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

Assimilation of Carbon Dioxide in Oxalate and Citrate by *Aspergillus Niger*

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When mycelial mats of *Aspergillus niger* are allowed to metabolize acetate or glucose in the presence of radioactive bicarbonate, assimilation of carbon dioxide occurs in citrate and oxalate; to a small extent in acetate, and negligibly in formate. The distribution of activity in the citrate carbons is what would be expected if assimilation of carbon dioxide occurs in the β-carboxyl of oxalacetate, followed by partial randomization of the activity between both carboxyls (probably by equilibration with fumarate). The data are in accord with the conception that oxalacetate splits to acetate and oxalate, but they also indicate that this is probably not the only source of oxalate. Neither direct oxidation of acetate nor splitting of oxalosuccinate appear to play a major role in oxalate biosynthesis; and coupling of formate appears to be eliminated from consideration.

Many pathways have been suggested for oxalate formation by fungi; for example, by direct oxidation of acetate through glycolic and glyoxylic acids,[2,3] by oxidation of hexoses or pentoses to 2-keto acids, followed by splitting off of oxalate,[4] by coupling of 2 molecules of formate[5,6] or by breakdown of the α-keto acids oxalacetate[3,7,8] or oxalosuccinate.[8] As yet, however, no definitive evidence for or against any single theory of oxalate formation has been advanced. In the course of experiments on citrate synthesis in *A. niger*[9] it was found that the citrate formed in the presence of labeled acetate was always accompanied by relatively large amounts of oxalate whose isotope content was similar in magnitude to that of the citrate. This finding established acetate as a precursor of oxalate but aside from emphasizing the close metabolic relationship between these three acids it otherwise shed little light on the mechanism of oxalate formation. To secure further information concerning the biosynthesis of oxalate, a series of experiments was carried out in which citrate and oxalate were formed by *A. niger* in the presence of C^{14}-labeled CO_2. It was expected that the extent of CO_2-assimilation in both products would yield information useful in establishing pathways of oxalate formation, and their relationships if any with citrate formation and breakdown.

Results

In all, eight experiments were carried out; four in which acetate, and four in which glucose was employed as substrate for citrate and oxalate formation. In each experiment approximately 3 g. (dry weight) of mycelium, well-washed to remove preformed acids and endogenous nutrients, was suspended in 100 ml. of water containing 10 mM. of substrate and about 2.5 mM. of radioactive sodium bicarbonate. The conditions and procedures of the experiments, their duration, and the methods of isolating the products were essentially the same as those described previously[9] and the yields of products were likewise the same as in the previous experiments; hence these data have been omitted. The isotopic data of all eight experiments are recorded in Table I. All of the values given represent the specific activities measured as barium carbonate and corrected, when necessary, for self-absorption. They are expressed as counts per minute per dish of 7.5 sq. cm. area, relative to an assumed value of 100 for the activity of CO_2 at the close of the experiments. The original actual activity (in counts per minute per dish) of the carbonate carbon was approximately 10,000, and during the course of the experiment it underwent a dilution of about tenfold by metabolic CO_2; so that the actual activity of the CO_2 at the close of the experiments was about 1000 counts/minute.

TABLE I

CO_2 ASSIMILATION BY *A. Niger* IN PRESENCE OF SODIUM ACETATE OR GLUCOSE. SPECIFIC ACTIVITIES ARE GIVEN AS COUNTS/MINUTE/DISH RELATIVE TO A VALUE OF 100 FOR THE METABOLIC CO_2 AT THE CLOSE OF THE EXPERIMENT

	Acetate Expt. no.				Glucose Expt. no.			
	1	2	3	4	1	2	3	4
Recovered acetate	1.9	0.6	0.3	0.4	0.4	..[a]	..[a]	2.4
Formate	0.0	0.02	0.0	0.6	0.0	..[a]	..[a]	..[a]
Oxalate	5.2	8.3	8.0	7.1	..[a]	7.5	5.2	9.5
Citrate	13.2	17.5	11.1	7.7	25.2	30.5	13.6	15.6
Primary COOH's	28.9	35.0	15.1	14.8	53.3	45.2	28.1	31.8
Tertiary COOH	19.4	28.9	18.8	14.5	47.8	37.8	25.0	30.4

[a] Not enough recovered for activity determination.

