CARBON DIOXIDE FIXATION AND BIOSYNTHESIS OF AMINO ACIDS IN YEAST (*SACCHAROMYCES CEREVISIAE*)

by

JAMES WENDELL DAVIS

A THESIS

submitted to

OREGON STATE COLLEGE

in partial fulfillment of
the requirements for the
degree of

DOCTOR OF PHILOSOPHY

June 1955

APPROVED:

[redacted]

Professor of Chemistry

[redacted]

Associate Professor of Chemistry

In Charge of Major

[redacted]

Chairman of Department of Chemistry

[redacted]

Chairman of School Graduate Committee

[redacted]

Dean of Graduate School

Date thesis is presented August 11, 1954

Typed by Evelyn Kanzelmeyer

ACKNOWLEDGEMENTS

The author wishes to express his sincere appreciation to Dr. Vernon H. Cheldelin and Dr. Chih H. Wang for their direction throughout the course of these studies. Dr. Cheldelin's generous gifts of time and suggestions pertaining to the research and the writing of the thesis were invaluable. Dr. Wang's personal direction of the research and stimulating discussions relevant to interpretation of the results made this work both possible and enjoyable.

The work was made finiancially possible through the generosity of the Atomic Energy Commission of the United States. I wish to express my gratitude to Dr. Richard C. Thomas and Mr. Charles T. Gregg for their assistance at times when more than two hands and one head were needed. I am also indebted to Mr. Kenneth G. Davis for the photography contained in these pages, as well as to Mr. Paul Kitos for the graph.

My wife's understanding and encouragement throughout the period of this research added greatly to the mental attitude requisite to a successful conclusion. For these, I am very appreciative.

TABLE OF CONTENTS

CARBON DIOXIDE FIXATION AND BIOSYNTHESIS OF AMINO ACIDS IN YEAST (*SACCHAROMYCES CEREVISIAE*)

INTRODUCTION

Investigation of non-photosynthetic carbon dioxide fixation has occupied a prominent place in biochemical research for the past two decades. Wood and Werkman (101) were the first to show that heterotrophic organisms were capable of incorporating CO_2.

Primary fixation reactions of CO_2. In the years following the first demonstration of CO_2 fixing ability on the part of *Propionibacterium* sp. more than a score of biochemical equilibria have been described in which carbon dioxide is a participant.

Carbon dioxide may enter C_4 dicarboxylic acids through at least three routes:

1) $\underline{CO_2} + CH_3\text{-}CO\text{-}COOH \rightleftharpoons HO\underline{OC}\text{-}CH_2\text{-}CO\text{-}COOH$

2) $\underline{CO_2} + CH_3\text{-}CO\text{-}COOH$[1] $+ TPN\cdot H + H^+ \rightleftharpoons$

$$HO\underline{OC}\text{-}CH_2\text{-}CHOH\text{-}COOH + TPN$$

3) $\underline{CO_2} + CH_3\text{-}CH_2\text{-}COOH \longrightarrow HO\underline{OC}\text{-}CH_2\text{-}CH_2\text{-}COOH$

Reaction 1 was proposed by Wood and Werkman (102) to account for their previous observation but a more complete

---

[1] More likely phosphoenolpyruvic acid ($CH_2{=}COPO_3H_2\text{-}COOH$) (87).

clarification of the system involved was afforded by isotopic tracer studies using $C^{13}O_2$ (41, 97, 98, 100). The reaction, catalyzed by oxalacetic acid decarboxylase, was found to be energy dependent (87), requiring adenosine triphosphate or inosine triphosphate (85).

The second of the foregoing reactions was first educed by Ochoa, Mehler, and Kornberg (58). The catalyst for this reaction has been given the name "malic enzyme" and its dependency upon reduced TPN and Mn^{++} has been established.

Reaction 3, found in Propionibacterium pentosaceum, (11), may also occur in rats, as evidenced by the findings of Marshall and Friedberg who find (50,p.784) that only thirty seconds is required after administration of $NaHC^{14}O_3$ to the intact rat for the liver succinate to accumulate 61% of the incorporated activity. In the same time malate and fumarate accumulate only 1 and 5 per cent, respectively, of the incorporated C^{14}.

Additional possibilities for entry of CO_2 involve other components of the Krebs cycle, as follows:

4) $\underline{CO_2} + CH_3\text{-}COOH \rightleftharpoons CH_3\text{-}CO\text{-}\underline{COOH}$

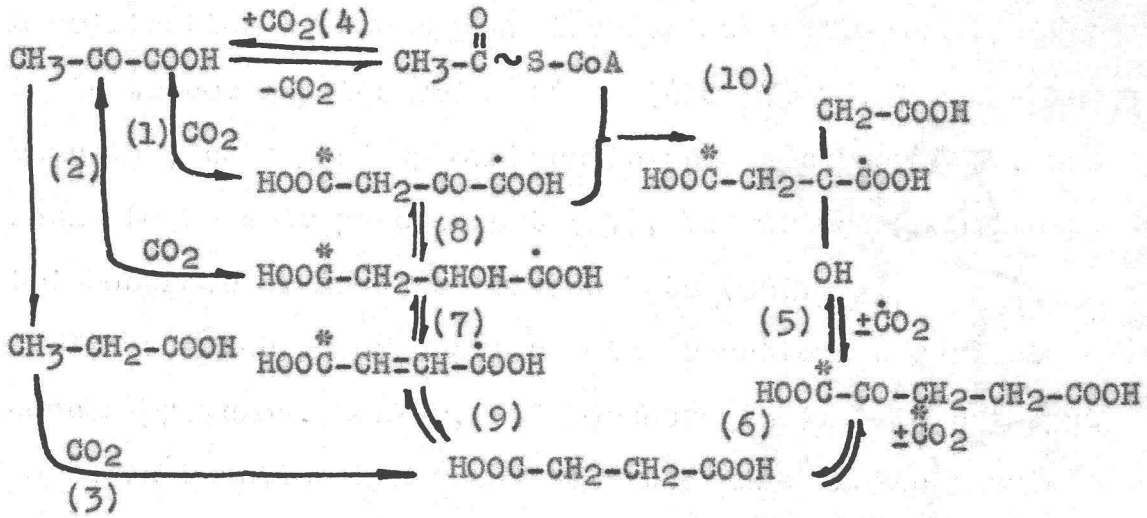
5) $\underline{CO_2} + HOOC\text{-}CH_2\text{-}CH_2\text{-}CO\text{-}COOH \rightleftharpoons HOOC\text{-}CH_2\text{-}\underset{\underline{COOH}}{C}(OH)\text{-}CH_2\text{-}COOH$

6) $\underline{CO_2} + HOOC\text{-}CH_2\text{-}CH_2\text{-}COOH \rightleftharpoons HOO\underline{C}\text{-}CO\text{-}CH_2\text{-}CH_2\text{-}COOH$

Evidence for reactions 4 and 6 is based on measured exchange of isotopic CO_2 with non-labeled pyruvic acid (in reaction 4) or ∝-ketoglutaric acid (in reaction 6). Because these studies were undertaken with crude enzyme systems and because the level of incorporation was usually low (86,p.92) the label may have been introduced by side reactions.

Reaction 5 is known to occur both in pig heart (57) and in parsley root (20). As with the malic enzyme, this system requires the presence of Mn^{++} and reduced TPN.

Scheme I reviews graphically the citric acid cycle in the presence of isotopic carbon dioxide ($\overset{*}{C}O_2$). The numbers in the scheme correspond to the numbered reactions in the text.



SCHEME I

Randomization of label in C_4 acids. The primary fixation into a C_4 acid via reactions 1 and 2 will yield dicarboxylic acids having the label in the C-4 position exclusively. However, the reversibility of reactions 7 and 8 provides for the formation of a symmetrical C_4 acid, fumaric acid. Mosbach, et al (53), have established that the formation of such a symmetrical intermediate will lead to the randomization of isotope in both carboxyl groups of malate and oxalacetate when these acids are reformed. The extent to which this occurs will depend upon the rapidity with which reaction 7 comes to equilibrium. The activity assumed by C-1 due to this randomization is noted as Ċ in Scheme I.

An idea as to the extent to which randomization proceeds in living systems may be gleaned from investigation of labeling patterns of key intermediates available from CO_2 fixation experiments.

In mammalian liver, Marshall and Friedberg (50) found that as much as 30% of the activity contained in oxalacetate appeared in the α-carboxyl group (C-1), within five minutes after injection of $NaHC^{14}O_3$ into the intact animal.

Swick (78,p.58) has demonstrated that over a longer period of time, complete equilibration of carbons 1 and 4 of aspartic acid is attained. The animals (rats) with which he worked were maintained for 85 days in contact with a low constant partial pressure of $C^{14}O_2$. During this time "radioactive saturation" in the liver was attained.

After a one hour incubation with $C^{14}O_2$, Escherichia coli had only 20% of the activity in C-1 of aspartate (3, p.170; 62,p.1015) but at the end of forty hours, Aspergillus niger could be shown to have 34% of the activity of malic acid in C-1 (54,p.439) indicating that randomization had proceeded more than half-way to completion in that length of time.

Appearance of isotope in C-1 of pyruvate may arise from two processes: first, through randomization of C_4 acids as just described, followed by reversal of reactions

1 or 2 (decarboxylation); or second, through combination of $C^{14}O_2$ with acetate (reaction 4). The carboxyl-labeled pyruvate produced in the latter reaction would also contribute isotope to C-1 of oxalacetate or malate through reactions 1 or 2. CO_2 incorporation into pyruvate C-1 has been observed directly in pigeon liver (99) and has been inferred by the finding of isotope in alanine isolated from bovine systems assimilating $NaHC^{14}O_3$ (12, p.367).

The three tricarboxylic acids, citric, cis-aconitic, and isocitric, are formed by way of reaction 10. Again, the extent to which randomization of the C_4 acids has occurred will be reflected in the labeling patterns of the C_6 acids. Thus, the ratio of the activities of primary COOH/tertiary COOH in citric acid from A. niger is about 2:1 (45, 51, 54), indicating that the randomization in the C_4 acids was about 2/3 complete. This conclusion is based on the assumption that all of the citrate arose from condensation of acetate with oxalacetate.

CO_2 fixation into amino acids. The biosynthesis of amino acids has been examined with fixation reactions involving CO_2 or other C_1 units.

Aspartic and glutamic acids bear a close relationship to oxalacetic and ∝-ketoglutaric acids, respectively, through amination and transamination reactions. It is not

surprising therefore that a large share of the isotope contributed by labeled CO_2 is usually found in these two amino acids. Examination of Scheme I suggests that aspartic acid will have activity confined to C-1 and C-4 in a ratio dictated by the extent to which randomization occurs. Previous consideration of these acids has confirmed this (3,p.170 and 62,p.1015).

Glutamic acid (and α-ketoglutaric acid) should have the activity of the entire molecule confined to C-1. This is confirmed by Delluva (28,p.744), Evans and Slottin (33, p.445), Roberts, et al (62,p.1015) and Abelson (3,p.170), although an important exception to this arises from the work of Hendler and Anfinsen (39). In hen oviduct preparations they found up to 30 per cent of the isotope in C-5 of glutamic acid.

Following exposure to $C^{14}O_2$, the guanido carbon of arginine is found to possess a high level of activity (28, p.744; 78,p.58; 3,p.169; 37) whereas it appears that histidine and lysine do not incorporate the label extensively. The histidine arising from CO_2 fixation in bovine tissues contains only small amounts of activity and this is confined entirely to the carboxyl group (12,p.368). The carboxyl group in lysine, on the other hand, contains only 15 to 30 per cent of the total activity in the molecule, when $C^{14}O_2$ is fixed by E. coli (3,p.170; 2,p.1023).

The proposed biosynthetic routes leading to lysine in yeast (74; 75 and 27,p.15) require that acetate carboxyl carbons become C-1 of lysine and, as may be observed from previously mentioned reactions, no activity of fixed CO_2^* may be expected in the acetate molecule. The differences between yeast and E. coli in this respect may be due to variations in the biosynthetic pathway to lysine in these organisms or may be based on the possible occurrence in E. coli of lysine decarboxylase, an enzyme system shown in Bacterium cadaveris by Hanke and Siddiqi (38).

