entirely unexpected.

Metabolism of Methyl-labeled Acetate by *A. niger*.—The essential correctness of the idea that the main pathway for acetate utilization and citrate formation is through the citric acid cycle has received support from experiments with methyl-labeled acetate. One of the characteristic features of the citric acid cycle is that acetate methyl carbons are not lost in the first round of the cycle. They first appear as central carbons of citrate, and only after getting into the carboxyls of C-4 acids are they liberated as CO_2 in subsequent rounds of the cycle. By following these methyl carbons through successive cycles one finds that a steady state is rapidly reached when the C-4 acids have 3 of 4 carbons labeled, the unlabeled one being a carboxyl carbon. As shown below in equation 1, further condensation with methyl-labeled acetate yields 2 varieties of citrate, depending on whether condensation occurs on the carbon adjacent to or one carbon removed from the labeled carboxyl carbon. Since each variety has an equal chance of being formed, the observed distribution will be the average of the 2 forms; *viz.*, labeled carbon in all 3 non-carboxyl carbons, in 1 of 4 primary carboxyls and in 1 of 2 tertiary carboxyls. One can therefore calculate that, assuming a value of 100 for the over-all activity of the 6 citrate carbons, the non-carboxyl carbons would have equal activities of 150, the primary carboxyls 38, and the tertiary carboxyl 75. The degree of correspondence between these calculated values and those observed in 2 experiments with methyl-labeled acetate is shown in Table IV. In two points the observed values are in extraordinarily good agreement with the calculated. The activities of the 3 non-carboxyl carbons are essentially equal, the differences noted being well within the experimental error of measurement. Second, the proportion of activity in these 3 carbons is almost exactly the calculated value. The agree-

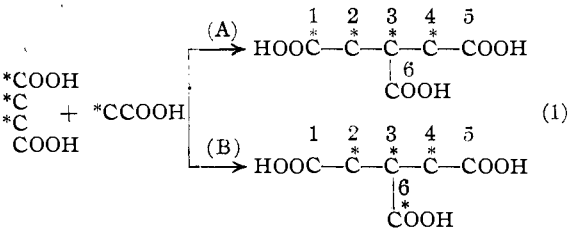


TABLE IV

DISTRIBUTION OF ACETATE METHYL CARBONS AMONG CARBONS OF CITRATE PRODUCED BY *A. niger*

Citrate carbon[a]	Form in which isolated	Relative specific activities[b] Expt. 1	Expt. 2[c]	Calcd.
Over-all	Ca citrate	100	100	100
1, 5	CO_2	51	45	38
6	CO	63	61	75
2, 3, 4	Acetone	146	149	150
2, 4	Iodoform	165	135	150
2, 3; 4, 3	Na acetate	152	148	150
3	$BaCO_3$	147	149	150

[a] Numbering as in equation 1. [b] Relative specific activity = activity of substance (measured as $BaCO_3$) × 100/activity of citrate (measured as $BaCO_3$). [c] Glucose also present in this experiment.

ment with calculated values for the primary and tertiary carboxyls is not so good, however, the former being too high and the latter too low. Inasmuch as, again, supplementary mechanisms must be operating to produce C-4 acids, and since their effects on the isotope distribution cannot be evaluated, better agreement between the observed values and those calculated on the basis of an "ideal" citric acid cycle may not be justified.

Metabolic Formation of COOH-Labeled from Methyl-labeled Acetate.—The interpretation of results in these experiments with methyl-labeled acetate is further complicated by the migration of methyl carbon to the carboxyl position of acetate. As shown in Table V this occurred to an

TABLE V

FORMATION OF CARBOXYL-LABELED FROM METHYL-LABELED ACETATE

	Specific activities[b] Expt. 1	Expt. 2[a]
Acetate methyl, start	100[b]	100
Acetate recovered, over-all	20.9	8.5
Acetate recovered, methyl	35.8	15.4
Acetate recovered, carboxyl	6.0	1.5