In all experiments assimilation of CO_2 was evident in both citrate and oxalate, and to a much smaller extent in acetate; but no appreciable activity was observed in formate. From 2 to 5% of the radioactive carbon originally added was assimilated in citrate and about 1% in oxalate.

Distribution of Assimilated Carbon Among Citrate Carboxyls.—A study of the distribution of C^{14} among the citrate carbons, carried out by the previously described chemical procedure,[9] revealed activity of approximately similar magnitude in both primary and tertiary carboxyls, in agreement with the recent results of Martin, Wilson and Burris.[10] In the eight experiments the ratio of primary to tertiary activity ranged from 0.8 to 1.5 with a mean

(1) Taken from the Ph.D. thesis of Katharine Lewis. Presented in part before the Biological Division of the American Chemical Society, Philadelphia, April, 1950. Aided by a grant from the National Cancer Institute, U. S. Public Health Service and an institutional grant from the American Cancer Society to the Institute for Cancer Research.

(2) F. Challenger, V. Subramanian and T. K. Walker, *J. Chem. Soc.*, **200**, 3044 (1927).

(3) F. F. Nord and J. C. Vitucci, *Arch. Biochem.*, **14**, 229 (1947).

(4) A. Allsopp, *New Phytologist*, **36**, 327 (1937).

(5) T. Chrzaszcz and M. Zakomorny, *Biochem. Z.*, **259**, 156 (1933).

(6) K. Bernhauer and F. Slanina, *ibid.*, **264**, 109 (1933); **274**, 97 (1934).

(7) H. Raistrick and A. B. Clark, *Biochem. J.*, **13**, 329 (1919).

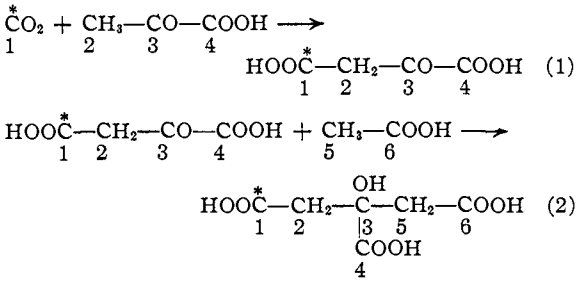
(8) F. Lynen and F. Lynen *Ann.*, **560**, 164 (1948).

(9) K. Lewis and S. Weinhouse, THIS JOURNAL, **73**, 2500 (1951).

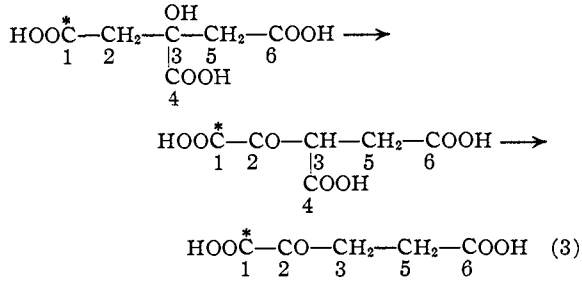
(10) S. M. Martin, P. W. Wilson and R. H. Burris, *Arch. Biochem.*, **26**, 103 (1950),

value of 1.1. Generally higher levels of activity were found in the citrate in those experiments in which glucose was employed as substrate.

"Asymmetry" of the Labeled Citrate.—If carboxylation of pyruvate represents a major pathway by which CO_2 is assimilated in citric acid according to the equations outlined, the labeled carbon would appear in only one of the two primary citrate carboxyls.



Although it was formerly thought impossible to make the distinction between the two primary carboxyls which was necessary to demonstrate the presence of activity in a particular primary carboxyl of citrate, Ogston[11] recently pointed out that a labeled citrate, formed enzymatically from labeled oxalacetate, would be expected to react asymmetrically in subsequent enzymatic reactions. This brilliant deduction was verified experimentally by Potter and Heidelberger,[12] who proved that citrate formed by rat liver in the presence of C^{14}, was broken down enzymatically to α-ketoglutarate with virtually all of the activity in the carboxyl adjacent to the keto group.



