breakdown of the substrate to small dextrins observed on one extreme with taka amylase and the relatively slow disappearance of long chain saccharides observed with swine pancreatic amylase on the other extreme appear to be definite properties of these amylases. It is challenging to speculate about and to seek the cause for these differences in the action of two amylases, both of which appear to be so-called simple proteins.[3,16-18,38]

NOTE ADDED OCTOBER 20, 1953.—Since this paper was submitted for publication, two additional series of experiments have been completed with results that confirm and strengthen the conclusions.

When all of the amylases in the comparison reacted with the substrate at the same hydrogen ion activity, at *p*H 7.2, the results were identical with those reported in Fig. 3 and in Table I for comparisons made when each amylase reacted with the substrate at the *p*H most favorable to its action. These results show that the differences observed in the action of the amylases are true differences and are not due to the influence of differences in the hydrogen ion activities of the substrate. An unfavorable hydrogen ion activity may decrease materially the concentration of active amylase but does not influence its unique action. It must be remembered that the comparisons given here for amylase action are independent of amylase concentration, Fig. 1.

Similarly, results identical with those reported in Fig. 3, curve 1, were obtained when suitable volumes of swine saliva that had been held at 100° for 5 minutes to inactivate the amylase were added to recrystallized swine pancreatic amylase. Thus, the substances that accompanied the unpurified swine salivary amylase had no influence on the action of recrystallized swine pancreatic amylase and presumably had no influence on the action of the swine salivary amylase they accompanied. These results give additional evidence that the differences observed above in the action of swine pancreatic amylase and of swine salivary amylase represent true differences in the specific mode of action of these two amylases.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY AND THE SCIENCE RESEARCH INSTITUTE, OREGON STATE COLLEGE]

Conversion of Acetate and Pyruvate to Tyrosine in Yeast[1]

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$CH_3C^{14}OCOOH$ and $CH_3C^{14}OOH$ have been compared as carbon sources for the formation of tyrosine in bakers' yeast. The intramolecular distribution of radioactivity in the isolated tyrosine indicated that the aromatic ring was formed from pyruvate *via* oxalacetate or a similar unsymmetrical C_4-acid as intermediate. The side chain appeared to arise from pyruvate as an intact C_3-unit.

Considerable interest has been focused recently upon the biosynthesis of tyrosine in microörganisms. Davis's studies[3] with mutant strains of *Escherichia coli* point to shikimic acid as a normal precursor of tyrosine and phenylalanine, as well as other aromatic compounds in that organism. Baddiley, *et al.*,[4] have suggested that when acetate is used as the sole carbon source for adapted Torula yeast, the carboxyl group gives rise to the carboxyl of tyrosine, as well as to carbon 4 of the ring; the other seven carbon atoms were considered derived chiefly from the methyl group of acetate. Finally, the side chain of tyrosine may arise from pyruvate as an intact unit in *E. coli* (Cutinelli, *et al.*[5]).

In this Laboratory, C^{14}-labeled pyruvate and acetate have been compared as carbon sources for yeast growth. The radioactivity distribution patterns in the isolated tyrosine suggest that oxalacetate or other unsymmetrical C_4 acid may function as an intermediate in the conversion of pyruvate to this amino acid.

Experimental

Use was made of the yeast samples obtained previously,[6] in which 20 mmoles each of $CH_3C^{14}OCOOH$ or $CH_3C^{14}OOH$ with a specific activity of 1.85×10^6 c.p.m. per mmole, were administered as the sole carbon source to Fleischmann's bakers' yeast that had been previously grown on glucose. In the pyruvate experiments, all of the labeled substrate was utilized in 4 hours aerobically, and 5 hours anaerobically. Acetate was employed only under aerobic conditions, 39% of the labeled substrate being used in 4 hours. Details of these fermentations have been presented elsewhere.[6]

