THE BIOSYNTHESIS OF AROMATIC AMINO ACIDS IN YEAST (*SACCHAROMYCES CEREVISIAE*)

by

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THE BIOSYNTHESIS OF AROMATIC AMINO ACIDS IN YEAST (*SACCHAROMYCES CEREVISIAE*)

INTRODUCTION

Considerable interest has been focused recently upon the biosynthesis of the aromatic amino acids in microorganisms which undoubtedly plays an important role in the overall understanding of amino acid metabolism. Recent availability of isotopic carbon, followed by the development of tracer methodology, has furnished workers with an extremely useful tool for attacking problems which until that time had appeared formidable. In reviewing the literature on this subject, one finds a great deal of confusion as well as apparent contradiction which, as in many other intensively attacked fields, is probably due to: (1) an overemphasis of the possible variation among microorganisms; (2) lack of systematic studies on the relationship between possible precursors and products; (3) lack of coordination among several different approaches to the problem; (4) the difficulty in evaluating the experimental findings of some studies which were published in a highly condensed version with no experimental details available to the public. The following is a brief review of the up to date information available on the biosynthesis of the aromatic amino acids, including tyrosine and phenylalanine, which has been arranged in a semi-historical but logical order.

Baddiley et al (2,pp.777-788) have made use of isotopic carbon in studying this problem by administering doubly labeled acetate, $C^{13}H_3$-$C^{14}OOH$, as the only carbon source, to yeast which had previously been adapted to acetate (1,pp.771-776). The tyrosine was isolated from the yeast hydrolysate and degraded in order to determine the distribution of the isotope in its carbon skeleton. From the degradation pattern observed, these workers concluded that both carbon atom 4 of the ring and the carboxyl carbon of tyrosine were derived by more or less direct routes from the carboxyl carbon atom of the administered acetate. The α and β carbon atoms of the side-chain as well as carbon atoms 1, 2 and 6 of the ring appeared to be derived from the methyl carbon of acetate. The mixed C^{14}, C^{13} content of carbon atoms 3 and 5 of the tyrosine ring makes the origin of these two carbon atoms uncertain. The mechanism proposed by Baddiley et al is presented in figure 1. Although this mechanism is too abstract to be of much value, the general information, and particularly the degradation method, reported are extremely helpful to other workers in this field.

Gilvarg and Bloch (20,pp.5791-5792) employed glucose-1-C^{14} as the principal carbon source for growth of baker's yeast. Both tyrosine and phenylalanine were isolated and partially degraded. Nearly all of the activity

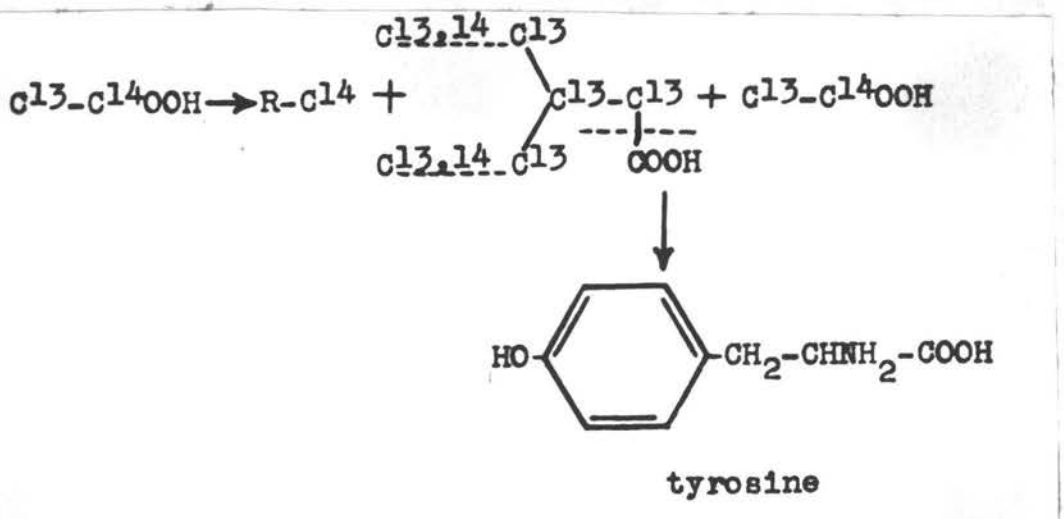


Figure 1

of the side-chain in each of the two amino acids was contained in the methylene carbon atom. Over 75 per cent of the activity of the aromatic ring was reported to be in carbon atoms 2 and 6 which are not distinguishable due to the symmetrical nature of the ring system. By assuming that the activity was present entirely in one or the other, and not distributed between the two atoms, these workers proposed that glucose cyclizes directly to form the aromatic ring with carbon atom one of glucose becoming carbon atom 2 or 6 of the ring. Furthermore, Gilvarg and Bloch concluded that the side-chain of tyrosine and phenylalanine could not arise from the carbon skeleton of pyruvic acid.

In contrast to their findings in the case of the glucose-1-C^{14} yeast fermentation experiments, Gilvarg and Bloch (21,pp.339-346) stated in a separate paper, concerning the growth of baker's yeast in the presence of $C^{14}H_3$-COOH and non-radioactive glucose as carbon sources, that the tyrosine and phenylalanine isolated contained very little radioactivity. Gilvarg and Bloch therefore concluded that only compounds higher in the glycolytic scheme than pyruvate can be involved in the biosynthesis of the aromatic amino acids. These results, however, are not in agreement with those of Baddiley et al (2,pp.777-788) who have observed that labeled acetate was readily incorporated into the tyrosine molecule in the case of Torula yeast.

Cutinelli et al (9,pp.353-371) administered $C^{13}H_3$-$C^{14}OOH$ as the sole carbon source to E. coli in order to compare acetate as a source of carbon in yeast and E. coli. On the basis of the partial degradation data of tyrosine and phenylalanine it was speculated: (1) the possibility that the side-chain of tyrosine could be derived from a 3-carbon unit such as pyruvate whereas the carboxyl carbon and the amino carbon of the phenylalanine side-chain seems to arise from an intact 2-carbon fragment such as acetate; (2) although these two amino acids seem to have a common biosynthetic pathway up to the ring formation, it is uncertain whether their respective side-chains were connected

to the same carbon atom of the ring.

Using an entirely different approach to the problem, Davis (12,pp.315-325) has made use of mutant strains of E. coli. The mutants were isolated by the penicillin method following ultraviolet irradiation of a wild strain (70,p.4267 and 11,pp.1-10). In one group of mutants, with a requirement for tyrosine, phenylalanine, tryptophan, and p-aminobenzoic acid plus a partial requirement for p-hydroxybenzoic acid, shikimic acid substituted for all these required aromatic compounds. Another group of mutants, blocked at later stages in aromatic synthesis was shown to accumulate shikimic acid in their culture filtrates. This author then concluded that in E. coli shikimic acid functions as a common precursor of several aromatic metabolites including tyrosine and phenylalanine. Davis (12,pp.315-325) also isolated a third strain having a requirement for tyrosine, phenylalanine, tryptophan, and p-aminobenzoic acid, which was shown to accumulate a precursor of shikimic acid. Another group of mutants with the same quadruple requirement was able to substitute this precursor for all four of the required metabolites. This compound was later identified as 5-dehydroshikimic acid (33,p.210 and 34, pp.5567-5571). Recently Weiss, Davis and Mingioli (51, pp.5572-5576) have isolated certain mutant strains of E. coli which can be shown to accumulate a precursor of

5-dehydroshikimic acid and certain other strains which can use this precursor as a growth factor. This compound has been identified as 5-dehydroquinic acid. Although quinic acid is closely related to this latter compound, and has been reported as an intermediate in aromatic biosynthesis in another microorganism (24,pp.427-430), Davis and Weiss do not believe that it is a normal precursor of 5-dehydroquinic acid (15,pp.1-15). On the basis of the foregoing information Davis and Weiss (15,pp.1-15) proposed that the sequence 5-dehydroquinic acid, 5-dehydroshikimic acid and shikimic acid as shown in figure 2 are intermediates in the biosynthesis of the aromatic amino acids in E. coli.

