THE FORMATION OF RADIOACTIVE LYSINE AND HISTIDINE FROM $CH_3C^{14}OOH$ AND $CH_3C^{14}O\ COOH$ IN YEAST

by

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TABLE OF CONTENTS

TABLE

THE FORMATION OF RADIOACTIVE LYSINE AND HISTIDINE FROM $CH_3C^{14}OOH$ AND $CH_3C^{14}O\ COOH$ IN YEAST

INTRODUCTION

The origin of lysine and histidine in living systems though studied for many years is still without complete elucidation. Amino acids in general have been shown (13) to arise from hydroxy- or keto analogues by transamination but the biosynthesis of lysine and histidine from simple one, two, and three carbon compounds is as yet unknown.

LYSINE. Nutritional studies (22) employing analogues of lysine have shown that α-hydroxycaproic acid, ε-aminocaproic acid, and α-hydroxy-ε-amino caproic acid are ineffective in relieving a lysine deficiency in rats. The discovery by Borsook (3; 4) that α-aminoadipic acid is a metabolic degradation product of lysine in guinea pig liver homogenates led Geiger and Dunn (14) to investigate the replacement of lysine by α-aminoadipic acid in the nutrition of rats and various microorganisms. In no instance did these workers find that α-aminoadipic acid could promote growth in place of lysine. However, in certain mutant strains of Neurospora it has been shown (23) that α-aminoadipic acid is equivalent to lysine for growth whereas α-ketoadipic acid is inactive.

Isotopic tracers have provided a new means of studying the problem starting with simple organic compounds ordinarily found in biological systems. Ehrensvärd and co-workers (11; 12) have used $C^{13}H_3C^{14}OOH$ as the simple carbon source administered to metabolizing Torulopsis utilis (Torula yeast). The ratio of C^{14} to C^{13} in the lysine molecule recovered from the protein hydrolysate was found to be 0.76 while the same ratio in the fatty acid fraction was found to be 0.80. The authors suggested that lysine may be connected with fat metabolism in yeast. This same group, using Escherichia coli as the test organism (10), has obtained results showing a much lower C^{14} to C^{13} ratio for lysine than found in Torulopsis. Gilvarg and Bloch (16) report very high incorporation of isotopes in the lysine molecule using Saccharomyces cerevisiae grown on $C^{14}H_3C^{13}OOH$.

HISTIDINE. The structures of histidine and arginine may be written to show a great degree of similarity. The possibility that these two compounds might be interconvertible was investigated (1; 15) and a tentative affirmative answer was given. However, it appears that these results were probably influenced by arginine contamination as brought out by Harrow and Sherwin (17) who found that methods available at that time were inadequate for removal of arginine. Subsequent work by Cox

and Rose (7) based on comparative growth rates in rats, completely ruled out any interconvertibility between arginine and histidine.

A number of analogues of histidine have been tried for growth promoting properties (8; 9; 17; 24) but among these only imidazole pyruvic acid and imidazole lactic acid displayed any ability to replace the amino acid.

An interrelationship between purines and histidine is suggested in the studies on histidine synthesis in lactic acid bacteria (5). It was found that while histidine requiring lactics would not grow on a medium deficient in both histidine and purines, either of these components would produce optimum growth when added separately to the medium. In the absence of excess quantities of the B_6 group of vitamins, however, the purine bases could not substitute for histidine.

Isotope studies (11; 12) in Torulopsis utilis have shown that the carboxyl group of histidine comes entirely from the methyl carbon of acetate whereas the non-carboxyl portion of the molecule contains a moderate amount of radioactivity.

Formate-C^{14} is found to be an excellent precursor of histidine in Saccharomyces cerevisiae (21) contributing only to the carbon atom at position 2 in the imidazole ring. Glycine-1-C^{14} and bicarbonate-C^{14} on the other hand

were incorporated into histidine to only a very slight extent. As glycine is a known precursor in the synthesis of purines in Saccharomyces cerevisiae it is evident that the biosyntheses of purines and histidine do not follow the same pathway. The suggestion is made by the authors that the purine nucleus may serve as a formate donor in the synthesis of histidine.

