

THE CARBOHYDRATE METABOLISM IN
PENICILLIUM DIGITATUM SACCARDO
USING RADIOACTIVE ISOTOPIC TRACERS

by

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INTRODUCTION

"The old order changeth, yielding place to new,
And God fulfills Himself in many ways,
Lest one good custom should corrupt the world."

Tennyson

In the last two decades, knowledge of the catabolic and anabolic reactions of carbohydrates in living systems has advanced by leaps and bounds. Newer concepts have arisen displacing or modifying the older ones. In many cases more questions have been raised than answered. Yet underlying this vast maze of complexity there seems to run a thread of unity.

Contributing greatly to the tremendous advances in the field of metabolism have been some rather basic techniques; among these, isotopic tracer methodology, chromatography and enzyme purification, to mention a few.

Pathways of glucose dissimilation. Fermentation of carbohydrates in yeast and a few other microorganisms, as represented by the over-simplified equation, $C_6H_{12}O_6 = 2CO_2 + 2C_2H_5OH$, has undergone some rather rigorous studies by various laboratories; and the wide chasm between the initial substrate and the final end products has been finally crystallized out as the Emden-Meyerhof glycolytic scheme.

It is true that the pioneering studies mainly dealt with yeast cells and muscle tissue; yet further studies, from the simpler unicellular organisms to the more complex tissue of the mammals, have shown the glycolytic process to be ubiquitous in practically all forms of life.

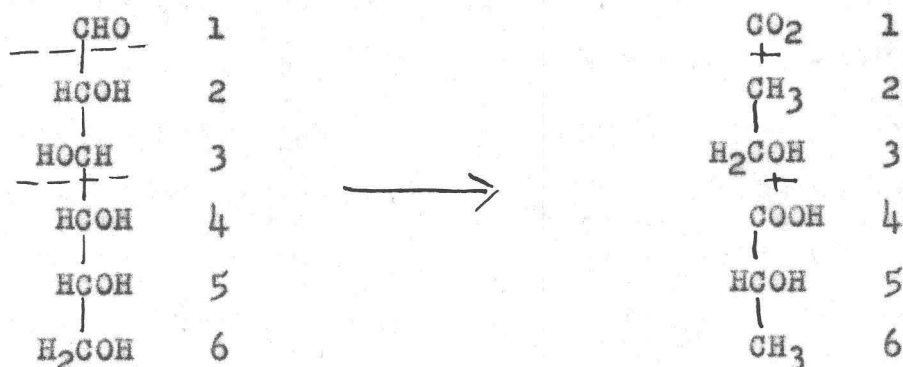
With the advent of tracer methodology, glucose has been differentially labeled with C^{14} . Using different test organisms in the study of carbohydrate utilization, a divergence from the pattern based on the glycolytic process has been observed.

If the glycolytic path is exclusively present in a given tissue, glucose-1- C^{14} and glucose-6- C^{14} should be equivalent as substrates for the formation of pyruvate-3- C^{14} .



A divergence from the glycolytic pathway in Leuconostoc mesenteroides (20) is observed. This organism is known to ferment 1 mole of glucose into 1 mole each of lactate, ethanol and CO₂. When glucose-1- C^{14} was degraded

by this microorganism, all the C^{14} label was found in the CO_2 . On the other hand when glucose-3,4- C^{14} was employed, the carbinol moiety of ethanol and the carboxyl of lactate were labeled, while no $C^{14}O_2$ was detected. The fate of the various carbon atoms from glucose as a result of fermentation by Leuconostoc mesenteroides are given below.



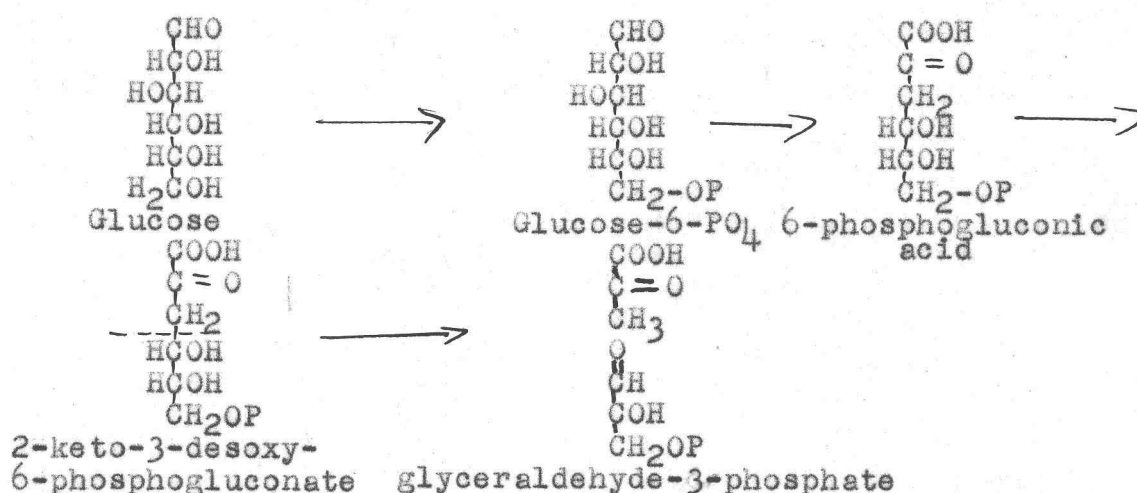
Similar types of preferential oxidation of C_1 of glucose has been observed in a wide variety of living systems. In fact, the existence of this oxidative pathway was first discovered by Warburg, et al (46,47), Lipmann (27), and Dickens (12) in the late thirties in the metabolism of glucose-6-phosphate. The earliest indication that this mechanism was a cyclic one came from the work of Dische (13) who found the degradation of pentose nucleosides in red cells to be accompanied by the appearance of hexose phosphate and triose phosphate.

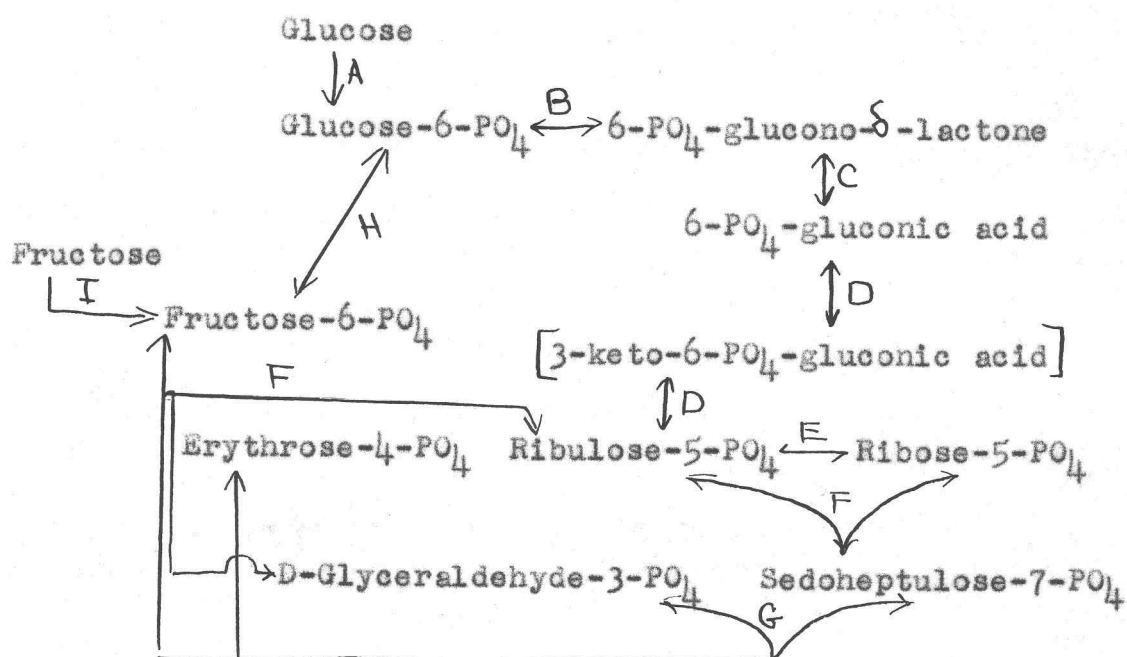
The discovery and isolation of two key enzymes, transaldolase and transketolase, by Horecker (24,23)

and Racker (32) together with the extensive use of C^{14} -labeled hexoses and pentoses in various fermentations has finally helped to establish the cyclic nature of this oxidative pathway.

On the following page is the pentose cycle (formerly known as the hexose monophosphate shunt) in diagrammatic form as it is conceived today.

Entner and Doudoroff (14) in an experiment with resting cells of Pseudomonas saccharophila, in which C_1 -labeled glucose was decomposed aerobically, it was found that the labeled carbon was almost quantitatively recovered as CO_2 . The isotope was entirely present in the carboxyl of pyruvate. The following scheme was hence proposed as pathway of glucose metabolism in Pseudomonas saccharophila.





The Pentose Cycle

- A. Glucokinase + ATP.
- B. Glucose-6-phosphate dehydrogenase + TPN⁺.
- C. Lactonase.
- D. 6-phosphogluconic acid dehydrogenase + TPN⁺.
- E. Phosphoriboisomerase.
- F. Transketolase + TPP.
- G. Transaldolase.
- H. Phosphofructoisomerase.
- I. Fructokinase + ATP.

The fate of the pentoses formed in the pentose cycle may include accumulation in the medium or cleavage into C_2 and C_3 units, the latter two entering the general metabolic pool of the organism.

The conversion of pentoses into hexoses in the pentose cycle brings about the possibility of utilization of the so formed hexoses by pathways other than the pentose cycle.

In studies on the metabolism in E.coli (8) it has been suggested that the ribose of RNA was derived mainly via the oxidative pathway, whereas the desoxyribose of DNA arose from triose phosphate generated from the Emden-Meyerhof scheme. As a test of this suggestion E.coli was grown on glucose-1- C^{14} . It was found that the activity per mole of ribose isolated from RNA was only 20 to 30 per cent of the activity of glucose (26) . Thus the ribose of RNA of E.coli was derived mainly from the pentose cycle.

Weimberg and Doudoroff (56) have oxidized, by crude cell-free preparations of Pseudomonas saccharophila, L-arabinose-1- C^{14} to α -ketoglutaric acid-1- C^{14} . They have found no evidence for the participation of any phosphorylated intermediates in the reaction. Results of isotope dilution experiments have indicated that the formation of α -ketoglutarate involved neither the

tricarboxylic acid cycle nor such intermediates as CO_2 , formate, acetate, glyoxalate, gluconate, or α -L-hydroxyglutarate.

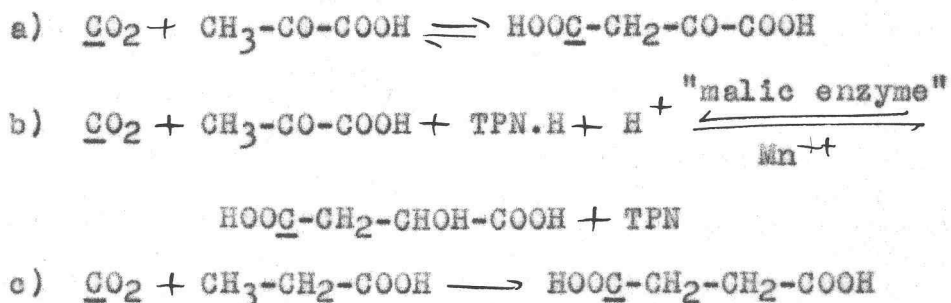
Pathways of terminal respiration. The tricarboxylic acid cycle (TCA) is probably the most important energy-producing mechanism in the terminal respiration of animal tissues. It was Krebs (25) who in 1937 proposed this cycle which elegantly explained the findings of Thunberg, Szent Györgyi and others. Although this scheme has passed through a battery of rigorous examinations, its present form is astonishingly similar to the original hypothesis. However, the evidence for the claim that the cycle is the terminal pathway of oxidation in different varieties of microorganisms is, in many cases, incomplete on the basis of lack of quantitative information.

C_4 dicarboxylic acids play a vital role in the biosynthetic processes and in the terminal oxidation of most living systems. There are various pathways for the mode of their formation.

1) The entrance of acetyl coenzyme-A into the TCA cycle by condensation with oxalacetate and the subsequent cycling process yields C_4 dicarboxylic acids. However, no net synthesis of C_4 acid can be realized by this process.

2) Another pathway whereby C_4 acids may be obtained is

by primary fixation reactions of CO_2 of the following types:



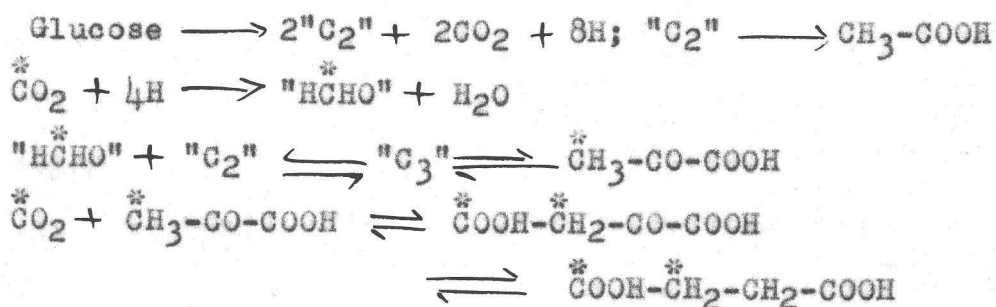
Reactions under 2 are discussed in detail in J. Wendell Davis's thesis (10).

3) The Thunberg-Wieland cycle of acetate condensation ("tail to tail") is still another route for C_4 dicarboxylic acid formation.

4) H. G. Wood (21) investigated the mechanism of CO_2 fixation in the fermentation of glucose by Clostridium thermoaceticum. The different molecular types of C^{13} -acetate that are formed from C^{13}O_2 were investigated by mass spectrometer. The data indicated that acetate was formed by two processes during the fermentation, one a synthesis of C_2 carbon skeleton from CO_2 with little or no dilution. (The mechanism of the incorporation of CO_2 into two adjacent carbons is at present unknown, but the author stipulates that it does not necessarily involve direct combination of CO_2 .) The major part of the singly labeled acid is probably formed by an exchange reaction

of CO₂ with the carboxyl of acetate. The possible role of formaldehyde, glycine and C₄ dicarboxylic acids in the fermentation had been considered; but the experimental results were inconclusive.

Utter and Wood (45, p.118) have considered several hypothetical mechanisms for fixations of CO₂ in adjacent carbons, and in the case of C. thermoaceticum one explanation was built around the reaction discovered by Sakami (36) in which formate presumably combines with glycine or some other C₂ unit to form β-labeled serine. Formaldehyde likewise is active in such a reaction (37). The C₁ scheme involving "formaldehyde" is given below.



Thus, this will provide an indirect pathway of CO₂ incorporation into the middle carbons of C₄ acids.

5) A. Clostridium kluyveri is an organism that lacks an active mechanism for complete oxidation of C₂ compounds. Tomlinson (43) in his study of this organism has presented isotopic data to indicate the net production of a 3-carbon unit from carbon dioxide and acetate. A C₄ dicarboxylic

acid is then formed by the addition of carbon dioxide to the other end of C₃-carbon unit, which is converted to oxalacetate and then to aspartate by transamination. Any extensive equilibration of the oxalacetate with a symmetrical compound such as fumaric acid is precluded by the virtual absence of acetate carboxyl activity from the β -carbon atom of aspartate.

B. The utilization of carbon dioxide and acetate in the photosynthetic Rhodospirillum rubrum in the synthesis of amino acids is very similar to that of C. kluyveri.

Cutinelli, et al (9), suggest that part of the CO₂ enters the system by condensation with a 2-carbon structure of acetate origin. A second entrance of CO₂ is indicated to occur via β -condensation with pyruvate to oxalacetate.

Studies on Penicillium digitatum. This mold (33) has been widely studied because of the destructive olive-green rot of citrus fruits produced by it. As one would expect, most of these studies have centered upon ways and means of reducing or preventing this rot. Nattrass (31) reported fruits dipped in cold saturated borax solution or in 1 per cent "shirlan" (cold) to remain almost free of infection. Childs and Siegler (7) used thiourea and thioacetamide in 5 per cent aqueous solutions and quinosol in 8 per cent solutions for momentary dips. Losses in

some varieties were reduced from 40 per cent to 2 per cent or lower.