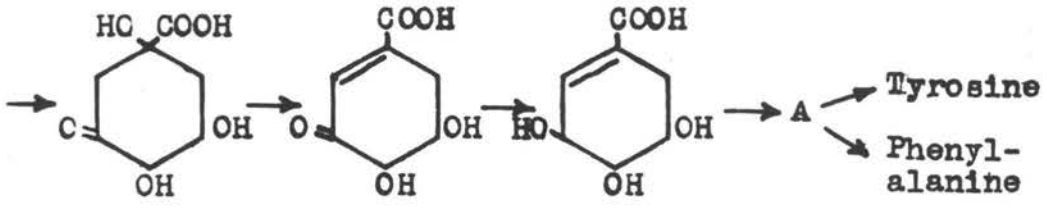


Figure 2

The origin of 5-dehydroquinic acid and the immediate fate of shikimic acid in the synthetic sequence leading to the aromatic amino acids, however, has not been elucidated in these studies. Furthermore, Davis (13,pp.729-748) has shown evidence that in the case of E. coli at least, phenylalanine is not a normal precursor of tyrosine. This view is not in agreement with that of Beerstecher and Shive (3,pp.53-61 and 4,pp.49-52), as concluded from their inhibition analysis experiments with E. coli, suggesting that tryptophan is a precursor of phenylalanine which is in turn a precursor of tyrosine. On the other hand Davis' conclusion does support the view of Simmonds et al (41, pp.91-101) who demonstrated that tyrosine does not exert a sparing action on the quantitative requirement of a phenylalanine requiring mutant strain of E. coli.

Thomas et al (46,pp.5554-5556) compared C^{14} labeled pyruvate and acetate as sole carbon sources for the biosynthesis of tyrosine in baker's yeast. The radioactivity distribution patterns in the isolated tyrosine samples suggested that oxalacetate or other unsymmetrical C_4 acids may function as an intermediate in the conversion of pyruvate to this amino acid via shikimic acid. This work also gave experimental verification to the suggestion of Cutinneli et al (9,pp.353-371) that the side-chain of tyrosine arises from a 3-carbon unit such as pyruvate.

It is interesting to note that, although Gilvarg and Bloch (21,pp.339-346) reported the failure of incorporation of acetate into the aromatic amino acids when yeast was grown on glucose and labeled acetate substrates, a considerable incorporation of acetate was observed by Thomas et al (46,pp.5554-5556) when labeled acetate was used as a sole carbon source for unadapted yeast.

Gilvarg and Bloch (23,pp.689-698), in publishing a detailed report of their experiments using glucose-1-C^{14} as a substrate for growth of baker's yeast, reversed their earlier proposal (20,pp.5791-5792) that the aromatic ring arises by the direct cyclization of glucose, as well as the possibilities that: (1) the ring arises from condensation of three 2-carbon units and (2) that the ring is formed by condensation of two isotopically equilibrated 3-carbon units. These authors pointed out, however, that their data are compatible with a speculative mechanism for aromatic ring biosynthesis involving condensation of a 3-carbon unit with some other product of glucose metabolism. Although the isotopic data obtained did not permit a clear interpretation of the course of aromatic biosynthesis, seven carbon sugars, such as sedoheptulose (5,p.2970 and 25,p.74) were considered by these authors as intermediates, possibly serving as a source of shikimic acid which Davis (12,pp.315-325) has shown to be an intermediate in the

biosynthesis of tyrosine and phenylalanine in E. coli. Furthermore, contrary to their preliminary discussion on the mechanism of the side-chain synthesis (20,pp.5791-5792), Gilvarg and Bloch agreed with the suggestion of Cutinelli et al (9,pp.353-371) that the side-chain of tyrosine and phenylalanine could arise from a 3-carbon glycolytic product such as pyruvic acid.

Shigeura and Sprinson (39,p.286) combined the use of isotopic carbon and mutant strains of E. coli in attacking the problem of aromatic biosynthesis. These authors made use of the shikimic acid accumulating E. coli mutant, having a quintuple aromatic requirement, which was mentioned earlier in connection with the work of Davis (12,pp.315-325). They found that little activity was incorporated into the accumulated shikimic acid when $NaHC^{13}O_3$, $HC^{14}OONa$ or $C^{14}H_3-C^{13}OONa$ was introduced into the growth medium, but the introduction of glucose-1-C^{14} to the growth medium resulted in shikimic acid having a specific activity close to that of the initial glucose. No conclusions were presented in this brief communication; and since no further details have appeared in the literature, it is very difficult to evaluate the significance of this finding.

Ehrensvärd and Reio (18,pp.229-234) reported the complete degradation data of tyrosine from E. coli and

Neurospora crassa which had been grown on $C^{13}H_3$-$C^{14}OOH$ as a sole carbon source. The growth conditions as well as partial degradation data of this amino acid from E. coli were described earlier in a paper by Cutinelli (9,pp.353-371). These workers proposed a speculative scheme for biosynthesis of the aromatic ring structure which includes the formation of glucose from the administered acetate followed by the production of one C_4 unit corresponding to glucose carbons 6, 5, 4, 3 or 1, 2, 3, 4 and one C_3 unit corresponding to glucose carbons 1, 2, 3 or 6, 5, 4 (as in a conventional glycolysis scheme) from two molecules of glucose. The condensation of the C_4 and C_3 units could then yield a C_7 compound analogous to the formation of seduheptulose (44,pp.591-604), which is subsequently cyclized to form the benzenoid structure. By assuming the labeling pattern of glucose, derived from acetate-1-C^{14}, as C-C-C^{14}-C^{14}-C-C (52,pp.201-210) the synthetic sequence leading to tyrosine labeled with the expected pattern, along with the pattern observed, is illustrated in figure 3. Ehrensvärd and Reio further pointed out that the above scheme will explain the tyrosine labeling pattern observed by Gilvarg and Bloch (20,pp.5791-5792 and 23,pp.689-698) when glucose-1-C^{14} was used as a yeast substrate. The scheme in figure 3 implies the preformation of the methylene carbon atom and subsequent condensation with a C_2