We have taken advantage of this possibility to determine the distribution of C^{14} in the primary carboxyls of some of the citrates isolated in these experiments. The method employed was that of Potter and Heidelberger, to whom we are indebted for supplying details of the degradation procedure before publication. Two degradations were performed with different samples of citrate: one obtained in an experiment in which acetate was employed as substrate, the other in which glucose was so employed. The distribution of activity in the α-ketoglutarates as given in Table II, shows that the preponderance of activity is in the carboxyl adjacent to the keto group. In one experiment 1029/(204 + 1029) = 83% of the activity was found in that carbon; and in the other experiment 679/(100 + 679) = 87% was in that position. We regard these data as leaving no doubt that carboxylation

(11) A. G. Ogston, *Nature*, **162**, 963 (1948).

(12) V. R. Potter and C. Heidelberger, *ibid.*, **164**, 180 (1949).

TABLE II

DISTRIBUTION OF C^{14} IN CITRATE PRIMARY CARBOXYLS AS DETERMINED BY ASYMMETRIC DEGRADATION OF α-KETOGLUTARATE. VALUES ARE SPECIFIC ACTIVITIES MEASURED AS $BaCO_3$

	Expt. number 1	2
α-Ketoglutarate 2,4-dinitrophenylhydrazone	647	364
CO_2 given off on oxidation with $KMnO_4$[a]	147	97
Estimated activity of α-carboxyl	1030	679
Succinic acid	51	25
Estimated activity of γ-carboxyl	204	100
Per cent. of total activity in α-carboxyl	83	87

[a] Ketoglutarate α-carboxyl plus 6 unlabeled hydrazine carbons.

of pyruvate represents a major if not exclusive pathway of CO_2 assimilation in *A. niger*.[13]

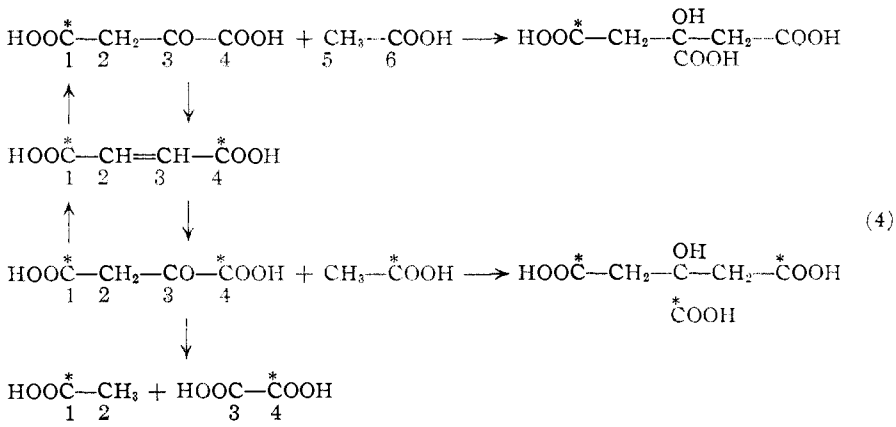
The remainder of the activity in the succinic moiety of α-ketoglutarate undoubtedly is present in the distal carboxyl, which is derived from the other (number 6) citrate primary carboxyl. The presence of a small amount of radioactivity in this position was not unexpected, inasmuch as some fixation was observed in acetate, whose carboxyl is the precursor of the citrate number 6 carbon (eq. 2).

Carbon Dioxide Assimilation in Oxalate.—As shown in Table I radioactive oxalate was obtained in every experiment, though the level of activity was below that in citrate. The substantial incorporation of CO_2 carbon in oxalate obtained from these experiments provides additional strong evidence for a close metabolic relationship between oxalate and citrate; possibly through a common precursor. At first glance it would appear that oxalacetate, though well-established as the gateway for CO_2 assimilation in citrate, cannot be a precursor of oxalate in the manner envisioned by Lynen[8] and others, namely, by splitting into oxalate and acetate. As shown in equation 1, labeled carbon appears in carbon 1 of oxalacetate, whereas oxalate would be derived from unlabeled carbons 3 and 4. Furthermore, reaction 2 with β-carboxyl-labeled oxalacetate would not give tertiary carboxyl-labeled citrate. It is possible, however, to bring the observations into harmony with the conception of oxalacetate as the precursor both of oxalate and citrate if we make the reasonable assumption that the β-carboxyl activity of oxalacetate becomes randomized in both carboxyls.[10] A possible mechanism for such randomization (see eq. 4) is equilibration of oxalacetate with the symmetrical C_4 acid, fumaric acid. Under these circumstances, as shown below, oxalacetate can be considered the source, not only of citrate, but of the labeled oxalate and acetate.