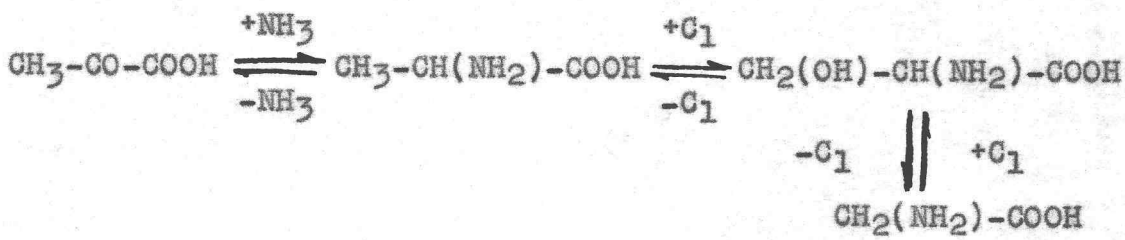
Proline has been shown to arise from glutamic acid via cyclization of glutamic-γ-semialdehyde (89) in an E. coli mutant. This finding has been supported by Abelson's isotopic competetion experiments (2,p.1022) using normal E. coli. Swick (78,p.58), on the basis of the low incorporation of CO_2 into proline as compared to glutamic acid in rats, concluded that this cyclization does not operate extensively in these animals.

The known interconversion of serine and glycine (10; 32; 46; 52; 69; 65; 66; 68; and 7) would lead to similarity in the labeling patterns in these compounds. It is further believed that the C_1 participating in this interconversion does not arise from CO_2 (86,p.99).

Serine has been demonstrated to be convertible to pyruvate in a number of microorganisms and in mammalian

livers (21), but the reversal of this reaction (i.e., pyruvate to serine) is not well established (67,p.165; 7,p.1345; 46,p.643).

The partial conversion of serine to alanine is shown by Lien and Greenberg (46,pp.641-3) to occur in rat liver cytoplasmic macrogranules as well as in the whole rat. The formation of alanine from pyruvic acid is meanwhile quite well established in pigeon breast muscle (23). Scheme II outlines these interconversions.



SCHEME II

In rats fixing $C^{14}O_2$ (78,p.59) it is shown that muscle serine and glycine probably do not acquire all of their respective activities from alanine while in the livers the activities are such as to make this conversion possible. Abelson (2,p.1021) has indicated that serine, alanine, and glycine are all devoid of activity in E. coli which has fixed $C^{14}O_2$.

An unusual example of $C^{14}O_2$ fixation into glycine is provided by Brucella abortus in which up to 40 per cent of the fixed activity appears in this amino acid. The corresponding alanine contains only 1 per cent of the fixed label.

Threonine has been presumed to arise from aspartate from the similarity in labeling observed in these two acids (3,p.169; 29; 48; 92) and from inhibition studies (60 and 61). Homoserine is indicated as the intermediate in this conversion (2,p.1023; 13; 22; 80).

Although interesting mechanisms for the formation of valine involving pyruvic acid in living systems have been proposed (47; 77; 79; 1; 92), no mention has been made of carbon dioxide fixation into this amino acid.

The lack of specific information on the mode of carbon dioxide fixation in Saccharomyces cerevisiae combined with the importance of supplementing work previously done with acetate and pyruvate in this organism (26, 42, 81, 90, 91, 92, 94 and 95) stimulated the research reported in this thesis. In the aerobic utilization of pyruvate it was noted that carbon dioxide fixation played a significant role (90,p.665) which could be more clearly explained employing the methods used in this study.

As will be noted later in the thesis, an unusually high incorporation of $C^{14}O_2$ into glycine and serine was observed in this work, shedding new light on the biosynthetic mechanism of these two amino acids.

EXPERIMENTAL

Time course studies on incorporation of $C^{14}O_2$ into amino acids in yeast. Preliminary studies were made into the number and order of labeling of amino acids in yeast grown on non-labeled pyruvic acid in the presence of $C^{14}O_2$. Representative samples of metabolizing yeast cells were removed from a closed system at intervals and acid hydrolyzed. Two dimensional paper chromatography of the hydrolysates followed by radioautography of the chromatograms provided a basis for evaluating the order in which the amino acids were formed in the yeast cell.

Fermentation of yeast in kinetic studies. A culture of Saccharomyces cerevisiae (baker's yeast) was grown in a malt extract medium. This was followed by a four hour "enrichment" growth on a glucose medium as described in earlier work (95,p.647). One hundred milliliters of the yeast suspension (equivalent to 400 mg of yeast, dry basis) was centrifuged, the cells washed, and added to 100 ml of a sterile nutrient medium having the following composition: NaCl, 200 mg; KH_2PO_4, 400 mg; $MgSO_4 \cdot 7H_2O$, 25 mg; $CaCl_2 \cdot 2H_2O$, 25 mg; H_3BO_3, 0.1 mg; $ZnSO_4$, 0.1 mg; $MnSO_4 \cdot 4H_2O$, 0.1 mg; $FeCl_3$, 0.1 mg; $TlCl_3$, 0.05 mg; $CuSO_4 \cdot 5H_2O$, 0.01 mg; KI, 0.01 mg; Bacto-yeast extract, 10 mg; distilled water to 100 ml; pH 4.0 to 4.5.

The medium containing the yeast was placed in sterilized flask A of the apparatus shown in Figure 1. The medium was oxygenated through tube B. After a period of 20-30 minutes the system was evacuated through tube C to a pressure of about 20 mm Hg. Two milliliters of a solution containing two millimoles freshly distilled pyruvic acid made to pH 3.5 with ammonia were injected into the system through vaccine port D. The $C^{14}O_2$ was generated in a separate apparatus shown in Figure 2. Fifty and five-tenths milligrams of $BaC^{14}O_3$ containing 1.82 millicuries C^{14} were introduced into chamber A. The apparatus was evacuated and 40 per cent perchloric acid solution was added through funnel B to react with the $BaC^{14}O_3$. This apparatus was connected to tube B of the growth flask and the $C^{14}O_2$ was swept into the system by a stream of oxygen until the pressure in the system became 20 cm Hg less than atmospheric pressure (measured by manometer connected to C).

The closed flask was agitated on a mechanical shaker at 30°C., except for brief intervals during the removal of samples for analysis. Samples of five ml each were taken by evacuating the filter flask E connected to the system via tube B. Each sample was received in a 10 ml centrifuge tube placed in the filter flask. The yeast cells were collected by centrifugation, washed, and their

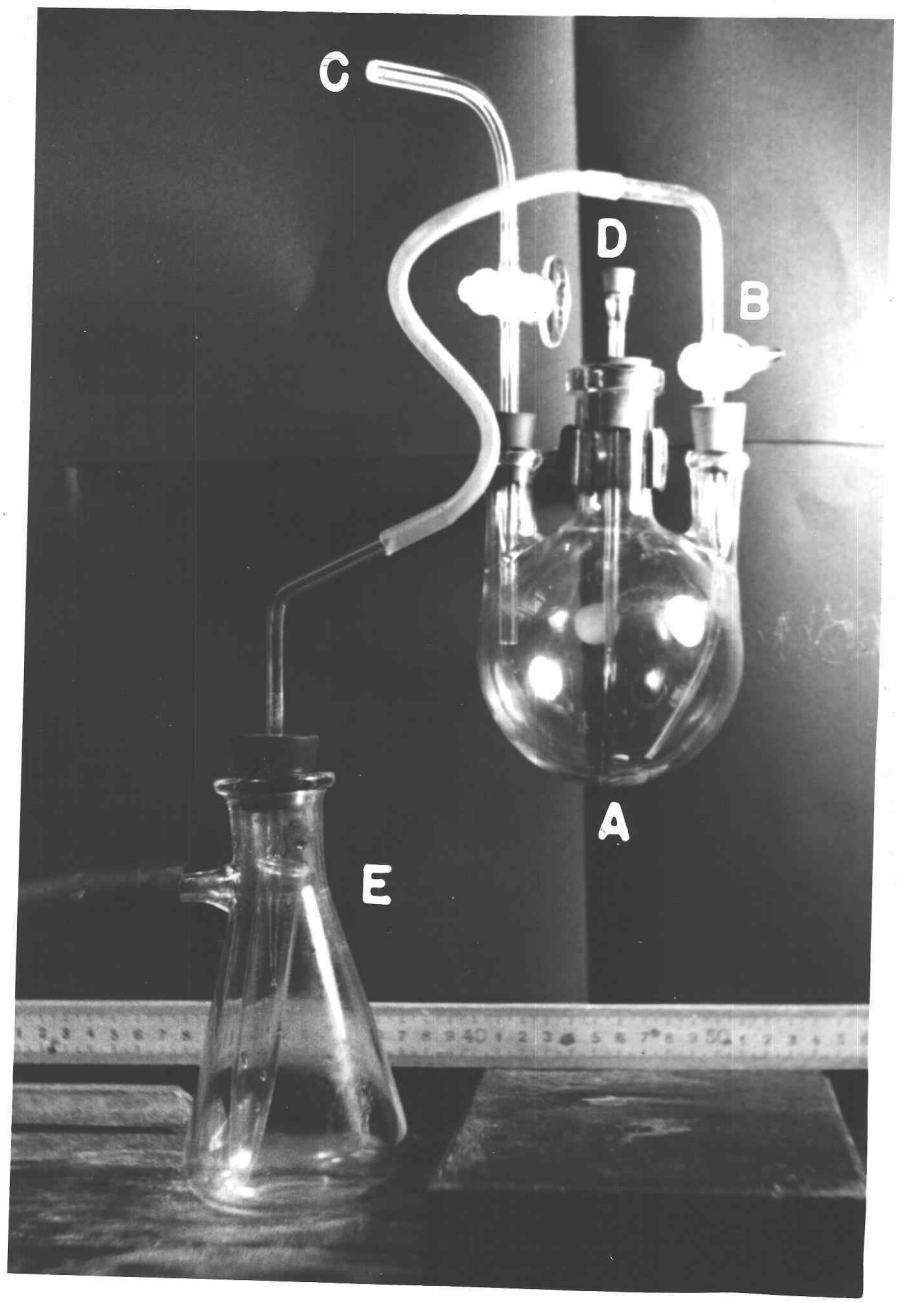


FIGURE 1

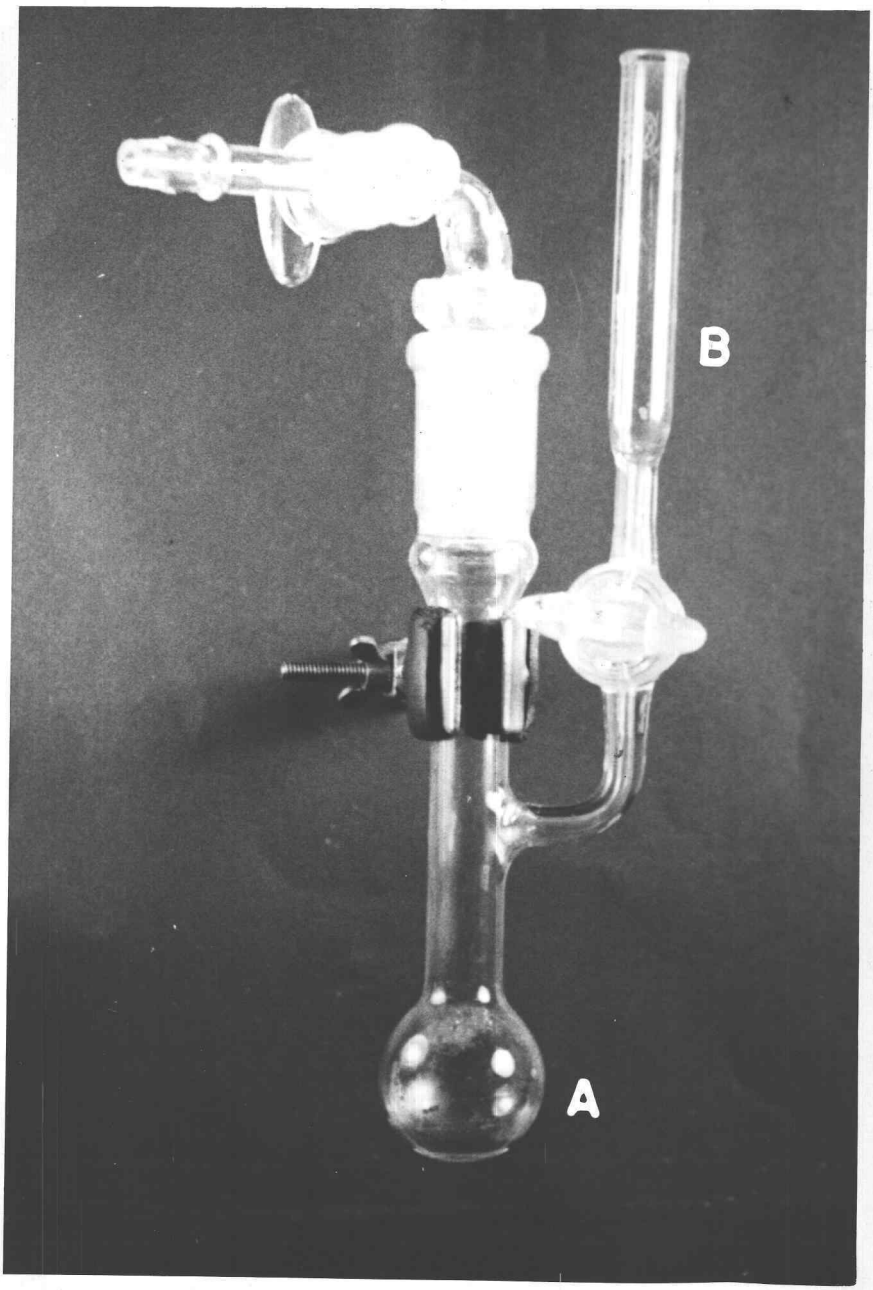


FIGURE 2

radioactivity measured as thin plates of aliquots in cupped planchets. The bulk of each sample of cells was killed in cold ethanol and dried in a vacuum desiccator. The activities on the plates were used as an indication of the rate of incorporation of $C^{14}O_2$ into the yeast and a curve of these activities was found to parallel very closely the specific activity curve of the hydrolyzed yeast, as shown in Figure 3.