The thesis here presented represents the isolation of radioactive lysine and histidine from Saccharomyces cerevisiae after growth on acetate-1-C^{14} and pyruvate-2-C^{14} as substrates. Preliminary investigations into the formation of these compounds by the yeast are also presented. This study is the first of its kind to investigate the formation of lysine or histidine from pyruvate as a sole carbon substrate. A new method of the separation of histidine as its oxalate is given.

EXPERIMENTAL

Yeast growth, hydrolysis and preliminary separations. The organism, Saccharomyces cerevisiae, used as source of protein in this work was grown (26), after a pre-growth on malt extract and glucose, in the three following manners: 1) under an atmosphere of oxygen in a medium containing $CH_3C^{14}OOH$ as the only source of carbon, 2) under an atmosphere of oxygen in a medium containing $CH_3C^{14}O$ COOH as the sole carbon source, and 3) under an atmosphere of nitrogen in a medium containing $CH_3C^{14}O$ COOH as the only carbon source. The specific radioactivity of each substrate was 1.87×10^6 cpm/m mole.

After growth, the yeast cells were harvested, de-fatted, and hydrolyzed with hydrochloric acid (20). Arginine, aspartic acid, and glutamic acid were isolated from the hydrolysate using IRA-400 and IR-4B (Rohm and Haas) ion exchange resin columns (20) by a modification of the method of Winters and Kunin (31). Tyrosine, after dilution with non-isotopic tyrosine as a carrier, was removed from the hydrolysate by adjusting the pH to 6.0 and concentrating in vacuo (25). The hydrolysate at this point contained the remainder of the neutral amino acids along with lysine and histidine.

Control of Separations. The effectiveness of the separations was checked at each step by measuring the radioactivity of the solutions and by microbiological assays (18).

Separation of Lysine. The hydrolysate was passed through an IRC-50 (Rohm and Haas) ion exchange resin column (sodium salt form) buffered with phosphate solution at pH 7 as described by Winters and Kunin (31). The resin used was subjected to a preliminary batchwise sodium hydroxide activation before being introduced into the columns for the final activation. After passage of the hydrolysate through this column, the lysine was eluted from the resin using four per cent hydrochloric acid solution. Progress of elution was followed by ninhydrin spot tests (31). The recoveries ranged from 57 to 69 per cent.

In the aerobic acetate and aerobic pyruvate hydrolysates, the lysine diluted with four parts of non-radioactive lysine as a carrier, was then precipitated, after removing the excess hydrochloric acid, as the phosphotungstate (2, pp.20-22). The phosphotungstic acid was liberated from a suspension of the lysine phosphotungstate, made acidic with sulfuric acid and extracted repeatedly, using a mixture of amyl alcohol, ether and ethanol. The sulfate ion was removed by treatment with

excess barium carbonate. In both samples, contamination with barium ion was evidenced by the precipitation of barium picrate to the total exclusion of lysine picrate, upon addition of picric acid to the filtered concentrate. The excess barium ion was therefore removed by titration with dilute sulfuric acid to the stoichiometric point. The lysine picrate then precipitated on standing twelve hours at 0°C. Yields obtained ranged from 3.5 to 15.7 per cent, with an unknown additional amount remaining in the unworked mother liquor.

In the hydrolysate of the anaerobically (pyruvate) grown yeast, the procedure for separation of the lysine differed from the foregoing. The yeast hydrolysate, after being freed from glutamic acid and aspartic acid on IR-4B ion exchange columns, was passed through an IRC-50 column buffered, as described above, at pH 7. Elution from this column gave a solution containing only arginine and lysine. The relative basicities of these two amino acids made possible their separation using an IRA-400 resin column in the form of the free base (31). On the first passage through the column the separation was incomplete, as attested by the low recovery of radioactivity in the lysine fraction. (This low recovery was later found due to the large amounts of sodium chloride in solution from the passage through IRC-50 resin). A second IRA-400

column was used and the eluates were combined, then appropriately diluted (five-fold) on the basis of the assay data with non-radioactive lysine as a carrier. A passage was made through an IR-4B column to remove interfering chloride ions. The recovery to this point was 45 per cent. The effluent was treated directly with picric acid yielding lysine picrate after twelve hours at 0°C. The yield of picrate was 53 per cent.