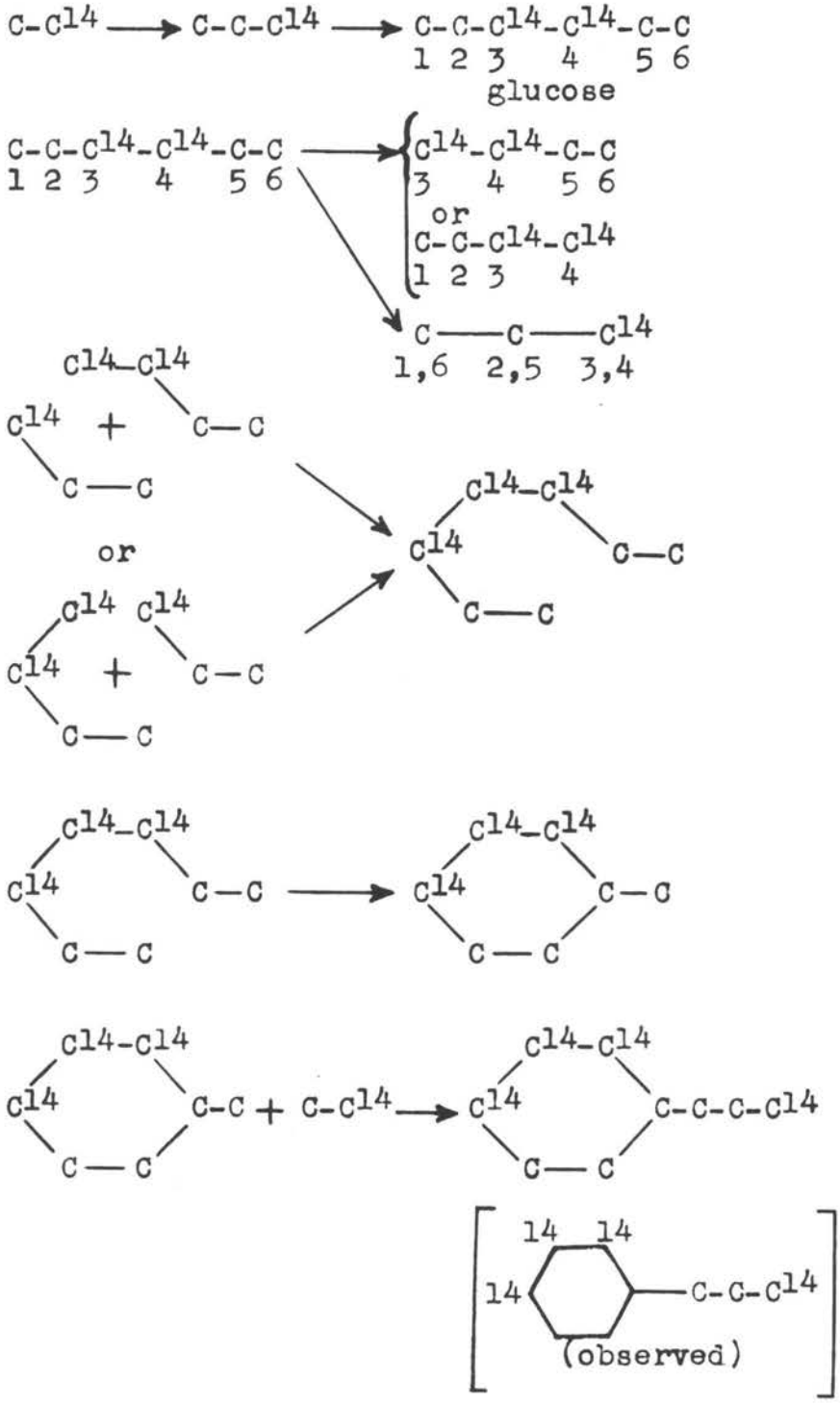

C-C14 → C-C-C14 → C-C-C14-C14-C-C
1 2 3 4 5 6
glucose
C-C-C14-C14-C-C
1 2 3 4 5 6
C14-C14-C-C
3 4 5 6
or
C-C-C14-C14
1 2 3 4
C—C—C14
1,6 2,5 3,4
C14-C14
C14 + C—C
C—C
or
C14 C14
C14 + C—C
C—C
C14-C14
C14 C—C
C—C
C14-C14
C14 C—C
C—C
C14-C14
C14 C-C + C-C14 →
C—C
C14-C14
C14 C-C-C-C14
C—C
14 14
14 C-C-C14
(observed)

Figure 3

unit, probably derived from acetate, to form the side-chain of tyrosine. This is not in line with the finding of Thomas *et al* (46,pp.5554-5556) that the side-chain of tyrosine appears to arise from pyruvate as an intact unit.

The biosynthesis of tyrosine in the photosynthetic microorganism *Rhodospirillum rubrum* was also studied by Ehrensvärd and Reio (19,pp.327-332). In their experiments $C^{13}H_3-C^{14}OOH + CO_2$ and $CH_3-COOH + C^{14}O_2$ were used separately as sole carbon sources for the microorganism. Based on the labeling patterns of tyrosine observed in these experiments, these authors pointed out the possibility of two mechanisms being involved in aromatic biosynthesis so far as this organism is concerned. Thus, in addition to the mechanism proposed for *E. coli* and *Neurospora crassa*, as described previously (figure 3), a second mechanism may be operating which involves condensation of a C_5 fragment derived from glucose with a C_2 fragment such as acetate in the manner illustrated in figure 4.

In a brief abstract, Shigeura, Sprinson and Davis (40,p.458) reported that the feeding of glucose, labeled at various unique positions, to a mutant strain of *E. coli* (obtained as discussed in a previous paper (12,pp.315-325)) resulted in definite labeling patterns for shikimic acid as observed by degradation of samples of this accumulated compound isolated from the culture filtrate. The results

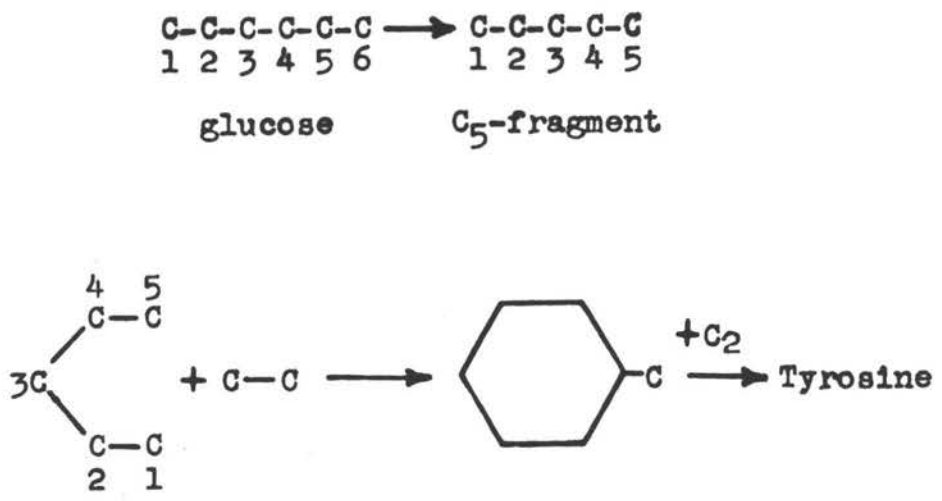


Figure 4

of their studies are given in figure 5. In this figure the number opposite a particular carbon atom of shikimic acid represents its specific activity based on the specific activity of the labeled carbon atom of the glucose substrate being assigned a value of unity. From these results Shigeura, Sprinson and Davis concluded that two or more fragments of glucose are utilized for the synthesis of

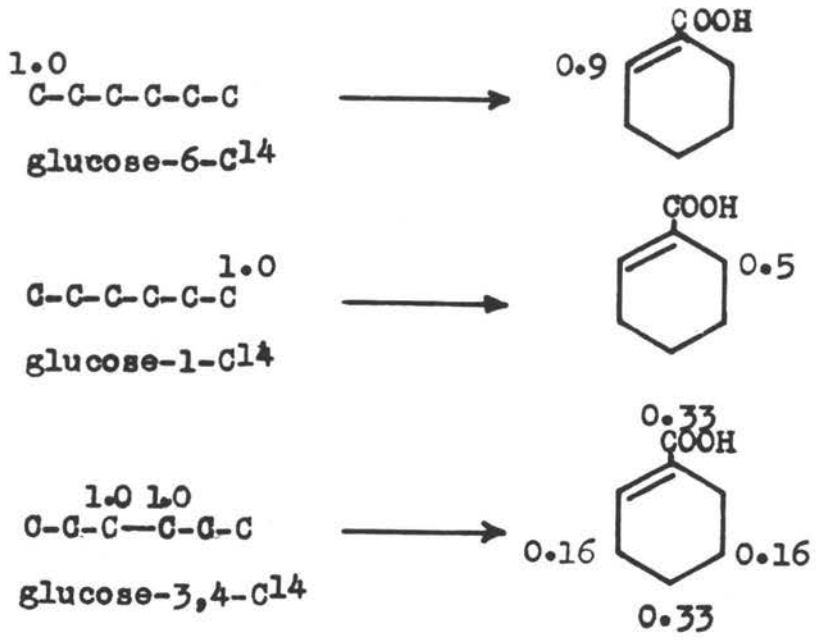


Figure 5

shikimic acid. Speculations on the nature of these fragments was not reported, but it was implied that they do not reflect the randomization reactions of glycolysis and the citric acid cycle or the loss of carbon atom number one of glucose in the hexosemonophosphate shunt.

Very recently Srinivasan, Sprecher and Sprinson (42,p.266) reported the results of their studies concerning the site of attachment and source of the side-chain of

tyrosine. Glucose-6-C^{14} was used as a source of carbon to a wild strain of E. coli in this work. Degradation studies on the tyrosine isolated from the cells revealed that carbon atom 2 or 6 of the ring was 0.9 as active as the labeled carbon atom of glucose while the methylene carbon of the tyrosine side-chain was 0.5 as active as the labeled carbon atom of glucose. These results are illustrated in figure 6 where the numbers refer to specific activities as explained previously for figure 5.