At two separate times during the course of the fermentation, stained slides were made of the medium. No contaminating organisms were recognizable. At the termination of the growth period, nutrient agar plates were streaked with varying dilutions of the remaining medium and these cultured at 30°C. Examination of these plates also revealed no contamination.

Chromatography and radioautography of the yeast hydrolysate. Hydrolysis of the samples of dried yeast cells was carried out individually in sealed pyrex tubes with one ml of 6 N HCl. These were autoclaved 6 hours at 15 p.s.i. pressure. The humin-free hydrolysates were evaporated to dryness in vacuo over P_2O_5 and NaOH.

To each of the dried hydrolysates was added the calculated amount of water to give a solution containing 700 µg amino acids in one-tenth milliliter of solution. An aliquot (0.01 ml) of each solution was plated on a

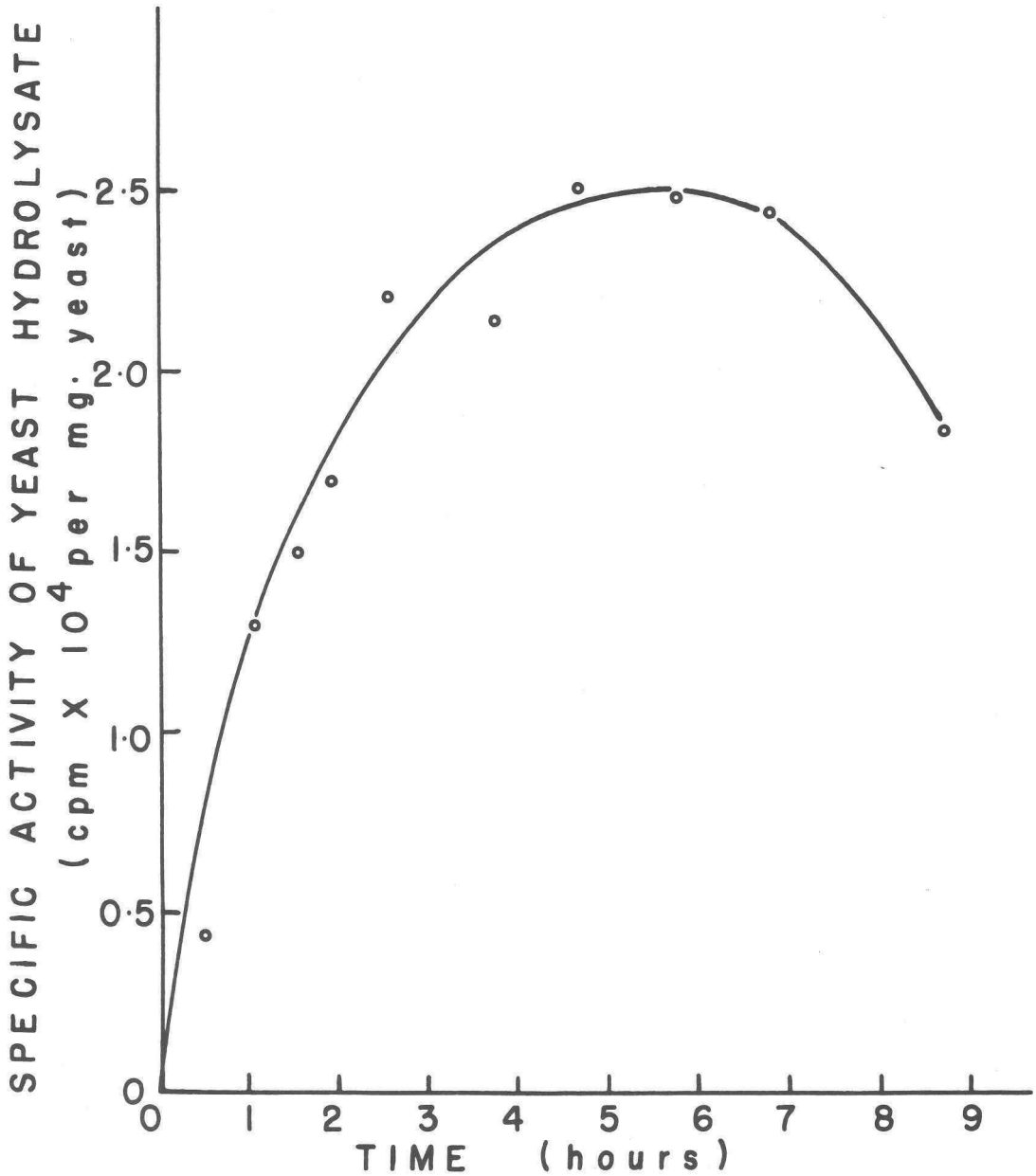

SPECIFIC ACTIVITY OF YEAST HYDROLYSATE
(cpm X 10⁴ per mg. yeast)
2·5
2·0
1·5
1·0
0·5
0
0
1
2
3
4
5
6
7
8
9
TIME (hours)

FIGURE 3

cupped stainless steel planchet, dried, and counted. The results of these countings are seen in Figure 3.

The remaining hydrolysate was used for single- and two-dimensional paper chromatography. Descending chromatography was carried out on Whatman no. 1 paper using n-butanol-acetic acid-water (4:1:5) and 80 per cent aqueous phenol containing a trace of 8-hydroxyquinoline. For resolution of the valine-leucine-isoleucine fraction a one dimensional paper chromatogram was run using Whatman no. 1 paper and 3:1 secondary butanol-ammonia (3 per cent) as the solvent system (63).

Chromatography of known amino acids using the same paper, solvents, and conditions served as the key in identifying spots on the chromatograms and radioautographs.

Radioautograms were obtained by placing x-ray film (Eastman, no-screen) in direct contact with the chromatogram for predetermined time intervals based on relative activity of the spots on the paper.

The radioautograms shown in later parts of this thesis depict isotope incorporation after growth for one-half hour to nine hours in the presence of $C^{14}O_2$.

The isotopic distribution of amino acids synthesized in the presence of $C^{14}O_2$. The intramolecular distribution of radioactivity in individual amino acids was also examined, using yeast samples that had been grown for

5 hours with $C^{14}O_2$. This time interval was found to permit maximum incorporation of isotope. Larger quantities of yeast were employed (see below) in order to permit satisfactory degradation of isotopic amino acids.

For the amino acid degradation experiments, 44 grams of moist yeast obtained from growth on malt medium followed by a four hour "energizing" growth on a glucose rich medium (95,p.647) were added to one liter of medium having the same composition as that mentioned earlier for the time course studies, but containing 2.5 grams $(NH_4)_2SO_4$ per liter in addition to the other constituents. The growth apparatus was similar to the one shown in Figure 1, but with a capacity of five liters. Measurements of pH were taken periodically during the growth with a pair of Beckman pH electrodes sealed into the flask.

Vigorous stirring was effected by means of a magnetic stirrer. Temperature was maintained at 30°C. in a well insulated air incubator. The pH was maintained between the limits of 4 and 4.5 by additions of either NaOH or HCl during fermentation.

The $C^{14}O_2$ (2.8 millicuries) was added as a solution containing 0.44 millimoles of $NaHC^{14}O_3$ in a total volume of 25 ml.

Samples were removed from the closed system hourly, using the same technique as that used in the time course

studies. Pyruvic acid assays were performed on these using the method of Friedemann and Haugen (36). A sufficient amount of pyruvic acid was added to the medium following each assay to restore or slightly exceed the initial level of this substrate and thus encourage maximum $C^{14}O_2$ fixation.

At the end of four hours, the yeast cells were centrifuged from the medium, washed twice with distilled water and once each with absolute alcohol and ether. The cells, dried in a vacuum desiccator overnight, weighed 7.52 grams.

Isolation of amino acids from the yeast. Three grams of the dry yeast were hydrolyzed by refluxing with 20% HCl for 26 hours. The hydrolysate, filtered free of humin, was extracted with ether for 20 hours (until fresh ether extract showed no radioactivity) in a liquid-liquid extractor. The residue was freed of HCl by repeated evaporation to dryness in vacuo.

The humin was also extracted with ether in a Soxhlet extractor to remove adsorbed fats. The two ether extracts were then combined, representing the fat fraction of the yeast. Table I gives the distribution of activity in various fractions of the yeast.

TABLE I

DISTRIBUTION OF ACTIVITIES IN FRACTIONS IN YEAST GROWN ON CH_3-CO-COOH AND $C^{14}O_2$

Fraction	Weight of Fraction (mg)	Specific Activity (cpm/mg)	Fraction of Total Activity (%)
Whole yeast	3010	1490	100
Humin	424	5	0.05
Fat (total)	222	84	0.41

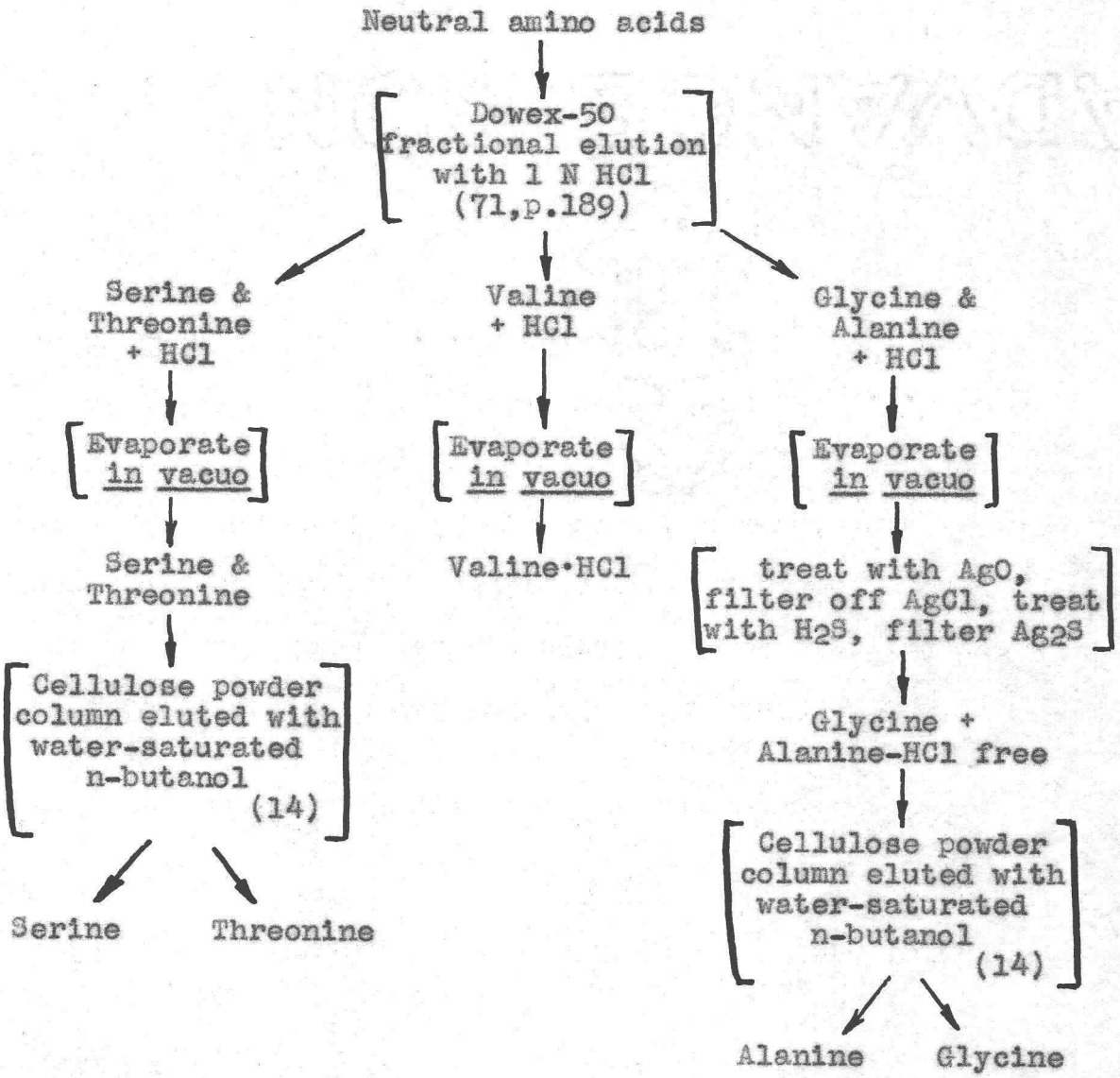
Sufficient non-labeled tyrosine was added to the hydrolysate to constitute a fifteen-fold dilution of the labeled compound as determined by microbiological assay. The tyrosine was removed from the hydrolysate by concentrating *in vacuo* and adjusting the pH of the concentrate to 6.0. The further treatment of this fraction is described in detail in the Ph.D. thesis of Richard C. Thomas (82,pp.19-32).