Exposure to ethylene gas has, for a considerable time, been known to be related to the ripening process in citrus fruits. Further, it was commonly observed that the fruit in a crate containing scattered fruits rotting with green mold appeared to "ripen" faster than in like crates containing only sound fruit. It was not until 1940, however, that Baile (3) and Miller, et al (30), discovered independently that ethylene was evolved by citrus fruits, and that decaying fruits produced more than sound ones. It was further shown in both investigations that Penicillium digitatum is capable of producing ethylene, thus further hastening the coloring process.

Wooster and Cheldelin (53) devised a synthetic medium for the growth of P. digitatum. They found that thiamin, or the thiazole moiety, was required for growth. Glucose afforded a more favorable carbon source than sucrose, and organic nitrogen sources such as asparagine or hydrolyzed casein were better than NaNO_3 and other inorganic salts. A synergistic effect was observed between P. digitatum and Oospora citri-aurantii in laboratory cultures by Gemmell (17), who demonstrated also that these fungi produced more rapid and extensive rotting when inoculated on the fruit together than when either one

or the other was present as the sole pathogen.

Birkinshaw, et al (4), have reported limited biochemical studies on P. digitatum. Considerable ethyl acetate was produced from glucose, and in addition some ethyl alcohol and a new polysaccharide which gave rise to glucose upon hydrolysis.

Fergus (15) studied the nutrition of this microorganism. D-Xylose, L-arabinose, D-glucose, D-fructose, D-mannose, D-galactose, sucrose, and cellobiose were utilized very efficiently as carbon sources; L-rhamnose and lactose allowed moderate growth; and D-mannitol, maltose, and dextrin were utilized poorly. Citric and malic were utilized to a certain extent, but fumaric, tartaric, lactic, acetic and oxalacetic were not.

In order to get a further insight into the nature of P. digitatum, we have endeavored to study the carbohydrate metabolism of this microorganism using isotopic tracer methods.

Warburg techniques, using as substrate differentially labeled glucoses, have been employed. In these studies a variety of conditions have been imposed on the mold cells so as to determine the effect of these factors on the contributions of the various catabolic pathways. Some of these conditions being age of mold, nature of medium, ethylene and indoleacetic acid.

Since C_2 is known to be the key unit in the terminal respiration of most microorganisms, the next mode of attack, despite the fact that acetate has been reported as not a normal carbon source for this mold, was directed on the kinetic pattern of $CH_3C^{14}OONa$ and $C^{14}H_3COONa$ utilization by the mold. A study of the fate of the C_2 units was studied also from the pattern of their unincorporation into amino acids.

Using various key amino acids as competitors of C^{14} activity in carboxyl and methyl labeled acetate, the various connections between amino acid families were studied next.

Finally, the pattern of incorporation of carbon dioxide was studied using $C^{14}O_2$ and non-labeled glucose as co-substrate. The isolation and degradation of key amino acids has provided further insight into the carbohydrate metabolism of Penicillium digitatum.

EXPERIMENTAL AND RESULTS

Cultural methods. Stock cultures of Penicillium digitatum Sacc. were grown on potato-glucose agar slants at room temperature for about a week, and then stored in the refrigerator.

For growth experiments the mold was transferred aseptically to a potato-glucose agar slant and was allowed to grow for 48 hours, after which time 5 ml of sterile water was added to it and the surface of the colony gently scraped with a loop. The fine suspension of mold was then added to the sterile growth medium; growth was then allowed to continue for a prescribed time at room temperature, which fluctuated between 23-27°C.

The synthetic medium employed was essentially the one devised by Wooster and Cheldelin (53) with the following exceptions; KH_2PO_4 was substituted with $(\text{NH}_4)\text{H}_2\text{PO}_4$, and glucose was used in place of sucrose. The sterilized medium had the following composition:

$(\text{NH}_4)\text{H}_2\text{PO}_4$, 1.0 g; NaNO_3 , 1.0 g; MgSO_4 , 0.25 g; KCl, 0.10 g; CaCl_2 , 0.10 g; FeCl_3 , 5.0 mg; MnCl_2 , 0.10 mg; ZnCl_2 , 0.05 mg; H_3BO_3 , 0.05 mg; CuCl_2 , 0.01 mg; KI, 0.01 mg; glucose, 26.4 g; asparagin, 3.0 g; ammonium tartrate, 5.0 g; casein hydrolysate, 40 ml (100 mg Casamino hydrolysate per ml water); 400 γ thiamine HCl, 800 γ pyridoxine HCl, 600 γ calcium pantothenate and 0.40 γ biotin. Distilled water was added to this mixture

to make 1 liter, and the pH was adjusted to 3.0 using HCl.

The medium containing the mold inoculum was placed in a sterilized flask as shown in Figure 1 and was vigorously aerated with purified air through air disperser tube A while a suction pressure was applied through tube S. The medium was also agitated by means of a ground glass-joint stirrer B. The air-purification train contained a NaOH solution scrubber followed by two soda-lime absorbers, a concentrated H_2SO_4 scrubber and a tube containing sterilized cotton.

The mold, after having attained the required growth phase, was harvested by centrifugation and washed four times with a carbon source-free medium. This medium contained all the ingredients as described above but was lacking in glucose, asparagin, ammonium tartrate and casein hydrolysate. A known weight of mold was then suspended in a measured volume of carbon source-free medium, and to this suspension was added the various C^{14} -labeled substrates used in the respective metabolism studies.

I. Warburg manometry

A. The detection of alternative catabolic pathway of glucose and the effects of aging and nature of medium on the distribution of catabolic pathways of glucose.



FIGURE 1

Two separate Warburg studies were carried out on glucose utilization using different batches of mold. For the sake of convenience we shall refer to the first group of studies as Experiment 1 and to the latter as Experiment 2.

Experiment 1. Two sets of three-necked flasks, each containing 300 ml of medium, were set up in series. Growth was initiated in the first flask by the addition of mold inoculum; 24 hours later growth was started similarly in the second flask. Four days later the 4- and 3-day-old cells in the first and second flasks respectively were harvested and washed with carbon source-free medium.

Seventy six milligrams of the 4-day-old cells were suspended in carbon source-free medium and added into each of the first and second 125 ml Warburg cups. The same amount of the 3-day-old cells were suspended in carbon source-free medium and added similarly to the third and fourth cups. The fifth and sixth cups each contained 76 mg of the 4-day-old cells, except they were suspended in 0.067 M phosphate buffer adjusted to pH 3.

Forty six and two tenths milligrams of glucose-1- C^{14} , having a total activity of 3.14×10^4 cpm, was added to the first, third and fifth cups, and 46.2 mg of glucose-6- C^{14} , having the same activity, was added to each of the remaining cups. Each cup was flushed for 5

minutes with oxygen and allowed to equilibrate for 15 minutes in the constant temperature bath maintained at 30°C.

The respiratory CO_2 evolved from each cup was trapped by the CO_2 -free NaOH in the center well, and after 7000 μl of O_2 were consumed by the mold the cells were killed by the addition of 6N HCl from the side-arm. The cells were separated from the medium by centrifugation in 4-inch test tubes and washed with water. The cells in the test tubes were then dried in vacuo over P_2O_5 . To each mg of mold 0.10 ml of 6N HCl was added; the test tubes were sealed and autoclaved for 6 hours at 15 p.s.i. pressure to effect complete hydrolysis of cellular proteins. The seals were broken and the hydrolysates were then evaporated at room temperature to dryness in vacuo over P_2O_5 and KOH. To each mg of cells which were hydrolyzed 0.10 ml of H_2O was added and 0.05 ml aliquots were taken for counting by direct plating. All the hydrolysate samples were counted as infinitesimal thickness samples by using a thin, end window Geiger-counter with corrections for background applied to the results in the conventional manner.

The supernatant medium was also assayed for radioactivity by direct plating. The contents and washings from the center well were combined and the carbonate was precipitated as BaCO_3 by the addition of a 1 M BaCl_2 -

1 M NH_4Cl solution. The precipitated barium carbonate was then mounted on aluminum planchets by the centrifugation technique, dried and counted by using a thin, end window Geiger-counter with appropriate corrections applied for self-absorption and background. The results are given in Table I.

Experiment 2. Under similar conditions as described in Experiment 1, three flasks were set up in series; and growth was initiated in each flask at 48-hour intervals. 5 1/2 days after the first inoculation, the two oldest batches of cells were harvested and washed with the carbon source-free synthetic medium. 94 mg of the 5 1/2-day-old cells were then suspended in carbon source-free medium and transferred to each of the first and second Warburg cups. The third and fourth cups contained 94 mg of 3 1/2-day-old cells suspended in carbon source-free medium. The results are given in Table II under cup number 1-4.

A much smaller amount of cells was obtained after 1 1/2 days of growth. The cells from this batch were harvested, one half washed and suspended in synthetic medium and the other half washed and suspended in 0.067 M phosphate buffer adjusted to pH 3. These were transferred respectively into two sets of two Warburg cups. Each of the four Warburg cups in this case contained only 29 mg of

mold cells.

Forty six and two tenths milligrams of glucose-1- C^{14} with a total activity of 2.77×10^4 cpm was added to each alternate Warburg cup; and to the remaining cups, 46.2 mg of glucose-6- C^{14} with the same total activity as glucose-1- C^{14} was added.

After 7000 μl of O_2 had been consumed by the mold, the incubation was stopped by the addition of 6N HCl. The cells, medium and the respiratory CO_2 were assayed for radioactivity following the procedure outlined in Experiment 1. A summary of the results are shown in Table II under cup number 5-8.

B. The effect of indoleacetic acid and ethylene on the distribution of catabolic pathways of glucose.

The 3 1/2-day-old cells from Experiment 2 were used. Four Warburg cups were used and each contained 94 mg of mold suspended in carbon source-free medium. 46.2 mg of glucose-1- C^{14} were added to cups 1 and 3 and an equivalent amount of glucose-6- C^{14} was added to the second and fourth cups. Each of the cups contained 2.77×10^4 cpm of glucose.

The first and second flasks were flushed with an atmosphere of 1 part ethylene in 1000 parts O_2 , while the

third and fourth cups each contained 28.3 μ l of indole-acetic acid and were swept only by O_2 .

When 7000 μ l of O_2 had been consumed by the mold the incubation was stopped by the addition of 6N HCl and the various fractions assayed for radioactivity.

The results are given in Table II under cup numbers 9-12.

C. Incorporation of glucose-1, -2, -6- C^{14}
into cellular amino acids.

Three 30 ml Warburg cups were used in this study, each cup containing 4.1 mg of the 4-day-old mold cells grown as described in Experiment 1 and suspended in 2 ml of carbon source-free medium. 3.40 mg of specifically labeled glucose having a total activity of 2.01×10^5 cpm was added to each of the three cups. The first flask contained the glucose-1- C^{14} ; the second, glucose-6- C^{14} and the third contained the glucose-2- C^{14} specie.

When 1500 μ l of O_2 had been utilized by the mold the incubation was stopped by the addition of 6N HCl and the medium and cells were assayed for radioactivity. Since the amount of CO_2 formed during fermentation in this series was relatively smaller, sodium carbonate was added as the carrier; and the combined carbonate was precipitated as barium carbonate by the addition of the $BaCl_2-NH_4Cl$

solution. The BaCO_3 was then centrifuged, plated, dried and counted.

Throughout this work, chromatography and radioautography of the amino acids in the hydrolysate were carried out in the following manner. Three systems of descending paper chromatography were employed. n-Butanol-acetic acid-water (4:1:5), (BAW), and 80 per cent aqueous phenol containing a trace of 8-hydroxyquinoline were used for single- and two-dimensional paper chromatography. Single-dimensional paper chromatograms were also obtained by using 3:1 secondary butanol-ammonia (3 per cent) as the solvent system (35). Whatman No. 1 filter paper, 57 cm by 46 cm, was used in all the above systems. Known amino acids were run in conjunction with all the unknowns and thus served as the key in the identification of the spots.

The cells from the glucose-1, -2, -6- C^{14} cups, respectively, were hydrolyzed and dried over P_2O_5 and KOH. 0.10 ml of water was added to each mg of cell. 50 μ l of each of these solutions were spotted on Whatman No. 1 paper for chromatography using phenol and BAW as solvents.

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.

Table I summarizes the results from the

differentially labeled glucoses; and figures 2, 3 and 4 are reproductions of the two-dimensional radioautograms of the cell hydrolysates.

D. Incorporation of C¹⁴-acetate into
P. digitatum proteins in the presence of
C¹²-amino acids in medium.

Three and one-half-day-old mold cells, grown in the usual manner, were used. After harvesting and washing, 15 mg of cells were suspended in carbon source-free medium and transferred to each of the ten 125 ml Warburg cups used in this series of experiments. 0.817 mg of carboxyl-labeled acetate with 0.640×10^6 cpm activity was added to each alternate cup, and to each of the remaining cups 0.817 mg of methyl-labeled acetate with 0.640×10^6 cpm activity was added. The first and second cups were used as controls. 200 μ moles of the desired unlabeled amino acid competitors¹ were added to each of the remaining cups. The third and fourth cups contained glutamic acid; the fifth and sixth contained aspartic acid; the seventh and eighth contained alanine; and, finally, the ninth and

¹ In their studies with E.coli, McQuillen and Roberts (29, p.87) define the terms competitor, competition, etc., "... to indicate that certain compounds, which can be synthesized by E.coli when supplied externally, reduce the amount of endogenously formed material appearing in cell substance."

tenth contained glycine.

The cups were flushed with an atmosphere of oxygen; and, after 10 minutes of equilibration period, readings on the manometers were taken. 4 3/4 hours later the experiment was terminated by tipping 6N HCl from the side arms into the reaction mixture. The cells, medium and respiratory CO₂ were assayed for radioactivity in the conventional manner.

Results of this experiment may be found in Table III. Single-dimensional paper chromatograms were made of 300 γ of each of the cell hydrolysates using BAW as solvent. This radioautogram of the protein hydrolysates may be found in Figure 5.

II. Kinetic studies

A. Kinetic pattern of glucose-1-C¹⁴ and glucose-6-C¹⁴ utilization.

Two suspensions, each containing 250 mg of 3 1/2-day-old mold cells in 223 ml of carbon source-free medium, were transferred respectively into two three-necked flasks as shown in Figure 6. A stream of purified and CO₂-free air was passed through A, the CO₂ emanating from the fermentation was swept through B and absorbed in the NaOH trap D. E was a safety trap also containing NaOH to

absorb any escaped CO_2 from D. Through S, samples of the suspension were drawn into the test tube contained in a suction flask. Suction was applied through the outlet of E. 125 mg of glucose-1- C^{14} with an activity of 2.85×10^5 cpm was added to one of the flasks and to the other was added 125 mg of glucose-6- C^{14} with an activity of 2.82×10^5 cpm.

Sterile and CO_2 -free air was passed at a maximum rate through the two suspensions and the CO_2 released during the fermentation of the C^{14} -glucoses was trapped in CO_2 -free NaOH solution and assayed for radioactivity as BaCO_3 . At various time intervals 2 ml aliquots were extracted from the reaction flasks. Cells were removed by centrifugation, and the activity left in the medium was determined by direct plating.

The experiment was allowed to continue for 8 hours; at the end of which time, 10 ml of concentrated HCl was added to each flask to kill the mold.

Table IV summarizes the rate of glucose utilization in the two media, the specific activity of the respiratory CO_2 and the total cumulative CO_2 activity. Graphical expressions of some of these results may be found in Figures 7 and 8.



FIGURE 2

Two dimensional radioautogram of isotopic amino acids from mold hydrolysate. Substrate glucose-1- C^{14} . 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, E = cystine, R = aspartic, N = glutamic, Y = glycine, K = arginine, lysine, O = threonine, B = ninhydrin sensitive unknown, A = alanine, D = valine, methionine, S = isoleucine, leucine, phenyl alanine, P = proline.

