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

Biosynthetic Pathways. III. The Biosynthesis of Lysine by *Torulopsis utilis*[1]

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RECEIVED NOVEMBER 17, 1952

Lysine isolated from yeast grown in glucose in the presence of tracer amounts of methyl- and carboxyl-labeled acetate had a distribution of activity which indicated that its carbon chain is built up through a combination of acetate with the succinate moiety of α-ketoglutarate. Similar experiments with methylene- and carboxyl-labeled glycine, carboxyl-labeled lactate, and formate are in accord with this assumption. Two possible mechanisms are suggested for the formation of the lysine carbon chain: (1) the methyl group of acetate may condense with the carbonyl carbon of an unsymmetrical succinate derivative such as succinyl–coenzyme A, or (2) the acetate methyl carbon may condense with the carbonyl carbon of α-ketoglutarate to give a 7-carbon homolog of citric acid, which by undergoing a sequence of reactions analogous to those of the citric acid cycle, could yield the lysine precursor, α-aminoadipic acid.

During the past several years we have been conducting experiments designed to explore biosynthetic pathways in fungi.[3,4] The experimental procedure consists in growing yeast (*Torulopsis utilis*) on glucose as essentially the only carbon source together with trace quantities of various labeled substances. By isolating and degrading products synthesized by the yeast so as to determine the distribution of labeled carbon therein, it is possible to obtain clues to mechanisms employed for their biosynthesis. In a preliminary note[5] evidence was reported from such studies indicating that the lysine carbon skeleton was derived by condensation of acetate with either α-ketoglutarate or a succinyl derivative. In the present communication these findings are described in further detail, and possible mechanisms are discussed in the light of present and previous studies of this problem.

Experimental

Details of the procedures employed in the cultivation of the strain of *Torulopsis utilis* used in these experiments, the radioactivity assays and in the isolation of the yeast amino acids are given in previous publications.[3,4] The hydrolysates from which the lysine was isolated were the same ones used for isolation of arginine, described in the previous study.[4] The mixture of amino acids was purified by Neuberg's mercuric salt precipitation method[6] and tyrosine was removed by precipitation; the dicarboxylic acids were precipitated as their barium salts from alcohol, and after removal of histidine as its Ag salt and arginine as its flavianate, lysine was precipitated, according to the method of Block and Bolling[7] by addition of a concentrated solution of phosphotungstic acid (15 g. in 50 ml. of 5% H_2SO_4). The lysine phosphotungstate was washed with a 2% solution of phosphotungstic acid in 5% H_2SO_4 and was decomposed by suspending it in 40 ml. of 1 *N* HCl and extracting the suspension several times with 50 ml. of an amyl alcohol–ether–ethanol solution. The aqueous layer was evaporated to dryness under reduced pressure and the residue was dissolved in 2 ml. of hot absolute ethanol. Lysine monohydrochloride was precipitated from the alcohol solution by the addition of 2 drops of pyridine and was allowed to crystallize in a refrigerator overnight. Yields of lysine monohydrochloride in the various experiments ranged from 65–140 mg. The purity of each sample was established by paper chromatography and in each case the lysine was found to be free from other amino acids.

The activities of the lysines, given in Table I, show that of the various labeled compounds tested, acetate is the most efficient supplier of lysine carbon and it can be seen that the carboxyl and methyl of acetate contribute to lysine carbon to about the same extent. The methylene group of glycine is incorporated into the lysine molecule to a much lesser degree and the carboxyls of glycine, lactate and formate appear to be incorporated in only relatively insignificant amounts.

TABLE I

ACTIVITIES OF LYSINE SAMPLES SYNTHESIZED BY TORULOPSIS UTILIS IN THE PRESENCE OF VARIOUS LABELED SUBSTRATES

Activities of lysine monohydrochloride are given in counts per minute per 7.5 sq. cm. dish at "infinite thickness"

Labeled substrate	Position of label	Activity of lysine, c./min.
Acetate	Carboxyl	7135
Acetate	Methyl	6380
Glycine	Methylene	1530
Glycine	Carboxyl	50
Lactate	Carboxyl	42
Formate	.....	38