The acidic and basic amino acids were removed from the hydrolysate in the same manner as has been used in previous work by Wang, *et al* (95,p.656) and Davis, *et al* (26,p.2252).

The neutral amino acids were then separated according to the method depicted in Diagram I.

DIAGRAM I

SEPARATION OF NEUTRAL AMINO ACIDS
FROM YEAST PROTEIN



The cellulose columns used for the final purification of threonine, serine, alanine, and glycine were made according to the method of Blackburn (14) by suspending Whatman No. 1 cellulose powder in 80% n-propanol made 0.25 N with ammonia. The amino acids to be separated were dissolved in 1.25 N ammonia and n-propanol was added to 80% before placing on the column. Elution of the column in each case was with water-saturated n-butanol.

Controls were maintained on column operations by radioactive assays, microbiological assays, ninhydrin spot tests, and paper chromatography.

Aspartic acid was purified as its copper salt, then free aspartate obtained from H_2S treatment of the salt in solution. Glutamic acid was crystallized from solution after addition of carrier.

Alanine, threonine, and serine were obtained chromatographically pure from the cellulose column eluates. Valine was obtained in free form from uncontaminated fractions of Dowex 50 eluate containing this acid. Fractions containing glycine with traces of alanine present were assayed for the former by the method of Alexander (5). Non-labeled glycine was added to make a 50 fold dilution and the diluted glycine crystallized from water and alcohol. Histidine was obtained as its oxalate salt from alcohol (26,p.2252; 27,p.9).

The purity of all amino acids was checked by paper chromatography.

Degradations of the C^{14} containing amino acids. The total activity of each amino acid molecule was determined by the wet oxidation method of Van Slyke and Folch using the combustion mixture cited by Calvin (18, p.93), but omitting the KIO_3.

The activities residing in the carboxyl groups of the various amino acids were determined by ninhydrin decarboxylations after the method of Frantz (18,pp.260-1) modified by Wang (93), using barium hydroxide in place of sodium hydroxide for trapping the CO_2 evolved and making use of a simplified apparatus.

The α-carboxyl group of aspartic acid was obtained by the following procedure:

One millimole of aspartic acid is mixed with one millimole $AgNO_2$ in ten ml of water. This is agitated with a magnetic stirrer at 0^oC. while a stoichiometric amount of 0.1 N HCl is introduced slowly over a period of about two hours. Stirring is continued at 0^oC. for an additional two hours, after which time the solution is brought to room temperature, filtered, and evaporated to a small volume under reduced pressure with gentle heating. The concentrate is then further evaporated to dryness in a vacuum desiccator. Deamination of aspartic acid to give

malic acid is known to proceed without isomerization (40, p.414). The malic acid thus obtained was degraded by the method of Utter (84,p.851) to yield the α-carboxyl group as CO. This was oxidized to CO_2 in a hot tube containing CuO. The overall yield was 85-100 per cent, with specific activity values agreeing within approximately ± 15 per cent in 4 replicate determinations.

Threonine was degraded with HIO_4 according to Ehrensvard's modification of the method of Shinn and Nicolet (30,p.99). The acetaldehyde obtained in this reaction was converted to iodoform which was in turn burned to CO_2 and plated as $BaCO_3$ to give the activity of carbon-4 of threonine.

Isobutyraldehyde was recovered from the ninhydrin decarboxylation of valine. The 2,4-dinitrophenylhydrazone of this was counted (direct plate) as a further check on the labeling of this compound.

All radioactivity measurements were carried out with a thin mica end window, G-M counter to a standard error of less than 5%. Unless otherwise stated, $BaCO_3$ was used as the counting form with appropriate corrections for background and self absorption.

DISCUSSION

Time course studies. The time course studies have been of value in indicating the order in which amino acids in yeast incorporate $C^{14}O_2$. Table II summarizes graphically the relative intensities of various spots on the radioautograms obtained from these studies (Figures 4, 5, 6, 7 and 8) at periods of growth of 1/2, 1, 1-1/2, 2, and 4-3/4 hours in the presence of $C^{14}O_2$.

TABLE II

APPEARANCE OF LABEL IN AMINO ACIDS FROM $C^{14}O_2$ IN TIME COURSE STUDIES

Amino acid	Relative intensity[2] of radioautogram spot at				
	1/2 hr	1 hr	1-1/2 hr	2 hr	4-3/4 hr
aspartic acid	+++	++++	++++	++++	+++++
glutamic acid	++	+++	+++	+++	++++
threonine	++	++++	++++	++++	+++++
cystine	?	++++	++++	++++	+++++
serine		++	+++	+++	++++
glycine		++	+++	+++	++++
arginine		+	++	++	++
tyrosine			+	+	+
valine			++	++	+++
leucine			++	++	+++
proline				++	+++
alanine					+

[2] Intensities expressed as follows: + trace, ++ light, +++ moderate, ++++ heavy, +++++ extensive.

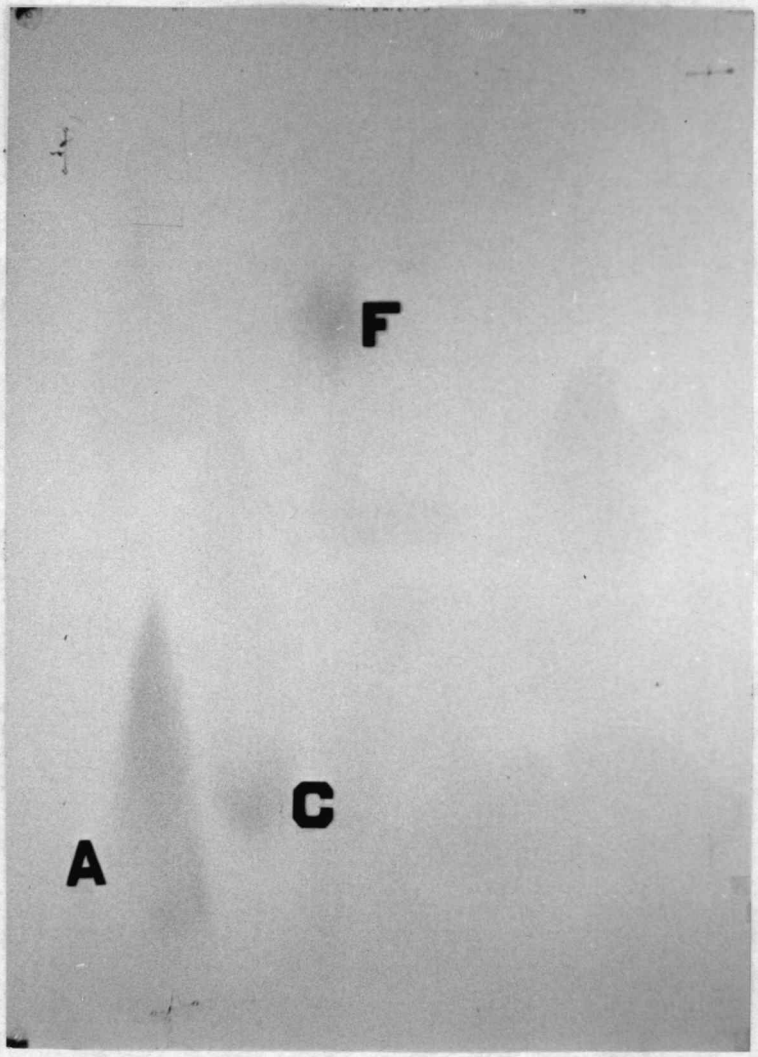


FIGURE 4

Two dimensional radioautogram of isotopic amino acids from yeast hydrolysate. Exposure of yeast to $C^{14}O_2$, 30 minutes. Solvent systems used, Butanol-acetic acid-water (4:1:5) moving from left to right, phenol-water (80%) moving from bottom to top. Identification of spots, A = aspartic acid, C = glutamic acid, F = threonine.

The early occurrence of labeling into aspartic acid, glutamic acid, and threonine observed in the one-half hour radioautogram (Figure 4) emphasizes the close relationships existing between aspartic acid and oxalacetic acid, glutamic acid and α-ketoglutaric acid, and threonine and aspartic acid (2,p.1023; 92). The rapid occurrence of activity in these acids serves to indicate that the amination and/or transamination reactions providing for the synthesis of aspartic and glutamic acids from their corresponding α-ketoacids comes rapidly to equilibrium in the yeast cell as has been observed in liver (24).[3]

By the end of one hour of growth, the yeast shows increasing incorporation of activity into aspartate, glutamate, and threonine while easily detectable amounts of incorporation of $C^{14}O_2$ into glycine, serine, arginine, and cystine are also noted (Figure 5).

Arginine, as pointed out earlier (28,p.744; 3,p. 169; 37; and 78,p.58) may incorporate $C^{14}O_2$ via the well established pathway through glutamic acid as well as

---

[3] Photosynthetic $C^{14}O_2$ fixation in higher plants is, however, much more rapid (19, 72), with only 30 seconds to one minute required for appreciable labeling. The first amino acids to appear labeled are, in order of the respective labeling levels: aspartic, alanine, asparagine, serine, β-alanine, and phenylalanine (19, 72).

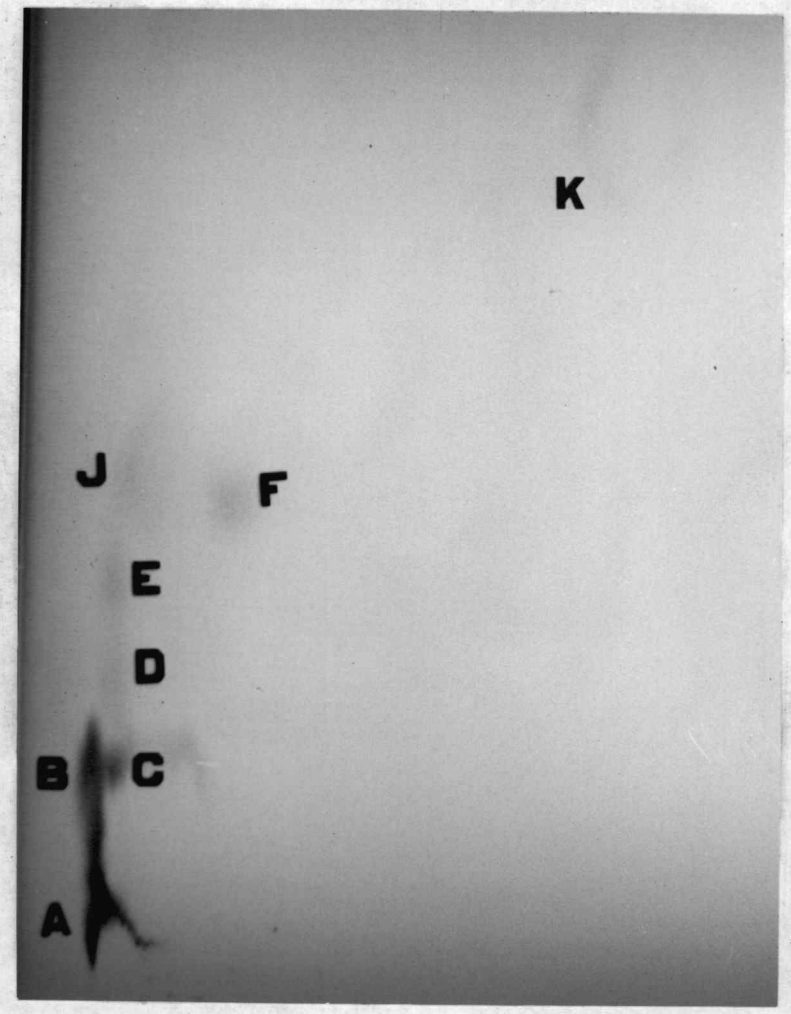


FIGURE 5

Two dimensional radioautogram of isotopic amino acids from yeast hydrolysate. Exposure of yeast to $C^{14}O_2$, 1 hour. Solvent systems used, Butanol-acetic acid-water (4:1:5) moving from left to right, phenol-water (80%) moving from bottom to top. Identification of spots, A = aspartic acid, B = cystine, C = glutamic acid, D = serine, E = glycine, F = threonine, J = arginine, and K = valine.

direct incorporation of $C^{14}O_2$ into the guanido-carbon atom. Hence, its relatively early incorporation of label is not surprising.