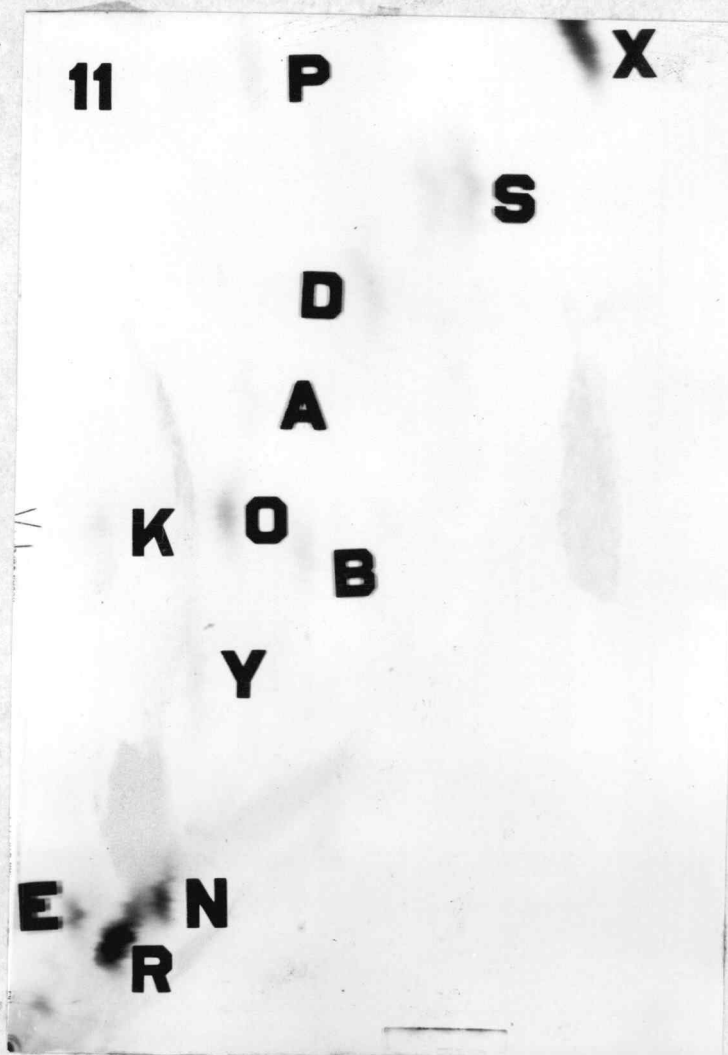


FIGURE 3

Two dimensional radioautogram of isotopic amino acids from mold hydrolysate. Substrate glucose-6- C^{14} . 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, E = cystine, R = aspartic, N = glutamic, Y = glycine, K = arginine, lysine, O = threonine, B = ninhydrin sensitive unknown, A = alanine, D = valine, methionine, S = isoleucine, leucine, phenyl alanine, P = proline, X = non-ninhydrin sensitive spot.

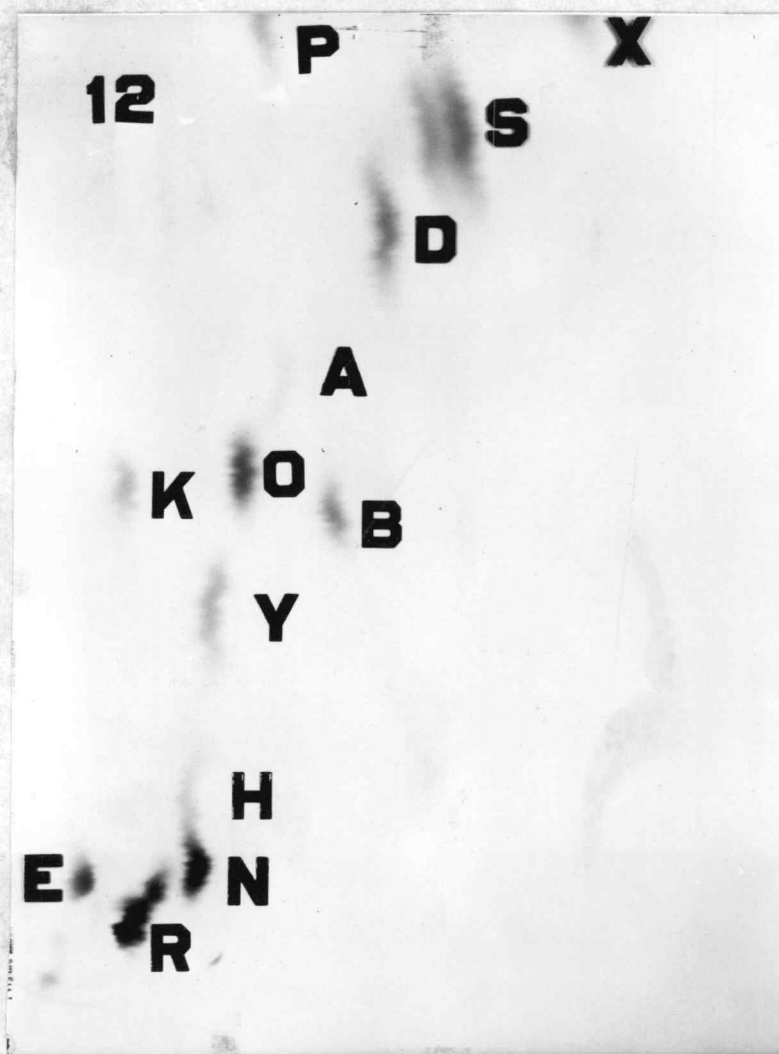


FIGURE 4

Two dimensional radioautogram of isotopic amino acids from mold hydrolysate. Substrate glucose-2-C¹⁴. 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, E = cystine, R = aspartic, N = glutamic, Y = glycine, K = arginine, lysine, O = threonine, B = ninhydrin sensitive unknown, A = alanine, D = valine, methionine, S = isoleucine, leucine, phenyl alanine, P = proline, H = serine, X = non-ninhydrin sensitive spot.

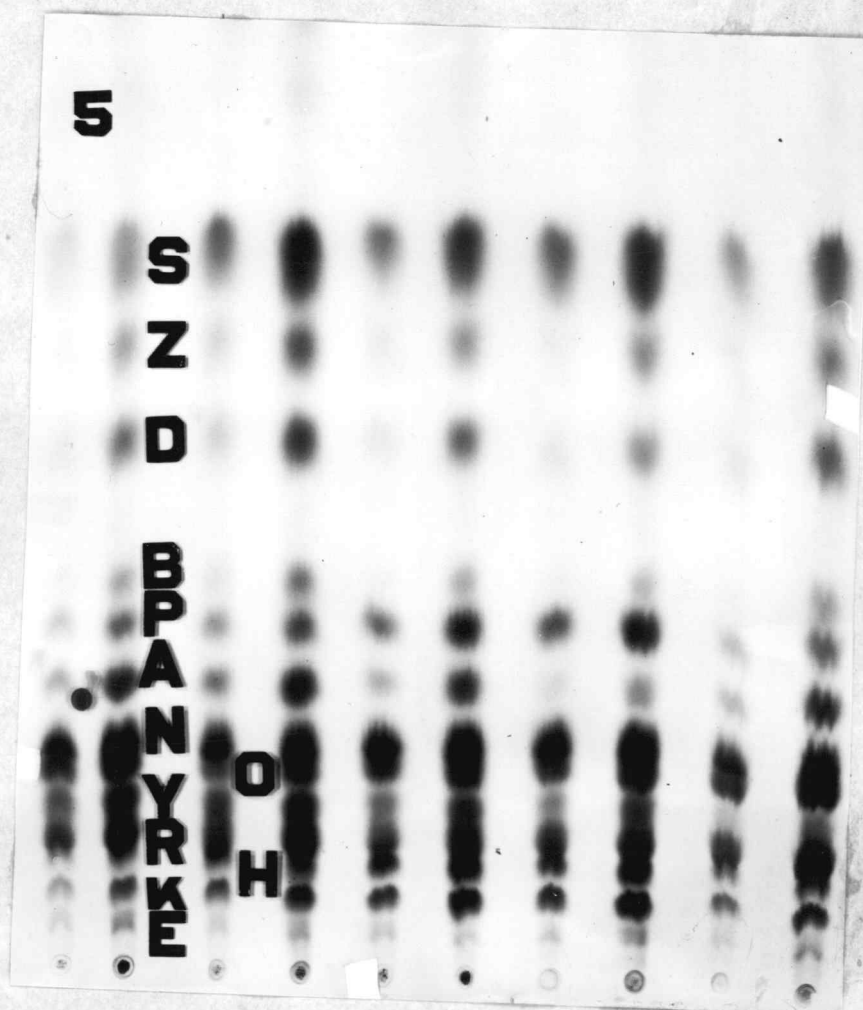


FIGURE 5

One dimensional radioautogram of time course samples. Solvent system used, Butanol-acetic acid-water (4:1:5) moving from bottom to top. Vertical spots by pairs from left to right (the first of the pair is from carboxyl labeled acetate and the second from methyl labeled acetate) represent control, glutamic acid, aspartic, alanine, and glycine as amino acid competitors. Identified spots are E = cystine, K = lysine, H = arginine, R = aspartic, serine, Y = glycine, O = threonine, N = glutamic, A = alanine, P = proline, B = tyrosine, D = valine, methionine, Z = phenylalanine, isoleucine, S = leucine.

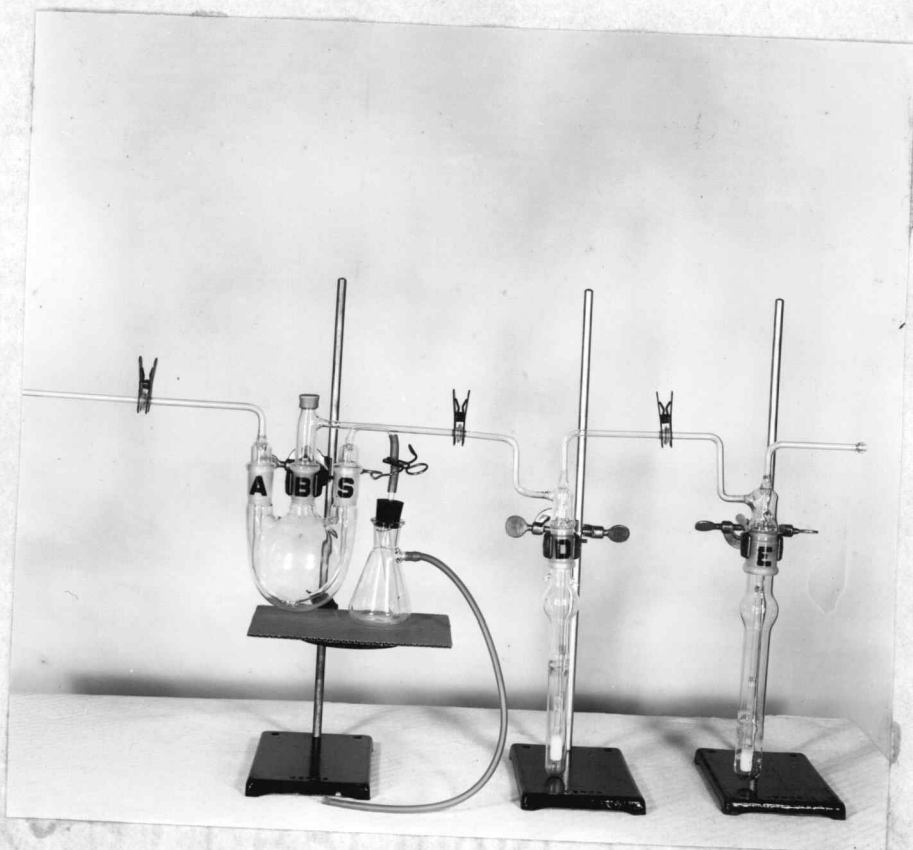


FIGURE 6

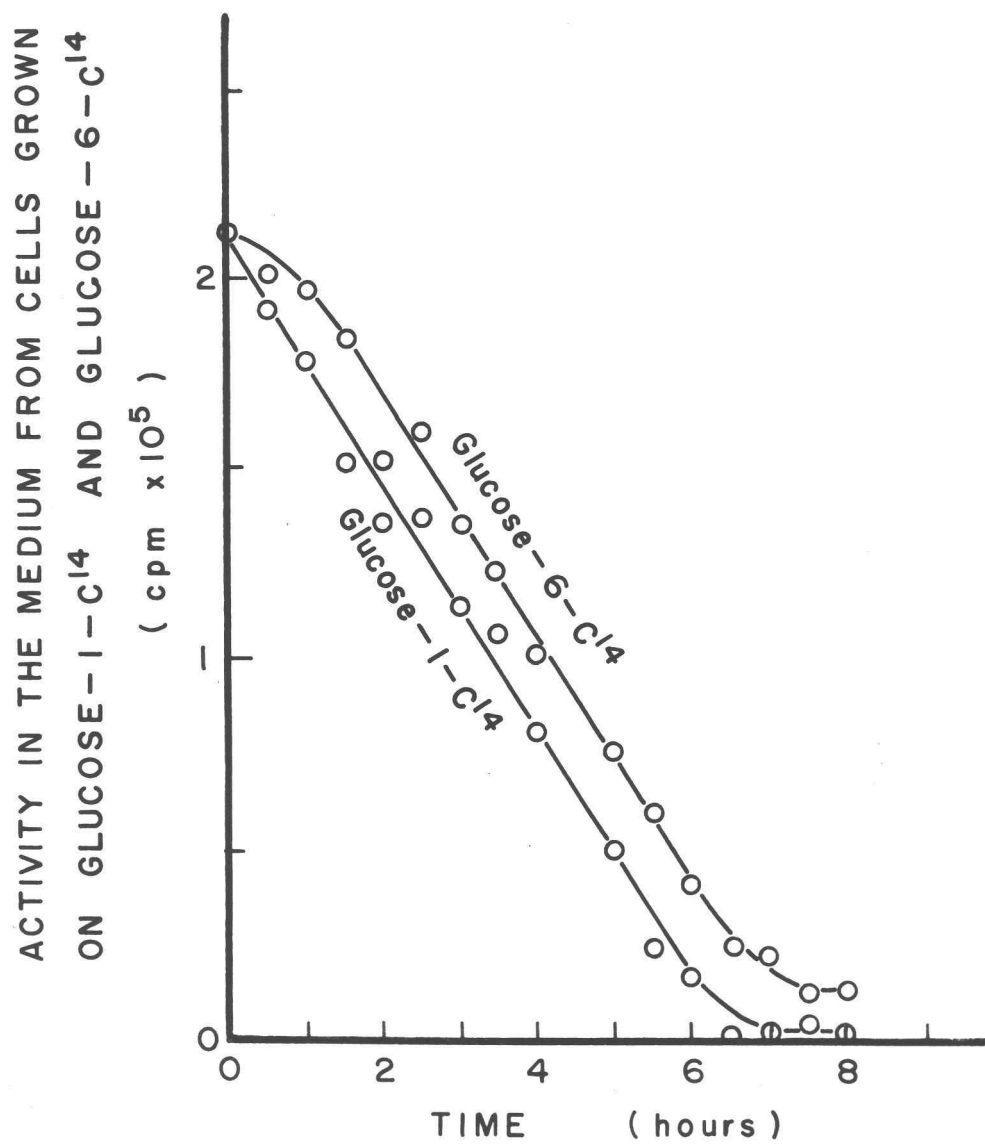


FIGURE 7

SPECIFIC ACTIVITY OF BaCO_3 FROM CELLS GROWN
ON GLUCOSE-1- C^{14} AND GLUCOSE-6- C^{14}

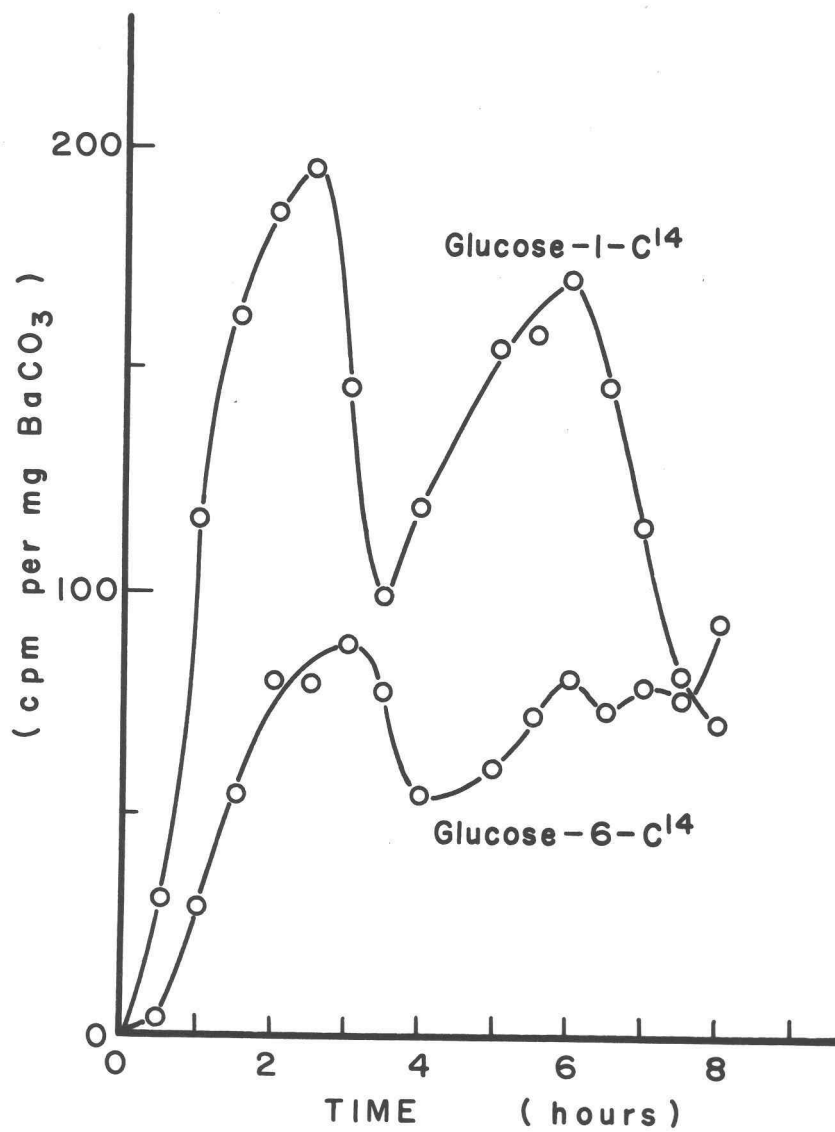


FIGURE 8

B. Kinetic pattern of $\text{CH}_3\text{C}^{14}\text{OOH}$ and $\text{C}^{14}\text{H}_3\text{COOH}$ utilization.

Two suspensions, each containing 200 mg of 3 1/2-day-old mold cells in 221 ml of carbon source-free medium, were transferred respectively into two-necked flasks (Figure 6). 12.8 mg of carboxyl-labeled acetate, having an activity of 22.4×10^6 cpm, was added to the first flask; and the same amount and activity of methyl-labeled acetate was added to the second flask.