Degradations Employed in Ascertaining Distribution of C^{14} Among Lysine Carbons.—The reactions by which the lysine carbon skeleton was broken down are shown in the following equations. In reaction 1, oxidation with dilute

$$\overset{6}{H_2N}\overset{5}{C}H_2\overset{4}{C}H_2\overset{3}{C}H_2\overset{2}{C}H\overset{1}{N}H_2COOH \longrightarrow H_2N\overset{6}{C}H_2\overset{5}{C}H_2\overset{4}{C}H_2\overset{3}{C}H_2\overset{2}{C}OOH + \overset{1}{C}O_2 \quad (1)$$

$$\overset{6}{H_2N}\overset{5}{C}H_2\overset{4}{C}H_2\overset{3}{C}H_2\overset{2}{C}OOH \longrightarrow H_2N\overset{6}{C}H_2\overset{5}{C}H_2\overset{4}{C}H_2\overset{3}{C}H_2\overset{2}{N}H_2 + \overset{2}{C}O_2 \quad (2)$$

$$\overset{6}{H_2N}\overset{5}{C}H_2\overset{4}{C}H_2\overset{3}{C}H_2CH_2NH_2 \longrightarrow \overset{6}{H}\overset{5}{O}\overset{4}{O}\overset{3}{C}CH_2CH_2COOH \longrightarrow \overset{3\&6}{CO_2} + H_2NCH_2CH_2COOH \quad (3)$$

$$\overset{6}{H_2N}\overset{5}{C}H_2\overset{4}{C}H_2\overset{3}{C}H_2\overset{2}{C}H\overset{1}{N}H_2COOH \longrightarrow HO\overset{6}{O}\overset{5}{C}\overset{4}{C}H_2\overset{3}{C}H_2\overset{2}{C}OOH + \overset{1}{C}O_2 \quad (4)$$

$$HO\overset{6}{O}\overset{5}{C}\overset{4}{C}H_2\overset{3}{C}H_2\overset{2}{C}OOH \longrightarrow \overset{2\&6}{CO_2} + \overset{5}{N}H_2\overset{4}{C}H_2\overset{3}{C}H_2CH_2NH_2 \quad (5)$$

$$\overset{6}{H_2N}\overset{5}{C}H_2\overset{4}{C}H_2\overset{3}{C}H_2\overset{2}{C}H\overset{1}{N}H_2COOH \longrightarrow \overset{1}{CO_2}\text{ (ninhydrin)} \quad (6)$$

(1) This work was done under contract with the U. S. Atomic Energy Commission (Contract No. AT(30-1)777) and was aided by grants from the American Cancer Society and the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service.

(2) This work will be included in a thesis to be submitted by Murray Strassman to the Graduate School of Temple University in partial fulfillment of the requirments for the Ph.D. degree.

(3) S. Weinhouse, R. H. Millington and M. Strassman, THIS JOURNAL, **73**, 1421 (1951).

(4) M. Strassman and S. Weinhouse, *ibid.*, **74**, 1726 (1952).

(5) M. Strassman and S. Weinhouse, *ibid.*, **74**, 3457 (1952).

(6) C. Neuberg and J. Kerb, *Biochem. Z.*, **40**, 498 (1912).

(7) R. J. Block and D. Bolling, "The Amino Acid Composition of Proteins and Foods," C. C. Thomas, Springfield, 1945, p. 287.

permanganate in acid solution yielded the lysine carboxyl carbon as CO_2 and the remaining five carbons as δ-aminovaleric acid, which was isolated by chromatography on Dowex 50.[8] This compound was degraded by the Schmidt reaction[9] to CO_2, representing carbon 2 of lysine, and 1,4-diaminobutane, representing carbons 3 to 6. The diaminobutane was not isolated, but was directly oxidized to succinic acid, which was decarboxylated by the Schmidt reaction to yield CO_2, representing carbons 3 and 6 of lysine. Carbons 4 and 5 were obtained by difference between the succinate activity and the activity of its carboxyl carbons. Another sample of lysine was oxidized with hot acid permanganate, yielding glutaric acid which, on decarboxylation by the Schmidt reaction, yielded CO_2, representing lysine carbons 2 and 6. The activity of carbon 6 could be calculated, since carbon 2 activity was obtained independently in reaction 2, and carbon 3 activity was obtained by calculation from the activity of carbon 6 and the activity of carbons 3 and 6 obtained in reaction 3. By these means, separate values were obtained for the activities of carbons 1, 2, 3 and 6, and an average value for carbons 4 and 5. The procedures to be described were adopted after considerable testing with respect to maximum yield and reproducibility.

