THE APPLICATION OF RADIOACTIVE TRACER TECHNIQUES TO THE ELUCIDATION OF THE INTERRELATIONS BETWEEN GLUCOSE METABOLISM AND AMINO ACID BIOSYNTHESIS IN *PENICILLIUM DIGITATUM* SACCARDO

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TO MY WIFE

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INTRODUCTION

Carbohydrates serve as a major source of energy and building stones for the growth of living cells. A knowledge of the transformations which carbohydrates must undergo to furnish the needs of the living cell adds to our understanding of the chemical basis of biological processes.

The pathways of carbohydrate metabolism in micro-organisms have been shown to be very similar to, however more diversified than, pathways in other living organisms. Up to date reviews on this subject have been presented in several articles appearing recently (4,36,47,79). At the present time, the complete enzymatic complement for carbohydrate metabolism by any of the known pathways has not been demonstrated for a single biological species.

In regards to glucose metabolism of the genus, *Penicillium*, fragmentary evidence exists which indicates that the Embden-Meyerhof-Parnas pathway is utilized for energy production and synthetic purposes. In support of this view, aldolase, triosephosphate isomerase and triose-phosphate dehydrogenase are present in the mycelium of a strain of *Penicillium notatum* (50). Kita and Peterson

(45) have isolated adenosine triphosphate, adenylic acid and mannose-1-phosphate from the mycelium of Penicillium chrysogenum strain Q 176. From P. chrysogenum strain NRRL 1951-B25, Casida and Knight (15) have prepared a soluble cytochrome-linked lactic dehydrogenase. A direct oxidative mechanism for glucose utilization is accompanying classical glycolysis in P. chrysogenum strain NRRL 1951-B25 as evidenced by the work of DeFriebre and Knight (19). Working with minced (sic) young mycelial cells, one mole of oxygen was consumed per mole of C^{14} glucose utilized; radioactive glucose-6-phosphate and 6-phosphogluconate were identified by paper chromatography of an alcohol extract of the mycelium.

Koffler and Heath (46) have made a quantitative estimation of the extent of the oxidative pathway in growing cells of P. chrysogenum strain Q 176 from the relative recovery of glucose-1-C^{14} and glucose-U-C^{14} in the respiratory CO_2. They have calculated that at least two-thirds of the glucose utilized is metabolized through a mechanism involving a primary oxidation of the first carbon atom to CO_2.

An even greater amount of evidence is accumulating in favor of an active tricarboxylic acid cycle and support has been given by Koffler and co-workers for P. chrysogenum strain Q 176 having this type of terminal

oxidation from their radioactive tracer studies (33). Extensive dilution of C^{14} labeled acetate in the incubation medium during the second and third day of an eight-day culture of P. chrysogenum Q 176 grown on a synthetic medium indicates a considerable amount of glucose is converted into acetate and that acetate is a normal intermediate in the breakdown of glucose in this microorganism (40,52). Evidence, when collected in experiments using cell free systems as well as whole cells, gives good support to a major role being played by the tricarboxylic acid cycle in the metabolism of carbohydrates (15).

Penicillium digitatum, a common green mold, is found extensively in nature on citrus fruits, and has been reported to stimulate the respiration and coloration of lemons (5). Interest in this effect, which is probably due to ethylene emanating from this fungus, caused Wooster and Cheldelin (81) to establish the optimum growth requirements on a medium of known chemical composition. Otherwise, only limited nutritional studies have been made on this organism. Birkenshaw et al (12) have shown the production of considerable amounts of ethyl acetate and ethyl alcohol as fermentation products in the utilization of glucose by P. digitatum. According to Fergus (24), carbohydrates which serve as efficient

carbon sources for P. digitatum in agitated and stationary cultures are D-xylose, L-arabinose, D-glucose, D-fructose, D-mannose, D-galactose, sucrose and cellobiose, while moderate growth is observed in experiments using L-rhamnose and lactose. D-mannitol, maltose and dextrin were reported to be poor carbon sources. With simple organic acids as substrates, citric and malic acids are moderately utilized, while fumaric, tartaric, lactic, acetic, and oxalic were not adequate carbon sources for the growth of this organism.