Although we have assumed thus far that CO_2-fixation occurs directly in the β-carboxyl of oxalacetate by way of the Wood–Werkman reaction it seems equally probable that fixation occurs in malate by way of the "malic" enzyme.[14] The

(13) Similar unpublished experiments on carbon dioxide fixation by Spirtes and Salles (quoted by Stern, Shapiro and Ochoa, *Nature*, **166**, 403 (1950)) with a different strain of *A. niger* and carried out under somewhat different experimental conditions gave results essentially the same as ours with respect to asymmetry of the citrate primary carboxyls.

(14) S. Ochoa, A. Mehler and A. Kornberg, *J. Biol. Chem.*, **167**, 871 (1947).



(4)

obligatory formation of malate during CO_2-fixation would explain more readily the randomization of the labeled carbon in terms of equilibration with fumarate. At present no evidence is available for a choice between these two reactions and a decision will probably have to await assays of the organism for the respective enzymes.

Inasmuch as the acetate carboxyl carbon is the one initially involved in the fixation reaction it might have been expected that fixation of CO_2 would be greater in acetate than in oxalate; however, all of the experiments were carried out under conditions in which a large dilution of acetate is unavoidable; that is, acetate was either present initially; or could be presumed to have been formed and utilized rapidly in those experiments in which glucose was used as a substrate.[9] Hence the relatively low activity of the recovered acetate cannot be taken to indicate that fixation of CO_2 was lower in acetate than in oxalate. The presumed equilibration of oxalacetate with fumarate evidently does not proceed to completion. As seen from Table I the primary citrate carboxyls have about the same activity as the tertiary carboxyl, and since nearly all of the primary carboxyl activity is in the number one carbon, its specific activity is twice that of the tertiary COOH (number 4 carbon), and hence the oxalacetate from which these carbons are derived must have had twice as much activity in the β-carboxyl as in the α-carboxyl.

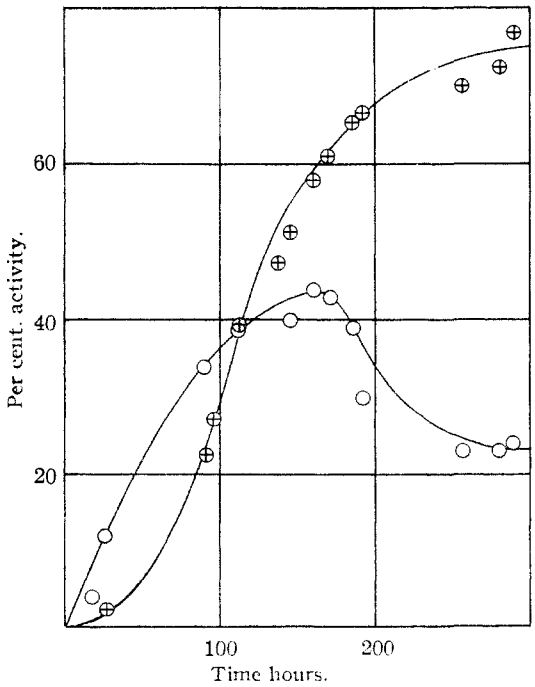
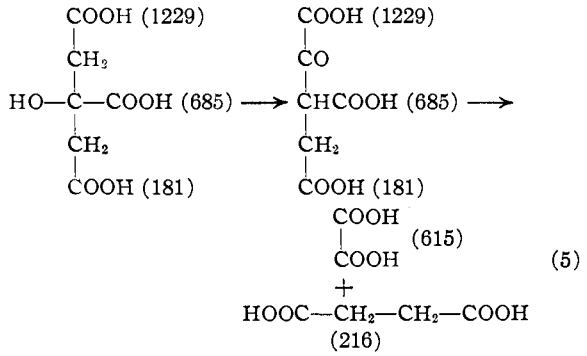


Fig. 1.—Evolution of tagged CO_2 from labeled citrate: crossed circles, per cent. of total activity liberated; open circles, specific activity of CO_2 relative to 100% in the labeled citrate.