Sterile and CO_2 -free air was passed at a maximum rate through the two suspensions. At definite time intervals the activity of the medium, cells and respiratory CO_2 were determined. These results are shown in Table V. Figures 9, 10, and 11 describe, in graphic form, the decrease of the C^{14} activity in the medium, the specific activity of the respiratory CO_2 and C^{14} activity incorporated into the cells.

The relatively higher level of radioactivity in this experiment permitted further examination of cellular constituents. Consequently, the cells were hydrolyzed and single-dimensional paper chromatograms and radioautograms using BAW, phenol and secondary butanol-ammonia were obtained. The radioautograms are shown in Figures 12-18.

Eighty five and three tenths per cent of the total

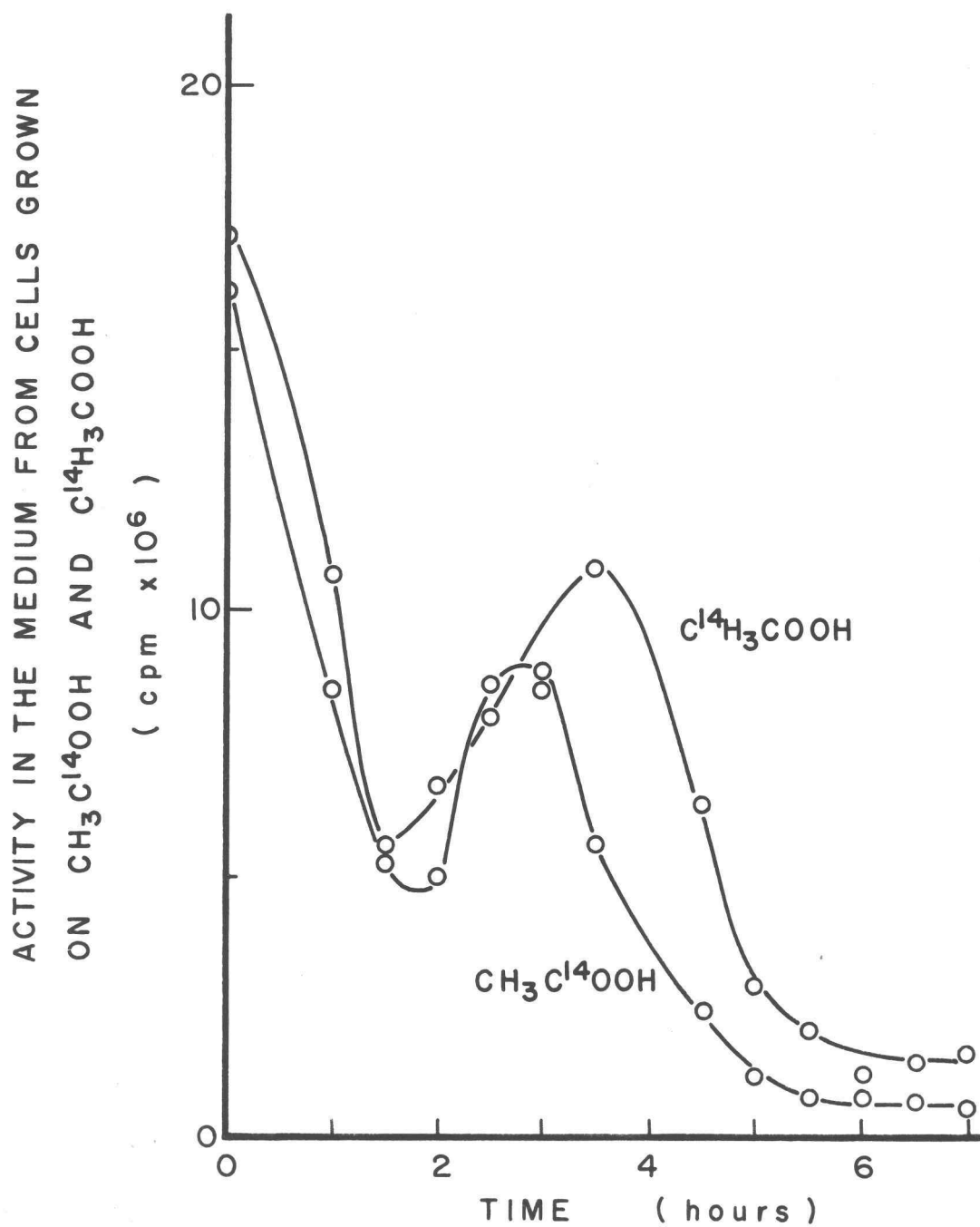
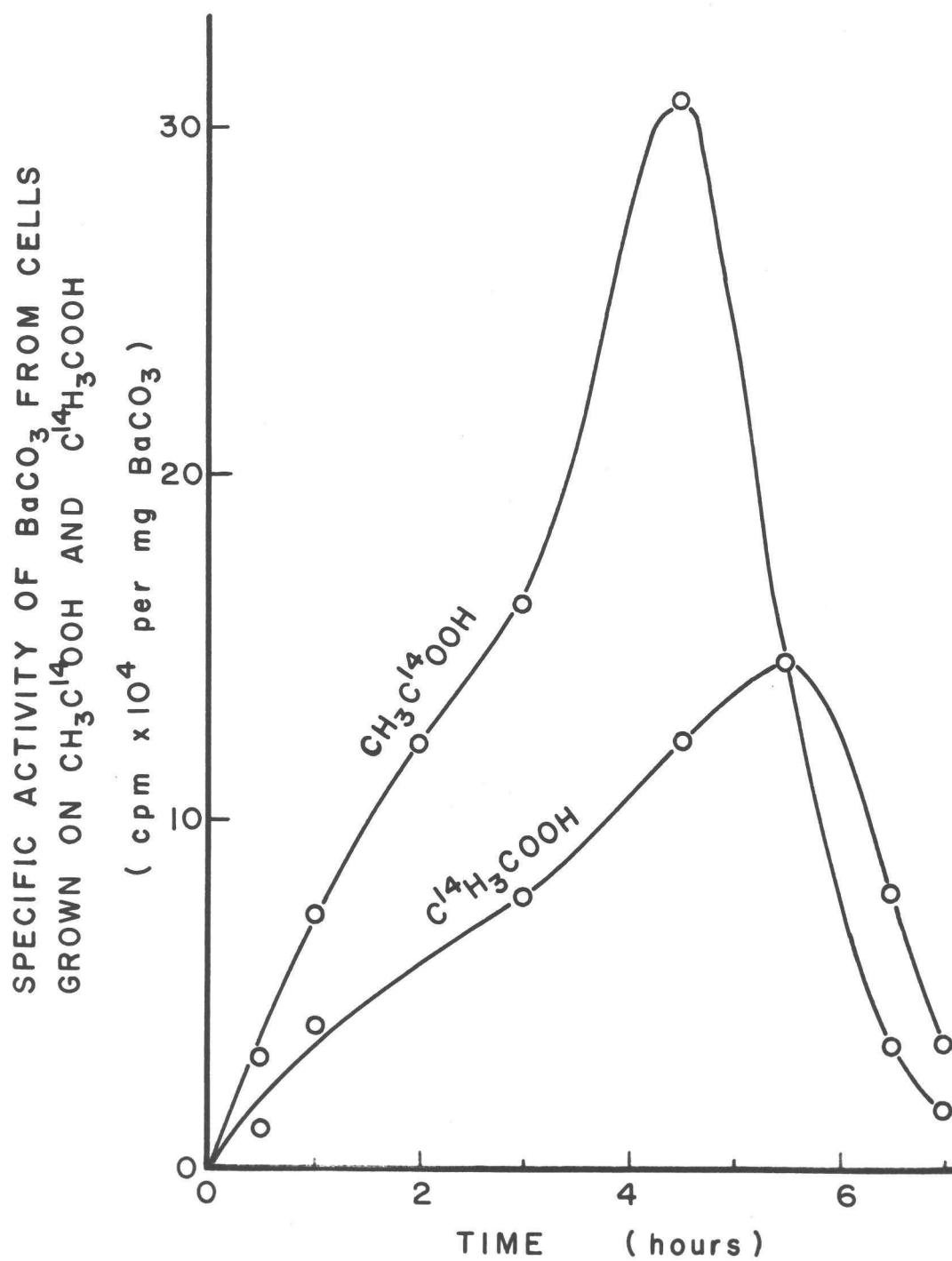


FIGURE 9

FIGURE 10



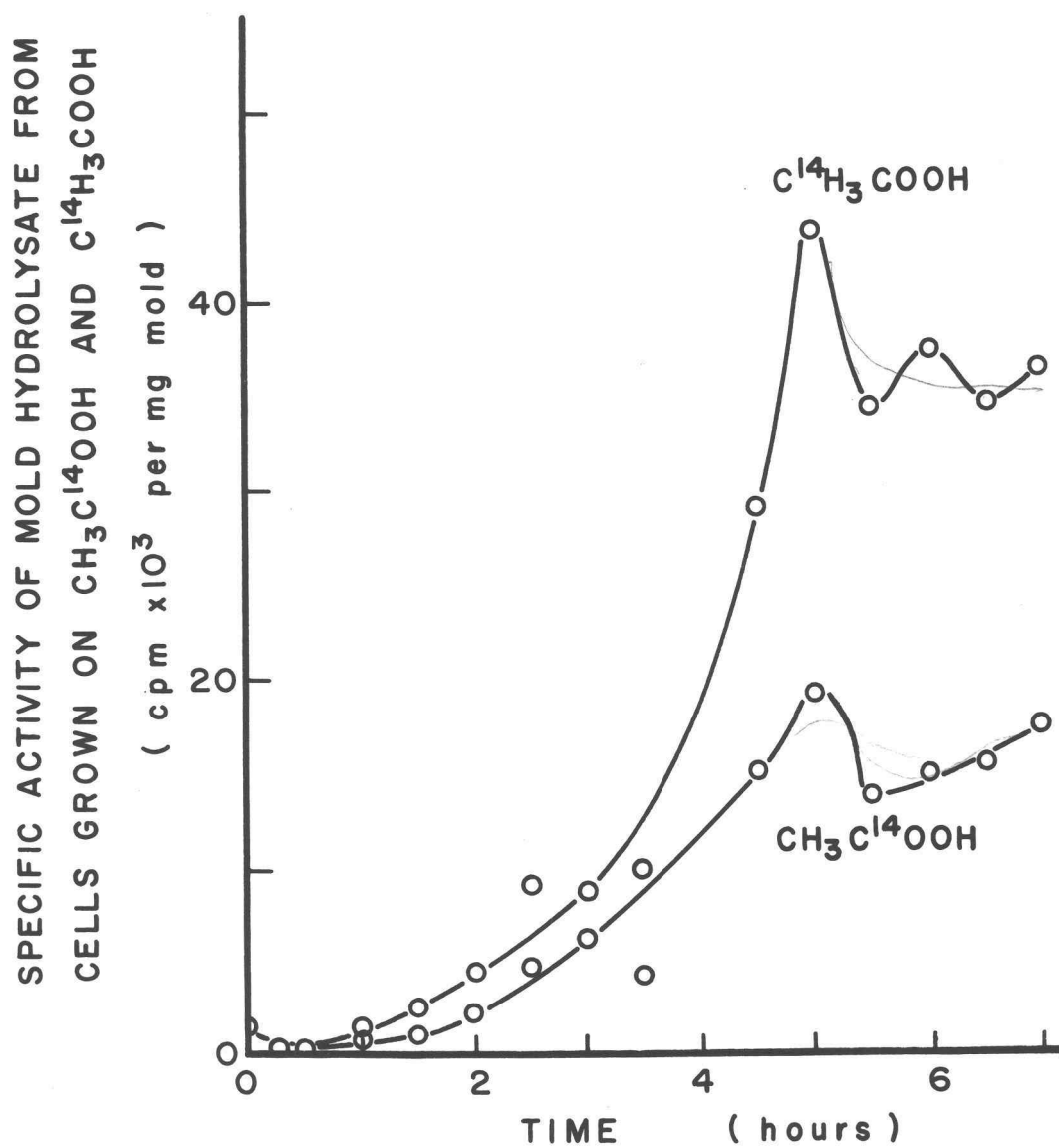


FIGURE 11

C^{14} activity from $CH_3C^{14}OOH$ was recovered as respiratory $C^{14}O_2$, while only 44.4 per cent of the C^{14} activity was recovered as $C^{14}O_2$ from $C^{14}H_3COOH$. This gives approximately a 2:1 ratio in favor of the carboxyl carbon in the combustion of the respective carbon atoms of acetate molecule into $C^{14}O_2$.

C. Kinetic study of $C^{14}O_2$ fixation.

Two and one-half grams of 3 1/2-day-old mold cells, grown in the usual manner were harvested, washed and suspended in 540 ml of carbon source-free medium. The cell suspension was transferred into a two-liter three-necked flask and to it was added 1.88 g of nonlabeled glucose. One neck of the flask was attached to a manometer; the other was equipped with a device for sampling aliquots of the cell suspension at various time intervals. The center neck contained a vaccine port to which was attached a vial containing 86.1 mg of $BaCO_3$ with 2.15 millicurie activity. (A photograph of this apparatus may be found in the Ph.D. thesis of J. Wendell Davis (10,p.12).)

The growth flask was oxygenated for 10 minutes; the system was then closed and evacuated to a pressure of 10 mm Hg. Through the vaccine port, 3 ml of concentrated H_2SO_4 was added to liberate the $C^{14}O_2$ from the $BaC^{14}O_3$.

Oxygen was then introduced into the system to give a pressure of about 600 mm Hg.