Degradation of Lysine–Oxidation to δ-Aminovaleric Acid.—Portions of the original samples were diluted with enough non-isotopic carrier lysine monohydrochloride to provide sufficient material of high enough activity to carry out the degradation (about 1–2 mM. with activity of 150–300 c./min.). The diluted material was recrystallized frome thanol to a constant activity, which in each instance corresponded closely to the value calculated from the original activity.

Approximately 2 mM. of the diluted lysine monohydrochloride was dissolved in 40 ml. of water in a 100-ml. 3-neck flask carrying a dropping funnel, a lead-in tube extending to the bottom, and a condenser surmounted with a tube leading to an absorption tower filled with glass beads. Ten ml. of 0.5 *N* CO_2-free NaOH was placed in the bead tower and 6 ml. of 9 *N* H_2SO_4 was added to the flask. After the slow addition of 12.2 ml. of 1.5 *N* $KMnO_4$ the system was shut off from the atmosphere by clamping the lead-in tube and the tube leading from the bead tower, and the solution was allowed to stand for 2 days. After this interval the CO_2 was collected in the bead tower with a current of air and the carbonate precipitated with 20% $BaCl_2$ solution.

The reaction mixture was filtered free of MnO_2, diluted to 150 ml. and heated to boiling; 130 ml. of saturated $Ba(OH)_2$ solution was then added until a *p*H of 10–11 was attained. The precipitate of $Mn(OH)_2$ and $BaSO_4$ was removed and washed thoroughly with hot water. The solution and washings were evaporated to about 60 ml. and CO_2 was slowly bubbled into it. The $BaCO_3$ was filtered off and the clear solution neutralized to *p*H 4–5 with 9 *N* H_2SO_4. The solution was evaporated to a small volume and sufficient water and concentrated HCl were added to give 24 ml. of a solution 1.5 *N* with respect to HCl. The acid solution was placed on a column of Dowex 50, 3.5 cm. in diameter and 45 cm. long. The solution was washed into the resin with several 20-ml. portions of 1.5 *N* HCl and the column was eluted with HCl of the same normality at a rate of 90–100 ml. an hour at atmospheric pressure. Fractions of approximately 20 ml. were collected and those (usually between numbers 70 and 90) containing δ-aminovaleric acid, as tested by paper chromatography, were combined and evaporated to dryness under reduced pressure.

The residue thus obtained, without further purification, had a melting point of 92–93° (recorded for δ-aminovaleric acid hydrochloride, 92–94°)[10] and on a paper chromatogram gave only one spot identical with that for known δ-aminovaleric acid. The yields were 50 to 60%.

Decarboxylation of δ-Aminovaleric Acid.—The degradation by means of the Schmidt reaction was carried out essentially as described previously by Phares.[9] δ-Aminovaleric acid hydrochloride in an amount of 1 to 1.3 mM. was dissolved in 1.0 ml. of cold 100% H_2SO_4 in a 25-ml. pear-shaped flask and 170 mg. of powdered NaN_3 were added in small amounts, with shaking, to the chilled solution. The flask was immediately connected to two bead towers in series, the first containing 10 ml. of 50% $KMnO_4$ in 1 *N* H_2SO_4 and the second containing 10 ml. of 0.5 *N* CO_2-free NaOH. A slow stream of CO_2-free air was drawn through the solution while the flask was heated to 55–65° for 4–5 hours. The bead tower containing the NaOH was washed down and the carbonate precipitated by addition of $BaCl_2$. The $BaCO_3$ produced in this step represents the α-carbon of lysine.