The lysine picrates were recrystallized from water until an explosion point of greater than 258°C. (corrected) was observed using a Berl and Kullmann copper block.

Separation of Histidine. The effluent from the IRC-50 columns following the removal of lysine was evaporated to dryness *in vacuo* to remove free hydrochloric acid and then passed through a second IRC-50 column which had previously been activated with sodium hydroxide and buffered with sodium acetate solution to pH 4.7 (31). The histidine remaining on the column was eluted with four per cent hydrochloric acid solution. Recoveries ranged from 66 to 91 per cent.

Using the Block microadaptation of the Kossel procedure (2, pp.17-18), modified to fit the amounts of material involved, the histidine was separated from the contaminating sodium chloride. The histidine was evaporated to dryness *in vacuo*, aqueous silver nitrate added until

an excess was established, the silver chloride removed by centrifugation and the pH adjusted to 7.4 to precipitate the silver salt of histidine. After washing a number of times by centrifugation, the silver salt of histidine, along with the silver oxide present, was suspended in water and decomposed by passing hydrogen sulfide through the suspension for 60 to 90 minutes. The silver sulfide was removed by filtration and the filtrate was evaporated to dryness in vacuo to yield crude, free histidine. Because of the unexpectedly high yield of histidine from the anaerobic (pyruvate) yeast, the above procedure was repeated on this sample before further purification was attempted.

The crude histidine was then converted to the oxalate salt by the procedure described below.

The histidine obtained from the previous step was dissolved in 2-4 ml of water and mixed with a 100 per cent excess of oxalic acid as a saturated solution. Alcohol was added to a concentration of approximately 80 per cent. The white solid that formed was allowed to stand for twelve hours at 0°C., then filtered and recrystallized by dissolving in water and reprecipitating with alcohol. The recrystallized material had a decomposition point beginning sharply at 223°C. (Fischer block, corrected).

The compound analyzed as follows:

	calculated for	histidine mono-oxalate found
Equivalent weight ($KMnO_4$ titration)	122.60	122.5
Histidine content (assay)	63.4%	66.6%
Carbon content*	39.2%	39.9%
Hydrogen content*	4.5%	4.8%

*The carbon and hydrogen analysis was done by William Schaeffer.

Yields in this operation, based on trial experiments with either histidine or the hydrochloride, ranged from 89 to 98 per cent. Recoveries of from 43 to 63 per cent were obtained on the yeast hydrolysates.

Radioactivity measurements of lysine picrate and histidine oxalate. The specific radioactivities (counts per minute per millimole of material) of the lysine picrate and histidine oxalate samples were measured by weighing a small sample (0.5 to 1.5 mg) directly onto a stainless steel, cupped planchet having an area of 5 cm^2, adding distilled water until the solution covered at least three-fourths of the area of the flat bottom of the planchet, and drying this slowly under a heat lamp to a thin, fairly uniform plate. When the amount of material is kept below one mg/cm^2, this method is found (28; 29, p.754; 30) to give values agreeing, within experimental error, with

results obtained on the same sample using barium carbonate plates.

The activity of each plate was counted at least twice, the plate being rotated 90° in its holder between countings. Statistical methods were used (6, p.288) to determine the appropriate counting times for each sample in order to maintain an over all precision of ± five per cent.

Measurement of the radioactivities in the carboxyl groups of histidine and lysine. The activity of the carboxyl group of the isolated amino acid was determined by ninhydrin decarboxylation of the picrate or oxalate salt after preliminary "cold" runs had established that satisfactory yields of barium carbonate could be obtained from these compounds. The method used was a modification by Wang (28) of the procedure of Frantz (6, pp.260-61) using barium hydroxide in place of sodium hydroxide and a simplified apparatus. Because of the instability of the imidazole ring toward long heating with ninhydrin (12, p.104), the decarboxylation of histidine oxalate was carried out at boiling water bath temperature for only ten minutes instead of the customary fifteen minutes. The $BaC^{14}O_3$ was plated and counted in the conventional manner.