In this laboratory, the interest in P. digitatum and its metabolic processes resulted from a study of the physiological effect of ethylene upon fruits and microorganisms. At the time, very little information was available concerning the catabolic pathways of carbohydrate utilization by any filamentous fungi.

In 1952, studies on P. digitatum using Warburg techniques and employing various C^{14} labeled substrates were carried out by Noble (54). A variety of conditions were imposed on the mold cells in order to determine the effect of these factors on the contributions of the various catabolic pathways. Some of these conditions were, age of the mold, nature of the medium and the presence of ethylene in the incubation atmosphere. A qualitative survey of glucose catabolism was also reported by Noble (54) for this microorganism

using glucose-1-C^{14}, glucose-2-C^{14} and glucose-6-C^{14} as substrates. Preferential loss of C-1 of glucose to respiratory CO_2 as compared to C-6 gave evidence for the operation of a direct oxidative pathway. Glucose-2-C^{14} was found to be incorporated into cellular constituents to a greater extent than glucose-6-C^{14}, suggesting that an active "C_2" unit may have been derived from carbons 2 and 3 of glucose. The role of "C_2" units as intermediates in terminal respiratory and general biosynthetic mechanisms was demonstrated with acetate-1-C^{14} and acetate-2-C^{14} as substrates to this microorganism. Both substrates were readily incorporated into all the cellular amino acids with very rapid incorporation into aspartic and glutamic acids, even though acetate has been shown to be an inadequate sole source of carbon for the growth of this organism.

Carbon dioxide fixation has been shown to be playing an important role in biosynthetic processes of many microorganisms (18, 17, 68, 69, 75) including P. chrysogenum (80). After 15 minutes of exposure in the latter case, $C^{14}O_2$ was incorporated into aspartic and glutamic acids and arginine of the mycelial protein. Interestingly, these amino acids are also the first ones to become labeled from assimilated $C^{14}O_2$ in the intact rat (20). This work however, only suggested

possible roles CO_2 fixation can play in biosynthesis by penicillia. Noble et al (54) utilized $C^{14}O_2$ in the presence of glucose and found extensive fixation of C^{14} isotope into cellular constituents of P. digitatum, especially glutamic and aspartic acids. Degradation studies to elucidate the distribution of radioactive isotopes were carried out on these amino acids and also those from acetate-1-C^{14} substrate. The labeling patterns indicate the possible occurrence of a double CO_2 fixation by a C_2 unit similar to that suggested by Tomlinson (68) for Clostridium kluyveri and by Cutinelli, et al for Rhodospirillum rubrum (17). It has also been pointed out that a C_3 plus C_1 condensation of the Wood-Werkman type (54) or malic enzyme reaction (77) is occurring in P. digitatum.

A tricarboxylic acid cycle appears to be the major terminal respiratory oxidation mechanism for this microorganism (54).

In the present work, efforts have been directed towards the quantitative estimation of the catabolic pathways involved for the conversion of carbohydrate to respiratory CO_2 in this organism and to gain further information concerning amino acid biosynthesis and the interrelations between amino acids and glucose metabolism.

For many years the field of intermediary metabolism

was dominated by workers demonstrating the occurrence in biological systems of 1) the anaerobic glycolytic breakdown of carbohydrates to lactate as postulated by Embden, Meyerhof and Parnas (25) and 2) the terminal oxidative cycle organized by Krebs (48). In recent years, workers in the field have demonstrated several alternate pathways for the metabolism of glucose in biological systems.

One of the primary alternate pathways, the oxidative pathway combined with pentose cycle has been illustrated to be a chain of reactions in which glucose-6-phosphate is oxidized to 6-phosphogluconic acid, decarboxylation of the latter to CO_2 and ribose-5-phosphate which is isomerized to ribulose-5-phosphate, on to xylulose-5-phosphate, which is a participant in the transketolase reaction. The action of the latter yields glyceraldehyde-3-phosphate and sedoheptulose-7-phosphate. By an analogous reaction of transaldolase, glyceraldehyde-3-phosphate and sedoheptulose-7-phosphate are transformed into fructose-6-phosphate and D-erythrose-4-phosphate. Fructose-6-phosphate is converted to glucose-6-phosphate by phosphogluconate decarboxylation. Thus, by a sequence of enzymatic reactions, glucose can be completely oxidized to CO_2, the final product.