Oxidation of Diaminobutane to Succinic Acid.—The 1,4-diaminobutane produced in the Schmidt reaction was not isolated as such but was oxidized directly to succinic acid. The reaction mixture from the previous step was dissolved in 25 ml. of water in a 100-ml. 3-neck flask, fitted as previously described, the *p*H was adjusted to 10–11 by addition of NaOH and 15 ml. of 1.5 *N* $KMnO_4$ was added. The solution was warmed to 55° and maintained at this temperature for 4 hours, after which 45 ml. of 5 *N* H_2SO_4 was added and the solution extracted continuously with ether for 48 hours. The ether solution was evaporated to dryness, the residue was taken up in 15 ml. of water, neutralized to a brom cresol blue end-point with dilute NH_4OH, and the silver salt precipitated by addition of 3 ml. of 10% silver nitrate. The silver succinate was decomposed by suspending in water and passing H_2S into the heated solution. The succinic acid obtained on evaporation invariably had the correct melting point, which was not depressed by admixture with authentic succinic acid.

The succinic acid was assayed for radioactivity and then diluted with an equal amount of normal succinic acid to yield sufficient material for the subsequent decarboxylation.

Decarboxylation of Succinic Acid.—The procedure employed for this step was the same as already described for the decarboxylation of δ-aminovaleric acid. Sixty mg. of succinic acid was dissolved in 0.6 ml. of cooled 100% H_2SO_4 and 100 mg. of powdered NaN_3 was added in small portions to the chilled solution. The flask was connected to the traps as before and, with a slow stream of air being drawn through it, was heated at 60° for 1.5 hours, then at 72° for 3 hours. The CO_2 representing the succinate carboxyls was collected in alkali, as described above, precipitated as $BaCO_3$, and assayed. Activity of the methylene carbons was calculated by difference between the activity of the carboxyl carbons and the over-all activity of the four succinate carbons.

Oxidation of Lysine to Glutaric Acid.—Between 1.5 and 2 mM. of lysine monohydrochloride was dissolved in 150 ml. of water in a 300-ml. 3-neck flask. Twenty ml. of 9 *N* H_2SO_4 was added, the solution was heated in a bath of boiling water and a few drops of 1.5 *N* $KMnO_4$ were added. When the reaction began the temperature of the bath was lowered to 85° and a total of 20 ml. of 1.5 *N* $KMnO_4$ was slowly added over a period of 1 hour. The solution was kept at this temperature for one hour longer and after cooling it was extracted continuously for 48 hours. The extract was evaporated to dryness, the residue was taken up in 15 ml. of water, neutralized to a brom cresol blue end-point with ammonia and the silver glutarate precipitated by addition of 3 ml. of 10% silver nitrate solution. The silver salt was decomposed with H_2S and the glutaric acid, isolated by evaporating the solution to dryness, was recrystallized from 1 ml. of hot benzene, with only very slight loss. The crystalline product thus obtained had the correct melting point, which was not lowered by mixture with pure glutaric acid.

The product was diluted with an equal weight of carrier glutaric acid and the activity of the diluted material was determined. This value corresponded closely with the sum of the activities determined for the α-carbon and the succinate moiety of the lysine.

Decarboxylation of Glutaric Acid.—This step was accomplished by means of the Schmidt reaction in the manner described in the two previous decarboxylations. One-third mmole of glutaric acid was dissolved in 0.4 ml. of 100% H_2SO_4 and 67 mg. of NaN_3 was added to the chilled solution. With a slow stream of air being drawn through it, the solution was heated to 55° for 3 hours and at 70° for one hour; the CO_2 representing the glutarate carboxyls was collected as $BaCO_3$ and assayed for its radioactivity.

Decarboxylation of Lysine with Ninhydrin.—About two-thirds mmole of lysine monohydrochloride was dissolved in

(8) W. Stein and S. Moore, *Cold Spring Harbor Symposia Quant. Biol.*, **14**, 279 (1949). We are greatly indebted to Drs. Stein and Moore for aid in chromatography procedures.

(9) E. F. Phares, *Arch. Biochem. Biophys.*, **33**, 173 (1951). We are indebted to Dr. Phares for supplying details of this method prior to publication.

(10) L. E. Schniepp and C. S. Marvel, THIS JOURNAL, **57**, 1557 (1935).

35 ml. of water in a 100-ml. 3-neck flask, set up for collecting CO_2, as already described. Concentrated H_3PO_4, 0.15 ml., was added, and after chilling the solution 350 mg. of ninhydrin was added. While drawing a slow stream of air through the solution the flask was heated in a bath of boiling water for one hour. The CO_2 was collected, precipitated with $BaCl_2$ and the $BaCO_3$ assayed.