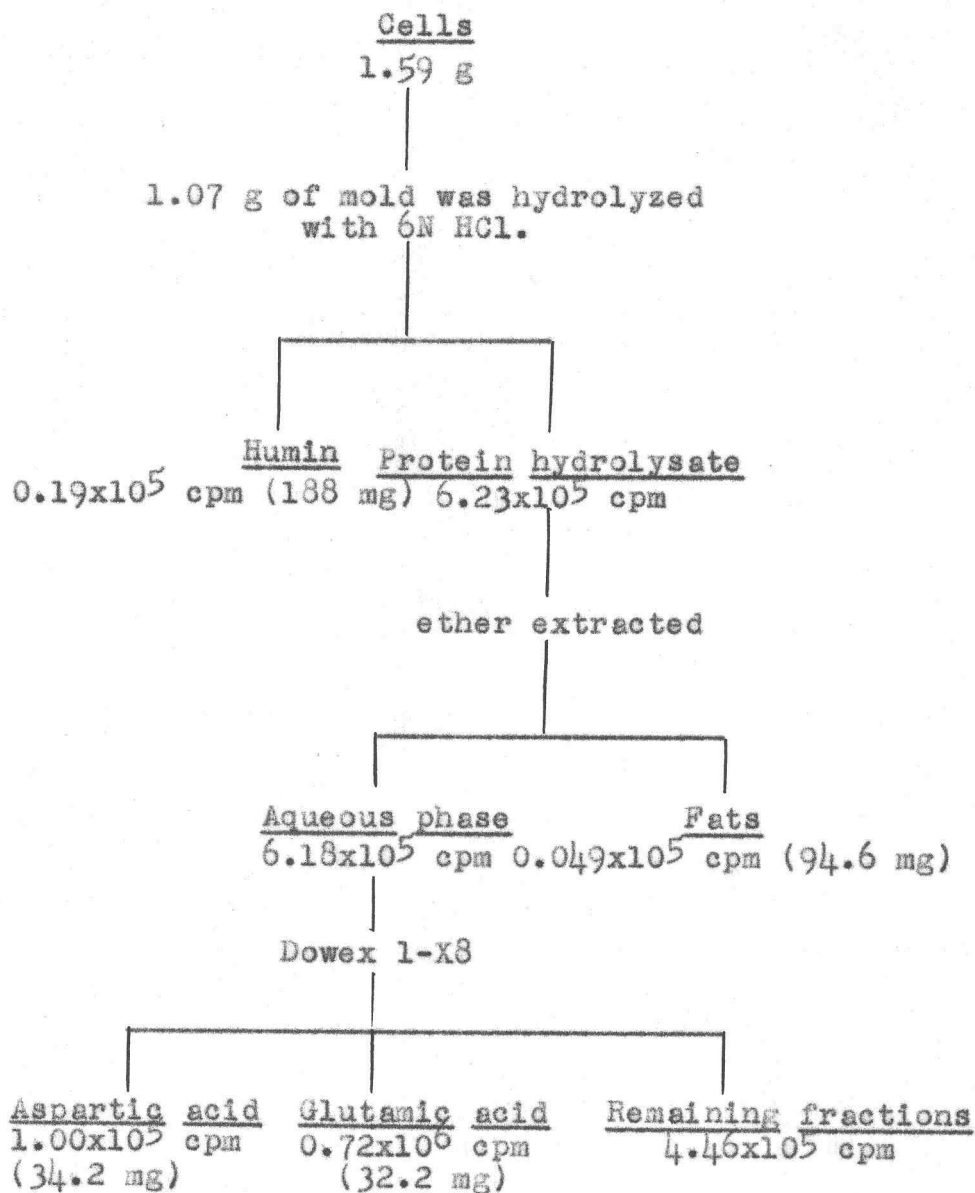
The closed flask was agitated on a mechanical shaker at 30°C, except for brief intervals during the removal of samples for analysis. 5 ml aliquots of cell suspension were taken out and the cells killed with 0.5 ml of 6N HCl. The cells were separated by centrifugation, washed with water, dried over P₂O₅, hydrolyzed and assayed for activity. The specific activity curve of the cell hydrolysates is shown in Figure 19.

Three hundred micrograms of the cell hydrolysates were spotted for single-dimensional paper chromatography. Phenol, BAW and secondary butanol-ammonia were respectively used as solvents to affect the separation of the amino acids. Radioautograms of the resolved amino acids were made and these are shown in Figure 20.

Six and three quarters hours after the introduction of C¹⁴O₂ the cells were killed by the addition of concentrated HCl. They were separated from the medium by centrifugation, washed twice with distilled water, and allowed to dry over P₂O₅ in a vacuum dessicator. At the end of the experiment, the medium contained 9.26×10^5 cpm activity. The distribution of C¹⁴-label in the various fractions of the mold may be found in Diagram I on the next page.

DIAGRAM I

DISTRIBUTION OF C^{14} -ACTIVITY IN THE
VARIOUS FRACTIONS OF MOLD GROWN
ON NON-LABELED GLUCOSE AND $C^{14}O_2$.



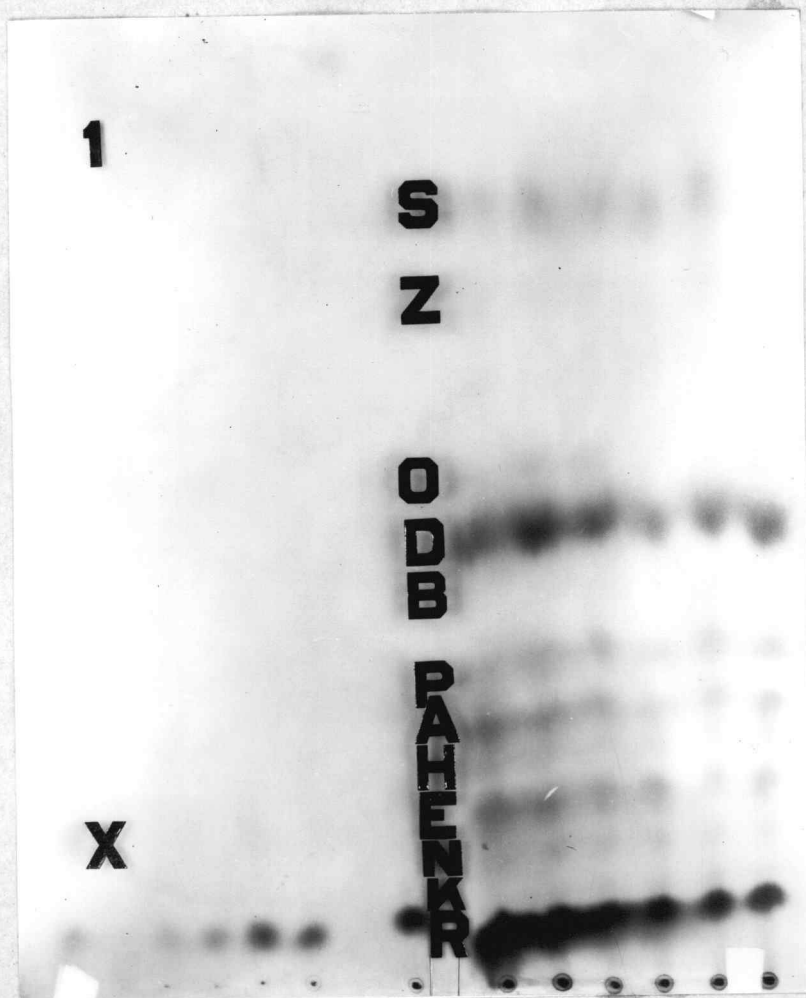


FIGURE 12

One dimensional radioautogram of time course samples. Solvent system used, secondary butanol-ammonia moving from bottom to top. Vertical rows of spots reading from left to right represent amino acids labeled after 0, 1/4, 1/2, 1, 1 1/2, 2, 2 1/2, 3 1/2, 4 1/2, 5 1/2, 6, 6 1/2, 7 hours exposure to $\text{CH}_3\text{C}^{14}\text{OONa}$. The spots are: R = aspartic, glutamic, cystine, K = lysine, N = arginine, E = glycine, serine, H = histidine, threonine, A = alanine, P = proline, B = tyrosine, D = valine, O = methionine, Z = isoleucine, S = phenylalanine, leucine, X = unknown.

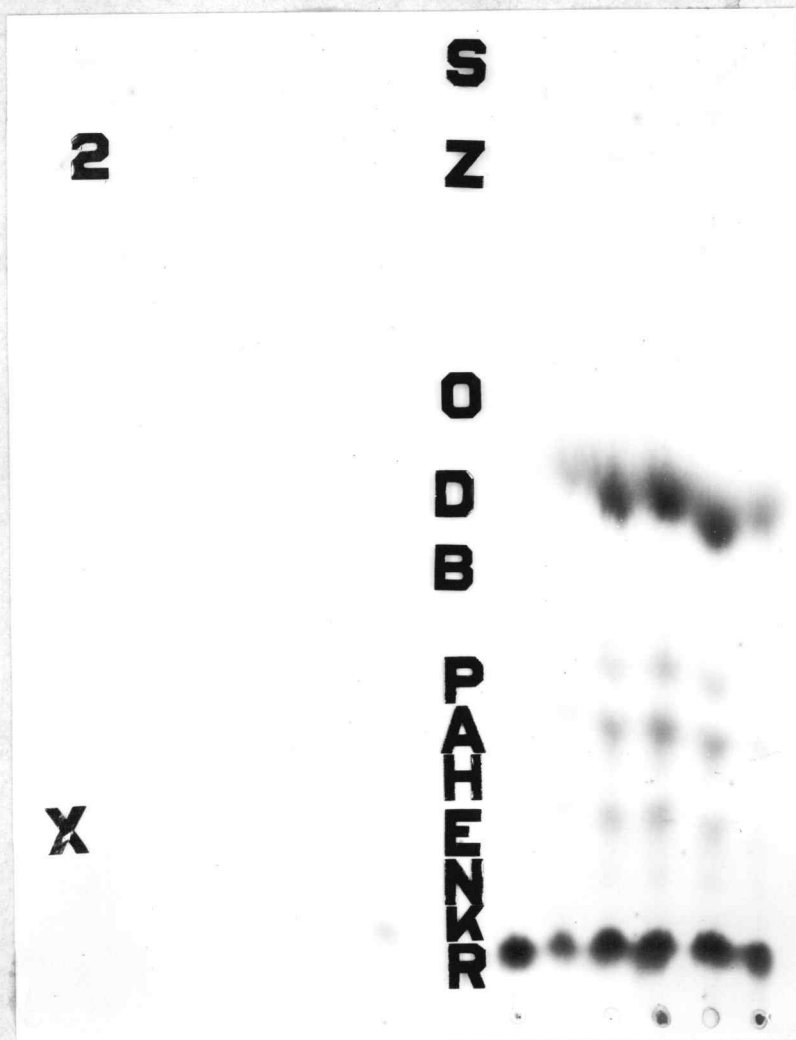


FIGURE 13

One dimensional radioautogram of time course samples. Solvent system used, secondary butanol-ammonia moving from bottom to top. Vertical row of spots reading from left to right represents amino acids labeled after 0, 1/4, 1/2, 1, 1 1/2, 2, 2 1/2, 3 1/4, 4 1/2, 5 1/2, 6 1/7, 7 hours exposure to $C^{14}H_3COONa$. The spots are the same as those identified on the preceding figure.

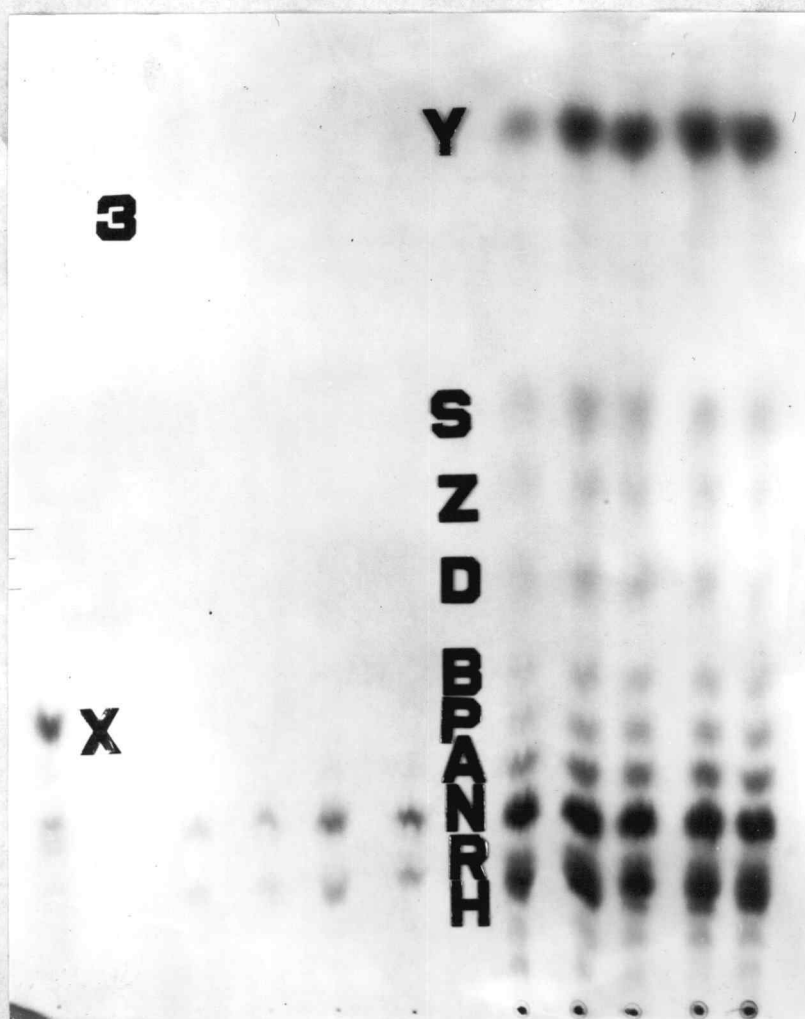


FIGURE 14

One dimensional radioautogram of time course samples. Solvent system used, Butanol-acetic acid-water (4:1:5) moving from bottom to top. Vertical spots reading from left to right represent amino acids labeled after 0, 1/4, 1/2, 1, 1 1/2, 2, 3 1/2, 4 1/2, 5 1/2, 6, 6 1/2 hours after exposure to $\text{CH}_3\text{C}^{14}\text{OONa}$. Identified spots are, E = cystine, K = lysine, H = arginine, R = glycine, serine, aspartic, N = glutamic, threonine, A = alanine, P = proline, B = tyrosine, D = methionine, valine, Z = isoleucine, phenyl-alanine, S = leucine, X and Y = unknown.

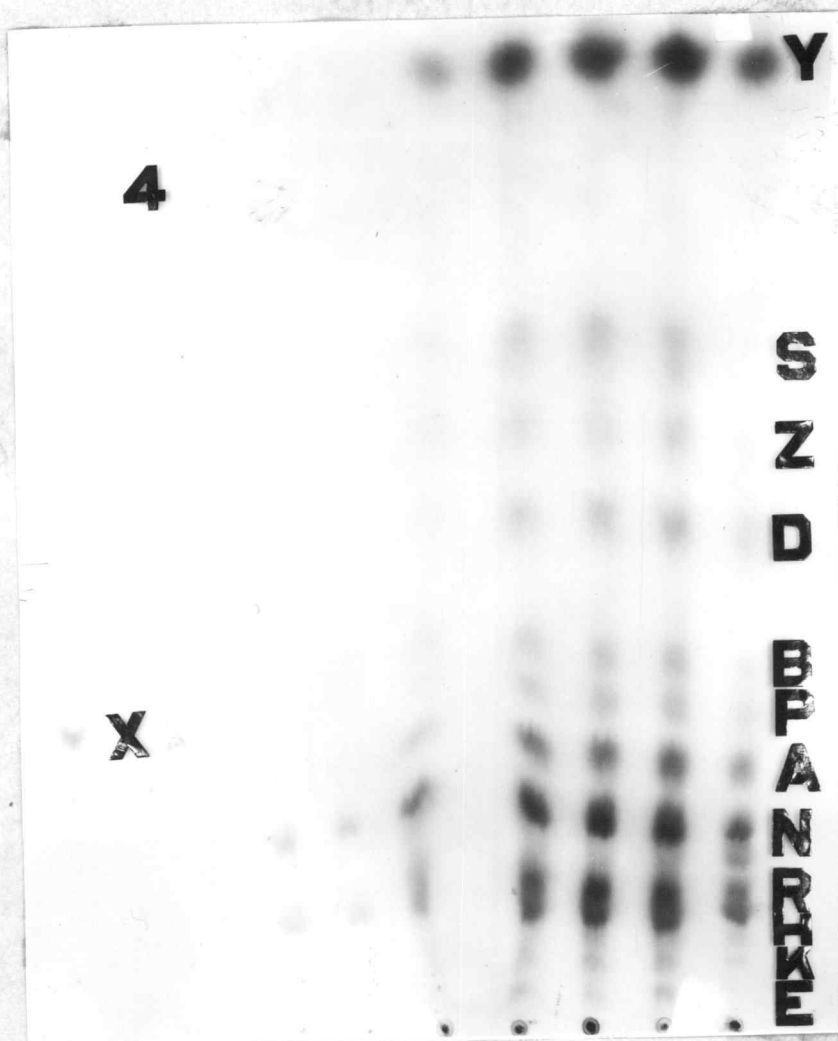


FIGURE 15

One dimensional radioautogram of time course samples. Solvent system used, Butanol-acetic acid-water (4:1:5) moving from left to right represent amino acids labeled after 0, 1/4, 1/2, 1, 1 1/2, 3 1/2, 4 1/2, 5 1/2, 6, 7 hours after exposure to $C^{14}H_3COONa$. Identified spots are the same as those on the preceding figure.