Tyrosine was isolated from the yeast hydrolysate[7] by concentration and crystallization at the isolectric point, following a fivefold dilution with non-isotopic L-tyrosine. The specific activities were thus one-fifth of the values given in Table I. 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 obtained was degraded according to the method of Baddiley, *et al.*,[4] on the same scale in the following manner: (1) combustion to CO_2 for the specific activity of the whole molecule; (2) decarboxylation with ninhydrin for 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 of tyrosine 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 converted to CO_2. This represented 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 to obtain the specific activity of the benzene ring as well as the specific activity of the methylene carbon atom of the side chain by difference. The remainder of the picric acid was oxidized with $Ca(OBr)_2$ to bromopicrin, which was oxidized to CO_2. This represented carbon atoms 1, 3 and 5 in the tyrosine ring. The average specific activity of carbon atoms 2, 4 and 6 could be thus obtained by difference.

Carbon atom 4 of the ring was not differentiated from

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(2) National Science Foundation Predoctoral Fellow.

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(6) C. H. Wang, R. F. Labbe, B. E. Christensen and V. H. Cheldelin, *J. Biol. Chem.*, **197**, 645 (1952).

(7) R. F. Labbe, R. C. Thomas, V. H. Cheldelin, B. E. Christensen and C. H. Wang, *J. Biol. Chem.*, **197**, 655 (1952).

carbon atoms 2 and 6 since only a very small amount of tyrosine was available.

The determination of the activity of the β-carbon in the side chain by difference, instead of by direct decarboxylation of *p*-hydroxybenzoic acid,[4] was also due to the limited amount of sample available in the present experiments.

All samples were counted as $BaCO_3$ with correction for background and self-absorption in the conventional manner.

Results and Discussion

The radioactivity distribution in tyrosine from each of the three yeast samples is given in Table I. As observed previously for aspartic acid[8] and glutamic acid,[9] the distribution patterns differed widely in the acetate and aerobic pyruvate samples. Also, as experienced before, the anaerobic pyruvate sample exhibited an intermediate pattern of activity.

TABLE I

DISTRIBUTION OF C^{14} IN TYROSINE FROM YEAST UTILIZING $CH_3C^{14}OOH$ OR $CH_3C^{14}OCOOH$

Conditions	Aerobic pyruvate C.p.m. per mmole × 10^4	Aerobic pyruvate Per cent. of total	Anaerobic pyruvate C.p.m. per mmole × 10^4	Anaerobic pyruvate Per cent. of total	Aerobic acetate C.p.m. per mmole × 10^4	Aerobic acetate Per cent. of total
Whole molecule	49.1	100	19.0	100	4.83	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}	11.8	46	3.8	36	0.74	29
C_{2+4+6}	5.7	22	5.0	48	1.74	72

With pyruvate as substrate (aerobic fermentation), 46% of the total tyrosine activity was located in the side chain, predominantly in the amino carbon atom. This atom presumably corresponded to the carbonyl carbon of the administered pyruvate. This observation, together with the complete absence of radioactivity in the β-carbon atom, strongly suggested that the side chain of tyrosine was derived from pyruvate as an intact unit. The small amount of activity in the carboxyl group can probably be accounted for by its partial equilibration with metabolic CO_2.[5,10] Origin of the tyrosine side chain from an intact C_3 unit is also suggested by Cutinelli, *et al.*,[5] as well as by the data of Gilvarg and Bloch.[11] In the latter, using glucose-1-C^{14} as the carbon source, the β-carbon of the tyrosine side chain contained nearly half of the total activity in the molecule. This carbon atom apparently corresponded to the labeled atom of glucose after glycolysis.

Over three-fourths of the remaining radioactivity (*i.e.*, in the aromatic ring) was found in positions 1, 3 and 5; C_1 was somewhat more active than the mean of C_3 and C_5. This distribution of activity cannot be explained by conversion of pyruvate to glucose and cyclization of the latter, since this would produce only two active centers in the ring, at para positions.