Results and Discussion

The activity data from the carboxyl- and methyl-labeled acetate, and the methylene-labeled glycine experiments are presented in Table II. To facilitate comparison, the specific activities of the individual carbons are given in percentages of the over-all specific activity of all of the lysine carbons. Since the incorporation of glycine, lactate and formate carboxyls into lysine was very low (Table I), the lysine samples obtained in these experiments were not degraded.

TABLE II

DISTRIBUTION OF ACETATE CARBONS IN LYSINE

Values are specific activities based on 100 for over-all activity of lysine

Lysine carbon number[a]	Precursor: Acetate methyl Obsd.	Acetate methyl Calcd.	Acetate carboxyl Obsd.	Acetate carboxyl Calcd.	Glycine methylene Obsd.	Glycine methylene Calcd.
1	18	0	360	300	170	100
2	206	150	0	0	103	100
3	121	150	7	0	120	100
4	116	150	−11	0	81	100
5	116	150	−11	0	81	100
6	7	0	226	300	137	100

[a] Numbering begins with carboxyl carbon.

From the observed distribution of activity in the various lysines shown in Table II, two points of interest are evident. First, the carboxyl of lysine from the carboxyl-labeled acetate experiment had high activity, whereas no activity was found in its α-carbon. Conversely the α-carbon of lysine from the methyl-labeled acetate experiment had high activity whereas comparatively low activity was present in the carboxyl position. It therefore appeared that an intact acetate molecule was incorporated in the lysine carboxyl and α-carbons. These conclusions are in accord with previously published data on lysine synthesis in yeast. Ehrensvaard, *et al.*,[11] noted a high incorporation of acetate carboxyl carbon in the carboxyl carbon of lysine synthesized by *Torulopsis* yeast and suggested that an intact acetyl group may be incorporated in the lysine carboxyl and α-carbons. Gilvarg and Bloch[12] also observed a relatively high and equal incorporation of acetate carboxyl and methyl carbons in lysine synthesized by yeast.

The second pertinent feature is that the distribution of activity in carbons 3, 4, 5 and 6 of lysine was very close to that expected in the succinate moiety of α-ketoglutarate arising from acetate by way of the citric acid cycle. The theoretical distribution of acetate carbons in ketoglutarate formed *via* the citric acid cycle has been discussed in a previous publication,[4] and is further outlined in Fig. 1, in which the acetate methyl carbons are in boldface type and the carboxyl carbons in ordinary type. It can easily be seen by tracing labeled acetate through successive rounds of the citric acid cycle that ultimately in the steady state of acetate oxidation two types of oxalacetic acid are formed, one whose β-carboxyl is representative of the carboxyl of acetate, and whose other three carbons are formed from the methyl of acetate; and the other whose β-carboxyl and two middle carbons are produced from the methyl of acetate and whose α-carboxyl is representative of the carboxyl of acetate. These two forms of oxalacetate, as shown in Fig. 1, can condense with acetate to produce two types of citrate, and ultimately two types of α-ketoglutarate, A and B. Both of these forms, on the loss of the α-carboxyl, will produce succinate. Because of the molecular symmetry of succinate, only one labeled species of this acid is formed. On oxidation through malate, however, *two* forms of labeled oxalacetate are regenerated because of the equal probability that oxidation will take place on a fumarate methine carbon adjacent to or one carbon removed from a methyl-labeled carboxyl carbon.