[a] Glucose added in this experiment. [b] Specific activities are given in per cent. of acetate methyl activity.

appreciable extent; in one experiment about one-sixth and in the other one-tenth of the total activity of the recovered acetate was in the carboxyl carbon. Although the exact pathway for this conversion is uncertain, 2 reasonable mechanisms suggest themselves. It has already been pointed out that the operation of the citric acid cycle yields C-4 acids from methyl-labeled acetate with 3 of 4 carbons labeled. Oxalacetate with this distribution can conceivably break down by 2 processes to yield carboxyl-labeled acetate as shown in equation 2.

$$\mathrm{HOO\overset{*}{C}\text{-}\overset{*}{C}H_2\overset{*}{C}OCOOH} \begin{cases} \xrightarrow{(A)} \mathrm{HOO\overset{*}{C}\text{-}\overset{*}{C}H_3} + \mathrm{HOO\overset{*}{C}\text{-}COOH} \\ \xrightarrow{(B)} \mathrm{\overset{*}{C}H_3\overset{*}{C}OCOOH} + \mathrm{\overset{*}{C}O_2} \end{cases} \quad (2)$$

$$\mathrm{\overset{*}{C}H_3\overset{*}{C}OCOOH} \longrightarrow \mathrm{\overset{*}{C}H_3\overset{*}{C}OOH} + \mathrm{CO_2}$$

Evidence for reaction A has been obtained by us in experiments to be published separately, which demonstrated that inorganic CO_2 can be assimilated into acetate by *A. niger*, presumably by the mechanism in equation 3.

$$\mathrm{CH_3COCOOH} + \mathrm{\overset{*}{C}O_2} \rightleftharpoons \mathrm{HOO\overset{*}{C}CH_2COCOOH} \longrightarrow \mathrm{HOOC\text{—}CH_3} + \mathrm{HOO\overset{*}{C}COOH} \quad (3)$$

Though no direct evidence for the stepwise decarboxylation of oxalacetate to yield acetate from the two central carbons is available, reaction B seems plausible, since it is based on known reactions of the substances concerned. At any rate, whatever the mechanism, the conversion of methyl to carboxyl carbon does not seem to occur at a sufficiently rapid rate to be a major reaction in this organism. Otherwise a greater migration of methyl carbon into acetate would have been observed.

Discussion

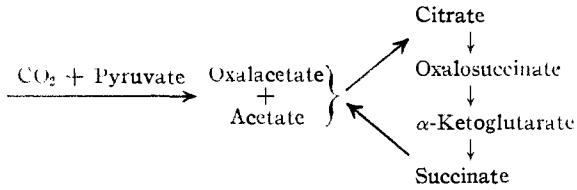
Alternate Pathways of C_4 Acid Formation.—It was already pointed out that citrate cannot accumulate unless auxiliary sources can supply C_4 acids.[7] Two plausible mechanisms for their formation can be envisioned: carboxylation of pyruvate with CO_2 to yield oxalacetate (the Wood and Werkman reaction) and dehydrogenative coupling of 2 acetates to yield succinate (the Thunberg reaction) as shown in equations 4 and 5.

$$\mathrm{CH_3COCOOH} + \mathrm{CO_2} \rightleftharpoons \mathrm{HOOCCH_2COCOOH} \quad (4)$$

$$\mathrm{2CH_3COOH} \longrightarrow \mathrm{HOOCCH_2CH_2COOH} + \mathrm{2H} \quad (5)$$

Assimilation of carbon dioxide into fumarate and citrate was observed by Foster, *et al.*,[13] and more recently fixation of carbon dioxide in citrate was reported by Martin, Wilson and Burris[14] which we have verified in unpublished experiments. Definite evidence for the occurrence of the Thunberg condensation was provided recently by Foster, *et al.*,[15] for the mold, *Rhizopus nigricans*.