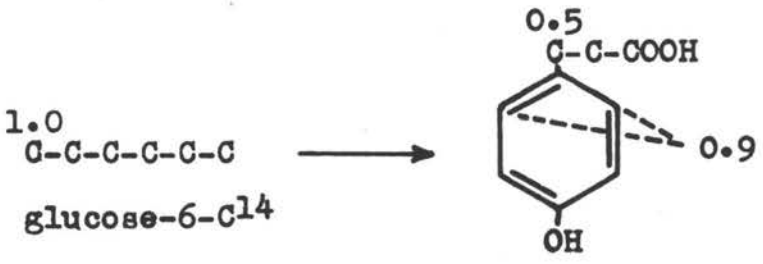
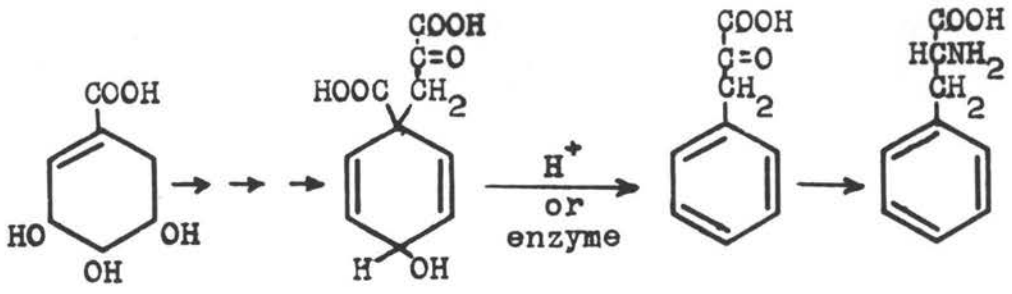


Figure 6

In comparing the labeling pattern of tyrosine as shown in figure 6 with that of shikimic acid formed from the same substrate (figure 5) Srinivasan, Sprecher and Sprinson concluded that in the biosynthesis of tyrosine: (1) the carboxyl of shikimic acid is eliminated; (2) the side-chain enters the site of decarboxylation as evidenced by the labeling pattern of shikimic acid as well as that of

tyrosine (as shown in figures 5 and 6); (3) the side-chain, in contrast to the ring, may be derived from a 3-carbon unit produced in glycolysis.

Davis et al (16,p.180) have recently reported the accumulation of a probable precursor of phenylalanine, and possibly tyrosine, in phenylalanine-requiring and tyrosine-requiring mutants of E. coli. The compound has been named prephenic acid by these workers. The accumulation of prephenic acid suggests that in the conversion of shikimic acid to phenylalanine, decarboxylation takes place in the same step as aromatization. The role of prephenic acid in aromatic biosynthesis, as proposed by Davis et al, is given in figure 7.



shikimic acid prephenic acid phenylpyruvic acid phenyl-alanine

Figure 7

It can be seen from figure 7 that decarboxylation and dehydration occur simultaneously with aromatization. These authors believe that the conversion of shikimic acid to prephenic acid involves more than one step.

EXPERIMENTAL AND RESULTS

Pyruvic acid was chosen as a sole carbon source for yeast fermentation since it is the principal product of glycolysis of carbohydrates and plays a key role for the entry of the latter into the citric acid cycle. The pyruvic acid was labeled with C^{14} (45,p.5914) in the carbonyl position for the following reasons: (1) to study the incorporation of the 3-carbon unit as a whole as compared to its incorporation via a 2-carbon unit and (2) in synthesizing labeled pyruvic acid higher yields (based on C^{14}) may be obtained by labeling the compound in the carbonyl position as compared to the methyl group. The fermentations were carried out under rigidly controlled conditions; pyruvate assays as well as radioactivity assays of the medium being maintained throughout the fermentations in order to follow the uptake of labeled substrate. All of the administered pyruvate was found to be utilized in four hours aerobically and five hours anaerobically.

Carboxyl-C^{14}-labeled acetic acid was also chosen as a yeast substrate in order to compare its incorporation with that of the carbonyl labeled pyruvate since the latter is known to be readily converted through decarboxylation to acetate or an equivalent 2-carbon unit. Acetate was employed only under aerobic conditions, 39 per cent of

the labeled substrate being used in four hours. Details of these fermentations have been presented elsewhere (47,pp.645-653).

After removal of arginine, glutamic acid and aspartic acid from the yeast hydrolysate by ion-exchange resin techniques, tyrosine was isolated (30,pp.655-661) by concentration of the eluate and precipitation at the isoelectric point using non-isotopic L-tyrosine as a carrier. A sufficient amount of carrier was added so as to dilute fivefold the original tyrosine as judged by a microbiological assay. Yields of the diluted tyrosine, obtained from three grams of dry yeast in each sample, were: from acetate, 81.0 mg; from pyruvate (aerobic), 91.2 mg; and from pyruvate (anaerobic), 66.1 mg. Purity of the isolated samples was established by paper chromatography.

The tyrosine samples obtained from aerobic pyruvate, anaerobic pyruvate and aerobic acetate respectively were degraded according to the method of Baddiley *et al* (2,pp.777-788) and on the same scale in the following manner: (1) combustion to CO_2 which was counted to obtain the specific activity of the whole molecule; (2) decarboxylation with ninhydrin to give CO_2 which was counted to obtain the specific activity of the carboxyl carbon; (3) fusion with KOH and NaOH to give p-hydroxybenzoic acid.

Combustion and radioactivity assay gave the specific activity of this compound directly and the specific activity of the amino carbon atom of the tyrosine side-chain by difference; (4) nitration of p-hydroxybenzoic acid to 3,5-dinitro-4-hydroxybenzoic acid. The latter was oxidized with $Ca(OBr)_2$ to bromopicrin which was in turn burned to CO_2 and counted in order to obtain the mean specific activity of carbon atoms 3 and 5 of the tyrosine ring; (5) nitration of p-hydroxybenzoic acid to picric acid. A small amount of the picric acid was burned to CO_2 and counted to obtain the specific activity of the benzene ring and that of the methylene carbon atom of the tyrosine side-chain by difference. The remainder of the picric acid was oxidized with $Ca(OBr)_2$ to bromopicrin, which was burned to CO_2 and counted to obtain the average specific activity of carbon atoms 1, 3 and 5 of the tyrosine ring. The average specific activity of carbon atoms 2, 4 and 6 could thus be obtained by difference. Carbon atom 4 of the ring was not differentiated from carbon atoms 2 and 6 due to the very small amount of sample available. The results of these degradations are given in table I. All samples were counted as $BaCO_3$ using a thin, end window Geiger-counter with corrections for self-absorption and background applied to the results in the conventional manner. The counting data were also corrected for dilution and are expressed in

Table I

Conditions	Aerobic pyruvate		Anaerobic pyruvate		Aerobic acetate	
	C.p.m. per mmole x 10^4	per cent of total	C.p.m. per mmole x 10^4	per cent of total	C.p.m. per mmole x 10^4	per cent of total
Whole molecule	49.1	100	19.0	100	4.84	100
COOH	4.3	9	2.8	15	2.43	50
$CHNH_2$	18.0	37	4.9	26	0	0
CH_2	0	0	0	0	0	0
C_6H_4OH	25.9	53	10.5	55	2.59	53
Distribution of C^{14} within the ring						
C_6H_4OH	25.9	100	10.5	100	2.59	100
C_1	8.3	32	1.6	15	0	0
C_{3+5}	11.4	46	3.8	36	0.74	29
C_{2+4+6}	5.7	22	5.0	48	1.74	72

counts per minute per millimole of original compound.

Phenylalanine was separated from the neutral amino acid fraction by Dowex-50 (strongly acidic ion-exchange resin) column chromatography using a slight modification of the method of Stein and Moore (43,pp.179-190). Due to the relatively small amount of phenylalanine present in the system, a sufficient amount of non-isotopic phenylalanine was added as a carrier prior to the column operation so as

to dilute the original phenylalanine ninefold as judged by a microbiological assay. Phenylalanine was isolated from the corresponding fractions by the method of Cutinelli et al (9,p.356) and its identity and purity were established by paper chromatography. Yields of diluted phenylalanine, obtained from three grams of dry yeast in each sample were: from acetate, 244 mg; from pyruvate (aerobic), 337 mg; and from pyruvate (anaerobic), 284 mg.