Entner and Doudoroff (23) working with Pseudomonas

fluorescens, reported the formation of pyruvate labeled in the carboxyl carbon using glucose-1-C^{14} as the substrate. Experiments with enzyme preparations indicate that two pathways are functioning for non-phosphorylative oxidation of glucose in this organism (35). One, glucose is oxidized to gluconate which is then phosphorylated to 6-phosphogluconate. Pyruvate and triosephosphate have been shown to be formed in the cleavage of 6-phosphogluconate by this organism. Two, glucose is oxidized to 2-ketogluconate before phosphorylation, phosphorylated by 2-ketogluconokinase and finally degraded to pyruvate and a triose. On the other hand, glucose can also be oxidized to 6-phosphogluconate by way of glucose-6-phosphate in this organism.

In the lactic acid fermentation by the heterotroph, Leuconostoc mesenteroides, one mole each of lactate, alcohol, and CO_2 are formed per mole of glucose fermented (79). Ethanol has been shown to be formed exclusively from carbons 2 and 3 of glucose, while carbon one is lost as CO_2 and carbons 4, 5, and 6 become the carbon skeleton of lactic acid. Thus, using glucose-3,4-C^{14} as the substrate to this organism, the CO_2 evolved was found to be unlabeled and the C^{14} isotope equally divided between the hydroxy carbon of ethanol and the carboxyl group of lactate (79).

To summarize, at the present time glucose can be metabolized in biological systems by way of one of the following pathways; 1) EMP glycolytic pathway, 2) the non-phosphorylated oxidation of glucose to gluconate followed by cleavage of the latter to pyruvate and triose, 3) the oxidation of glucose-6-phosphate to 6-phosphogluconic acid, subsequent decarboxylation of the latter gives rise to a pentose which is in turn participating in the pentose cycle reactions.

Numerous methods have been reported in the literature, in which specifically labeled substrates are employed to determine the quantitative significance of each individual oxidative pathway for the metabolism of glucose in various biological systems (2, 9,10,11, 44,47,76). It is evident that many limiting assumptions are required to quantitate the relative contribution of the Embden-Meyerhof-Parnas pathway and the more direct oxidative pathway. These assumptions have been well discussed in a review article by Wood (79).

The principal assumptions, which are common to all methods of estimation of this type, are as follows: (1) glucose is metabolized to CO_2 either by phosphogluconate decarboxylation or the Embden-Meyerhof-Parnas pathway in combination with the tricarboxylic acid cycle (3), (2) any triose formed via the EMP pathway has an equal opportunity to be derived from glucose

carbons 1 to 3 or 4 to 6 and these trioses are equivalent to each other in respect to further metabolic reactions (25), (3) carbons 3 and 4 of glucose are rapidly converted to respiratory CO_2 via the glycolytic pathway, (4) phosphogluconate decarboxylation of carbon 1 of nonglycolytic glucose to respiratory CO_2 is also a rapid process, (5) in the course of the rapid utilization of glucose by a biological system, there cannot be appreciable reverse aldolase activity to reform hexose from trioses (76), and (6) the pentose formed from phosphogluconate decarboxylation is only slightly metabolized, by way of either the pentose cycle or degradation to C_2 and C_3 intermediates (76).

In the application of radioactive tracers to estimate the catabolic pathways of carbohydrate metabolism, various approaches can be employed that make use of the above assumptions. First, calculations from the specific activity of the respiratory CO_2 from specifically labeled glucoses, second, an analysis of the fermentation products in regards to their specific activities and labeling patterns, and third, the use of radioactive substrates other than glucose which can be assumed to be metabolized by a single pathway to respiratory CO_2.

An estimation of pathways of glucose catabolism

in yeast has been reported by Blumenthal *et al* (11) and Lewis *et al* (49), which involves the radioactive assay of the metabolic intermediates. Essentially, the procedure consists of carrying out simultaneous experiments with the same cell preparations, using glucose-1-C^{14} and glucose-U-C^{14} as the substrates. A 3-carbon or 2-carbon metabolic intermediate is isolated from the incubation medium of each substrate, purified and assayed for radioactivity. The data from glucose-1-C^{14} provide an indication of the relative incorporation of this carbon into the intermediate, and the data from uniformly labeled glucose provide a correction factor for the endogenous metabolism, whereby "true" values may be calculated for the specific activities of compounds derived from glucose-1-carbon.