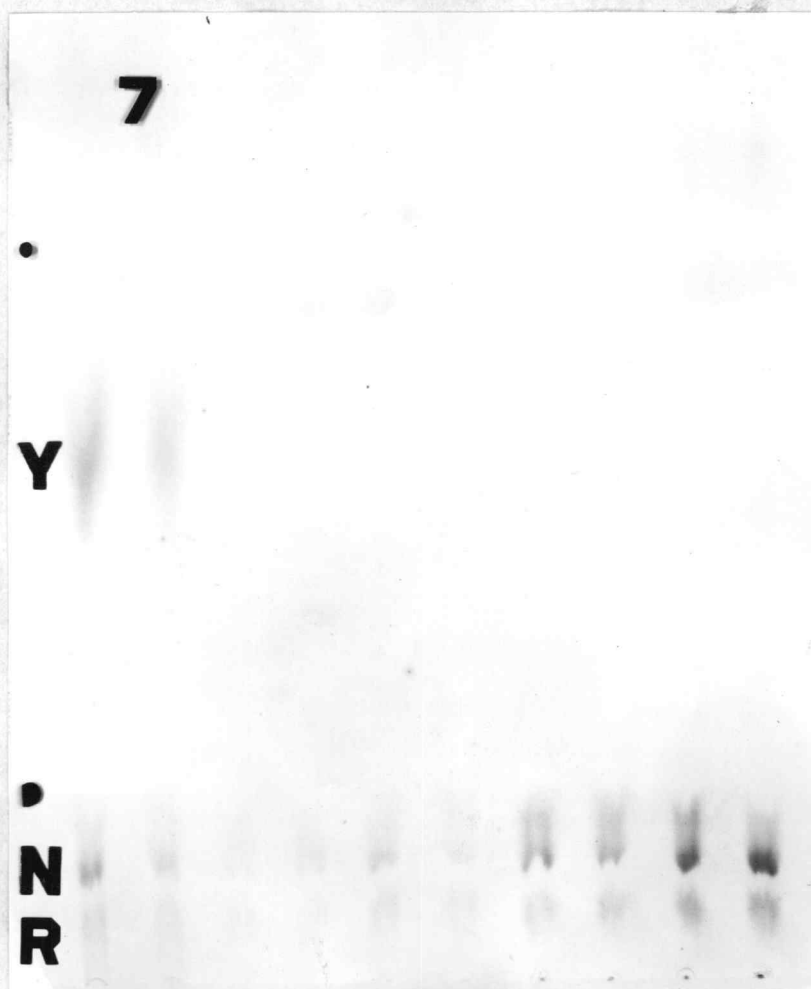


FIGURE 16

One dimensional radioautogram of time course samples. Solvent system used, phenol (80%) moving from bottom to top. Vertical spots reading by pairs from left to right (the first of each pair is from carboxyl labeled acetate and the second from methyl labeled acetate) 0, 1/4, 1/2, 1, 1 1/2 hours. Y = unknown, N = glutamic, R = aspartic.

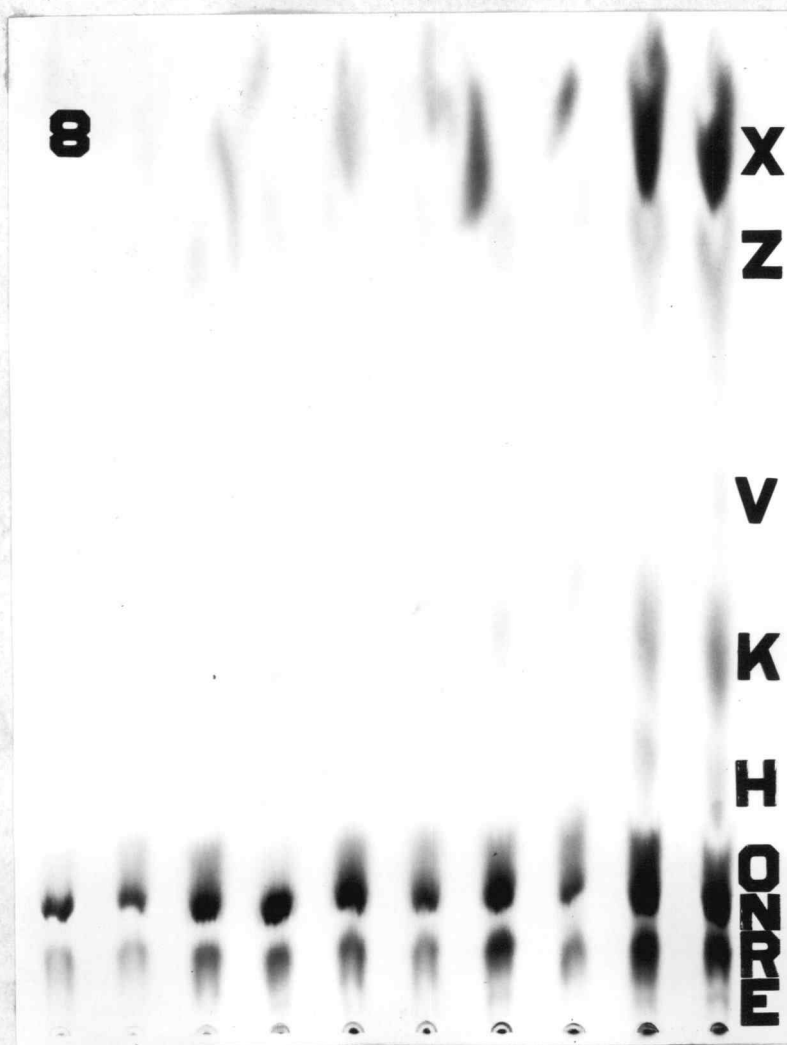


FIGURE 17

One dimensional radioautogram of time course samples. Solvent system used, phenol (80%) moving from bottom to top. Vertical spots reading by pairs from left to right (the first of each pair is from carboxyl labeled acetate and the second from methyl labeled acetate) 2, 2 1/2, 3, 3 1/2, 4 1/2 hours. E = cystine, R = aspartic, N = glutamic, serine, O = glycine, H = threonine, tyrosine, K = lysine, histidine, alanine and arginine, V = valine, Z = leucine, isoleucine, phenyl, alanine, X = unknown and proline.



FIGURE 18

One dimensional radioautograms of time course samples. Solvent system used, phenol (80%) moving from bottom to top. Vertical spots reading by pairs from left to right (the first of each pair is from carboxyl labeled acetate and the second from methyl labeled acetate) 5, 5 1/2, 6, 6 1/2, 7 hours. Identity of spots are the same as on the preceeding figure.

FIGURE 19

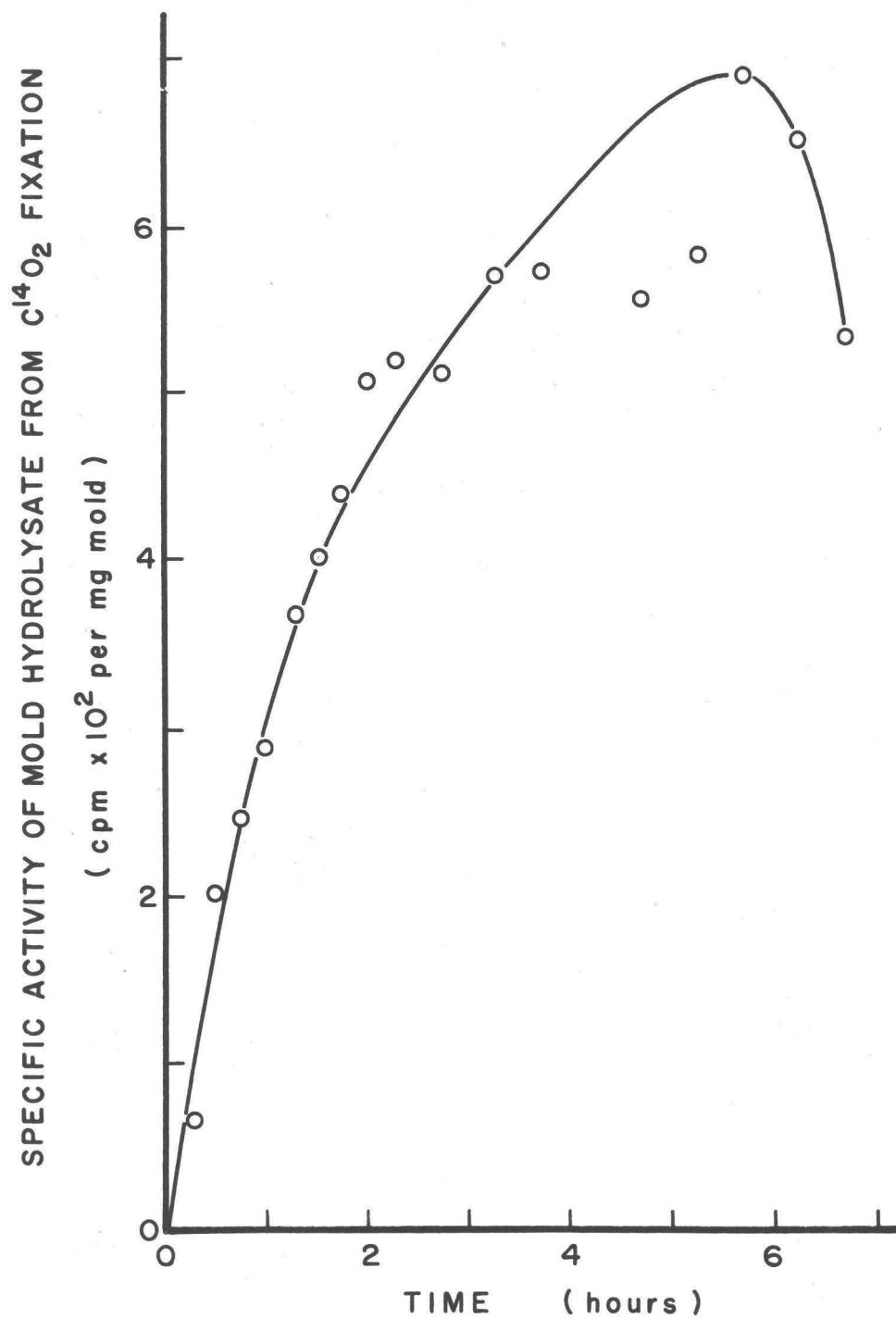




FIGURE 20

One dimensional radioautogram of mold cells from $C^{14}O_2$ fixation experiments. The three pairs are amino acids obtained from the kinetic studies of $C^{14}O_2$ fixation. The first of each pair of chromatograms (starting from the left side) represent labeling in amino acids after $5 \frac{3}{4}$ hours of exposure to $C^{14}O_2$. The last radioautogram is labeling obtained from the high level $C^{14}O_2$ fixation experiment. The first pair of radioautograms (phenol used as solvent) R = aspartic acid, N = glutamic acid. The second pair, (BAW used as solvent) R = aspartic acid and serine, E = glycine and threonine, N = glutamic, A = alanine, P = alanine, P = proline, Z = phenylalanine and isoleucine, S = leucine. The third pair (secondary butanol-ammonia) R = aspartic, glutamic, cystine, N = arginine, E = glycine, serine, A = threonine, P = proline. In the last strip, R = aspartic, glutamic and cystine, K = lysine, N = arginine, E = glycine, serine, A = threonine, P = proline, B = tyrosine, D = valine, O = methionine, X = ninhydrin negative spot, Z = isoleucine, S = phenyl alanine and leucine.

Isolation and degradation of aspartic and glutamic acid. One and seven hundredths gram of the dried mold was hydrolyzed with 6N HCl in Carius tube by autoclaving the mixture at 15 p.s.i. for six hours. The hydrolysate, filtered free of humin, was repeatedly evaporated to dryness to remove the HCl. The protein hydrolysate was then taken up in water and extracted exhaustively with ether in a liquid-liquid extractor to remove fatty substances.

Following the method of C. H. W. Hirs, et al (22), the aspartic and glutamic acids were separated by the passage of the protein hydrolysate through a Dowex 1-X8 column. 34.2 mg of aspartic acid and 32.2 mg of glutamic acid were isolated. They were found to be chromatographically pure.

Appropriate amounts of the isolated pure amino acids were diluted with the non-labeled corresponding amino acid, and the mixture obtained was subjected to degradative studies.

The total activity of the amino acids were determined by the wet-combustion method of Van Slyke using the combustion mixture cited by Calvin (6, p.93) but omitting the KIO_3 .

The ninhydrin method of Frantz (16, pp.260-1) modified by Wang (49) was used to determine the activity

of both carboxyl groups of aspartic acid and the α -carboxyl group of glutamic acid.

The α -carboxyl group of aspartic acid was obtained by nitrous acid ceamination of aspartic acid to malic acid (10,p.24). The malic acid thus obtained was degraded by the method of letter (44,p.851) to yield the α -carboxyl group as CO, which was in turn oxidized to CO₂ by passing through a hot tube containing coarse copper oxide.

The C¹⁴ labeling pattern of these two amino acids are given in Table VI.

III. Utilization of carboxyl-labeled acetate by Penicillium digitatum.

Five hundred milligrams of a 3 1/2-day-old mold cells grown in the usual manner were harvested, washed and added to 250 ml of a carbon source-free medium contained in the three-necked flask shown in Figure 6. 20.4 mg (0.25 m moles) of carboxyl-labeled acetate, having a total activity of 26.5×10^6 cpm was added to this suspension and sterile CO₂-free air passed through this mixture.

At definite time intervals 5 ml samples were taken out of the reaction flask and the activity in the medium

TABLE VI

ISOTOPIC DISTRIBUTION PATTERNS OF ASPARTIC
AND GLUTAMIC ACID FROM MOLD GROWN
ON NON-LABELED GLUCOSE AND $C^{14}O_2$.

<u>Amino acid group</u>	<u>Radioactivity</u> (cpm $\times 10^{-6}$ /m mole amino acid)	<u>Per cent</u> <u>of total</u>
Aspartic acid		100
COOH -----	1.13	23.8
HCNH ₂ } -----	0.85	18.0
CH ₂ }		
COOH -----	2.76	58.2
Glutamic acid		100
COOH -----	2.86	84.9
HCNH ₂ } -----		
CH ₂ }		
CH ₂ } -----	0.51	15.1
COOH }		

and the cells were determined in the usual manner. The activity of the respiratory CO_2 was determined by direct plating and counting of 0.05 ml aliquots from the CO_2 trap. These results are shown in Table VII.

At the end of four hours, the experiment was terminated by the addition of concentrated HCl to the mold suspension. The rest of the mold was separated from the medium by centrifugation, washed with water and alcohol and allowed to dry over P_2O_5 in a vacuum dessicator.

Isolation and degradation of aspartic and glutamic acid. Three hundred and fifty five milligrams of the dried mold was hydrolyzed with 6 N HCl in a Carius tube by autoclaving the mixture at 15 p.s.i. for nine hours. The hydrolysate, filtered free of humin, was repeatedly evaporated to dryness to remove HCl. The protein hydrolysate was then dissolved in water and extracted exhaustively with ether in a liquid-liquid extractor. The distribution of activity in the various parts of the mold is given in Table VIII.

The aspartic and glutamic acids were separated and removed from the hydrolysate in the same manner as has been used in previous work by Wang, et al (48). The isolated amino acids were diluted with appropriate amounts of the corresponding non-labeled amino acids, and

TABLE VII

CARBOXYL LABELED ACETATE
UTILIZATION BY PENICILLIUM DIGITATUM

<u>Time</u> (hours)	<u>Medium</u> (cpm $\times 10^{-6}$)	<u>Total respiratory</u> <u>CO₂ activity</u> (cpm $\times 10^{-7}$)	<u>Activity per</u> <u>mg cell</u> (cpm $\times 10^{-3}$)
0	16.0		
0.5	15.5	0.18	0.73
1.0	11.4	0.41	1.46
1.5	6.81	0.66	2.79
2.5	3.04	1.13	3.36
3		1.23	
3.5	1.28	1.36	3.46
4	0.89	1.38	1.77

20.4 mg of carboxyl-labeled acetate having a total activity of 26.5×10^6 cpm were added to 500 mg mold.