The phenylalanine samples obtained from aerobic pyruvate, anaerobic pyruvate and aerobic acetate respectively were degraded according to the method of Gilvarg and Bloch (23,pp.689-698) on a slightly larger scale in the following manner: (1) combustion to CO_2 which was counted to obtain the specific activity of the whole molecule; (2) decarboxylation with ninhydrin to give CO_2 which was counted to obtain the specific activity of the carboxyl carbon; (3) oxidation with $K_2Cr_2O_7$ in 25 per cent sulfuric acid to give benzoic acid. Combustion and radioactivity assay of the latter gave the specific activity of the compound directly and the specific activity of the amino carbon of phenylalanine by difference; (4) decarboxylation of the benzoic acid with hydrazoic acid to give aniline. The reaction was carried out by adding a twofold excess of HN_3 in chloroform over a period of one hour to a chloroform solution of the benzoic acid containing concentrated H_2SO_4

as a catalyst. The reaction took place over a period of three hours at 40° C during which time vigorous stirring was maintained. After making the reaction mixture alkaline with NaOH, it was extracted with ethyl ether in a continuous liquid-liquid extractor for 16 hours. Following removal of the ether, the aniline was converted to its hydrochloride and acetylated with acetic anhydride and sodium acetate in an aqueous medium. A small sample of the acetanilide was burned to CO_2 and counted to give the specific activity of the ring directly and the specific activity of the methylene carbon atom of the phenylalanine side-chain by difference; (5) nitration of a portion of the acetanilide to p-nitroacetanilide. The latter compound was in turn oxidized with $Ca(OBr)_2$ to bromopicrin which was converted to CO_2 by combustion and counted to obtain the specific activity of carbon atom number 4 of the phenylalanine ring. It is very important that the temperature be kept below 20° during the nitration and that the crude product be washed thoroughly until the filtrate is neutral or extensive decomposition of the p-nitroacetanilide results on drying; (6) a portion of the acetanilide was brominated to p-bromoacetanilide with bromine using glacial acetic acid as solvent[1]. The p-bromoacetanilide was then

[1] In the original directions of Gilvarg and Bloch for bromination of acetanilide 0.2 ml of bromine was prescribed. It was found in the present work that 0.02 ml is the correct amount to use based on the amount of acetanilide which they employed.

nitrated to 2-nitro-4-bromoacetanilide. After drying, the latter compound was purified by passing its benzene solution through an activated alumina column followed by removal of the solvent and recrystallization from aqueous ethanol. The 2-nitro-4-bromoacetanilide was oxidized with $Ca(OBr)_2$ to bromopicrin which was in turn converted to CO_2 and counted to obtain the average specific activity of carbon atoms 2 and 6 of the aromatic ring of phenylalanine. Since the specific activities of the ring as well as carbon atom 4 of the phenyl substituent were obtained in the previously described procedures, it is thus possible to calculate the average specific activity of carbon atoms 1, 3 and 5 by difference. The results of these degradations are given in table II. All samples were counted as $BaCO_3$ using a windowless, gas-flow Geiger-counter with corrections for background and self-absorption applied to the results in the conventional manner. The counting data given in table II were also corrected for dilution and are expressed in counts per minute per millimole of original compound. In order to compare the degradation data of phenylalanine and tyrosine, it was necessary to apply a correction factor of 1/11.6 to obtain the specific activities presented in table II. This factor arises because different counters were used in the two degradations, the windowless flow counter being 11.6 times as sensitive as

Table II

Conditions	Aerobic pyruvate		Anaerobic pyruvate		Aerobic acetate	
	C.p.m. per mmole x 10^4	per cent of total	C.p.m. per mmole x 10^4	per cent of total	C.p.m. per mmole x 10^4	per cent of total
Whole molecule	38.8	100	19.0	100	4.58	100
COOH	2.7	6.9	2.7	14.2	1.33	29
$CHNH_2$	14.8	38.2	6.5	34	0	0
CH_2	.9	2.2	0.8	4.2	0	0
C_6H_5	20.4	52.7	9.0	47.6	3.25	71
Distribution of C^{14} within the ring						
C_6H_5	20.4	100	9.0	100	3.25	100
C_{1+3+5}	16.5	81	6.0	66.6	2.50	77
C_{2+6}	2.2	10.8	1.6	17.8	.17	5.1
C_4	1.7	8.3	1.4	15.6	.58	17.6

the end window counter after correction for background and self-absorption.

Examination of the degradation data of these aromatic amino acids has revealed the possibility that a C_4 acid such as oxalacetate could play an important role in the biosynthesis of the aromatic structure (75,pp.5554-5556). To further verify and evaluate this mechanism, a series of fermentations were carried out to study the incorporation of

$C^{14}O_2$ into yeast. In order to reproduce the conditions used in previous fermentations, non-isotopic pyruvic acid was administered together with the $C^{14}O_2$ as carbon sources to two grams of baker's yeast which had previously been grown on glucose in a synthetic medium. The fermentation was carried out at 30° C in a closed system containing an oxygen atmosphere at slightly reduced pressure. Stirring was maintained by means of a magnetic stirrer and the pH kept at 4.5 as determined by a set of electrodes enclosed in the fermentation flask. The progress of the fermentation was followed by periodical assay for pyruvic acid and radioactivity of the cells and medium. Pyruvic acid was administered at intervals in order to maintain a constant concentration of about 8 millimoles per liter. Five per cent of the radioactive $C^{14}O_2$ was found to be incorporated into the yeast cells in five hours. This fermentation study was carried out in cooperation with Mr. James Wendell Davis in whose thesis details will be described.

The isolation of tyrosine from this yeast sample was carried out by a somewhat different procedure than was used for the previous tyrosine samples (30,pp.655-661). All of the amino acids except arginine in the hydrolysate of two grams of dry yeast were absorbed on an amberlite IRA-400 (strongly basic) resin column, allowing the neutral and basic impurities to pass through in the effluent. Upon

elution of this column, inert L-tyrosine was added to the eluate as a carrier and the diluted tyrosine isolated by concentration and precipitation at the isoelectric point. Sufficient carrier was added to dilute the original tyrosine (based on a microbiological assay) tenfold. The diluted tyrosine thus obtained was found to be inferior in purity. Consequently it was further purified by repeated recrystallization followed by passage of its aqueous solution through two consecutive amberlite IR4-B (weakly basic) resin columns. The purity of the sample thus obtained was established by paper chromatography. The yield of diluted tyrosine obtained from two grams of dry yeast was 128 mg.

At this time an article by Reio and Ehrensvärd (32, pp.301-311) appeared in literature describing an improved method for the degradation of tyrosine. The only difference between this procedure and the previous one (2, pp.777-788) is that a new method is given for determining the mean specific activity of carbon atoms 2 and 6 of the tyrosine ring on a significantly smaller scale. It was thus possible to degrade the present sample of tyrosine completely including differentiating the specific activity of carbon atom 4 of the ring from that of carbon atoms 2 and 6 with the new procedure: (1) p-hydroxybenzoic acid was decarboxylated to phenol by heating with KHF_2; (2) the

phenol was condensed with isobutyl alcohol using 70 per cent H_2SO_4 as a catalyst to give para-t-butylphenol; (3) the para-t-butylphenol was oxidized to trimethylpyruvic acid with alkaline permanganate; (4) the trimethylpyruvic acid, its carboxyl carbon corresponding to carbon atoms 2 and 6, and its carbonyl carbon corresponding to carbon atom 4 of the tyrosine ring, was decarboxylated oxidatively with $K_2Cr_2O_7$ to give CO_2. The CO_2 was counted in order to determine the mean specific activity of carbon atoms 2 and 6 of the original tyrosine ring. Since the specific activities of the ring, of carbon atoms 1+3+5, as well as that of carbon atoms 2+6 were known, it was possible to obtain the specific activity of carbon atom 4 of the aromatic ring by difference. The results of this degradation are given in table III. All samples were counted as $BaCO_3$ using a windowless, gas-flow Geiger-counter with corrections for background and self-absorption applied to the results in the conventional manner. The specific activities given in table III have been corrected for dilution and are expressed in counts per minute per millimole of original compound. The specific activities have been related to the same end window counter by application of the correction factor (1/11.6) mentioned previously.