Because there is equal probability of the formation of types A and B of labeled α-ketoglutarate, its C^{14}-distribution will be represented by the average of the two forms. Thus, equal numbers of acetate methyl and carboxyl carbons will be present in the α-carboxyls; and there will be twice as many acetate carboxyl carbons in the γ-carboxyls than in the α-carboxyls, but no acetate methyl carbons in the γ-carboxyl. There will be no acetate carboxyl carbons in positions 2, 3 and 4, but there will be equal numbers of acetate methyl carbons in these positions. On the basis of an over-all specific activity of 100 for α-ketoglutarate, the distribution pattern for its five carbons produced from methyl-labeled acetate will be 72, 143, 143, 143 and 0, respectively, and from carboxyl-labeled acetate will be 167, 0, 0, 0 and 333, respectively. The essential correctness of this picture has been borne out in a previous study[4] in which it was found that the carbon skeleton of arginine, whose synthesis is generally considered to proceed through glutamate, had an activity distribution closely corresponding to these calculated ones. In examining the activity distributions in the lysine we were struck by the close similarity of carbons 3 to 6 with the activity distributions in the corresponding carbons 2 to 5 of the α-ketoglutarates. Thus, there were high and equal activities in carbons 3 to 5 and very little in carbon 6 of lysine from methyl-labeled acetate, and none in carbons 3 to 5 and high activity in carbon 6 of lysine from carboxyl-labeled acetate. In Table II the observed values are accompanied by values calculated for lysine on the assumption that of its six-carbon chain, carbons 1 and 2 are derived from an acetate carboxyl and methyl carbon, and carbons 3 to 6 are derived from carbons 2 to 5 of α-ketoglutarate. Despite some discrepancy, the distribution patterns are strikingly similar. The higher-than-calculated activities in lysine carbons 1 and 2 are probably due to incomplete equilibration of acetate with citric acid cycle components.

(11) G. Ehrensvaard, L. Reio, E. Saluste and R. Stjernholm, *J. Biol. Chem.*, **189**, 93 (1951).

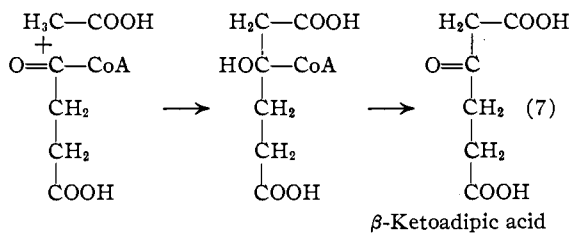
(12) C. Gilvarg and K. Bloch, *J. Biol. Chem.*, **193**, 339 (1951). In private communications these investigators have informed us that their results with respect to distribution of acetate carbons in lysine are in essential agreement with those reported herein.

Since the calculations were made assuming such an equilibrium carbons 3, 4 and 5 in lysine from the methyl-labeled acetate experiment and carbon 6 in lysine from the carboxyl-labeled acetate experiment have lower than expected values. The existence of a small amount of activity in carbons 1 and 6 of lysine derived from methyl-labeled acetate is probably due to recycling whereby methyl activity migrates to the carboxyl of acetate.

Mechanism of Formation of Lysine Carbon Skeleton.—Inasmuch as succinate is a symmetrical molecule, the possibility that the lysine carbon skeleton is built up by condensation of acetate with succinate as such has to be rejected. If this occurred the activities of positions 3 and 6 of lysine would have been randomized owing to the fact that both succinate carboxyls are equivalent. The asymmetric distribution of activity in carbons 3 to 6 of lysine indicates that carbon 3 could have arisen from carbon 2 of ketoglutarate but could not have arisen from both positions 2 and 5.

Two possibilities remain to be considered; either an unsymmetrical derivative of succinate is involved, or condensation occurs with α-ketoglutarate, followed by loss of the α-carboxyl. Regarding the first possibility, the thiolester of coenzyme A and succinic acid (succinyl–CoA) is now recognized to be a direct product of the oxidation of α-ketoglutarate.[13,14] The participation of an unsymmetrical derivative of succinic acid has already been suggested in the synthesis of the porphyrins[15] and the principal reason for its consideration was a situation similar to that encountered in the present study, *viz.*, the necessity of accounting for the unsymmetrical distribution of succinate carbon in the protoporphyrin structure.

It is possible that a condensation of acetate (or acetyl–CoA) with succinyl–CoA might yield β-ketoadipic acid as shown in equation 7.



However, it is difficult to visualize a reasonable biochemical mechanism for conversion of this product to a logical precursor of lysine.