The possible cleavage of carbon atoms 1 and 2 from fructose-6-phosphate (37), to cause randomization of the label in the three upper carbon atoms of the reformed hexose, or any other randomization among the carbon atoms of the glucose skeleton, can cause considerable error in these calculations, since it has been assumed that the EMP pathway is the only one which yields labeled C_2 units from glucose-1-C^{14}. Thus, a major discrepancy has been shown between the data from glucose-1-C^{14} and glucose-

6-C^{14} experiments, which points to the fact that the described schemes are unable to account for the true metabolic events occurring in such systems.

In mammalian tissues, Bloom et al (8, 9,10) have made extensive studies on the participation of the phosphogluconate oxidative pathway in glucose metabolism. In addition to the use of specifically labeled glucoses, specifically labeled intermediates, such as lactate and gluconate, were employed under the assumption that they are formed in the medium as intermediary products by individual pathways. The specific activities of the respiratory CO_2 and the fatty acids were examined. An expression was derived which allows a calculation of the relative contribution of the two pathways from the observed specific activity of the respiratory CO_2. A second derivation was employed on the basis of fatty acid labeling under the assumption that fatty acid synthesis from carbon 1 of glucose proceeds only by way of glycolytic intermediates. Values calculated by these two equations are in accord with the noticeable operation of the phosphogluconate oxidative pathway in liver slices.

Katz et al (43,44) and Abraham et al (2) have used rat mammary glands to carry out similar studies. In the use of these equations to determine the relative contribution of the phosphogluconate oxidative pathway

one cannot ignore the utilization of the C-2 carbon of glucose via the pentose cycle when the contribution of the latter pathway becomes appreciable.

Korkes has presented equations to calculate the participation of the phosphogluconate oxidative pathway from specific activity data obtained from the use of C^{14}-labeled glucoses as substrates to a biological system (47). These equations take into consideration the relative rates of the reactions involved and corrects for this by extrapolation of the specific activity data to zero time. It is necessary to assume no randomization of the isotope by triose recombination and an equal rate of formation of CO_2 from carbons 1, 2, and 3 of pyruvate.

Wang et al (76) have studied carbohydrate metabolism in bakers' yeast by a time study of the utilization of specifically labeled glucoses. The rate of change of specific activity in respiratory CO_2 from the respectively labeled substrate allows calculations on the basis of cumulative radiochemical recoveries of metabolic CO_2 from each labeled glucose.

The interrelations of glucose metabolism and amino acid biosynthesis can provide information concerning terminal oxidation of the breakdown products of glucose and the pathways of carbon assimilation.

The metabolism of amino acids has been reviewed recently in a symposium edited by McElroy and Glass (53). Emphasis in the field today is directed towards the establishment of the origin of the carbon skeletons of the amino acids, and the documentation of the enzymatic transformations of their metabolic pathways which have been suggested by tracer studies. It has been shown in many biological systems that alanine, aspartic acid and glutamic acid can be formed by transamination of their respective keto analogs which are known to be key intermediates in the Krebs cycle processes. CO_2 fixation onto C_3 and C_2 units appears to play an important role in the biosynthesis of these amino acids.

The interconversion of glycine and serine has been well studied (53,60). Serine may be formed by transamination of either phosphohydroxypyruvic acid or hydroxypyruvic acid and subsequently an alpha-beta cleavage can account for the formation of glycine in animal cells. In yeast and E. coli, glycine may result from the alpha-beta cleavage of C_4 acids and serine can in turn be synthesized by means of hydroxymethylation (47,74).

EXPERIMENTAL AND RESULTS

Cultural methods

The spore stock cultures of Penicillium digitatum Saccardo, a filamentous fungi, were maintained on potato-dextrose agar slants under refrigeration. As needed, the spores were transferred to fresh potato-dextrose agar slants and cultured for 4-5 days at 30°C. The spores were then carefully suspended in a few ml. of sterile growth medium and asceptically inoculated into sterile growth medium.