TABLE VIII

DISTRIBUTION OF C^{14} ACTIVITY
IN PENICILLIUM DIGITATUM GROWN ON
CARBOXYL-LABELED ACETATE.

<u>Fraction</u>	<u>Weight of fraction (mg)</u>	<u>Total activity (cpm$\times 10^{-5}$)</u>	<u>Fraction of total activity</u>
Whole mold	355	19.4	100
Humin	47.3	0.87	4.5
Fat (total)	31.4	1.24	6.4

the mixture obtained was subjected to degradation studies using procedures given previously under the CO_2 fixation experiment. The results for the isotopic distribution patterns of aspartic and glutamic acid are given in Table IX.

IV. High level C^{14}O_2 fixation.

One hundred milligrams of 3 1/2-day-old mold cells were suspended in 25 ml of carbon source-free medium contained in a 250 ml Erlenmeyer flask. 18 mg of glucose were added to this suspension, and the Erlenmeyer was closed. Through a vaccine port, concentrated H_2SO_4 was added into a suspended vial which contained 2.79 mg of BaCO_3 having an activity of $100\mu\text{C}$. The Erlenmeyer was then placed on a shaker at room temperature, and the suspension was agitated for four hours. At the end of this time alkali was introduced into the vial to absorb the C^{14}O_2 . The system was opened and the mold separated from the medium by centrifugation. The mold was washed twice with distilled water and dried over P_2O_5 in a vacuum dessicator. After hydrolysis with 6N HCl in a Carius tube, the mold hydrolysate was dried over P_2O_5 and KOH.

Single-dimensional paper chromatograms were made of the hydrolysate using 300 γ equivalent of the original mold for spotting, using secondary butanol-ammonia system.

A radioautogram of this protein hydrolysate is found in Figure 20.

TABLE IX

ISOTOPIC DISTRIBUTION PATTERN OF ASPARTIC ACID
AND GLUTAMIC ACID FROM MOLD GROWN ON
CARBOXYL LABELED ACETATE.

<u>Amino acid group</u>	<u>Radioactivity</u> (cpm $\times 10^{-3}$ /m mol of diluted amino acid)	<u>Per cent</u> <u>of total</u>
Aspartic acid		100
COOH -----	0.87	24.8
HCNH ₂ } -----	1.25	35.6
CH ₂ } -----		
COOH -----	1.39	39.6
Glutamic acid		100
COOH -----	0.45	32.6
HCNH ₂ } -----		
CH ₂ } -----	0.93	67.4
CH ₂ } -----		
COOH -----		

DISCUSSION

The occurrence of alternate catabolic pathways of glucose in *Penicillium digitatum*. The finding of higher specific activity of respiratory $C^{14}O_2$ from glucose-2- C^{14} than that from glucose-6- C^{14} revealed the occurrence of the Emden-Meyerhof (EM) pathway in these organisms. Since an exclusive pentose cyclic process will yield methyl labeled acetate from either glucose-2- C^{14} or glucose-6- C^{14} and hence the same specific activity of respiratory $C^{14}O_2$ in the terminal respiration processes (38). In the Emden-Meyerhof scheme in combination with TCA cyclic activity one would find a higher specific activity in the respiratory $C^{14}O_2$ from glucose-2- C^{14} by way of carboxyl labeled acetate rather than glucose-6- C^{14} , which would give rise to methyl labeled acetate in the EM pathway.

The fact that the specific activity of the respiratory $C^{14}O_2$ from glucose-1- C^{14} is considerably higher than that from either glucose-2- C^{14} or glucose-6- C^{14} is indicative of the extensive operation of the pentose cycle in this organism. That the Entner-Doudoroff pathway (14) is probably not the cause of higher specific activity of $C^{14}O_2$ from glucose-1- C^{14} is evident by the significantly less incorporation of glucose-1- C^{14} activity into alanine than from either of the other two glucose species.

Since the Entner-Doudoroff scheme will give rise to pyruvate-1-C¹⁴, a known precursor of alanine, which should have a similar labeling level as compared to pyruvate-3-C¹⁴ derived from glucose-6-C¹⁴, is also indicative of the significant extent of the operation of pentose cycle, in which case glucose-1-C¹⁴ activity will be lost in the preferential decarboxylation reaction with non-labeled C-4, 5, 6 furnishing the C₃ intermediates in the alanine biosynthesis. This is in direct contrast to the equal contribution of activity in alanine from either glucose-1-C¹⁴ or glucose-6-C¹⁴ via the EM scheme.

In kinetic studies on glucose-1-C¹⁴ and glucose-6-C¹⁴ utilization (Figure 8) the very rapid rise of CO₂ activity of the first peak in the glucose-1-C¹⁴ curve and its predominance over a similar peak in glucose-6-C¹⁴ could then represent the occurrence of a more rapid decarboxylation reactions via the pentose cycle. The second peak in the CO₂ activity of both species of glucose is possibly due to the operation of the EM scheme.

A. The effect of age of mold, nature of medium, ethylene and indoleacetic acid on the distribution of catabolic pathways of glucose.

1. Effect of aging. The effect of aging through 3, 3 1/2, 4 and 5 1/2-day-old mold cells were studied.

The ratio of respiratory CO_2 from glucose-1- C^{14} to the respiratory CO_2 from glucose-6- C^{14} (referred to from now on as $\frac{\text{Glucose-1}}{\text{Glucose-6}}$) are 3.42, 2.70, 1.98 and 1.89 respectively (Tables I and II).

The logarithmic phase of cell growth and division is 72 hours, according to the studies of Wooster and Cheldelin (53). The experimental evidence presented points strongly to the pentose cycle as being directly related to active biosynthesis. This would reflect the need of active pentose supply for the formation of RNA units, or related energy requirements in active growth.

2. Nature of medium. The $\frac{\text{Glucose-1}}{\text{Glucose-6}}$ ratio of 4-day-old cells were practically identical in growing medium and buffer (1.98 and 2.00 respectively, Table I); this is also true in so far as the total activity recovered as C^{14}O_2 is concerned.

Similarly, the $\frac{\text{Glucose-1}}{\text{Glucose-6}}$ ratio of 1 1/2-day-old cells in growing medium and buffer were again very much the same (2.16 and 2.12 respectively, Table II). However, in this case almost twice as much CO_2 was formed from glucose in the buffer medium as from the growing medium. The comparison of specific activity and total activity recovered as C^{14}O_2 are summarized as the following:

<u>Medium</u>	<u>Substrate</u>	Total Respiratory $C^{14}O_2$ Activity (cpm)	<u>Glucose-1</u> <u>Glucose-6</u>
growing	glucose-1- C^{14}	4250	2.18
growing	glucose-6- C^{14}	1950	
buffer	glucose-1- C^{14}	6480	2.38
buffer	glucose-6- C^{14}	2720	

Fifty milligrams of $BaCO_3$ were obtained per cup from the glucoses in growing medium, and 92 mg $BaCO_3$ per cup was obtained from the glucoses in buffer medium.

These results thus show that with 1 1/2-day-old cells the glucose in phosphate buffer medium is primarily used for respiratory activity, hence a higher recovery in CO_2 and its activity. With carbon source-free medium, on the other hand, a considerable amount of glucose has been incorporated into cell constituents.

When the exponential phase of growth is over, the less active biosynthetic functions tend to reduce the difference between the growing medium and buffer. Thus the ratio of $\frac{\text{Glucose-1}}{\text{Glucose-6}}$ for older cells come out very much the same in growing and buffer medium.

3. Indoleacetic acid. Indoleacetic acid is known to stimulate the Emden-Meyerhof scheme in some micro-organisms . However, no evidence has been found in P. digitatum (Table III) to indicate this nature of

stimulation. At a level of 100 γ IAA per 100 ml no stimulatory effect has been demonstrated as the ratio of $\frac{\text{Glucose-1}}{\text{Glucose-6}}$ in the control (2.70) was not much different from the ratio (2.62) of the cups which contained IAA.

4. Ethylene. Since ethylene is known to be produced by P. digitatum (3,30), the effect of the mold metabolizing preferentially labeled glucose under ethylene enriched atmosphere was studied. The results in Table II show that the specific activity of CO_2 from glucose-6- C^{14} is not too different in the control (0.59×10^4 cpm/m mol BaCO_3) and the ethylene set (0.61×10^4 cpm/m mol BaCO_3). However, a significant decrease in the activity of CO_2 was found with glucose-1- C^{14} in the ethylene set (1.37×10^4 cpm/m mol BaCO_3) over the control (1.59×10^4 cpm/m mol BaCO_3). The resulting lower $\frac{\text{Glucose-1}}{\text{Glucose-6}}$ ratio in ethylene atmosphere may then indicate the effect of ethylene in suppressing the pentose cycle but displaying no effect on the Emden-Meyerhof pathway.

B. Kinetic studies of glucose catabolism.

Figure 7 shows the rate of utilization of glucose. This rate is calculated out to be 0.071 mg glucose used by 1 mg mold per hour.

The over-all specific activity of BaCO_3 from

glucose-1- C^{14} was 1.32×10^2 cpm/mg $BaCO_3$, and of glucose-6- C^{14} was 0.635×10^2 cpm/mg $BaCO_3$. When these values are compared with 3.43×10^2 cpm/mg $BaCO_3$, the equivalent specific activity of the glucoses used, a high endogenous value of the mold is revealed.

Thirty four and four tenths per cent and 13.6 per cent of the respective glucose-1- C^{14} and glucose-6- C^{14} activity were recovered as carbon dioxide. These values again point to the extensive operation of the preferential loss of C_1 over C_6 carbon.

The over-all recovered activity ratio of $\frac{\text{Glucose-1}}{\text{Glucose-6}}$ recovered as CO_2 is 2.53, which compares favorably with the value obtained in Warburg studies (2.70) for molds of the same age.

It is interesting to note that the time-course curve of $C^{14}O_2$ (Figure 8) specific activity from glucose-6- C^{14} displayed an early peak. If one assumed that the second peak which appeared at 6 hours as due to the turnover of C-6 of glucose via the EM and TCA scheme, then the first peak might be the result of a possible relocation of C-6 to C-1 position of glucose and the appearance of $C^{14}O_2$ activity via the pentose cycle. A simultaneous peak from glucose-1- C^{14} occurs at this stage also.

Similar observations have been made in Baker's yeast

The incorporation of C^{14} from various differentially

labeled glucose into cellular constituents also provides an interesting lead in the utilization of glucose breakdown products in the mold. Thus, the C^{14} activity in cells grown on glucose-2- C^{14} is higher than that grown on glucose-6- C^{14} .

The preferential incorporation of C-2 of glucose into cellular constituents thus points to the non-equivalence of acetate (or related C_2 unit) derived from glucose carbon atoms 1, 2, 3 and 4, 5, 6 (2). Inasmuch as incorporation of the respective carbon atoms of glucose into cellular compounds via EM and TCA cycle should have resulted in a lesser incorporation from glucose-2- C^{14} (Diagram I) as compared to that from glucose-6- C^{14} ; also with extensive pentose cyclic process and accepting the observation by Sprinson, et al (38), that pentose-1- C^{14} sugars give rise to C_2 and C_3 units with the majority of the activity residing in the methyl group of the C_2 moiety. The analogy could be carried to 2- C^{14} which could conceivably give the same pattern as pentose-1- C^{14} , after having been first converted to a pentose via the pentose cycle. From either pathway, one would then expect either equal incorporation from C-2 or C-6 of glucose or a higher incorporation in favor of C-6 glucose.

The observed preferential utilization of C-2 of glucose in cellular biosynthesis as evidenced by the

significantly higher level of amino acid labeling (Figures 2, 3, 4) in the glucose-2-C¹⁴ experiment, points to a unique entry of the C₂ unit into the biosynthetic mechanism of this organism. The fate of the C₂ unit in this respect will be discussed later under acetate utilization.

C. CH₃C¹⁴OOH and C¹⁴H₃COOH utilization by mold.

Although acetate was found not to be a good carbon source for the growth of this organism (53), preliminary experiments have indicated a considerable amount of radioactivity from C¹⁴ labeled acetates was incorporated into cellular constituents, particularly amino acids. It was thought that this type of studies would have provided a way of tracing the fate of a typical C₂ unit in active biosynthesis.

Figure 11 shows the rate of utilization of carboxyl and methyl labeled acetate. Accumulation of labeled compounds in the medium from both acetate experiments were observed prior to complete substrate utilization. Paper chromatography of an aliquot of the medium identified the labeled compound to be amino acids, consisting mainly of aspartic and glutamic acid. A similar type of observation has been reported in Streptomyces griseus (18).

From an equal amount and activity of specifically

labeled acetates utilized by mold, 85.3 per cent of the acetate-1- C^{14} activity was recovered as $C^{14}O_2$; whereas only 44.4 per cent of the acetate-2- C^{14} activity was recovered as $C^{14}O_2$. This pointed favorably to the evidence of the operation of TCA cycle.

Cells, on the other hand, incorporated a higher amount of activity from the methyl labeled acetate than from carboxyl labeled acetate. This was again in line with TCA cycle operation, which calls for conservation of the methyl of acetate over the carboxyl group of acetate in the cyclic processes.

It is interesting to note that glutamic and aspartic acids are among the first amino acids to be labeled (Figures 12-18). An unknown X which gave a positive ninhydrin test, but whose R_f value did not coincide with any known amino acid, was also labeled at the earlier stage. This unidentified amino acid soon disappeared. No attempt was made in identifying the nature of this compound.

The level of labeling in these initial components were lowered in the next hour or so; activity began to spread into the other amino acids at the second hour.

The high incorporation of acetate-2- C^{14} into glycine and serine is in line with the known mechanism of serine biosynthesis, since the β -carbon of serine is

established as derived from C-2 of acetate (52). Similar labeling in the case of alanine is indicative of the operation of TCA cyclic process, since the C-2 carbon of acetate tends to be conserved in the recycling process.

In order to elucidate the mechanism of acetate utilization in the organism, a larger amount of mold cells were grown so as to isolate key amino acids for degradation studies. The radioactive substrate used in this case was carboxyl labeled acetate.

The isotopic distribution pattern obtained (Table IX) reveals the surprisingly high incorporation of acetate carboxyl activity into the middle carbon atoms of aspartic acid. This situation provides evidence of the occurrence of double fixation of CO_2 by acetate (or a related C_2 unit) as reported for R.rubrum by Cutinelli, et al (9), and Tomlinson for C.kluyveri (43). The higher activity in C-4 of aspartic acid probably reflects the dilution of the C_3 unit by pyruvic acid derived from endogeneous carbohydrates.

In the case of glutamic acid, 32.6 per cent of its activity was found to be located at the α -carboxyl carbon atom which is probably derived directly from the acetate carboxyl carbon by way of the conventional TCA cyclic process. As indicated in Diagram II, one would expect considerably higher activity in C-2, 3, 4, 5 of glutamic

acid. The observed low value, in view of the expected labeling in carbon atom 2, 3 and 5, is probably the result of limited recyrcing processes. This is also supported by observed glutamic acid excretion in the medium.

D. The incorporation of $C^{14}O_2$ into amino acids of *P. digitatum*.

The observed unique pathway of C_2 (as acetate) entry into the TCA cycle as discussed in the foregoing section led to the study of the role played by CO_2 in growing cells of mold. Unlabeled glucose was used as co-substrate to ensure a normal growth.

In the time-course study of $C^{14}O_2$ fixation, one finds the C^{14} activity was readily incorporated (1/2 hour) into aspartic and glutamic acid. This is expected from the scheme given in Diagram II.

Labeling subsequently occurred in the remaining amino acids except alanine, valine, leucine and phenylalanine. This was demonstrated in a high-level experiment (Figure 20).

The early labeling in glycine and serine from $C^{14}O_2$ is in good agreement with the observation made in $C^{14}O_2$ fixation experiments using *Saccharomyces cerevisiae* (10) and *Streptomyces griseus* (5) and can be readily explained by the biosynthetic scheme for these amino acids as

proposed by Wang, et al (51).