RESULTS AND DISCUSSION

Table I is a compilation of the radioactivities found in the lysine and histidine fractions derived from the three yeast samples employed. The specific radioactivities are expressed as counts per minute (c.p.m.) per millimole of lysine or histidine in the original yeast. Since the utilization of pyruvate was much greater (20 m moles) than that of acetate (7.8 m moles) (26) for the same duration of growth, the radioactivities have also been expressed as c.p.m./m mole/m mole of substrate (pyruvate or acetate) consumed, for a more direct comparison.

The specific activities of the carboxyl groups, given in Table I, are expressed as c.p.m./m mole of lysine or histidine. The contents of the two amino acids in each yeast sample are also given.

Lysine. It may be seen from Table I that the overall radioactivity of the lysine molecule is greatest in the aerobic pyruvate sample whereas the relative activity of the carboxyl group is greatest in the lysine derived from acetate. The latter observation suggests that there may be fundamental differences in the pathways of formation of lysine from the two substrates, since if pyruvate were equivalent to acetate (through oxidative decarboxylation)

TABLE I

RADIOACTIVITY OF LYSINE AND HISTIDINE FRACTIONS FROM YEAST GROWN ON $CH_3\ C^{14}OOH$ AND $CH_3\ C^{14}O\ COOH$

Fraction	Substrate and method of growth: Acetate, aerobic	Pyruvate, aerobic	Pyruvate, anaerobic
Lysine:			
Content in yeast, %	4.6	5.8	4.0
Specific activity of total molecule (c.p.m./m mole)	1.28×10^5	5.61×10^5	1.81×10^5
Specific activity -COOH group (c.p.m./m mole lysine)	0.72×10^5	1.94×10^5	0.76×10^5
Per cent of total a ctivity residing in -COOH group	56.2	34.6	41.8
Specific activity of lysine (c.p.m.) per m mole of substrate utilized	1.64×10^4	2.8×10^4	0.90×10^4
Histidine:			
Content in yeast, %	1.1	1.2	1.3
Specific activity of total molecule	1.38×10^4	17.4×10^4	12.1×10^4
Specific activity -COOH group (c.p.m./m mole)	0	0.97×10^4	1.33×10^4
Per cent of total activity residing in -COOH group	0	5.59	9.1
Specific activity of histidine (c.p.m.) per m mole of substrate utilized	0.18×10^4	0.87×10^4	0.61×10^4

as a precursor of the carbon skeleton of lysine, the percentages incorporated into the carboxyl groups should be similar among the three yeasts.

On the basis of the data in Table I, some speculations may be offered regarding the mode of formation of lysine. Thus, the carboxyl group of lysine, because of its high activity (56 per cent of the total within the molecule), appears to arise almost directly from acetate carboxyl. The presence of so much activity in carbon 1 of lysine also rules out a direct C_2-C_2-C_2 condensation in its formation. This finding disagrees with that of Ehrensvärd et al (11), who observed only slightly more than one-third of the lysine activity in the carboxyl group and proposed that the synthesis of lysine from acetate might resemble the synthesis of fatty acid skeletons. Such a mechanism would require the lysine activities per millimole of substrate to be in the same ratio among the three yeast samples as were the respective fat fractions. This was not observed.

The formation of lysine through a C_3-C_3 condensation is also precluded, since such a molecule would possess little activity in carbon 1.

The radioactive pattern observed for the three differently derived lysine molecules may be satisfactorily explained by a "head to tail" condensation between C4 and

C_2 units to produce a six carbon straight chain skeleton. These results are substantiated by the radioactive patterns known for the C_4 acids (27), as described below.

The labeling of the C_4 acids (formed from acetate -1-C^{14} proceeding through one revolution of the Krebs tricarboxylic acid cycle) is known (27) to be equal on the two end groups. Because this activity came originally from one acetate molecule, the sum of the activities of these groups will be equivalent to that of one acetate. The condensation of this C_4 unit with a new acetate molecule will produce a six carbon entity having roughly 50 per cent of the activity in the carboxyl group. This is in agreement with the results found for the acetate grown yeast.