Cystine shows appreciable incorporation of radioactivity after one hour. Because the large spot identified as aspartic acid in the one-half hour chromatogram may have contained two amino acids, the earlier appearance of this amino acid cannot be obviated.

Figure 6 pictures the incorporation of $C^{14}O_2$ into yeast amino acids after one and one-half hours. One can discern newly acquired activity in tyrosine, valine, and the "leucines" (leucine, isoleucine, and phenylalanine). Absence of activity in the alanine spot (H in Figure 6) is not surprising, since its probable precursor (pyruvate, non-isotopic) had been added in high concentration at the start of the experiment.

As the growth period reached two hours duration, proline appeared to have incorporated C^{14} (Figure 7). If cyclization of glutamic acid to form proline were a reaction occurring extensively, one would expect the density of the proline spot to increase proportionally with that of glutamic acid. The delay in proline labeling suggests that in yeast the turnover time for this amino acid is somewhat longer than for other metabolic intermediates. This is in agreement with studies in the rat (78,p.59).

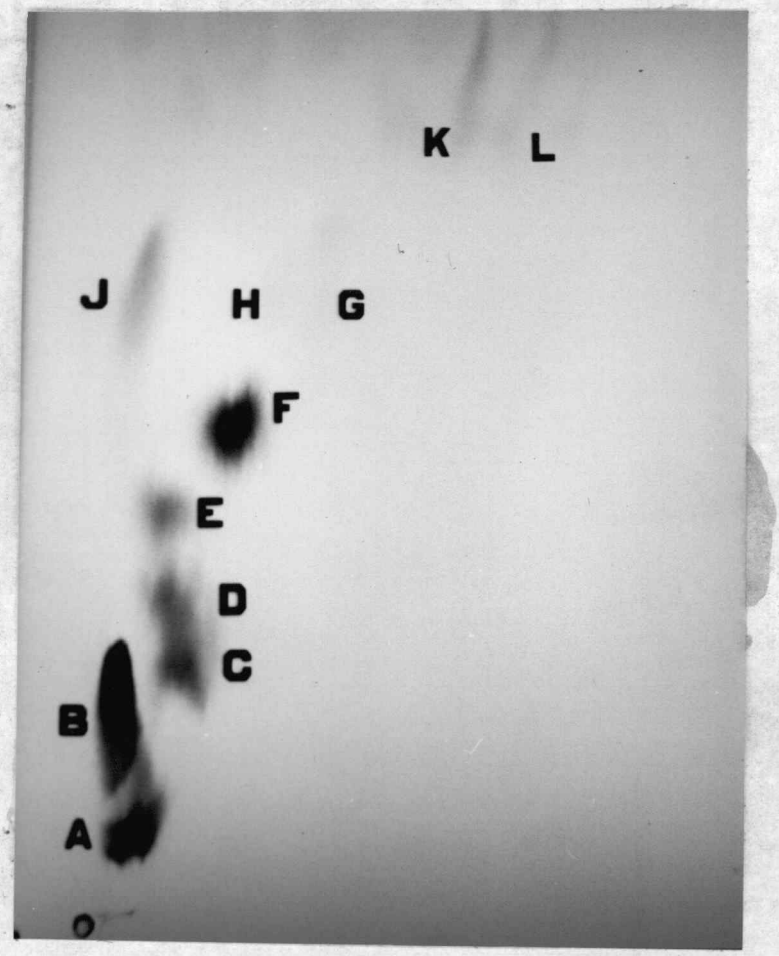


FIGURE 6

Two dimensional radioautogram of isotopic amino acids from yeast hydrolysate. Exposure of yeast to $C^{14}O_2$, 1-1/2 hours. Solvent systems used, Butanol-acetic acid-water (4:1:5) moving from left to right, phenol-water (80%) moving from bottom to top. Identification spots, A = aspartic acid, B = cystine, C = glutamic acid, D = serine, E = glycine, F = threonine, G = tyrosine, H = alanine (no spot showing), J = arginine, K = valine and L = "leucines". Other unidentified spots are non-amino acid components in hydrolysate.

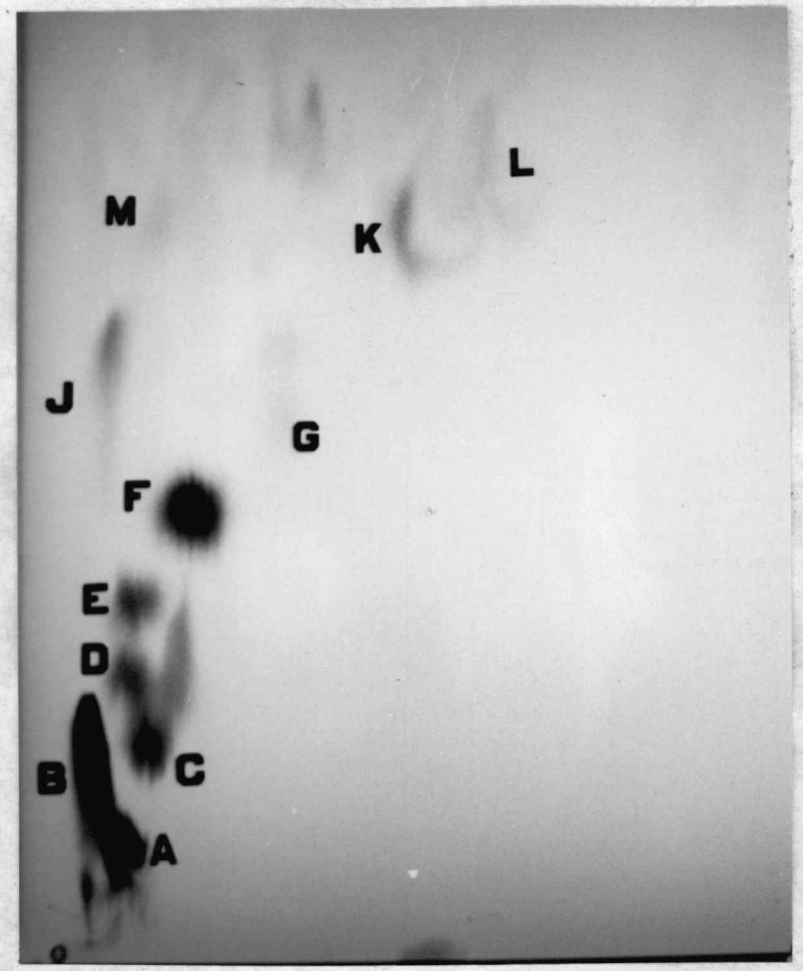


FIGURE 7

Two dimensional radioautogram of isotopic amino acids from yeast hydrolysate. Exposure of yeast to $C^{14}O_2$, 2 hours. Solvent systems used, Butanol-acetic acid-water (4:1:5) moving from left to right, phenol-water (80%) moving from bottom to top. Identification of spots, A = aspartic acid, B = cystine, C = glutamic acid, D = serine, E = glycine, F = threonine, G = tyrosine, J = arginine, K = valine, L = "leucines", M = unidentified amino acid, possibly a hydrolytic breakdown product. Other spots of radioactivity due to non-amino acid components in the hydrolysate are likewise unidentified.

Figure 8 is a radioautogram of the yeast after four and three-quarters hours growth. This interval represents the time necessary for isotopic saturation of the yeast (see Figure 3) and this radioautogram depicts the approximate relative isotopic distributions into the amino acids of the yeast used for the large-scale degradation studies.

Incorporation of isotope into amino acids in this sample has reached a high level in aspartic acid, cystine, glutamic acid, glycine, serine, and threonine with smaller, though significant amounts in arginine, proline, valine, and the "leucines".

A salient feature of the four and three-quarters hours yeast samples is the appearance of C^{14} into alanine. The length of time necessary for alanine to acquire measurable isotope may be due either to a slow attainment of equilibrium of the transamination reaction between alanine and pyruvate or to the presence of non-labeled pyruvic acid in the medium from which most of the alanine to meet the yeast's requirements was formed.

The one-dimensional radioautograph in Figure 9 shows the overall trend in $C^{14}O_2$ incorporation into various amino acid fractions during time periods ranging from one-half hour to eight and three-quarters hours growth of the yeast. This radioautogram, while not providing optimum separation, has the advantage of comparing

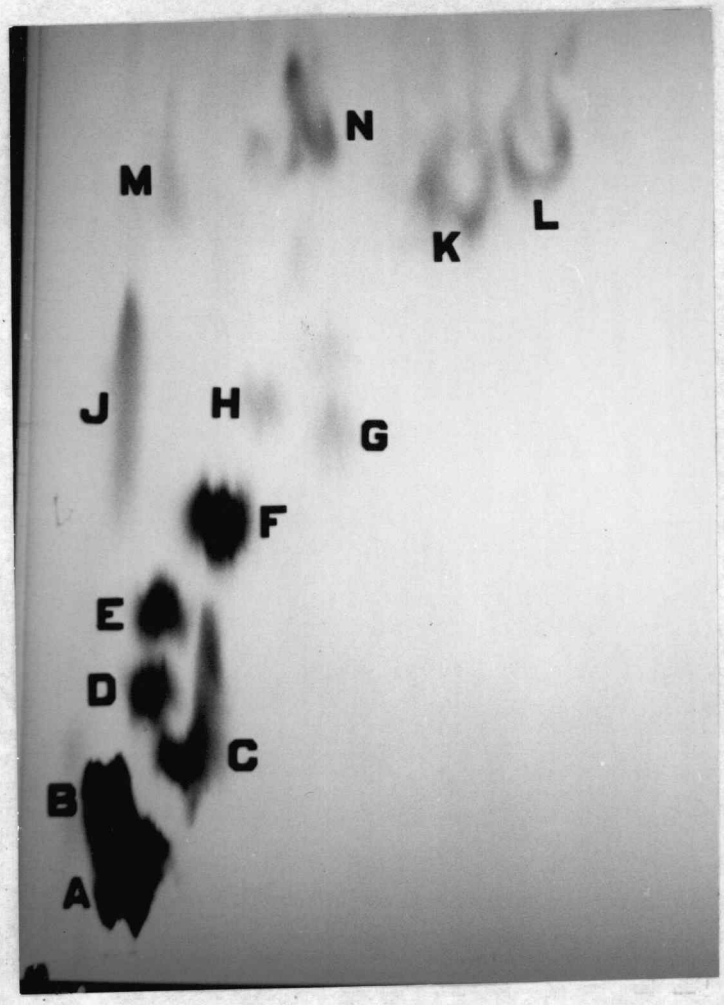


FIGURE 8

Two-dimensional radioautogram of isotopic amino acids from yeast hydrolysate. Exposure of yeast to $C^{14}O_2$, 4-3/4 hours. Solvent systems used, Butanol-acetic acid-water (4:1:5) moving from left to right and phenol-water (80%) moving from bottom to top. Identification of spots, A = aspartic acid, B = cystine, C = glutamic acid, D = serine, E = glycine, F = threonine, G = tyrosine, H = alanine, J = arginine, K = valine, L = "leucines", M = unidentified amino acid, possibly a hydrolytic breakdown product, N = proline. Unidentified spots are non-amino acid components of the hydrolysate.