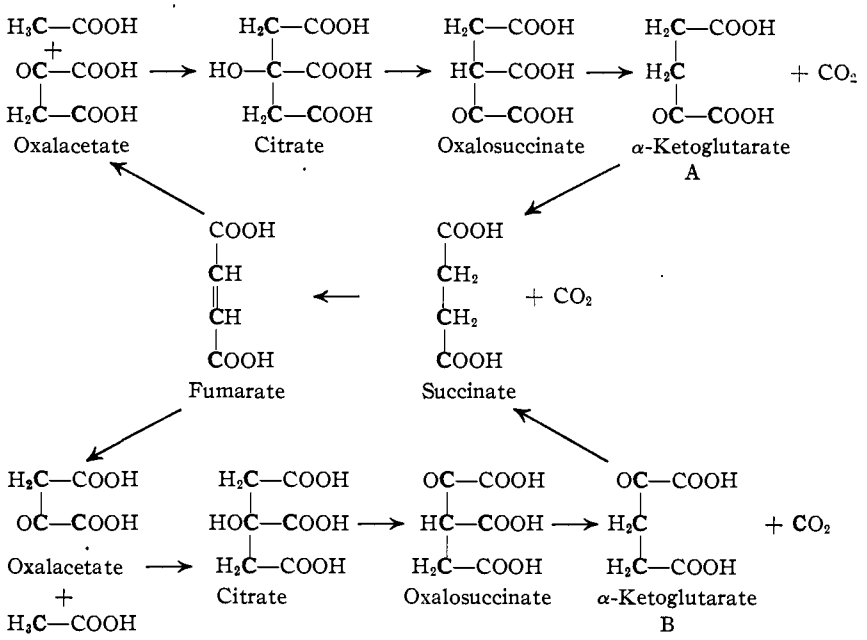
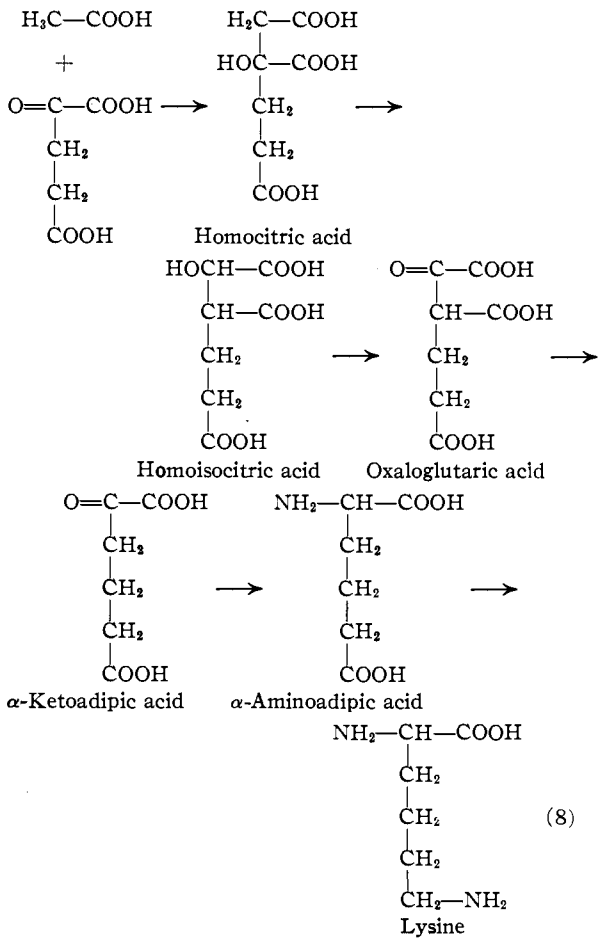


Fig. 1.—Distribution of acetate carboxyl and methyl carbons in intermediates of citric acid cycle.

A more likely, though highly speculative possibility, is that an acetyl methyl carbon condenses with the carbonyl carbon of α-ketoglutarate in the same manner that acetate condenses with oxalacetate to yield citrate (equation 8).



(13) S. Kaufman, Abstracts, Amer. Chem. Soc. Meeting, Atlantic City, N. J., Sept., 1952, p. 32c.
(14) D. E. Green, *ibid.*, p. 31c.
(15) D. Shemin, *ibid.*, p. 35c.