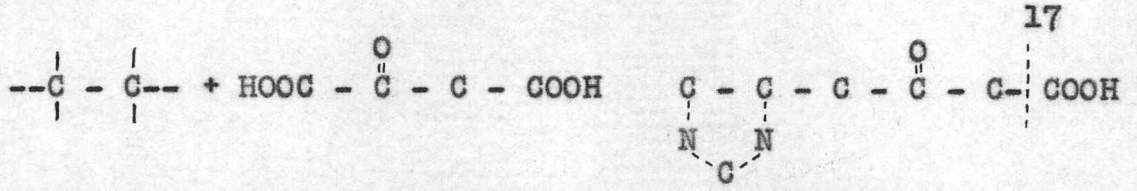
In the lysine from the aerobic pyruvate sample, the same explanation may serve. However, the C_4 acids here show two very active central groups (due to extensive Wood-Werkman or other C_3-C_1 condensation (27)), with moderate activities in the end groups. The total activity approaches more closely that of two acetate molecules. The condensation of another acetate (from pyruvate) with a C_4 unit so labeled could produce a C_6 straight chain, in which the carboxyl group may thus be expected to have an a ctivity more nearly 33 per cent of the total molecule. Again, this agrees with the values for the aerobic pyruvate

yeast in Table I.

The activity of the lysine carboxyl from anaerobic pyruvate is intermediate between the activities of the carboxyl groups from the other two lysine fractions. This is in line with the lower activity in the C_4 acids in this sample as compared to the aerobic pyruvate sample.

The disagreement of the present results with those of Ehrensvärd *et al* (11) may likely be due to the fact that their yeast, which was first adapted to acetate growth, showed a much higher incorporation of radioactivity into C_4 acids, i.e., aspartic acid (12). The yeast used in this study (27), which was not previously adapted to acetate as a substrate, did not grow and incorporated only 9 per cent of the radioactivity administered into the protein fraction.

Histidine. The results presented for histidine in Table I confirm the previous suggestion (11) that the carboxyl group of acetate plays no direct role in the formation of the carboxyl group of this amino acid. The finding of a non-radioactive carboxyl group also rules out the condensation of a biosynthetic C_3-unit (from acetate) with an imidazole ring to form histidine from pyruvate. Instead, the data in Table I indicate an asymmetric condensation between oxalacetate and a two carbon moiety, as follows:



Decarboxylation, amination and formylation (21) would then give the carbon skeleton of histidine.

Again, taking into consideration the relative labelings on the aspartic acid molecule, the results presented earlier fit directly into this scheme: the oxalacetate derived from acetate has two inner carbons devoid of radioactivity. After the C_2 - C_4 condensation, decarboxylation, amination, and formylation, a non-radioactive carbon becomes the carboxyl group of the histidine molecule. This explains not only the lack of radioactivity in the carboxyl group in the histidine grown on acetate but also the finding of Ehrensvärd (11) that the carboxyl group is derived entirely from the methyl group of acetate.

On the other hand, oxalacetate from pyruvate (aerobic experiment) possesses a weakly radioactive carbon in the 3 position. This presumably becomes the carboxyl group of histidine, which furnishes a low percentage of the overall radioactivity of the molecule.

The oxalacetate from anaerobic pyruvate shows greater randomization on the two central carbon atoms (27). It should therefore (as observed) present a carbon atom for the carboxyl group of histidine that has slightly higher activity than was found from the aerobic pyruvate.

SUMMARY

A description has been given of the separation of lysine and histidine from yeast protein hydrolysate, using ion exchange resin columns. Lysine was isolated as its picrate and histidine was purified as its oxalate. The preparative procedure for histidine oxalate, previously unmentioned in the literature, is also described, and some physical measurements are listed.

The results of the determinations of the radioactivities of the total molecules (by direct plating method) and of the carboxyl groups of each molecule (ninhydrin reaction and barium carbonate plates) are presented.

It is proposed, on the basis of preliminary information available, that the carbon skeleton of lysine may be formed by the condensation of a C_4 acid of the Krebs cycle with acetate. The condensation of oxalacetate with a C_2 intermediate together with amination, formylation, and decarboxylation, is suggested as a means by which the carbon structure of histidine may be formed.

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