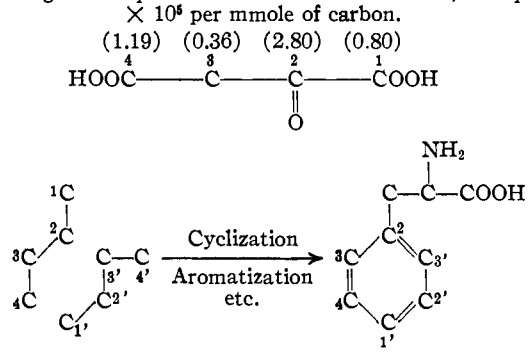
A study of other possible metabolic pathways to tyrosine *via* shikimic acid revealed a good reconciliation of the observed radioactivities, through the use of two molecules of oxalacetate or other C_4-acids with similar isotopic patterns (formed from pyruvate *via* C_3-C_1 condensation[8]) as the starting materials. Thus, in this speculative sequence (Scheme I), the hypothetical condensation product is cyclized, aromatized, condensed with pyruvate (or its equivalent) and transaminated. When oxalacetate was assigned the radioactivity distributions observed for aspartic acid in this yeast,[8] the calculated activities within the ring agreed closely with those observed, as follows: with C_1 of the ring considered equivalent to C_2 of oxalacetate, the value of 8.33×10^4 c.p.m./mmole carbon should be accompanied by counts of 11.8×10^4 in C_{3+5}, and 4.5×10^4 in C_{2+4+6}. (Found: 11.8 and 5.7×10^4 in these positions, respectively). The activity of C_1 of the ring could not, of course, be expected to equal that of the amino carbon, since the relative dilution of labeled pyruvate and C_4-acids with cellular constituents was unknown

The present proposal that tyrosine may arise from unsymmetrical C_4-acids, differs from the earlier conclusion[12] that this amino acid is produced from glucose *via* cyclization. Later work with glucose as substrate[11] has ruled out direct cyclization, and the authors have pointed out the possibility of condensation of a C_3 unit with some other product of glucose metabolism. However, the isotopic data obtained did not permit a clear interpretation of the course of tyrosine synthesis. C_7 sugars were considered as intermediates, based on the work of Benson, *et al.*,[13] and Horecker and Smyrniotis.[14]

SCHEME I

POSTULATED CONVERSION OF AN UNSYMMETRICAL C_4-ACID TO TYROSINE

Values given in parentheses are for oxalacetate, in c.p.m. × 10^5 per mmole of carbon.



Very recently, Shigeura, *et al.*,[15] in a study of the biosynthesis of shikimic acid (hence presumably also tyrosine) in *E. coli*, have suggested that glucose is not utilized *per se*, but instead undergoes fragmentation to produce two or more unsymmetrical units

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(10) G. Ehrensvärd, L. Reio, E. Saluste and R. Stjernholm, *ibid.*, **189**, 93 (1951).

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which recombine to form the appropriate cyclic compounds. Both their work and the present study, in which the observed labeling patterns are compatible, demonstrate the need for intermediates that do not equilibrate with symmetrical products of glycolysis or the Krebs cycle. From pyruvate, this is more easily envisioned, since randomization or interaction with glycolysis products does not occur extensively; from glucose, the possible pathway is less clear. C_4-acid formation through $C_3 + C_1$ condensation is favored by pyruvate (as distinguished from acetate,[8,9]) and the failure of tyrosine to be formed from C^{14}-bicarbonate in the absence of pyruvate or other substrate[16] may reflect the previously observed importance of the C_3-C_1 condensation reaction in yeast and its dependence upon pyruvate.[8]