In a series of experiments (18,pp.229-234 and 19, pp.327-332) using $C^{13}H_3$-$C^{14}OOH$ as a sole carbon source for

Table III

	C.p.m. per mmole x 10^4	per cent of total
Whole molecule	29.7	100
COOH	12.4	42
$CHNH_2$	0	0
CH_2	0	0
C_6H_4OH	17.3	58
Distribution of C^{14} within the ring		
C_6H_4OH	17.3	100
C_1	0	0
C_{2+6}	4.3	25
C_{3+5}	5.9	34
C_4	7.1	41

growth of various microorganisms, Ehrensvärd and Reio proposed a mechanism for the biosynthesis of aromatic amino acids which is in disagreement with the mechanism revealed by the foregoing studies. The mechanism of Ehrensvärd and Reio involves fragments of glucose in such a manner that the aromatic ring of tyrosine and phenylalanine should have a specific activity not greater than 3/2 that of glucose. Since these workers did not isolate glucose in any of their experiments, it seemed pertinent to compare the specific

activities of glucose and tyrosine in yeast when $C^{14}O_2$ was incorporated into the yeast in the presence of pyruvic acid. Since a considerably greater quantity of yeast was required for the isolation of glucose, an experiment similar to the previously described $C^{14}O_2$ incorporation study was carried out on a larger scale.

A portion of the yeast was hydrolyzed in the normal manner with 20 per cent HCl and the tyrosine isolated and burned to CO_2 for determination of its specific activity. Since such drastic hydrolysis destroys the carbohydrate material, another portion of this same yeast was treated according to the method of Gilvarg and Bloch (21,pp.339-346) and Gilvarg (22,pp.57-64) in order to isolate the carbohydrate fraction in the following manner: (1) the cells were defatted with alcohol and ether; (2) the protein was extracted from the defatted cells with 0.2 per cent NaOH; (3) the resulting residue containing yeast polysaccharides was hydrolyzed with 1N HCl for one hour; (4) the cell residue was removed and the hydrolysate passed successively through an amberlite IR-112 (strongly acidic) ion-exchange column and an amberlite IR4-B (weakly basic) resin column; (5) the effluent from the column operation was evaporated in vacuo, made up to volume and assayed for reducing sugars by the method of Shaffer and Somogyi (36, pp.695-713). Three grams of dry yeast were found to yield

345 mg of reducing sugars based on glucose. Paper chromatography showed approximately equal amounts of mannose and glucose to be present. Since mannose has been shown to be derived directly from glucose in baker's yeast (22,pp.57-64), the specific activities of the two sugars and the labeling patterns of their carbon skeletons should be essentially identical. Therefore an aliquot of the solution containing the mixed sugars was converted to glucosazone directly. After repeated recrystallization from aqueous methanol, a sample of this compound was burned to CO_2 by dry combustion and counted to obtain the specific activity of the glucosazone. The specific activities of tyrosine and glucosazone are given in table IV. Although the specific activity of the tyrosine obtained in the present experiment was too low for degradation studies, as a result of the greater amount of yeast involved, preliminary experiments showed that the C^{14} labeling patterns of tyrosine from $C^{14}O_2$ incorporation in yeast do not vary appreciably in different runs. It is therefore reasonable to assume the specific activity of various carbon atoms in the present tyrosine sample based on the data given in table III. The estimated specific activity of the tyrosine ring in the present experiment in included in table IV. Samples were counted as $BaCO_3$ using a windowless, gas-flow Geiger-counter with corrections for background and self-absorption

Table IV

	C.p.m. per mmole
glucosazone	930
tyrosine, total	5300
tyrosine ring	3070

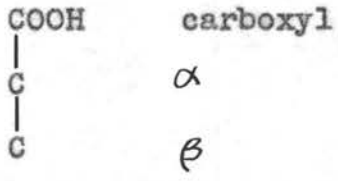
applied to the samples in the conventional manner. The specific activities in table IV have also been corrected for dilution and are expressed in counts per minute per millimole of original compound. The specific activities have been related to the end window Geiger-counter used in the earlier studies by application of the correction factor (1/11.6) discussed previously.

DISCUSSION

The inherent complexity of the mechanism of aromatic biosynthesis, as enhanced by the confusion created by various contradictory experimental findings in the literature, makes it a very difficult task to speculate on possible pathways leading to the total biosynthesis of tyrosine and phenylalanine from simple substrates. Furthermore, the symmetrical nature of the benzenoid ring structure makes it impossible to distinguish carbon 2 from 6 and 3 from 5 in degradation studies involving such isotopically labeled compounds. Consequently, the present studies are designed on the basis of the principles that: (1) efforts should be concentrated on one species of microorganism in combination with a selected group of simple carbon sources known to play important roles in natural biosynthetic processes and (2) in order to trace the pathway of these substrates the latter should be labeled with isotopic carbon in selected positions aimed at differentiating carbon 2 from 6 and 3 from 5 of the aromatic ring by means of deductive elimination.

In view of the difference adherent to the nature of the aliphatic side-chain and the aromatic ring structure, it seems desirable to discuss the mechanism of formation of the side-chain separately from ring synthesis. Figure 8

is presented in order to summarize the observations obtained on the origin of each carbon atom of the tyrosine and phenylalanine side-chains based mainly on the results reported in tables I, II, and III.



(side-chain of tyrosine and phenylalanine)

Carbon atom of side-chain	Labeled carbon atom of substrate contributing significant activity to the corresponding carbon atom in the side-chain	
	tyrosine	phenylalanine
Carboxyl	carboxyl of acetate (table I) CO_2 (table III)	carboxyl of acetate (table II)
α	carbonyl of pyruvate (table I)	carbonyl of pyruvate (table II)
β	carbon one of glucose (21,pp.339-346) carbon six of glucose (42,pp.302-303)	carbon one of glucose (21,pp.339-346)

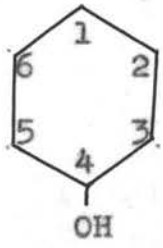
Figure 8

The heavy incorporation of pyruvate carbonyl carbon into the α carbon of the tyrosine and phenylalanine side-chains

strongly suggests that the latter arise from an intact C_3-unit such as pyruvate. This is in perfect agreement with the labeling patterns of the respective side-chains arising from acetate, CO_2 and glucose, as shown in Figure 8, if one visualizes that: (1) carboxyl labeled acetate or labeled CO_2 gives rise to pyruvate labeled in the carboxyl position and (2) glucose-1-C^{14} and glucose-6-C^{14} upon glycolysis would yield pyruvate labeled in the methyl carbon. The proposed mechanism is further supported by the recent finding of Davis (16,p.18C) that prephenic acid, formed by condensation of shikimic acid and a C_3 unit, is an intermediate in the biosynthesis of phenylalanine as shown in figure 7. It is also interesting to note that, although a labeling pattern for the tyrosine side-chain similar to the one reported in the present studies was obtained by Ehrensvärd and Reio (18,pp.229-234) in their E. coli experiments using carboxyl labeled acetate, these authors proposed that a C_2-unit (intact acetate) condenses with a C_1 side-chain, pre-attached to the ring system, to complete the tyrosine side-chain. It implies that the α carbon of the side-chain is derived directly from the methyl carbon of acetate and consequently can not account for the α carbon atom of the tyrosine side-chain being heavily labeled when carbonyl-C^{14}-pyruvate is used as a yeast substrate.

On the basis of the results obtained in the present studies as well as findings in the literature, the possible origin of each carbon atom of the aromatic ring component of tyrosine, as related to the original carbon source, is summarized in figure 9. It is understood that the substrate may have undergone a number of reactions including randomization and re-incorporation via metabolic CO_2 before being incorporated (secondarily) into the tyrosine structure.