The heavy labeling in arginine is in line with the biosynthetic scheme proposed by Strassman and Weinhouse (39).

The incorporation of activity from $C^{14}O_2$ into threonine, tyrosine and isoleucine is again in good agreement with their respective biosynthetic mechanisms, which all involved oxalacetate as a key intermediate (50,41, 40).

The labeling in proline could be understood in view of its direct relationship to glutamic acid (28, pp.277-289).

Failure of C^{14} activity incorporation into alanine and valine is not surprising considering the fact that the bulk of the C_3 unit (pyruvic acid) was derived from the non-labeled glucose, which was used as co-substrate in this experiment. The much diluted activity of the C_3 unit is evidenced by the non labeling of alanine, which is related to pyruvic acid by transamination, and valine, which is synthesized from two pyruvic acid units according to Strassman's scheme (40).

It is interesting to note that no $C^{14}O_2$ activity was incorporated into the phenylalanine skeleton in contrast to the relatively higher labeling in tyrosine. This might render additional support to the suggestion

made by Thomas, et al (42), that these two amino acids may not share the same biosynthetic pathway.

Leucine is also not labeled in the present experiment; this is best explained by the biosynthetic scheme proposed by Reed, et al (34), since the only labeled carbon in the key intermediate, α -carboxyl carbon of α -ketoglutarate, would be expected to be lost in the decarboxylation processes postulated.

The bulk of the cell crop in the $C^{14}O_2$ fixation experiment was recovered and hydrolyzed for the isolation and degradation studies of some key compounds. The distribution of $C^{14}O_2$ activity in the various fractions of cellular constituents is given in Diagram I. The major portion of the activity was found in the amino acids fraction, particularly aspartic and glutamic acids. The small amount of activity detected in the fat fraction could imply that some $C^{14}O_2$ activity was incorporated into the acetate unit via some unknown pathway. That this might be an important pathway is evidenced by the significant amount of $C^{14}O_2$ activity observed in the middle carbon atoms of aspartic acid (Table VI).

The isotopic distribution patterns given in Table VI for aspartic acid and glutamic acid are in good agreement with the scheme given in Diagram II. Thus, one would expect heavier labeling in the β -COOH carbon of aspartic

acid (43), since the experiment was carried out in the presence of non-labeled glucose and hence unlabeled pyruvic acid was also fed into the C_3 pool resulting in lower specific activity of the α -COOH carbon of aspartic acid. In the case of glutamic acid, as would be expected, the labeling is practically confined to the α -COOH carbon (10). The minor amount of $C^{14}O_2$ activity detected in C-2, 3, 4, 5 of glutamic acid again suggests the existence of an unknown pathway leading to the incorporation of C^{14} activity from $C^{14}O_2$ into the acetate molecule.

E. Isotopic competition with carboxyl and methyl labeled acetate.

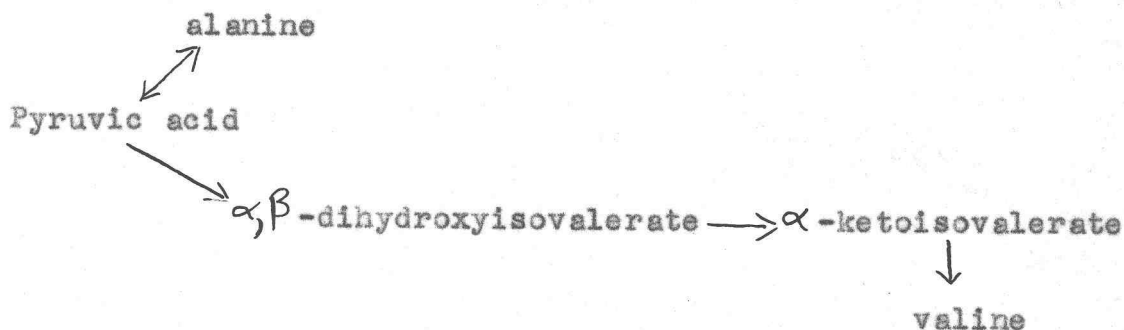
The results of these studies were complicated by the fact that with the addition of the unlabeled amino acids to the medium, a significantly greater utilization of the labeled acetates were obtained (only exception to this was glycine). Nevertheless, some definite observations were made.

When glutamic acid was present as a competitor, the acetate incorporation into the rest of the amino acids were stimulated; nevertheless, a significant decrease in labeling was observed in glutamic acid. Similar decrease in labeling was observed in proline and arginine. This compares favorably with the similar work reported by

Abelson (1) for E.coli. The known interrelationship between above mentioned amino acids is well established (16,39).

With aspartic acid as competitor the expected decrease of acetate labeling in this amino acid was also observed.

Using alanine as a competitor a very decisive block of incorporation of C^{14} into the alanine moiety was observed. In both acetate experiments a block of activity incorporation from the two labeled acetates into the valine structure was observed. Similar results were obtained by Abelson with E.coli (1). This result was in support with the scheme proposed by Strassman, et al (40), that pyruvic acid is an intermediate in valine synthesis.



Glycine, unlike the above discussed three amino acids, did not stimulate the incorporation of labeled acetate into the other amino acids; however, a significant block in the activity of glycine was observed.

TABLE I

THE EFFECTS OF AGING AND NATURE OF MEDIUM ON THE OXIDATION
OF DIFFERENTIALLY LABELED GLUCOSE² BY CELLS OF PENICILLIUM DIGITATUM

	Warburg Cup No.	Mold Age	Medium	Glucose Label	Activity Left in Medium (cpmX10 ⁻⁴)	Activity per mg Cell (cpm)	Activity per m mol BaCO ₃ (cpmX10 ⁻⁴)	Respiratory CO ₂ Ratio
Large Cups	1	4 days	Carbon source-free	-1-C ¹⁴	1.46	29.8	1.73	1.98
	2	4 days	Carbon source-free	-6-C ¹⁴	1.51	69.9	0.87	
	3	3 days	Carbon source-free	-1-C ¹⁴	1.63	22.4	2.00	3.42
	4	3 days	Carbon source-free	-6-C ¹⁴	1.62	54.3	0.59	
	5	4 days	Phosphate buffer	-1-C ¹⁴	1.10	29.4	2.15	2.00
	6	4 days	Phosphate buffer	-6-C ¹⁴	1.43	48.5	1.07	
Small Cups	1	4 days	Carbon source-free	-1-C ¹⁴	5.05	0.80X10 ⁴	8.21	
	2	4 days	Carbon source-free	-6-C ¹⁴	3.73	1.04X10 ⁴	4.75	
	3	4 days	Carbon source-free	-2-C ¹⁴	5.91	1.51X10 ⁴	5.79	

²46.2 mg of differentially labeled glucose having a specific activity of 1.23×10^5 cpm per millimole were added to 76 mg of cells in each of the large cups. 3.40 mg of differentially labeled glucose with a specific activity of 1.11×10^7 cpm per millimole were added to 41 mg in each of the small cups.

TABLE II

THE EFFECTS OF AGING, NATURE OF MEDIUM, ETHYLENE AND INDOLE ACETIC ACID
ON THE OXIDATION OF DIFFERENTIALLY LABELED GLUCOSE³ BY CELLS OF PENICILLIUM DIGITATUM

Warburg Cup No.	Mold Age	Medium	Glucose Label	Activity Left in Medium (cpmX10 ⁻³)	Activity per mg Cell (cpm)	Activity per m mol BaCO ₃ (cpmX10 ⁻⁴)	Respiratory CO ₂ Ratio
1	5½ days	Carbon source-free	-1-C ¹⁴	11.5	9.3	1.94	1.89
2	5½ days	Carbon source-free	-6-C ¹⁴	9.61	15.1	1.03	
3	3½ days	Carbon source-free	-1-C ¹⁴	4.76	16.1	1.59	2.70
4	3½ days	Carbon source-free	-6-C ¹⁴	5.83	31.2	0.59	
5	1½ days	Carbon source-free	-1-C ¹⁴	9.10	24.7	1.70	2.16
6	1½ days	Carbon source-free	-6-C ¹⁴	7.06	46.7	0.79	
7	1½ days	Phosphate buffer	-1-C ¹⁴	7.69	27.5	1.30	2.12
8	1½ days	Phosphate buffer	-6-C ¹⁴	7.96	49.3	0.61	
9	3½ days	Carbon source-free ethylene ⁴	-1-C ¹⁴	5.99	14.2	1.37	2.26
10	3½ days	Carbon source-free ethylene	-6-C ¹⁴	8.09	35.8	0.61	
11	3½ days	Carbon source-free IAA ⁵	-1-C ¹⁴	5.23	18.9	1.63	2.62
12	3½ days	Carbon source-free IAA	-6-C ¹⁴	6.02	40.8	0.62	

³46.2 mg of differentially labeled glucose having a specific activity of 1.08×10^5 cpm per m mol glucose were added to each of the above cups. Warburg cup numbers 1-4 and 9-12 each had 94 mg of cells, while 5-8 had only 29 mg of cells.

⁴The cups indicated were flushed with an atmosphere of 1 part ethylene per 1000 O₂ before equilibration.

⁵The cups indicated each contained 28.3 of indole acetic acid (IAA).

TABLE III

INCORPORATION OF $\text{CH}_3\text{C}^{14}\text{OOH}^6$ AND $\text{C}^{14}\text{H}_3\text{COOH}^6$ INTO P.DIGITATUM
 PROTEINS IN PRESENCE OF C^{12} -AMINO ACIDS IN MEDIUM

Warburg Cup No.	Acetate Label	Amino Acid Competitor	Activity Left in Medium (cpmX10 ⁻⁴)	Activity per mg Cell (cpmX10 ⁻⁴)	Activity per m mol BaCO ₃ (cpmX10 ⁻⁶)	Respiratory CO ₂ Ratio
1	-C ¹⁴ OOH	Control	6.65	0.57	6.28	1.40
2	C ¹⁴ H ₃ -	Control	12.5	1.42	4.49	
3	-C ¹⁴ OOH	Glutamic	6.24	0.68	4.61	1.98
4	C ¹⁴ H ₃ -	Glutamic	16.0	1.72	2.32	
5	-C ¹⁴ OOH	Aspartic	5.09	0.73	5.06	1.74
6	C ¹⁴ H ₃ -	Aspartic	14.8	1.64	2.92	
7	-C ¹⁴ OOH	Alanine	6.60	0.86	4.93	1.87
8	C ¹⁴ H ₃ -	Alanine	12.8	1.79	2.64	
9	-C ¹⁴ OOH	Glycine	4.13	0.55	5.52	2.31
10	C ¹⁴ H ₃ -	Glycine	12.6	1.43	2.38	

⁶Each cup contained 15 mg of 3 1/2-day-old cells suspended in carbon source-free medium. 0.817 mg of differentially labeled acetate having a specific activity of 6.43×10^7 cpm per m mol was added to each cup.

TABLE IV

KINETIC STUDY OF GLUCOSE -1-C¹⁴ AND GLUCOSE -6-C¹⁴ UTILIZATION BY P.DIGITATUM

Time (Hours)	Medium		Respiratory CO ₂			
			Activity per m mol BaCO ₃		Cumulative Total Activity	
	Glucose-1-C ¹⁴ (cpmX10 ⁻⁵)	Glucose-6-C ¹⁴ (cpmX10 ⁻⁵)	Glucose-1-C ¹⁴ (cpmX10 ⁻⁴)	Glucose-6-C ¹⁴ (cpmX10 ⁻⁴)	Glucose-1-C ¹⁴ (cpmX10 ⁻³)	Glucose-6-C ¹⁴ (cpmX10 ⁻³)
0	2.10	2.12				
0.5	1.92	2.02	0.58	0.10	2.4	0.4
1	1.78	1.97	2.30	0.54	5.9	1.0
1.5	1.52	1.85	3.19	1.09	12.0	2.8
2	1.36	1.53	3.64	1.59	21.0	5.9
2.5	1.37	1.60	3.86	1.58	30.0	8.9
3	1.14	1.36	2.88	1.76	38.3	13.0
3.5	1.07	1.23	1.96	1.54	43.6	15.5
4	0.81	1.01	2.34	1.10	50.1	18.1
5	0.50	0.76	3.05	1.18	65.9	22.3
5.5	0.24	0.60	3.11	1.44	72.9	25.0
6	0.17	0.41	3.37	1.61	82.6	28.3
6.5	0.01	0.24	2.90	1.47	89.7	31.6
7	0.03	0.22	2.27	1.58	93.6	33.9
7.5	0.05	0.13	1.63	1.51	96.2	35.8
8	0.01	0.14	1.35	1.84	98.1	38.3

⁷125 mg of Glucose -1-C¹⁴ with an activity of 4.11 X 10⁵ cpm per m mol and a similar weight and activity of Glucose -6-C¹⁴ were inserted in each flask.

TABLE V

KINETIC STUDY OF $\text{CH}_3\text{C}^{14}\text{OOH}^8$ AND $\text{C}^{14}\text{H}_3\text{COOH}^8$ UTILIZATION BY P. DIGITATUM

Time (Hours)	$\text{CH}_3\text{C}^{14}\text{OOH}$ (cpmX10 ⁻⁶)	$\text{C}^{14}\text{H}_3\text{COOH}$ (cpmX10 ⁻⁶)	Respiratory CO ₂				Cells	
			Activity per m mol BaCO ₃		Total Cumulative Activity		$\text{CH}_3\text{C}^{14}\text{OOH}$ (cpm per mgX10 ⁻³)	$\text{C}^{14}\text{H}_3\text{COOH}$ (cpm per mgX10 ⁻³)
			$\text{CH}_3\text{C}^{14}\text{OOH}$ (cpmX10 ⁻⁶)	$\text{C}^{14}\text{H}_3\text{COOH}$ (cpmX10 ⁻⁶)	$\text{CH}_3\text{C}^{14}\text{OOH}$ (cpmX10 ⁻⁶)	$\text{C}^{14}\text{H}_3\text{COOH}$ (cpmX10 ⁻⁶)		
0	16.1	17.1					1.68	1.97
0.25							0.16	0.27
0.50			6.33	2.42	0.70	0.30	0.32	0.45
1.0	8.56	10.7	14.5	8.16	1.41	0.56	0.82	1.39
1.5	5.28	5.52					1.21	2.62
2.0	4.97	6.67	24.0		3.37		2.37	4.65
2.5	8.62	8.01					4.73	9.22
3.0	8.87	8.56	32.1	15.6	6.36	2.04	6.14	8.91
3.5	5.62	10.91					4.14	10.0
4.5	2.43	6.39	60.7	24.2	15.9	5.64	15.3	29.0
5.0	1.22	2.87					19.3	43.9
5.5	0.80	2.04	28.8	29.0	18.5	8.34	13.9	34.4
6.0	0.80	1.23					14.9	37.5
6.5	0.72	1.47	7.23	15.7	19.0	9.59	15.7	34.5
7.0	0.59	1.62	3.59	7.29	19.1	9.95	17.5	36.4

⁸12.8 mg of carboxyl labeled acetate with an activity of 1.44×10^8 cpm per m mol and a similar weight and activity of methyl labeled acetate were inserted in each flask.

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SUMMARY

A study of carbohydrate metabolism in Penicillium digitatum was carried out by using C^{14} labeled carbon sources such as: glucose-1- C^{14} , glucose-2- C^{14} , glucose-6- C^{14} , $CH_3C^{14}OONa$, $C^{14}H_3COONa$ and $C^{14}O_2$.

1. Evidences supporting the occurrence of the Emden-Meyerhof scheme and the pentose cycle for glucose utilization were presented.
2. The occurrence of the tricarboxylic acid cycle with limited cycling was indicated.
3. Double fixation of CO_2 by C_2 units has been detected as one of the major biosynthetic pathways in C_4 acid formation.
4. The incorporation of C^{14} activity from $CH_3C^{14}OONa$ and $C^{14}O_2$ into amino acids in this organism was studied. This renders support to various speculative biosynthetic pathways proposed for amino acids in the literature.

In conclusion, it seems that glucose was utilized by P. digitatum via mixed catabolic pathways; namely, Emden-Meyerhof scheme and pentose cycle. The C_2 and C_3 units derived from these schemes entered subsequently conventional tricarboxylic acid cyclic processes.

The synthesis of C_4 acids by way of single CO_2 fixation ($C_3 + C_1$) and double CO_2 fixation ($C_1 + C_2 + C_1$) is also demonstrated.

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