Though all of the data thus far considered are in complete accord with the conception that oxalacetate is a direct source of oxalate, there is also good reason for the belief that this is not the only source of oxalate. On the basis of the reactions outlined above, the oxalacetate number 4 carbon is the precursor of both the citrate tertiary COOH and one of the oxalate carbons; hence the oxalate should have approximately one-half the activity of the citrate tertiary COOH. Of seven experiments (see Table I) in only two did the ratio reach the anticipated value of 0.5; in the others the ratio was in the neighborhood of 0.2 to 0.3. It seems highly probable, therefore, that other mechanisms than splitting of oxalacetate are functioning in oxalate formation.

Citrate as Precursor of Oxalate.—Recently Lynen and Lynen[8] suggested that oxalate may be formed by a hydrolytic splitting of both oxalosuccinic and oxalacetic acids. Although both reactions are reasonable chemically, the evidence presented in their favor seems hardly definitive, being based on the fact that citrate, succinate and acetate gave yields of oxalate proportional to the respective carbon contents; that is, in the ratio 3:2:1. Although this type of evidence suggests that there are common mechanisms for oxalate formation from the three substrates, no insight is provided concerning the chemical steps. In an attempt to answer more specifically the question whether oxalate is derived directly from citrate an experiment was carried out in which a thoroughly washed mycelial felt of *A. niger* was allowed to metabolize 1 mM. of labeled citrate. This material, obtained biosynthetically by $C^{14}O_2$-fixation, had an over-all activity of 349 counts/minute, with individual activities of 1230 and 181 c./m. in the respective primary carboxyls and 665 c./m. in the tertiary carboxyl. During 12 days at room temperature there was a slow, steady evolution of CO_2 at a rate of about 1.2 mM. per day. The specific activity of the CO_2 rose to a peak of 173 c./m. in 6 days, and dropped gradually thereafter to 83 c./m. after 12 days (Fig. 1). At the close of this experiment oxalate was isolated in the amount of 0.51 mM. with a specific activity of 89 c./m. This experiment illustrates the difficulties

in eliminating the endogenous metabolism of the molds. Despite reasonable efforts to wash out endogenous nutrients the average specific activity of the metabolic CO_2 was 114 c./m. which is only $^1/_3$ of that of the citrate carbons. Apparently endogenous substrates supplied the major quantity of carbon for complete combustion to CO_2. As shown in equation 5, if breakdown of citrate proceeded by way of oxalosuccinate to oxalate and



(5)

succinate the former would have an over-all specific activity of 615 as compared with an over-all activity of 216 for the succinate. Although interpretation is rendered somewhat ambiguous by the high endogenous metabolism, the fact that the oxalate activity of 90 c./m. is even less than that of the average of the metabolic CO_2 (114 c./m.) is taken to indicate that a direct conversion of oxalosuccinic to oxalic acid is not a major pathway of oxalate biosynthesis.[15]

Direct Oxidation of Acetate.—Although the relatively low activity in acetate isolated from these experiments as compared with that of oxalate apparently excludes acetate as the sole or even major precursor of oxalate, the possibility remains that a minor source of oxalate is a direct oxidation of acetate *via* glycolic and glyoxylic acids, as suggested by Challenger, *et al.*,[2] and more recently by Nord and Vitucci.[3] This process is now under investigation by us. The very low relative activity of formic acid isolated from these experiments quite definitely eliminates formate as a direct precursor of oxalate.[5,6]

(15) In a recent study of α-ketoglutarate metabolism in the wood-destroying molds *Trametes cinnabarina* and *Lentinus lepideus*, De Baun, Kudzin and Schubert, *Arch. Biochem.* **26**, 375 (1950), conclude that the conversion of this keto acid to oxalate occurs *via* succinate, fumarate and malate, in substantial agreement with the data of this paper.