The semi-synthetic growth medium employed in all experiments was prepared according to the following procedure: Glucose, 13.2 g.; asparagine (monohydrate), 3.0 g.; Casamino acids (Difco), 4.0 g.; ammonium tartrate, 5.0 g.; 20.0 ml. of salts mixture consisting of: $(NH_4)H_2PO_4$, 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.; KI, 0.01 mg.; $ZnCl_2$, 0.05 mg.; H_3BO_3, 0.05 mg.; $CuCl_2$, 0.01 mg.; 1.0 ml. of vitamin solution containing: Thiamine HCl, 400γ; Ca pantothenate, 600γ; pyridoxine HCl, 800γ. Dilute to one liter with distilled water, adjust to pH 3.5-3.6 with 6 N HCl and autoclave for 30-40 minutes.

The initial incubation was carried out in a 3-liter, 3-necked flask equipped with a gassing sparger, sampling

tube and a gas outlet. The incoming air was passed through a sulfuric acid scrubber and a sterile cotton plug before being sparged into the growth medium. Growth was continued for a period of 40-48 hours depending on the size of the inoculum and the rate of growth.

After the required growth phase was reached, the mycelia were harvested by centrifugation, washed three times, and finally suspended in a salts carbon-free medium (identical to the growth medium except that glucose, asparagine, ammonium tartrate and the Casamino acids are excluded). An aliquot of the suspension was taken in order to adjust the concentration of the cell suspension to one mg. per ml. (dry weight basis) for the time course experiments.

Determination of radioactivity

The respiratory CO_2, from cells in the time course experiments, was trapped with CO_2-free NaOH, precipitated as $BaCO_3$ by the addition of $BaCl_2$-NH_4Cl solution and quantitatively plated onto aluminum planchets by centrifugation for radiochemical assay. Radioactivity incorporation into the mycelia was determined by the Van Slyke-Folch wet combustion method. Fermentation products in the medium were assayed for radioactivity by the persulfate wet combustion method.

The activities of the $BaCO_3$ plates were counted by an end-window G-M counter to an accuracy within 3% standard error. Corrections for background and self-absorption were applied to the data in the conventional manner.

The determination of the radioactivity in the $BaCO_3$ obtained from the degradation of amino acids was carried out by the use of cupped stainless steel planchets counted by an end-window G-M counter. The samples were counted to an accuracy within 2% standard deviation; corrected for background and self-absorption in the conventional manner.

C^{14} labeled substrates

Glucoses-1,-2, and -6-C^{14} were furnished by the Bureau of Standards through the kind cooperation of Dr. Isbell. Glucose-U-C^{14}, acetate-1-C^{14} and acetate-2-C^{14} were obtained from Tracerlab Inc. Pyruvates-1, -2, and -3-C^{14} were procured from Nuclear·Chicago. Glucose-3,4-C^{14} was prepared in this laboratory according to the method of Wood and co-workers (80). The specific activity of each substrate was adjusted to a prescribed level with nonisotopic substrates.

TIME COURSE STUDIES

Apparatus

The apparatus shown in Figures 1 and 2 was developed in this laboratory to conduct time course studies on the utilization of labeled substrates by microorganisms. By the use of this method, it is possible to obtain interval radiochemical recovery with respect to the respiratory CO_2, the fermentation products in the medium, and the cells, at any interval time throughout the experiment.

As seen from Figure 1, six flasks can be employed per set under identical conditions to study the metabolism of six different substrates at the same time. Two-way stopcocks (S) are used to connect each flask (I) with the master flowmeter (F) to ensure a uniform flow rate of the sweeping CO_2-free air in each flask. Sampling of the incubation medium is accomplished by the use of the sampling device (D). The CO_2 trap (T) has been shown to have less than 2% leakage at a flow rate of 200 ml. per minute using 25 ml. of 0.5 N CO_2-free NaOH solution with a suitable antifoaming agent (6).

Experimental procedure

The procedures for time course studies have been

reported by Wang et al (76). With Penicillium digitatum, the concentration of cell suspension was adjusted to 250-300 mg. (dry weight basis) per 250 ml. of carbon-free salts medium. The cells were depleted under vigorous aeration (200-400 ml. CO_2-free air per minute) for 1.5-2.0 hours, after which, labeled substrates with an activity level of 0.5 microcurie each were added to each of 12 flasks. Glucose level in each flask was 90 mg. Sample aliquots of the incubation mediums were taken at regular intervals for reducing sugar assay according to the method of Shaffer and Somogy (62,64). The CO_2-free NaOH solutions in the respiratory CO_2 traps were replaced every hour throughout the experiment.