On the basis of the data presented herein, our conception of citrate formation is presented in the following outline. It envisages a condensation of



acetate with oxalacetate, with the latter being supplied from 3 sources; the citric acid cycle (breakdown of citrate through α-ketoglutarate), the Wood and Werkman reaction, and the Thunberg condensation.[16] The extent of participation of these 3

(13) Foster, Carson, Ruben and Kamen, *Proc. Natl. Acad. Sci. U. S.*, **27**, 590 (1941).

(14) Martin, Wilson and Burris, *Arch. Biochem.*, **26**, 103 (1950).

(15) Foster, Carson, Anthony, Davis, Jefferson and Long, *Proc. Natl. Acad. Sci. U. S.*, **35**, 663 (1949).

(16) Foster and Carson (THIS JOURNAL, **72**, 1865 (1950)) have recently found (as we have reported here) that in *A. niger*, methyl labeled acetate yields citrate labeled equally in the non-carboxyl carbons and concluded thereform that citrate arises by successive C_2 condensations, *i.e.*, $2C_2 \rightarrow C_4$; $C_2 + C_4 \rightarrow C_6$. These reactions alone cannot account for the isotope distribution we have observed in citrate derived from COOH-labeled acetate; nor does it account for the appearance of acetate methyl carbon in citrate carboxyls. To account for the latter, these authors assume a "very active C_4 respiratory cycle," presumably $C_4 \rightarrow C_3 \rightarrow C_2$; $2C_2 \rightarrow C_4$; a combination of reactions 2B described above, and the Thunberg condensation. We pointed out above, however, that although such a process does account for the metabolic formation of carboxyl-labeled from methyl-labeled acetate, this finding can also be explained by splitting of oxalacetate to acetate and oxalate; besides the extent of the transformation is not compatible with a major reaction. These authors have apparently not considered the citric acid cycle as a mechanism for migration of acetate methyl into citrate carboxyl positions.

processes in C_4 acid formation would be expected to vary under different conditions. In our experiments citrate accumulation was rather low; in the experiment of Table I, for example, only about 4% of the acetate was accounted for in the citrate isolated, whereas 85% was completely oxidized to carbon dioxide. In no experiment did the yield of citrate exceed 25%. We interpret these results as indicating under our conditions a rapid turnover of citrate and C_4 acids *via* the cycle, the Thunberg condensation and the Wood–Werkman reaction occurring to a minor extent. Under conditions of rapid citrate accumulation, as in the commercial production from sugars, it must be assumed that the major sources of C_4 acids would be the Wood and Werkman or the Thunberg reaction. From these results and those of other investigators it seems fairly certain that citric acid is not merely an end product of metabolism in molds, but is a normal intermediate which accumulates under certain circumstances because of some as yet unknown interference in its further metabolism.

Experimental

The mold used in this study was a commercial citric acid-producing strain of *Aspergillus niger*, number 337, obtained through the kindness of Dr. S. Beesch of the Publicker Industries, Philadelphia. After many trials the following procedure was found to give adequate, though not particularly uniform yields of citric acid when acetate was the only carbon source. Mats of mycelium were produced by inoculating spores from previous cultures of the organism on malt extract broth into 500 ml. of sterile 14% sucrose-salts medium[17] in 2-liter wide-mouth culture flasks. After standing at 20–24° for 7 to 13 days, the medium was drawn off and the mat of mycelium washed with six 100-ml. portions of distilled water, care being taken to maintain sterility and to avoid undue disintegration of the mat. All experiments were run with 10 mM. of labeled sodium acetate, but some contained, in addition, 20 mM. of glucose, in the presence of which citric acid production was enhanced. The substrates were added in 100 ml. of distilled water, and the flasks stoppered and allowed to stand undisturbed for 2 to 3 days. At approximately 10-hour intervals the air in the flask was displaced by fresh air, the metabolic carbon dioxide being collected by drawing the air through a bead tower containing carbon dioxide-free NaOH. This served the double purpose of renewing the oxygen supply and collecting the metabolic carbon dioxide for isotope analysis. After 2–3 days, when about 0.5 to 1.5 mM. of citrate had accumulated, the medium together with washings of the mycelium was acidified to 1 *M* with sulfuric acid and submitted to steam-distillation for collection of unused acetate and small quantities of ethanol and formic acid. The residual solution was extracted continuously with ether for at least 72 hours and citrate isolated from the extract. In some experiments it was obtained as the quinidine salt after preliminary purification *via* the silver salt. In others, it was isolated as the calcium salt after prior renewal of oxalate, essentially according to Lynen and Lynen.[18] The mycelial mat was separated into lipide and non-lipide portions by extraction by alcohol and ether.[6] Formate was separated from the acetate in the distillate by selective oxidation with mercuric ions[19] and alcohol was determined by oxidation to acetate. The isotopic acetate was labeled in the carboxyl carbon either with C^{13} or C^{14}; and in the methyl group with C^{14}. Preparation of the C^{13} COOH-labeled acetate and C^{13} analyses were carried out as previously described.[20] C^{14} activities were determined either with a flow counter or a thin window Geiger tube. Specific activities are reported as relative values, based on a value of 100 for the labeled acetate position, except where otherwise noted. The organic substances were oxidized to carbon dioxide by wet combustion, using either chromic–sulfuric acids, or persulfate.