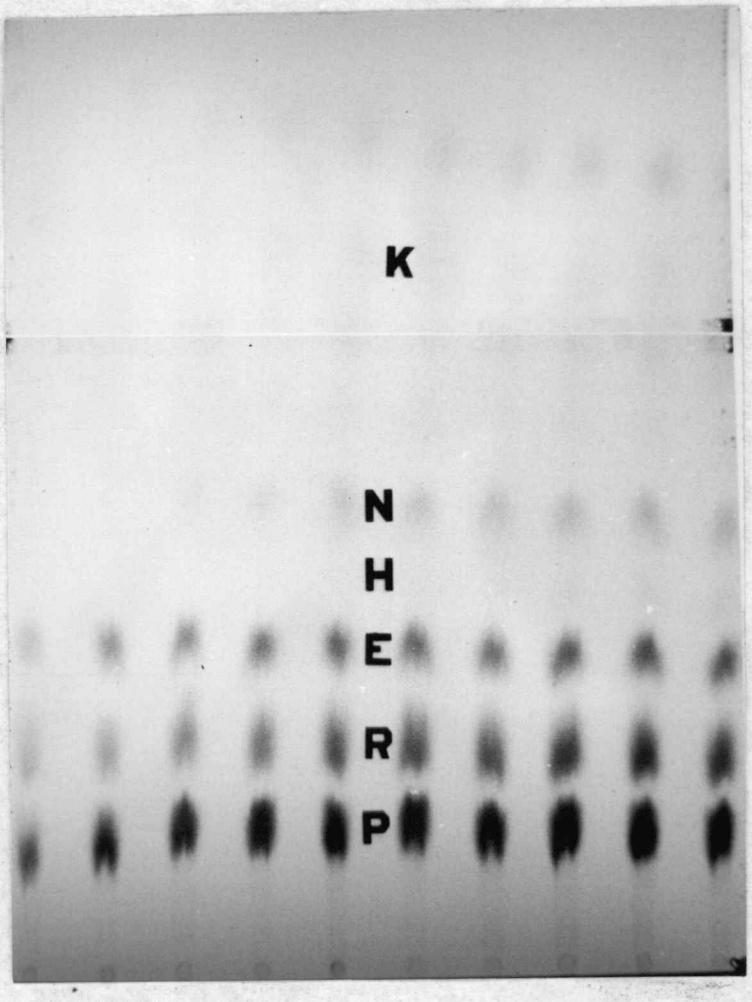


FIGURE 9

One-dimensional radioautogram of time course samples. Solvent system used, Butanol-acetic acid-water (4:1:5) moving from bottom to top. Vertical rows of spots reading from left to right represent amino acids labeled after 1/2, 1, 1-1/2, 2, 2-1/2, 3-3/4, 4-3/4, 5-3/4, 6-3/4, and 8-3/4 hours exposure to $C^{14}O_2$. Identified spots are, P = aspartic acid, cystine, basic amino acids, R = glutamic acid, glycine, serine, E = threonine, H = alanine, N = proline and tyrosine, K = valine.

the relative activities of the amino acids under the same conditions of exposure and development. It may be noted that there is no dimunition of activity in a given amino acid or amino acid group, during the progress of the experiment. This is in spite of the fact that the overall specific activity of the yeast cells decreased slightly after six hours.

In summarizing the time course studies the following points have been observed:

1) Aspartic acid, arising from oxalacetate represents a primary fixation of carbon dioxide into an amino acid. Its predominance may possibly be due in part to the addition of pyruvate at the start of the experiment to encourage a Wood-Werkman or malic type condensation. Glutamic acid and threonine are also readily formed from pyruvate and $C^{14}O_2$ in yeast.

2) Glycine and serine, which become markedly labeled within one hour and increase in activity at a rate roughly paralleling the rate of increase of activity in glutamic acid, provide an unexpected observation which permits speculation on their possible modes of biosynthesis.

3) Cystine becomes labeled early and increases in activity at a rate paralleling closely that of aspartate. If it arose directly from serine (1,p.340) then cysteine

(cystine after hydrolysis) should display an activity similar to, and increasing in a manner paralleling that of serine which is found to have a lower activity than either aspartate or cystine.

4) Arginine slowly increases in activity at a rate similar to the increase in activity in glutamic acid from which the ornithine moiety arises in yeast (76). This suggests that the guanido carbon atom does not fix $C^{14}O_2$ to an appreciable extent but that its origin is in some non-labeled C_1 unit under these conditions.

5) The occurrence of labeling into valine before that into alanine (the latter presumably reflecting labeling in pyruvate, a proposed direct precursor of the valine carbon chain) suggests that a pathway leading to valine biosynthesis might have a possible entrance for $C^{14}O_2$ as in the proposal by Wang, Christensen and Cheldelin (92 and 94).

6) Incorporation of $C^{14}O_2$ into proline is delayed, suggesting that its formation from glutamic acid, a moderately heavily labeled molecule, takes place slowly under the conditions imposed in these studies.

7) Activity also appears slowly in the "leucines". A supplementary radioautogram of a single dimensional chromatogram of this fraction (not shown in thesis) served to determine that isoleucine possessed the entire activity

of the leucine-isoleucine pair for periods up to and including eight and three-quarters hours in the presence of $C^{14}O_2$. Yeast does not incorporate $C^{14}O_2$ into the carbon chain of leucine.

8) Alanine does not show measurable C^{14} activity until nearly five hours have passed. This may be attributed to the presence of a high level of non-labeled pyruvate in the medium.

9) Histidine appears to incorporate little, if any activity via $C^{14}O_2$ fixation.

10) Lysine does not incorporate activity from $C^{14}O_2$, in line with proposed biosynthetic routes to this amino acid in yeast (74, 75).

Information from degradation of C^{14}-labeled amino acids. In Tables III, IV, and V are listed the isotope distributions in several of the foregoing amino acids.

Aspartic acid. From the degradation data on aspartic acid (Table III) it may be observed that C-1 and C-4 of this molecule picture the occurrence of randomization between oxalacetate and a symmetrical C_4 acid to the average extent of about 20 per cent over the four hours growth of the yeast in the presence of $C^{14}O_2$. The occurrence of 90 per cent of the activity of the whole molecule in C-4 indicates that the formation of aspartate from oxalacetate is relatively more rapid than equilibration

TABLE III

ISOTOPE CONTENT OF ASPARTIC ACID, GLUTAMIC ACID AND THREONINE DERIVED FROM CH_3-CO-COOH AND $C^{14}O_2$ IN YEAST

Amino acid group	Radioactivity (cpmx10^5/m mole amino acid)	Per cent of total
Aspartic acid		
Whole molecule	5.78	100
C-1	0.61	11
C-4 (by difference)[4]	5.18	89
Glutamic acid		
Whole molecule	12.8	100
C-1	12.2	96
Threonine		
Whole molecule	1.00	100
C-1	0.15	15
C-4	0.78	78

[4] A duplicate determination on aspartate from another yeast sample grown under identical conditions showed C-2 and C-3 to be devoid of activity.

of oxalacetic acid with symmetrical C_4 acids via counter-clockwise Krebs cycle activity. Forward operation of the cycle would eliminate all incorporated $C^{14}O_2$ in a single turn (see reactions 5 and 6, Scheme I).

Glutamic acid. Glutamic acid, as mentioned earlier, should possess the entire activity of the molecule in C-1 when $C^{14}O_2$ is the exclusive source of labeling. This is confirmed, within the limits of experimental error, by the data on glutamic acid labeling presented in Table III.

In a paper by Hendler and Anfinsen (39) the occurrence of 30 per cent of the activity of glutamic acid in C-5 from $C^{14}O_2$ in hen oviduct preparations is reported. This could be introduced from Krebs cycle activity only via the carboxyl group of acetate which cannot accumulate $C^{14}O_2$ activity directly but must depend upon some reaction involving probable cleavage of a C_4 acid between C-2 and C-3. Possible similar activity in this yeast may have been prevented by the non-isotopic pyruvate present. The low level of $C^{14}O_2$ incorporated into the fat fraction (Table I) indicates that, as expected, only small amounts of activity would reside in acetate.

A further notable finding in Table III is the much higher specific activity in C-1 of glutamate (1.22×10^6 cpm/μ mole) as compared to C-4 of aspartate (5.18×10^5 cpm/μ mole) from which it would arise by the reactions of

the Krebs cycle. Previous findings (42,pp.659-660) in this yeast have shown that when pyruvate is used as a substrate the glutamate content within the cell increases from 50 per cent to 80 per cent over normal (42,p.660). This extra glutamate may thus serve to trap C^{14}, and would accumulate higher than expected specific activities.

Alanine. The specific activity of alanine (Table IV) is low by comparison to most of the amino acids studied. This finding is in line with observations made previously for the time course studies and, again, may be due to the added, unlabeled pyruvate. The incorporation of label, to the extent that it occurred, was found in the carboxyl group of alanine. This would be predicted from reactions leading to the reformation of pyruvic acid from C_4 acids (Scheme I).

Threonine. If threonine arose from aspartic acid as has been suggested (13) the isotopic distribution patterns of these two would be similar. The slightly greater degree of randomization displayed by C-1 and C-4 of threonine (30%) as compared to that shown by the corresponding carbons of aspartate (20%) in Table III, may, if significant, question the necessity of having aspartate on the biosynthetic route to threonine. Such a difference in labeling patterns would be permissible if threonine were separately in equilibrium with some other

TABLE IV

ISOTOPE CONTENT OF ALANINE, SERINE, GLYCINE, AND VALINE DERIVED FROM CH_3-CO-COOH AND $C^{14}O_2$ IN YEAST

Amino acid group	Radioactivity (cpmx10^5/m mole amino acid)	Per cent of total
Alanine		
Whole molecule	0.28	100
C-1	0.30	100+
Serine		
Whole molecule	4.0	100
C-1	3.9	96
Glycine		
Whole molecule	5.2	100
C-1	4.7	92
Valine		
Whole molecule	0.041	100
C-1		100 (by difference)
C-2 + C-3 + C-4 + C-5	0	0

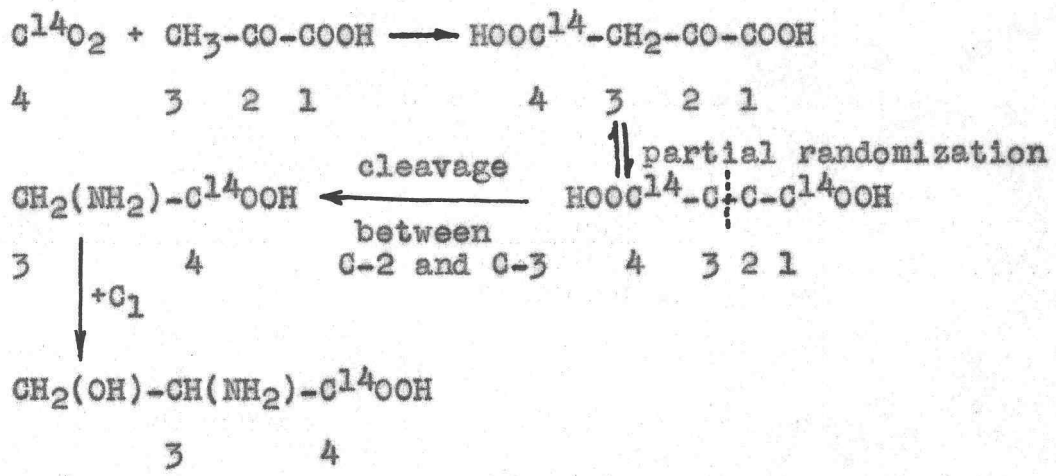
compound, as indicated by the incorporation of acetate carboxyl activity into C-2 and C-3 of threonine in Torula yeast (30,p.105) and E. coli (25,p.361).

Serine and glycine. It will be noted from Table IV that the isotopic content of serine and glycine are comparable, confirming the ease of interconversion of these two compounds (69). The slightly higher activity in glycine (5.15×10^5 cpm/m mole) over that of serine (4.01×10^5 cpm/m mole) may indicate that glycine is the first formed of this pair. The activity of glycine compared to the inferred activity of acetate (fat fraction of Table I) makes evident the lack of a direct conversion of acetate to this amino acid as a major pathway of biosynthesis in yeast.

Another equivocally demonstrated pathway, the conversion of alanine to serine in animals (67,p.165; 7,p. 1345 and 46,p.643), is also ruled out as a major source of this pair of amino acids by virtue of the comparatively low specific activity of the alanine.

Using the data on $C^{14}O_2$ fixation to augment other findings from $CH_3C^{14}OCOOH$ metabolism in yeast, it is suggested that glycine arises from the symmetrical cleavage of a C_4 acid as depicted in Scheme III.