In this instance the product would be a 7-carbon analog of citric acid. In the same manner that citrate yields α-ketoglutarate through the intermediary formation of *cis*-aconitate, isocitrate and oxalosuccinate, this 7-carbon "homocitrate," by an analogous reaction sequence, should yield α-ketoadipic acid. Though none of the suggested 7-carbon tricarboxylic acids are known, the plausibility of this mechanism is heightened by the fact that α-aminoadipic acid, which bears the same relationship to α-ketoadipate as glutamate does to α-ketoglutarate, is already known to be a precursor of lysine in certain microorganisms. Mitchell and Houlahan[16] have shown that α-aminoadipic acid can replace lysine for growth of a lysine-requiring mutant of Neurospora; and this observation has been confirmed by Windsor[17] who demonstrated the direct conversion of ε-C^{14}-labeled aminoadipate to lysine in this mutant. Presumably the transformation of α-aminoadipate to lysine involves a sequence of reactions analogous to that involved in the conversion of glutamate to ornithine.

Incorporation of Methylene Carbon of Glycine in Lysine.—It was shown in earlier papers that the methylene carbon of glycine in these same experiments was incorporated into fatty acids[3] and into the ornithine moiety of arginine[4]; and the distribution of the activity in these substances was consonant with their formation from acetate labeled approximately equally in both carbons. On the assumption that the glycine α-carbon is incorporated in both acetate carbons we would expect the lysine to have a distribution of activity approximating that of a mixture of lysines produced from the separately labeled acetates. The results were in good accord with this expectation; as shown in Table II. The activity distribution in this experiment was fairly close to the average of the distributions in lysine of the carboxyl and methyl carbons of acetate.

Incorporation of Lactate, Glycine and Formate Carboxyl Carbons into Lysine.—By referring to Fig. 1 it can be seen that no direct route exists by which the carboxyl of lactate can enter α-ketoglutarate. Fixation of radioactive CO_2 by carboxylation of pyruvate should produce oxalacetate labeled in its β-carboxyl and this would yield α-carboxyl-labeled ketoglutarate. Since this carbon of ketoglutarate does not enter the succinate moiety but is lost by decarboxylation, it cannot be incorporated into the lysine molecule according to either of the mechanisms proposed for lysine formation.

(16) H. K. Mitchell and M. B. Houlahan, *J. Biol. Chem.*, **174**, 883 (1948).

(17) E. Windsor, *J. Biol. Chem.*, **192**, 595 (1951).

The low activity observed in the lysine may be explained by two reactions which can conceivably occur to a small extent. First, it is possible that oxalacetate produced by CO_2-fixation can be directly reduced to succinate, through malate and fumarate. Second, it is possible that oxalacetate may break down to oxalate and acetate.[18] Fixation of labeled CO_2 would thus lead to formation of labeled acetate which can enter the succinate moiety of α-ketoglutarate via the citric acid cycle. The extremely low incorporation of lactate carboxyl in lysine (Table I) is thus in full accord with the mechanisms postulated. Similar considerations apply to the low incorporation of glycine carboxyl and of formate carbon. Glycine carboxyl carbon could enter the carboxyl of pyruvate *via* serine[3] or could enter oxalacetate *via* CO_2-fixation; and formate carbon likewise could enter the cycle *via* transformation to carboxyl and methyl-labeled acetate by way of α- and β-labeled serine and pyruvate.[19,20]

It would appear from present information that two biosynthetic pathways for lysine exist. Work[21] has shown the presence of α,ε-diaminopimelic acid in *Escherichia coli*, and Dewey and Work[22] have recently shown the presence of a decarboxylase in certain strains of this microörganism which converts this dicarboxylic acid to lysine. The absence of this enzyme from lysine-requiring mutants suggested that diaminopimelic acid may be a precursor of lysine in *E. coli*. Positive evidence for this role of diaminopimelic acid has been provided by Davis[23] in a study of the requirements of this amino acid and of lysine in a number of lysine auxotrophs of *E. coli*. Davis has pointed out, on the basis of the findings of Mitchell and Houlahan,[16] that *Neurospora crassa* differs from *Escherichia coli* in its pathway of lysine synthesis. The present study is in accord with this conception and suggests, moreover, that *Torulopsis utilis* resembles *Neurospora* in this respect more closely than *E. coli*. It is recognized, however, that the present results are not decisive with respect to mechanism. It is hoped that extensions of these studies, which are now under way, to other substrates and to other organisms such as *E. coli* will further clarify our picture of lysine biosynthesis.

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(18) K. F. Lewis and S. Weinhouse, THIS JOURNAL, **73**, 2500, 2906 (1951).

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