The conversion of acetate to tyrosine may also proceed through the C_4-acids, although quantitative agreement between observed and expected values is less satisfactory than in the pyruvate sample. The high activity of the carboxyl group, as well as the high ring activity at C_{2+4+6}, confirm the intramolecular distribution reported by Baddiley, *et al.*,[4] as far as the present degradation studies were carried out. It may thus be assumed that C_4 of the ring contains most of the activity designated in Table I as C_{2+4+6}. This would be expected if oxalacetate were the precursor of tyrosine, since aspartic acid derived from acetate in this yeast contained C^{14} only in the carboxyl groups.[8] It would also account for the absence of isotope in C_1 of tyrosine. The deviation between expected and observed values occurs at C_{3+5}; the activity of these atoms should equal the activity of C_4. An inspection of the C^{14} distribution obtained by Baddiley, *et al.*[4] (using $C^{13}H_3C^{14}OOH$ as substrate), reveals a similar observed deficiency of radioactivity at C_{3+5}, although the C^{13} distribution agrees well with expectations based on the foregoing scheme.

The present results, as well as others,[12,15,17] suggest that the citric acid cycle does not participate directly in the formation of tyrosine from pyruvate, acetate or glucose, although the over-all effect of Krebs cycle intermediates is to increase tyrosine synthesis by yeast.[18] Studies are in progress to relate further the behavior of glucose and pyruvate in these transformations.

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The Preparation of Optically-active Peptides Using Mixed Carbonic–Carboxylic Acid Anhydrides

BY JAMES R. VAUGHAN, JR., AND JOYCE A. EICHLER

RECEIVED JUNE 11, 1953

The study of racemization by this method of synthesis has been continued. Using N-carbobenzoxyamino acid or peptide–isobutylcarbonic acid anhydrides and amino acid or peptide esters, L-lysyl-L-valyl-L-phenylalanylglycine was prepared by two routes and shown to have almost identical properties in each case. Therefore, very little, if any, racemization occurred. When these mixed anhydrides in anhydrous solution were caused to react with the sodium salts of amino acids (or peptides) in aqueous solution no racemization was observed if the mixed anhydride was formed from an optically-active *N-carbobenzoxy*-amino acid. Partial racemization was observed, however, if the mixed anhydride was formed from an optically-active *N-acyl*amino acid, *i.e.*, an optically-active N-carbobenzoxy dipeptide, etc.

In a preliminary report[1] it was demonstrated that, in the reaction employing mixed carbonic–carboxylic acid anhydrides for the formation of carbobenzoxy peptide esters, racemization can be avoided or greatly minimized by the proper choice of reaction solvents and conditions. This investigation has now been extended to the preparation of the tetrapeptide L-lysyl-L-valyl-L-phenylalanylglycine and to a study of the reaction of mixed carbonic–carboxylic acid anhydrides with salts of amino acids or peptides under aqueous conditions. Interest in the above-mentioned tetrapeptide was initiated by the announcement[2] that the sequence on the amino end of the single polypeptide chain of lysozyme is lysylvalylphenylalanylglycyl-, and it was hoped that a direct comparison of a synthetic material with the natural product might be made. This has now been accomplished through the cooperation of Dr. Walter A. Schroeder of the California Institute of Technology, who compared the synthetic material prepared here with his natural product in the form of their dinitrophenyl (DNP) derivatives, and found them to be essentially identical.[3] No conclusions concerning the optical identity of the individual amino acids could be drawn, however.

For comparison of optical purity, the derivatized tetrapeptide was prepared by two routes. In the first of these, dicarbobenzoxy-L-lysyl-L-valine was prepared by saponificaton of its ethyl ester and coupled through its mixed isobutylcarbonate anhydride with ethyl L-phenylalanylglycinate to give the dicarbobenzoxy tetrapeptide ester in 54% yield m.p. 199–200°, $[\alpha]^{24}D$ $-21.4 \pm 0.6°$ (c 2, glacial acetic acid).

By the second route carbobenzoxy-L-valine was first condensed with ethyl L-phenylalanylglycinate to give the tripeptide derivative, which was then hydrogenated to ethyl L-valyl-L-phenylalanylglycinate. This was coupled with dicarbobenzoxy-L-lysine by the standard method[1] to give ethyl dicar-

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