In an early report (46,pp.5554-5556) covering a portion of the results obtained in the present studies a speculative mechanism for the biosynthesis of the aromatic ring of tyrosine was suggested which involves condensation of two C_4-acids such as oxalacetate. This mechanism together with the carbon skeleton of oxalacetate, as the possible C_4-acid involved, are given in figure 10. For purposes of identification one molecule of oxalacetate will be designated A and the other B, which may be some other unit.



(ring component of tyrosine)

Carbon atom of ring	Labeled carbon atom of substrate contributing significant activity to the corresponding carbon atom in the ring	References
1	carbonyl of pyruvate	Table I
2,6	carbon one of glucose	(21,pp.339-346)
	carbon six of glucose	(42,pp.302-303)
	carboxyl of acetate	(18,pp.229-234) and Table I
	CO_2	Table III
3,5	carbonyl of pyruvate	Table I
	carboxyl of acetate	Table I
	CO_2	Table III
4	CO_2	Table III
	carboxyl of acetate	(18,pp.229-234) and Table I

Figure 9

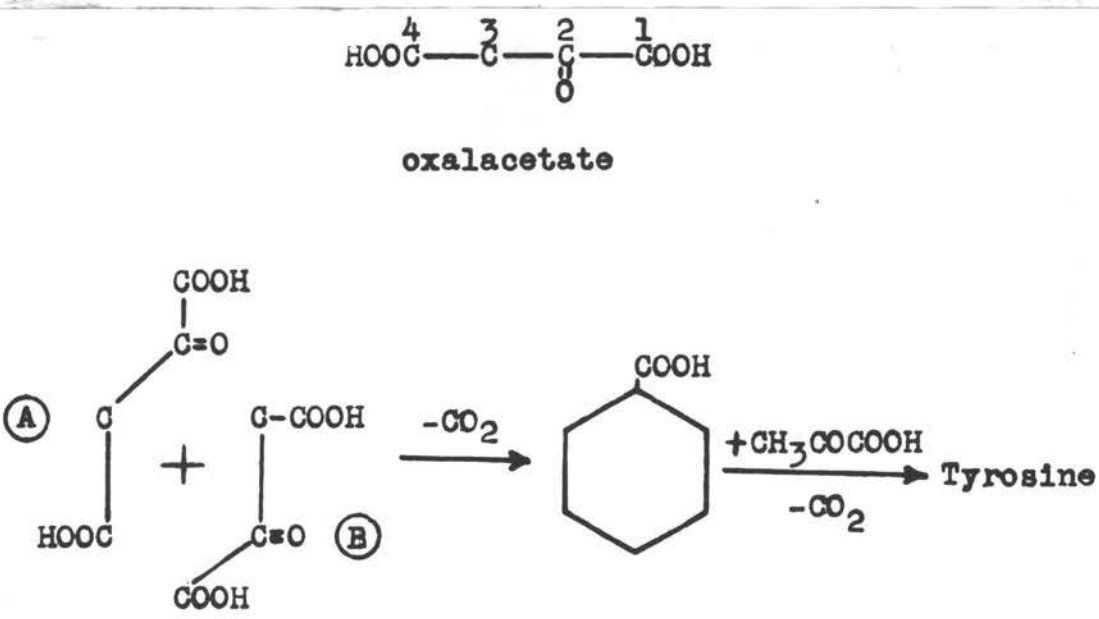


Figure 10

Thus, in this speculative sequence the hypothetical condensation product is cyclized, aromatized, condensed with pyruvate and transaminated to form tyrosine. When oxalacetate is assigned the labeling pattern observed for aspartic acid in yeast grown on carbonyl labeled pyruvate (48,pp.663-667), the quantitative distribution of activity within the ring synthesized according to this mechanism, agrees closely with that observed by degradation of tyrosine in the case of the aerobic pyruvate fermentation. The actual specific activities of the ring carbon atoms would not, of course, be expected to equal those of the corresponding carbon atoms of aspartic acid since the relative dilution of labeled pyruvate, C_4-acids and tyrosine by cellular constituents was unknown. This mechanism

also explains the results obtained by Gilvarg and Bloch (21,pp.339-346) using glucose-1-C^{14} as a substrate for yeast growth. On the other hand this mechanism fails to account quantitatively for the labeling pattern of tyrosine observed in the anaerobic pyruvate experiment, particularly in respect to the heavy labeling of carbons 2+4+6 in the ring. The discrepancy becomes greater in the case of tyrosine from the acetate experiment where the activity of carbons 2+4+6 in the ring was found to be twice as great as that of carbons 3+5, although the proposed mechanism calls for activities of equal magnitude.

On re-examination of the C_4 condensing components in figure 10, one finds that the nature of component A is quite well defined by the experimental finding of heavy labeling in ring carbon one from carbonyl labeled pyruvate (figure 9) and on ring carbons 2+6 from glucose-1-C^{14} (23,pp.689-698). Thus, the oxalacetate carbon skeleton would seem to be furnishing carbons 1, 6 and 5 of the tyrosine ring as shown in figure 10. The above discrepancy in both the acetate and anaerobic pyruvate experiments could then mean that condensing component B in figure 10 is not oxalacetate. This was considered in designing and carrying out the $C^{14}O_2$ experiment. A considerable amount of activity in ring carbons 2+6 and heavier labeling of ring carbon 4, as compared to carbons 3+5, were

found in the $C^{14}O_2$ experiment. These observations thus provide valuable clues toward understanding the nature of condensing component B which furnishes carbons 2,3 and 4 of the tyrosine ring as shown in figure 10. Meanwhile, Ehrensvärd and Reio (18,pp.229-234), using refined degradation methods, also reported that carbons 2+6 in the ring of tyrosine contained significant activity when several species of microorganisms were grown on carboxyl labeled acetate.

These new findings thus led to speculation on the nature of a new condensing component to replace B in figure 10. Such a compound should contain carboxyl groups, or other groups arising readily from metabolic CO_2, oriented in positions meta to each other, so as to give rise to carbon atoms 2 and 4 of the tyrosine ring. Since the activity of carbon 2 does not equal that of carbon 4 in the ring of tyrosine from either the $C^{14}O_2$ or the carboxyl labeled acetate experiment, a head to tail condensation of two C_2-acids is ruled out as a source of component B. The condensation of the carboxyl carbon of a C_4-acid with the non-carboxylic carbon of a C_2-acid may therefore be considered, since in this case the activities of the carboxyl groups of the two acids need not be equal in magnitude. Considering this a possibility and by postulating the carboxyl group of the C_2-acid as the

origin of carbon 4 in the ring (with its activity derived from either acetate carboxyl or CO_2) the next problem becomes one of understanding the nature of the non-carboxylic carbon which should give rise to carbon 3 of the tyrosine ring. In the case of the pyruvate experiment ring carbon 3 must be labeled since carbon 5 could not furnish sufficient activity alone to account for that observed as C_{3+5} in table I. Examination of known labeling patterns of possible C_2 intermediates reveals that the glycine carbon skeleton (50) fulfills the above conditions. Degradation studies of this amino acid indicate that its carboxyl carbon originates from either acetate carboxyl or CO_2 and its methyl carbon is derived primarily from either the carbonyl carbon of pyruvate or the methyl carbon of acetate. The exact nature of the C_4-acid mentioned is not too important at this step since 3 of its 4 carbon atoms would be removed in the speculative sequence to follow.

Few compounds occurring in biological systems meet the requirements just discussed. Recent reports of Shemin (37,pp.4873-4874 and 38,p.295), however, have shown the importance of a condensation product of glycine and succinic acid, α -amino-β -keto-adipic acid, in the biosynthesis of the pyrrole ring of porphyrins. A speculative compound of this nature, formed by the condensation of the

β-carboxyl carbon of oxalacetate and the non-carboxylic carbon of the glycine skeleton, could be the condensing component B for the biosynthesis of the aromatic ring of tyrosine as shown in figure 11.