After the completion of each experiment, the mycelia were separated from the media by filtering through sintered glass crucibles and drying in a vacuum desiccator over phosphorus pentoxide. The media were kept frozen in a deep-freeze for radiochemical assay and further processing.

Utilization of specifically labeled glucoses

The relative rates of conversion of various labeled glucose carbons to respiratory CO_2 are shown in Figure 3, which is a plot of the percent-interval-recovery of respiratory CO_2 vs. time in hours. The respiratory CO_2 curve in the glucose-3,4-C^{14} experiment showed an

abrupt decrease at 3-4 hours, and at which time it is reasonable to assume substrate glucose has been exhausted in the cells. Just prior to this point of glucose exhaustion, there should be a steady state between the labeled glucose in the cell and the respiratory CO_2. It is at this point on the percent-cumulative-recovery graph, (Figure 4) that the relative cumulative recoveries in $C^{14}O_2$ from the labeled glucoses are taken for the calculation of the distribution of pathways.

In Table 1 is presented a radiochemical inventory of the radioactivity in respiratory CO_2, fermentation products (medium) and cells at the end of experiment.

Metabolism of tracer amounts of labeled acetates and pyruvates in the presence of nonisotopic glucose

Earlier experiments have shown that although acetate cannot serve as a sole carbon source to P. digitatum, yet, it appears to be a key intermediate in the metabolism of this mold (54). Therefore, the present experiment was designed to investigate the rate of utilization and the route of metabolism of acetate by this organism in the presence of glucose. The study was carried out under conditions identical to those described under the labeled glucose experiments, except that the glucose in this experiment was non-isotopic and 1.5 microcuries of acetate-1-C^{14} or

acetate-2-C^{14} was added which is equivalent to tracer amounts chemically. The interval recoveries of substrate activity in CO_2 are given in Figure 5 and a radiochemical inventory of the metabolized substrates is shown in Table 1 for this time course study.

Since pyruvate is also a key intermediate in glycolysis, it was desirable to measure the rate of utilization of pyruvate from the medium in the presence of glucose. The results of experiments using tracer amounts of pyruvate-1-C^{14}, pyruvate-2-C^{14} or pyruvate-3-C^{14} (0.5 microcurie level) as the labeled substrates in the presence of nonisotopic glucose is given in Figure 6. Again the conditions for this experiment were identical to the other time course studies. Table 1 contains a radiochemical recovery analysis of the various fractions derived from the substrate observed in these experiments.

Utilization of specifically labeled glucoses by Penicillium chrysogenum

A penicillin producing strain of penicillia, Penicillium chrysogenum Q 176, has been under rather extensive investigation by Koffler et al (32, 33, 46, 66, 83, 82). It was felt that a time course study of the utilization of carbon sources by this microorganism under identical conditions as those employed for P.

digitatum would be of great interest. A spore culture of Penicillium chrysogenum strain Q 176 was kindly supplied by Dr. Koffler. The techniques of culturing, nature of the medium, etc. employed in these experiments were the same as those used with P. digitatum, which have been previously described. The interval and cumulative radiochemical recoveries of substrate activity in respiratory CO_2 are given in Figures 7 and 8. The radiochemical inventory for the respective substrate utilization is given in Table 2.

Metabolism of tracer amounts of labeled acetates and pyruvates in the presence of nonisotopic glucose by Penicillium chrysogenum

The work of Koffler et al (33), with P. chrysogenum gives good evidence for the view that resting cells of this organism which have been grown on an acetate medium oxidize acetate by way of a Krebs cycle. This experiment was designed to show the utilization of tracer amounts of acetate in the presence of glucose by proliferating cells of P. chrysogenum. The interval radiochemical recovery of substrate activity in respiratory CO_2 is given in Figure 9.

It has been shown by Koffler et al (46) that this organism metabolizes glucose by both an oxidative and a glycolytic pathway. Therefore, it was of interest to

study the utilization of pyruvate from the medium of proliferating cells in the presence of glucose, and compare this utilization with that of P. digitatum. The interval radiochemical recovery of substrate activity in respiratory CO_2 from tracer amounts of pyruvate-2-C^{14} and pyruvate-3-C^{14} is given in Figure 9.