The forward operation of the cycle denies C^{14} label to the C_4 acid (succinate) first formed from α-ketoglutarate



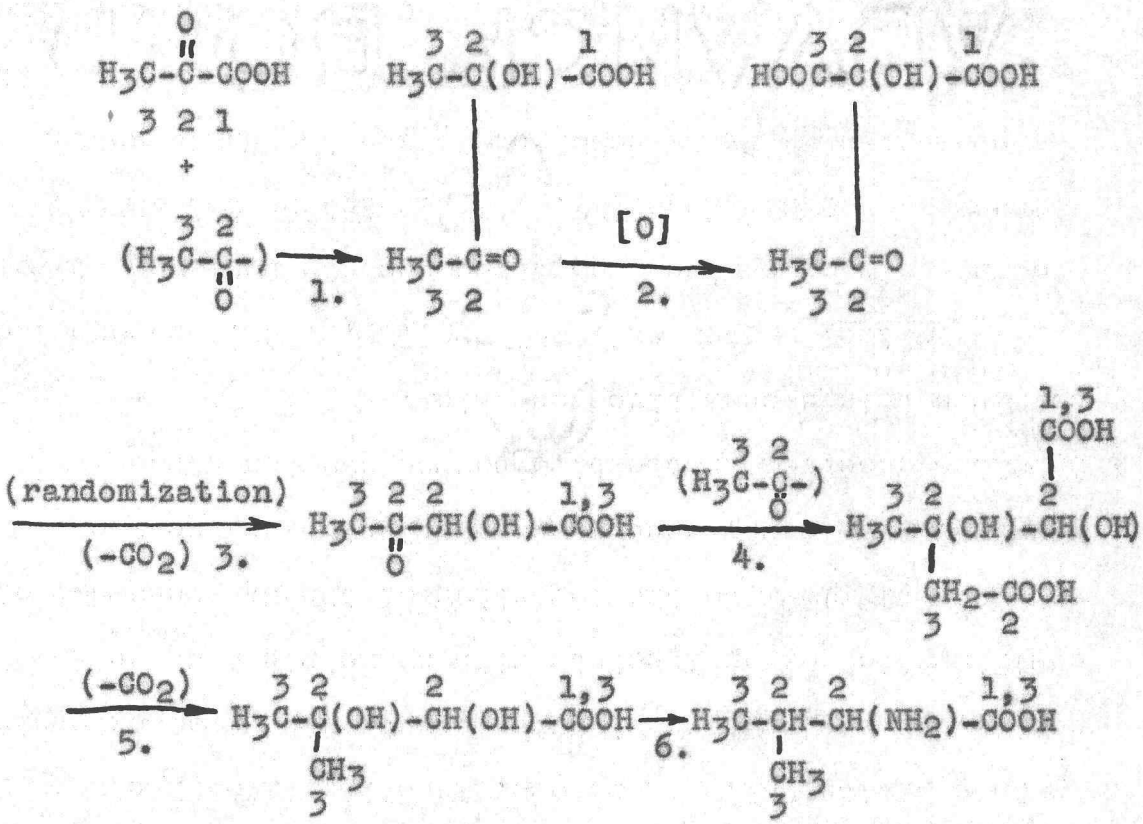
SCHEME III

by loss of its C-1. Therefore, labeling into glycine and serine, by the process pictured, must arise from extensive equilibration of oxalacetate with symmetrical C_4 acids and subsequently lead to the formation of the C_4 precursor of glycine. The early occurrence of glycine and serine labeling observed in the time course studies strongly confirms this speculative mechanism.

Valine. Tatum and Adelberg (79,p.849) propose the biosynthesis of valine from three acetate residues while Strassman and Weinhouse (77,p.5135) picture the valine skeleton arising from acetolactate via the condensation of two C_3 moieties followed by loss of CO_2. In the first proposal, no $C^{14}O_2$ would be permitted to enter the valine molecule while in the second mechanism, the $C^{14}O_2$ activity

residing in C-1 of pyruvate would be found in the C-1 of valine exclusively. If the alanine molecule provides an accurate representation of the specific activity of pyruvate then labeling in C-1 of valine in this yeast would be permitted up to a level of 3×10^4 cpm/m mole (alanine C-1 from Table IV).

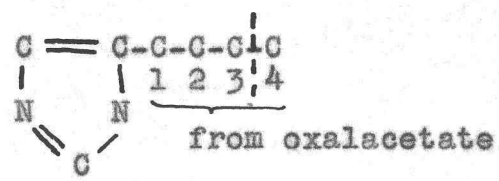
A recent proposal (92, 94) for the formation of valine is outlined in Scheme IV.



SCHEME IV

As noted in reaction 3 of Scheme IV, this mechanism provides for the randomization between two carbons of one of the intermediates in the biosynthesis of valine. If this randomization is complete, a maximum activity of 1/2 that of the alanine C-1 (1.5×10^4 cpm/m mole) would be permitted, provided that alanine gives an accurate picture of the C_3 condensing components. The observation of an even lower activity in valine C-1 (4.08×10^3 cpm/m mole from Table IV) indicates extensive dilution in various stages of this synthesis. Furthermore, the previously noted early incorporation of $C^{14}O_2$ activity into valine as compared to alanine can be explained by possible limited reversibility of reaction 3 in Scheme IV.

Histidine. The failure of the yeast to incorporate $C^{14}O_2$ into the histidine carboxyl group is in line with proposals on the biosynthetic origin of the side chain (C-1, C-2 and C-3) of this molecule (26 and 27,p.17). This involves C_3 of oxalacetate serving as the origin of the carboxyl group of histidine as shown:



If this were true, C-2 and C-3 of histidine (arising from C-2 and C-1, respectively, of oxalacetate) would have only slight activity (10 per cent of the total activity of aspartate at most).

However, comparison of the specific activity of histidine with that of C-1 of aspartic acid from the same yeast (Table V) indicates that a considerable amount of $C^{14}O_2$ activity has been incorporated into the imidazole ring. In view of the reported origin of C-6 (the ureido carbon) from formate (44) and the known non-conversion of CO_2 to formate, it is reasonable to suppose that the remaining two carbons of the imidazole ring (C-4 and C-5) could have arisen in part from $C^{14}O_2$.

TABLE V

ISOTOPIC CONTENT OF HISTIDINE AND ASPARTIC ACID DERIVED FROM $CH_3COCOOH$ AND $C^{14}O_2$ IN YEAST[5]

Amino acid group	Radioactivity (cpm x 10^5/mmole)	Per cent of total
Histidine		
Whole molecule	19.	100
C-1	0.48	3.3
Aspartic acid		
Whole molecule	21.8	100
C-1	1.66	8
C-4 (by difference)	20.1	92

5 This yeast is a different sample than those depicted in Tables I, III and IV. It was grown under similar conditions as that from which the other data is taken but incorporated a total of 1.34×10^7 cpm instead of the 4.48×10^6 cpm represented in the other yeast.

SUMMARY

1. The fixation of $C^{14}O_2$ into growing cultures of baker's yeast has been examined, with non-isotopic pyruvate added to enhance the process. It appears from the data given that this incorporation occurs with pyruvate, predominantly according to a Wood-Werkman or malic acid type condensation.

2. Equilibration between oxalacetate and aspartate favors formation of the latter. This is emphasized by the limited randomization pictured between C-1 and C-4 of aspartate.

3. Surprisingly heavy isotope incorporations into glycine and serine have been observed. Degradation studies indicate that this labeling is confined to C-1 in each amino acid. Based on these observations, a new biosynthetic mechanism for their formation has been proposed. The scheme involves the α - β cleavage of a C4 acid derived from oxalacetic acid to provide the glycine skeleton; the latter is readily interconvertible with serine.

4. Relatively early labeling in valine as compared to alanine, combined with the labeling levels found in the respective carboxyl groups, suggests possible involvement of a reversible decarboxylation.

5. The low incorporation of $C^{14}O_2$ into the histidine carboxyl group supports a previously proposed synthesis of histidine whereby the alanine side chain is thought to arise from carbons 1, 2 and 3 of oxalacetate. In addition, the relatively high specific activity of this amino acid after $C^{14}O_2$ incorporation could indicate the latter's deposition in C-4 and C-5 of the imidazole ring.

BIBLIOGRAPHY

1. Abelson, Philip H. Amino acid biosynthesis in Escherichia coli: Isotopic competition with C^{14}-glucose. Journal of biological chemistry 206: 335-343. 1954.

2. Abelson, Philip H., et al. Synthesis of the aspartic and glutamic families of amino acids in Escherichia coli. Proceedings of the national academy of science 39:1020-1026. 1953.

3. Abelson, Philip H., Ellis T. Bolton and Elaine Aldous. Utilization of carbon dioxide in the synthesis of proteins by Escherichia coli, I. Journal of biological chemistry 198:165-172. 1952.

4. Abelson, Philip H., Ellis T. Bolton and Elaine Aldous. Utilization of carbon dioxide in the synthesis of proteins by Escherichia coli, II. Journal of biological chemistry 198:173-178. 1952.

5. Alexander, Benjamin, Greta Landwehr and Arnold M. Seligman. A specific micro method for the colorimetric determination of glycine in blood and urine. Journal of biological chemistry 160:51-59. 1945.

6. Amos, H. and G. N. Cohen. Amino acid utilization in bacterial growth, 2. A study of the threonine-isoleucine relationships in mutants of Escherichia coli. Biochemical journal 57:338-343. 1954.

7. Anker, H. S. Some aspects of the metabolism of pyruvic acid in the intact animal. Journal of biological chemistry 176:1337-1352. 1948.

8. Anfinsen, Christian B., Ann Beloff and A. K. Solomon. The incorporation of radioactive carbon dioxide and acetate into liver proteins in vitro. Journal of biological chemistry 179:1001-1013. 1949.

9. Anfinsen, Christian B., et al. The in vitro turnover of dicarboxylic amino acids in liver slice proteins. Journal of biological chemistry 168:771-772. 1947.

10. Arnstein, H. R. V. and A. Neuberger. The synthesis of glycine and serine by the rat. Biochemical journal 55:271-280. 1953.

11. Barban, Stanley and Samuel J. Ajl. Interconversion of propionate and succinate by Propionibacterium pentosaceum. Journal of biological chemistry 192: 63-72. 1951.

12. Black, Arthur L., Max Kleiber and Arthur H. Smith. Carbonate and fatty acids as precursors of amino acids in casein. Journal of biological chemistry 197:365-370. 1952.

13. Black, Simon and Nancy G. Wright. Enzymatic phosphorylation of L-aspartate and formation of homoserine and threonine. Federation proceedings 13: 184. 1954.

14. Blackburn, S. The separation of α-amino acids on powdered cellulose columns. Chemistry and industry, April 14, 1951, pp.294-295.

15. Block, Richard J. and Diana Bolling. The amino acid composition of proteins and foods. Second edition. Springfield, Illinois, Charles C. Thomas, 1951. 576p.

16. Block, Richard J., Raymond LeStrange and Gunter Zweig. Paper chromatography, a laboratory manual. New York, Academic Press, 1952. 195p.

17. Bonstein, R. A. and M. J. Johnson. The mechanism of formation of citrate and oxalate by Aspergillus niger. Journal of biological chemistry 198:143-153. 1952.

18. Calvin, Melvin, et al. Isotopic carbon. New York, John Wiley, 1949. 376p.

19. Calvin, Melvin and A. A. Benson. The path of carbon in photosynthesis. Science 107:476-480. 1948.

20. Ceithaml, Joseph and Birgit Vennesland. The synthesis of tri-carboxylic acids by carbon dioxide fixation in parsley root preparations. Journal of biological chemistry 178:133-142. 1949.

21. Chargaff, Erwin and David B. Sprinson. Studies on the mechanism of deamination of serine and threonine in biological systems. Journal of biological chemistry 151:273-280. 1943.

22. Cohen, Georges N. and Marie-Louise Hirsch. Threonine synthase, a system synthesizing L-threonine from L-homoserine. Journal of bacteriology 67:182-190. 1954.

23. Cohen, Philip P. Transamination with purified enzyme preparations (transaminase). Journal of biological chemistry 136:565-584. 1940.

24. Cohen, Philip P. Transamination. Federation proceedings 1:273-280. 1942.

25. Cutenelli, C., et al. Acetic acid metabolism in Escherichia coli. Acta chemica scandinavica 5: 353-371. 1951.

26. Davis, J. Wendell, et al. On the origin of the carboxyl group of histidine in yeast. Journal of the American chemical society 75:2252-2253. 1953.

27. Davis, James Wendell. The formation of radioactive lysine and histidine from $CH_3C^{14}OCOOH$ in yeast. Master's thesis. Corvallis, Oregon state college, 1952. 22 numb. leaves.

28. Delluva, Adelaide M. and D. Wright Wilson. A study with isotopic carbon of the assimilation of carbon dioxide in the rat. Journal of biological chemistry 166:739-746. 1946.