Degradation Procedures.—The primary and tertiary carboxyls of citric acid were split off by treatment of the calcium or quinidine salt with concentrated sulfuric acid as already described.[20] In this procedure the primary carboxyl carbon comes off as carbon dioxide and the tertiary carboxyl as carbon monoxide. The mixture of gases was swept, by a stream of nitrogen, through a bead tower containing carbon dioxide-free sodium hydroxide to absorb the carbon dioxide; then over wire-form copper oxide at 300° where the carbon monoxide was quantitatively converted to carbon dioxide, and then absorbed in a second bead tower. The carbonates were then precipitated as barium carbonate by addition of barium chloride to the alkaline solution. The 3 central, non-COOH carbons were obtained as acetone (isolated as the Deniges complex) by oxidation with dilute dichromate solution.

Degradation of Acetone Obtained from Citrate Formed from Methyl-labeled Acetate.—Calcium citrate in the amount of 369 mg. was refluxed in 135 ml. of solution containing 35 ml. of 10% $HgSO_4$, 10 ml. of 50% H_2SO_4 and 10 ml. of 5% potassium dichromate for 90 minutes. The precipitated mercury–acetone complex was filtered off, dissolved in 20% HCl and the acetone distilled into ice-water and reprecipitated with mercuric sulfate yielding 523 mg. of Deniges complex.

A hydrochloric acid solution of 206 mg. of this complex was distilled into 20 ml. of 0.1 *N* iodine solution in 5 *M* NaOH. The iodoform was collected by centrifugation, washed thoroughly, and then converted to carbon dioxide by wet combustion. The solution and washings was acidified and treated with excess silver sulfate to remove excess iodine. One mM. of sodium acetate was added as a carrier and the acetate recovered and purified by repeated steam-distillation. A total of 1.23 mM. was recovered. This was converted to the barium salt by distilling the acid with steam and neutralizing the barium hydroxide. After thorough drying the salt was thermally degraded to acetone and barium carbonate. The salt was sealed into one arm of an inverted U-tube, which was attached to a high vacuum system and evacuated to a low pressure. The U tube was then closed and while the arm containing the barium acetate was heated to 400° for 1 hour in a furnace, the other arm of the U was cooled in liquid nitrogen so as to remove instantly the acetone formed by pyrolysis. After cooling, the tube was broken and the barium carbonate (91 mg.) counted as described above.

PHILADELPHIA, PENNA. RECEIVED OCTOBER 23, 1950

(17) Shu and Johnson, *Ind. Eng. Chem.*, **40**, 1202 (1948).

(18) Lynen and Lynen, *Ann.*, **560**, 149 (1948).

(19) Weinhouse and Millington, *J. Biol. Chem.*, **181**, 645 (1949).

(20) Weinhouse, Medes and Floyd, *ibid.*, **166**, 691 (1946).