INCORPORATION OF GLUCOSE-2-C^{14} AND GLUCOSE-6-C^{14} INTO THE CELLULAR CONSTITUENTS OF *PENICILLIUM DIGITATUM*

Incorporation experiment

Cells grown in the manner described previously, were harvested, washed three times and suspended in a carbon-free salts medium. Two 1-liter growth flasks were equipped in the same manner as the 3-liter growth flask described previously, and connected to the manifold of the time course apparatus. To each of the flasks was added 1500 mg. (dry weight basis) of cells suspended in 500 ml. of carbon-free salts medium and depleted under vigorous aeration (400-600 ml. per minute) with CO_2-free air for 1.5-2.0 hours. To one of the flasks was administered 750 mg. of glucose-2-C^{14} containing 50 microcuries of radioactivity. An identical amount of glucose-6-C^{14} was administered to the other flask.

The CO_2-free NaOH solution in the CO_2 traps was replaced every hour and aliquots of the solution taken for radiochemical assay. Direct-plate counting of hourly medium samples was employed to follow glucose utilization. The glucose in the incubation medium was exhausted after 4 hours and the experiment was terminated after 7 hours.

The cells in each flask were harvested by filtration through a sintered glass funnel, washed with 100-200 ml.

of distilled water, followed by an equal amount of 80% alcohol, and last by a small amount of ether. The cells were then dried in a vacuum desiccator over P_2O_5.

The distribution pattern of radioactivity among the respiratory CO_2, medium, cells, and sample aliquots is summarized in Table 3.

Isolation of amino acids from glucose-2-C^{14} and glucose-6-C^{14} P. digitatum mycelia

In the following procedures, glucose-2-C^{14} and glucose-6-C^{14} cells were hydrolyzed by 0.05 HCl in the presence of Dowex-50 (H form) resin in a manner similar to that described by Paulson et al (55) for the hydrolysis of protein. By preliminary experiments, it was found that nearly all of the hydrolyzable polysaccharide in the mycelia could be obtained in the supernatant as monosaccharides after refluxing for 10-12 hours at 100° C. After the removal of the supernatant containing the sugars by means of centrifugation, the hydrolysis was continued until essentially exhaustive hydrolysis of the cellular protein was effected. The resulting amino acids were then eluted from the Dowex-50 resin with 1.5 N HCl followed with 6 N HCl. The basic amino acids, arginine and lysine were recovered in somewhat reduced yields due to the

difficulty of eluting them from the resin. The eluted amino acids were freed of excess HCl by repeated evaporation to dryness *in vacuo*. They were then dissolved in a small volume of 0.5 N acetic acid and placed onto a Dowex-1-X 8 (acetate form) column and fractionally eluted with 0.5 N acetic acid according to the procedure of Hirs *et al* (39).

Tyrosine, glutamic acid and aspartic acid, were found to be chromatographically pure in the respective fractions from this column operation. The glutamic acid fractions were combined and taken to dryness *in vacuo* several times to remove the acetic acid followed by dilution with the appropriate amount of unlabeled L-glutamic acid. The diluted glutamic acid was isolated as the hydrochloride. The aspartic acid fractions were treated in the same manner, except, the diluted aspartic acid was obtained as the free acid.

The neutral and basic amino acids fractions from the Dowex-1 column were combined and evaporated to dryness *in vacuo*, to remove the excess acetic acid. The amino acids were then dissolved in a small volume of 1 N HCl and placed on a Dowex-50 X 4 column which had been previously equilibrated with 1 N HCl. The amino acids were fractionated by means of gradient elution using a hydrochloric acid solution ranging from

1 normal to 4 normal in concentration.

The alanine fractions from the above Dowex-50 column were shown to be chromatographically pure, then they were combined and evaporated to dryness *in vacuo* several times to remove the excess HCl. Alanine hydrochloride was converted to the free acid in the following manner; the crude acid was dissolved in a small amount of distilled water and absorbed onto a small Dowex-1 (OH form) column, followed by washing the column well with distilled water. Alanine was then eluted from the column with 0.5 N acetic and the solution evaporated to dryness to remove the excess solvent. Finally, the crystallized alanine was diluted to 3 mM with unlabeled L-alanine and recrystallized from a water-alcohol mixture.