29. Delluva, Adelaide M. Some aspects of threonine and serine biosynthesis in Escherichia coli. Archives of biochemistry and biophysics 45:443-446. 1953.

30. Ehrensvärd, Gösta, et al. Acetic acid metabolism in Torulupsis utilis. Journal of biological chemistry 189:93-108. 1951.

31. Ehrensvärd, Gösta. Amino acid metabolism in Torulopsis utilis. Cold Spring Harbor symposia on quantitative biology 14:81-87. 1948.

32. Elwyn, David and David B. Sprinson. Certain interrelationships in the metabolism of glycine and serine. Journal of biological chemistry 207:459-465. 1954.

33. Evans, E. A., Jr. and Louis Slotin. Carbon dioxide utilization by pigeon liver. Journal of biological chemistry 141:439-450. 1941.

34. Evans, E. A., Jr. and Louis Slotin. The utilization of carbon dioxide in the synthesis of α-ketoglutaric acid. Journal of biological chemistry 136:301-302. 1940.

35. Floyd, Norman F., Grace Medes and Sidney Weinhouse. Fatty acid metabolism, VI. Conversion of acetoacetate to citrate in animal tissues studied with isotopic carbon. Journal of biological chemistry 171:633-638. 1947.

36. Friedemann, Theodore E. and Gladys E. Haugen. Pyruvic acid. The determination of keto acids in blood and urine. Journal of biological chemistry 147:415-442. 1943.

37. Grisolia, Santiago, R. H. Burris and Philip P. Cohen. Carbon dioxide and ammonia fixation in the biosynthesis of citrulline. Journal of biological chemistry 191:203-209. 1951.

38. Hanke, Martin E. and M. S. H. Siddiqi. Fixation of radioactive carbon dioxide in lysine and tyrosine by decarboxylase-enzymes of these amino acids. Federation proceedings 9:181-182. 1950.

39. Hendler, Richard W. and Christian B. Anfinsen. The incorporation of carbon dioxide into both carboxyl groups of glutamic acid. Journal of biological chemistry 209:55-62. 1954.

40. Karrer, P. and A. Schlosser. Untersuchungen über die Konfiguration der Aminosäuren, I. Helvetica chimica acta 6:411-418. 1923.

41. Kramptiz, L. O., Harland G. Wood and Chester H. Werkman. Enzymatic fixation of carbon dioxide in oxalacetate. Journal of biological chemistry 147: 243-253. 1943.

42. Labbe, Robert F., et al. Radioactive yeast fractions derived from C^{14}-labeled pyruvate and acetate. Journal of biological chemistry 197:655-661. 1952.

43. Labbe, Robert F. Unpublished analytical data on the yeast used in these studies.

44. Levy, Louis and Minor J. Coon. The role of formate in the biosynthesis of histidine. Journal of biological chemistry 192:807-815. 1951.

45. Lewis, Katherine F. and Sidney Weinhouse. Assimilation of carbon dioxide in oxalate and citrate by Aspergillus niger. Journal of the American chemical society 73:2906-2909. 1951.

46. Lien, Oliver G. and David M. Greenberg. Chromatographic studies on interconversion of amino acids. Journal of biological chemistry 195:637-644. 1952.

47. McManus, I. Rosabelle. The biosynthesis of valine by Saccharomyces cerevisiae. Journal of biological chemistry 208:639-644. 1954.

48. McQuillen, Kenneth and Richard B. Roberts. The utilization of acetate for synthesis in Escherichia coli. Journal of biological chemistry 207:81-95. 1954.

49. Marr, A. G. and J. B. Wilson. Fixation of $C^{14}O_2$ in amino acids by Brucella abortus. Archives of biochemistry and biophysics 34:442-448. 1951.

50. Marshall, Lawrence M. and Felix Friedberg. On carbon dioxide fixation in vivo. Journal of biological chemistry 199:783-787. 1952.

51. Martin, S. M., Perry W. Wilson and R. H. Burris. Citric acid formation from $C^{14}O_2$ by Aspergillus niger. Archives of biochemistry 26:103-111. 1950.

52. Mitoma, Chozo and David M. Greenberg. Studies on the mechanism of the biosynthesis of serine. Journal of biological chemistry 196:599-614. 1952.

53. Mosbach, E. H., E. F. Phares and S. F. Carson. Conversion of α-ketoglutaric-1,2-C^{14} to malic acid in pigeon breat muscle. Archives of biochemistry and biophysics 34:449-452. 1951.

54. Mosbach, E. H., E. F. Phares and S. F. Carson. The role of one-carbon compounds in citric acid biosynthesis. Archives of biochemistry and biophysics 35:435-442. 1952.

55. Najjar, Victor A. and Jean Fisher. Studies on L-glutamic acid decarboxylase from Escherichia coli. Journal of biological chemistry 206:215-219. 1954.

56. Nakada, Henry I. and Sidney Weinhouse. Studies of glycine oxidation in rat tissues. Archives of biochemistry and biophysics 42:257-270. 1953.

57. Ochoa, Severo. Biosynthesis of tricarboxylic acid by carbon dioxide fixation. Journal of biological chemistry 174:133-157. 1948.

58. Ochoa, Severo, Alan H. Mehler and Arthur Kornberg. Reversible oxidative decarboxylation of malic acid. Journal of biological chemistry 167:871-872. 1947.

59. Racusen, D. W. and S. Aronoff. Metabolism of soy bean leaves, VI. Exploratory studies in protein metabolism. Archives of biochemistry and biophysics 51:68-78. 1954.

60. Ravel, Joanne M., Barbara Felsing and William Shive. Glutamic acid inhibition of aspartic acid utilization in threonine biosynthesis. Journal of biological chemistry 206:791-796. 1954.

61. Ravel, Joanne M., et al. Some interrelationships of aspartic acid, threonine and lysine. Journal of biological chemistry 206:391-400. 1954.

62. Roberts, R. B., et al. The role of the tricarboxylic acid cycle in amino acid synthesis in Escherichia coli. Proceedings of the national academy of science 39:1013-1019. 1953.

63. Roland, J. F., Jr. and A. M. Gross. Quantitative determination of amino acids using monodimensional paper chromatography. Analytical chemistry 26:502-505. 1954.

64. Ruben, S. and M. D. Kamen. Radioactive carbon in the study of respiration in heterotrophic systems. Proceedings of the national academy of science 26: 418-422. 1940.

65. Sakami, Warwick. The conversion of formate and glycine to serine and glycogen in the intact rat. Journal of biological chemistry 176:995-996. 1948.

66. Sakami, Warwick. The conversion of glycine into serine in the intact rat. Journal of biological chemistry 178:519-520. 1949.

67. Shemin, David. Some aspects of the biosynthesis of amino acids. Cold Spring Harbor symposia on quantitative biology 14:161-167. 1949.

68. Shemin, David. The biological conversion of L-serine to glycine. Journal of biological chemistry 162:297-307. 1946.

69. Siekevitz, Philip and David M. Greenberg. The biological formation of serine from glycine. Journal of biological chemistry 180:845-856. 1949.

70. Sprinson, David B. The utilization of the α-carbon atom of glycine for the formation of acetic and aspartic acids. Journal of biological chemistry 178:529-530. 1949.

71. Stein, William H. and Stanford Moore. Chromatographic determinations of the amino acid composition of proteins. Cold Spring Harbor symposia on quantitative biology 14:179-190. 1949.

72. Stepka, W., A. A. Benson and Melvin Calvin. The path of carbon in photosynthesis, II. Amino acids. Science 108:304. 1948.

73. Stettin, Marjorie Roloff. Mechanism of the conversion of ornithine into proline and glutamic acid in vivo. Journal of biological chemistry 189:499-507. 1951.

74. Strassman, Murray and Sidney Weinhouse. Biosynthetic pathways, III. The biosynthesis of lysine by Torulopsis utilis. Journal of the American chemical society 75:1680-1684. 1953.

75. Strassman, Murray and Sidney Weinhouse. Lysine biosynthesis in Torulopsis utilis. Journal of the American chemical society 74:3457-3458. 1952.

76. Strassman, Murray and Sidney Weinhouse. The biosynthesis of arginine by Torulopsis utilis. Journal of the American chemical society 74:1726. 1952.

77. Strassman, Murray, Alice J. Thomas and Sidney Weinhouse. Valine biosynthesis in Torulopsis utilis. Journal of the American chemical society 75:5135. 1953.

78. Swick, Robert W., Donald L. Buchanan and Akira Nakao. The normal content of fixed carbon in amino acids. Journal of biological chemistry 203:55-61. 1953.

79. Tatum, E. L. and Edward A. Adelberg. Origin of the carbon skeletons of isoleucine and valine. Journal of biological chemistry 190:843-852. 1951.

80. Teas, H. J., N. H. Horowitz and Marguerite Fling. Homoserine as a precursor of threonine and methionine in Neurospora. Journal of biological chemistry 172:651-658. 1948.

81. Thomas, Richard C., Jr., et al. Conversion of acetate and pyruvate to tyrosine in yeast. Journal of the American chemical society 75:5554-5556. 1953.

82. Thomas, Richard Clarence. The biosynthesis of aromatic amino acids in yeast (Saccharomyces cerevisiae). Ph.D. thesis. Corvallis, Oregon state college, 1954. 53 numb. leaves.

83. Topper, Yale G. and DeWitt Stetten, Jr. Formation of "acetyl" from succinate by rabbit liver slices. Journal of biological chemistry 209:63-71. 1954.

84. Utter, Merton F. Interrelationships of oxalacetic and L-malic acids in carbon dioxide fixation. Journal of biological chemistry 188:847-863. 1951.

85. Utter, Merton F. and K. Kurahashi. Mechanism of action of oxalacetic carboxylase from liver. Journal of the American chemical society 75:758. 1953.

86. Utter, Merton F. and Harland G. Wood. Mechanisms of fixation of carbon dioxide by heterotrophs and autotrophs. Advances in enzymology 12:41-151. 1951.

87. Utter, Merton F. and Harland G. Wood. The fixation of carbon dioxide in oxalacetate by pigeon liver. Journal of biological chemistry 164:455-476. 1946.

88. Vennesland, Birgit, T. T. Tchen and Frank A. Loewus. Mechanism of enzymatic carbon dioxide fixation into oxalacetate. Journal of the American chemical society 76:3358-3359. 1954.

89. Vogel, Henry J. and Bernard D. Davis. Glutamic γ-semialdehyde and Δ^1-pyrroline-5-carboxylic acid, intermediates in the biosynthesis of proline. Journal of the American chemical society 74:109-112. 1952.

90. Wang, Chih H., et al. Conversion of acetate and pyruvate to aspartic acid in yeast. Journal of biological chemistry 197:663-667. 1952.

91. Wang, Chih H., Bert E. Christensen and Vernon H. Cheldelin. Conversion of acetate and pyruvate to glutamic acid in yeast. Journal of biological chemistry 201:683-688. 1953.

92. Wang, Chih H., Bert E. Christensen and Vernon H. Cheldelin. Conversion of $CH_3C^{14}OCOOH$ to some aliphatic monoamino acids in yeast. Journal of biological chemistry, in press.

93. Wang, Chih H. Personal communication.

94. Wang, Chih H., et al. The origin of glycine and serine in baker's yeast. Nature, in press.

95. Wang, Chih H., et al. Utilization of C^{14} labeled pyruvate and acetate by yeast. Journal of biological chemistry 197:645-653. 1952.

96. Weissbach, Arthur and David B. Sprinson. The metabolism of 2-carbon compounds related to glycine, I. Glyoxylic acid. Journal of biological chemistry 203:1023-1030. 1953.

97. Wood, Harland G., et al. Heavy carbon as a tracer in heterotrophic carbon dioxide assimilation. Journal of biological chemistry 139:365-376. 1941.

98. Wood, Harland G., et al. Mechanism of fixation of carbon dioxide in the Krebs cycle. Journal of biological chemistry 139:483-484. 1941.

99. Wood, Harland G., Birgit Vennesland and E. A. Evans. The mechanism of carbon dioxide fixation by cell free extracts of pigeon liver: Distribution of labeled carbon dioxide in the products. Journal of biological chemistry 159:153-158. 1945.

100. Wood, Harland G., et al. The position of carbon dioxide in succinic acid synthesized by heterotrophic bacteria. Journal of biological chemistry 139:377-381. 1941.

101. Wood, Harland G. and Chester H. Werkman. The utilization of CO_2 by the propionic acid bacteria. Journal of bacteriology 30:332. 1935.

102. Wood, Harland G. and Chester H. Werkman. CLXVIII. The utilization of CO_2 by propionic acid bacteria. Biochemical journal 32:1262-1271. 1938.