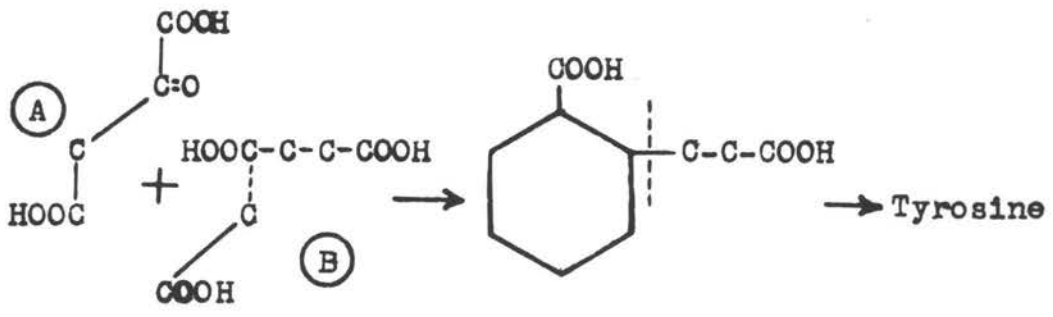


Figure 11

The origin of each carbon atom of the tyrosine ring, as observed experimentally and as required by the mechanism shown in figure 11, are presented in table V. From table V it can be seen that the new speculative sequence is in good agreement with the findings of the pyruvate, acetate and $C^{14}O_2$ experiments of the present studies. It also explains the results of Ehrensvärd and Reio (18,pp.229-234) using carboxyl labeled acetate and Gilvarg and Bloch using labeled glucose (23,pp.689-698) in

their respective experiments.

Table V

Carbon atom of tyrosine ring	Origin of each carbon atom of tyrosine ring from experiments (Figure 9)	Predicted origin of each carbon atom of tyrosine ring from figure 11
1	carbonyl of pyruvate	carbonyl of pyruvate via oxalacetate
2	carboxyl of acetate	carboxyl of acetate via C_4-acid
	CO_2	CO_2 via carboxyl of C_4-acid
3	carbonyl of pyruvate	carbonyl of pyruvate via methyl carbon of glycine
4	carboxyl of acetate	carboxyl of acetate via carboxyl of glycine
	CO_2	CO_2 via carboxyl of glycine
5	carboxyl of acetate	carboxyl of acetate via carboxyl of oxalacetate
	CO_2	CO_2 via carboxyl of oxalacetate
6	carbon one of glucose	carbon one of glucose via methylene carbon of oxalacetate
	carbon 6 of glucose	carbon 6 of glucose via methylene carbon of oxalacetate

It is interesting to compare the scheme presented in figure 11 with the mechanism of tyrosine biosynthesis proposed by Ehrensvärd and Reio (18,pp.229-234) as illustrated in figure 3. A careful study of figure 3 reveals that these authors have actually proposed several slightly different mechanisms, varying in the orientation of the C_4 and C_3 fragments at the time of condensation. However, according to any of these slightly different variations, two adjacent carbon atoms of the tyrosine ring would arise as a unit from carbon atoms 3 and 4 of glucose. From carboxyl labeled acetate or from $C^{14}O_2$, carbon atoms 3 and 4 of glucose would be equal in activity, consequently two adjacent carbon atoms of the tyrosine ring should also have equal activities. Examination of the data in table III for the $C^{14}O_2$ experiment of the present studies, together with the data of Ehrensvärd and Reio (18,pp.229-234), reveals that such is not the case. However, in the mechanism resulting from the present studies, adjacent carbon atoms of the ring would <u>not necessarily be equally labeled</u> in the case of the $C^{14}O_2$ and carboxyl labeled acetate experiments.

It will be recalled that in the $C^{14}O_2$ experiment described earlier the concentration of pyruvic acid was maintained at a constant level by periodic addition of non-radioactive pyruvate during the fermentation. For

this reason, and as subsequently confirmed by the extremely low activity of alanine observed in the same yeast sample[2], it seems likely that glucose would have a specific activity comparable in magnitude to alanine. Although comparison of specific activities does not absolutely prove precursor-product relationship it may be indicative. The experimental finding that glucose has a much lower specific activity than tyrosine (table V) when both were isolated from the same yeast sample provides strong evidence that glucose is not likely to be directly involved in tyrosine biosynthesis.

The present studies on the degradation pattern of phenylalanine formed from different labeled substrates reveal a very interesting situation. As shown in tables I and II, the labeling patterns of tyrosine and phenylalanine are very similar in the case of the aerobic pyruvate experiment. This indicates that the speculative scheme presented in figure 11 for tyrosine is also applicable for the biosynthesis of phenylalanine from this substrate. However, in comparing the labeling patterns of the two amino acids when labeled acetate was the carbon source, a sharp contrast was observed. Thus carbons 2+4+6 of the tyrosine ring account for over 70 per cent of the ring

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[2] Personal communication from Mr. James Wendell Davis.

activity while the majority of the phenylalanine ring activity was found in carbons 1+3+5. The contrast is also indicated, but to a lesser extent, in the anaerobic pyruvate experiment. This is understandable since considerable oxidative decarboxylation of pyruvate to acetate seems to occur under these conditions.

Without carrying out additional experiments it is impossible at the present time to offer an explanation for the observed shift of activity (carbons 2+4+6 to 1+3+5). However, if one visualizes that the intermediate in the proposed scheme shown in figure 11 has two possible positions for attachment of the side-chain, namely positions a and b, it then appears that the observed shift in labeling patterns could well be the result of differences in the mechanism of side-chain formation as shown in figure 12. In view of the fact that acetate may not be a normal metabolite in the yeast system (as evidenced by its slow rate of utilization) and that the exact nature of the intermediates in aromatic biosynthesis are not known, one could speculate that the acetate causes a slight variation in the respective tyrosine and phenylalanine biosynthetic intermediates. This may require different mechanisms for side-chain synthesis which in turn causes the observed shift in labeling patterns. If this is true, then in the case of phenylalanine the side-chain may be

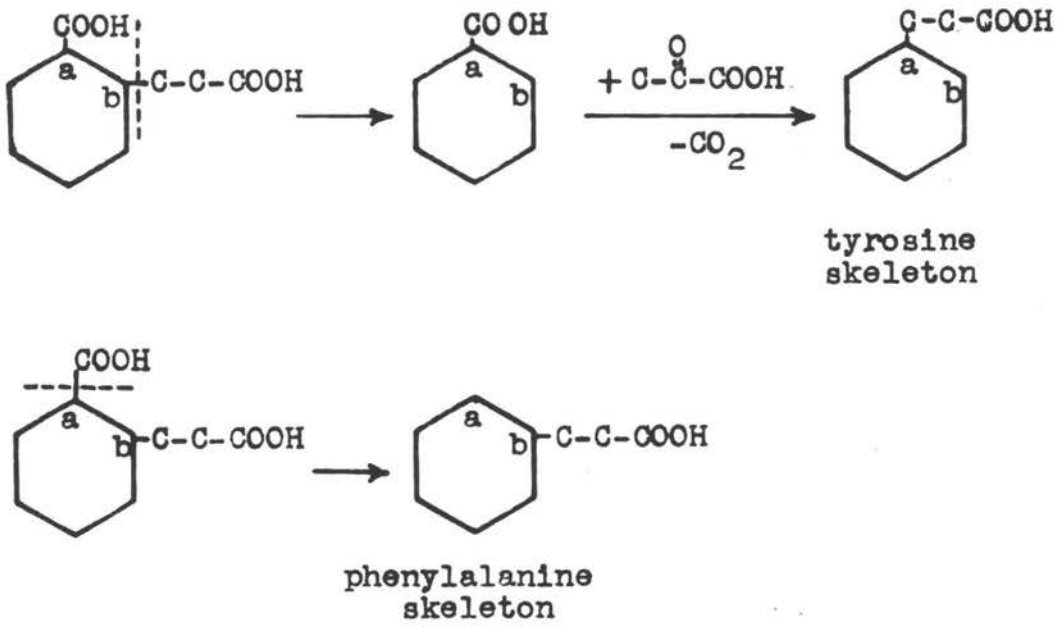

Figure 12

formed simultaneously with the ring as indicated in figure 12.

Because of the unavailability of the experimental details, including the all-important degradation method for shikimic acid in the E. coli mutant work of Shigeura (39,p.286 and 40,p.458), no attempt was made to correlate the present biosynthetic schemes with the findings of this author.

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