Experimental

Enzymatic Degradation of Citric to α-Ketoglutaric Acid.—The procedure used for citrate breakdown was essentially that of Potter and Heidelberger.[12] A washed homogenate of rat liver was prepared by the method of Lehninger and Kennedy[16] and 2.0 ml. suspended in 3 ml. total volume of a *p*H 7.4 phosphate-buffered 0.1 *M* KCl solution containing Mg^{++} ions, 0.005 *M*, adenosine triphosphate, 0.001 *M*, arsenite, 0.001 *M* and 20 μM. of radioactive citrate. After incubation of this mixture at 37° in air for one hour, approximately 7 μM. of α-ketoglutarate is formed; the arsenite prevents further breakdown of the α-ketoglutarate, so that virtually all of the citrate which disappears under these conditions can be accounted for as the keto acid. In the degradation of labeled citrate the contents of 4 such flasks were combined, and after centrifugation, the supernatant solution and washings were treated with an excess of 2,4-dinitrophenylhydrazine. There was then added 100 mg. of non-isotopic α-ketoglutarate 2,4-dinitrophenylhydrazone as carrier and the mixture exhaustively extracted with ethyl acetate. The hydrazone was separated from the excess hydrazine by extraction with 10% sodium carbonate and was recovered by reëxtraction in ethyl acetate and evaporation to dryness. The residue was then crystallized 5 times to constant specific activity. Approximately 100 mg. of the hydrazone was oxidized to CO_2 and succinic acid by the procedure of Krebs.[17] The CO_2 was recovered in essentially quantitative yield by absorption in a bead tower with CO_2-free sodium hydroxide and was isolated by precipitation as barium carbonate. The residual solution was filtered from manganese dioxide, 60 mg. of non-isotopic succinic acid was added as carrier, and the solution extracted with ether. The succinate was recovered in pure form by successive precipitations as the silver salt and barium salt. Its activity was established by oxidation with persulfate and counting as barium carbonate. All activities given in Table II are corrected for added carrier.

(16) A. L. Lehninger and E. P. Kennedy, *J. Biol. Chem.*, **173**, 753 (1948).

(17) H. A. Krebs, *Biochem. J.*, **32**, 108 (1938).

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[CONTRIBUTION FROM THE CHEMOTHERAPY SECTION, NATIONAL CANCER INSTITUTE, NATIONAL INSTITUTES OF HEALTH]

Components of Podophyllin. V. The Constitution of Podophyllotoxin[1]

BY JONATHAN L. HARTWELL AND ANTHONY W. SCHRECKER[2]

New evidence is given for the location of the free hydroxyl group in picropodophyllin at C_1, as in formula II. Podophyllotoxin is formulated as a diastereoisomer, not a structural isomer, of picropodophyllin, differing only in the configuration around C_3. Podophyllotoxin chloride and bromide are described and their hydrolysis and ethanolysis studied. Two new diastereoisomers of podophyllotoxin are reported.

The finding that podophyllin N.F. exerts a strong destructive action against sarcoma 37 in mice[3] has led to the isolation of three active components, α-peltatin, β-peltatin and podophyllotoxin. The first two of these are new,[4] but podophyllotoxin has been known for seventy years[5] and has been the subject of many investigations. Certain aspects of the chemistry of the peltatins[4] have suggested to us that revision of the generally accepted structural formula for podophyllotoxin might be indicated. New experimental data have been obtained which are difficult to explain by the older structure. The evidence for a new formula forms the subject of this communication.

(1) This paper was presented in part before the Medicinal Chemistry Division of the American Chemical Society, in Philadelphia, April 10, 1950, and summarized in a Communication to the Editor (Paper IV, J. L. Hartwell and A. W. Schrecker, THIS JOURNAL, **72**, 3320 (1950)).

(2) Postdoctoral research fellow, National Cancer Institute.

(3) J. L. Hartwell and M. J. Shear, *Cancer Research*, **7**, 716 (1947).

(4) J. L. Hartwell and W. E. Detty, THIS JOURNAL, **72**, 246 (1950).

(5) V. Podwyssotzki, *Arch. exp. Path.*, **13**, 29 (1881).