The glycine fractions were found to be contaminated with small amounts of glutamic acid, possibly as a result of the hydrolysis of 2-pyrrolidonecarboxylic acid which could have been formed in the initial cell hydrolysis. The contaminant was removed from these fractions by means of the operation of a Dowex-1 column (acetate form). Glycine was then diluted to a 3 mM level with unlabeled glycine for degradation studies.

The pure fractions of serine from the Dowex-50

column were combined and converted to the free amino acid in the same manner as the alanine samples. After dilution of the isolated free serine with L-serine (3 mM scale) the diluted serine was crystallized from a water-alcohol mixture.

Degradation of amino acids isolated from glucose-2-C^{14} and glucose-6-C^{14} P. digitatum mycelia

Aspartic and glutamic acid

Degradation methods for these two amino acids were essentially those of Wang et al (71). For aspartic acid, combustion of the whole molecule gave the total activity of the compound. The ninhydrin reaction was used for the determination of the sum of the activity in the alpha and beta carboxyl groups. Direct determination of the specific activity of the alpha carboxyl group was done by conversion of aspartic acid to malic acid, followed by a von Pechmann decarboxylation to give CO which was oxidized to CO_2 by passing through a hot copper oxide tube (3). The haloform reaction on aspartic acid by a mixture of KOH-K_2 CO_3-KI_3 gave rise to iodoform corresponding to the methylene carbon of aspartic acid.

Glutamic acid was oxidized to CO_2 for total activity of the molecule. Ninhydrin reaction on this amino acid converted the alpha carboxyl to CO_2 for

the specific activity of carbon 1. The Schmidt decarboxylation (71) was used to determine the C^{14} in the gamma carboxyl group. The specific activity of the gamma carboxyl group was also verified by isolating the alpha, gamma-diamino butyric acid, produced in the Schmidt reaction. The bulk of the excess H_2SO_4 in the reaction mixture was removed by the addition of $Ba(OH)_2$ and the subsequent removal of the $BaSO_4$ by centrifugation. The supernatant and washings were passed through a Dowex-3 (OH form) column to remove the remaining traces of H_2SO_4 and unreacted glutamic acid. The soluble alpha, gamma-diamino butyric acid was isolated and crystallized as the hydrochloride salt upon the addition of ethanol. For radioactive assay of this compound, a small amount of concentrated H_2SO_4 was added to remove the HCl, and then the amino acid oxidized to CO_2 by means of wet combustion. The specific activity of the middle carbons of glutamic acid was assayed for by difference.

Glycine, alanine and serine

Glycine was degraded by combustion to CO_2 for total activity of the entire molecule, and ninhydrin decarboxylation for the activity of the carboxyl carbon. The activity of the amino carbon was obtained by difference.

Alanine was degraded by total combustion to CO_2 for the specific activity of the entire molecule. The ninhydrin decarboxylation gave the carboxyl group as CO_2 and acetaldehyde which was trapped and immediately oxidized to acetic acid in a 1 N $K_2Cr_2O_7$-H_2SO_4 solution. Purification of the acetic acid by steam distillation and titration with NaOH gave sodium acetate which was isolated and degraded according to the method of Phares (56). By this method, the carboxyl carbon of acetate was removed by HN_3 decarboxylation to CO_2 and the methyl amine formed was oxidized to CO_2 by alkaline $KMnO_4$. The carboxyl carbon of acetate is equivalent to the amino carbon of alanine and the methyl carbon of acetate corresponds to the methyl carbon of alanine.

The degradation of serine was carried out according to the method of Sakami (60) which consists of a $NaIO_4$ oxidation to give the carboxyl group as CO_2, the amino carbon as formic acid and the beta carbon as formaldehyde. The formic acid was oxidized to CO_2 in the presence of the formaldehyde by mercuric chloride. The formaldehyde was then oxidized to formic acid with sodium hypoiodite and then to CO_2 by mercuric chloride.

DIAGRAM 1

DISTRIBUTION OF C^{14} ACTIVITY IN VARIOUS CELLULAR CONSTITUENTS OF PENICILLIUM DIGITATUM GROWN ON GLUCOSE-2-C^{14}

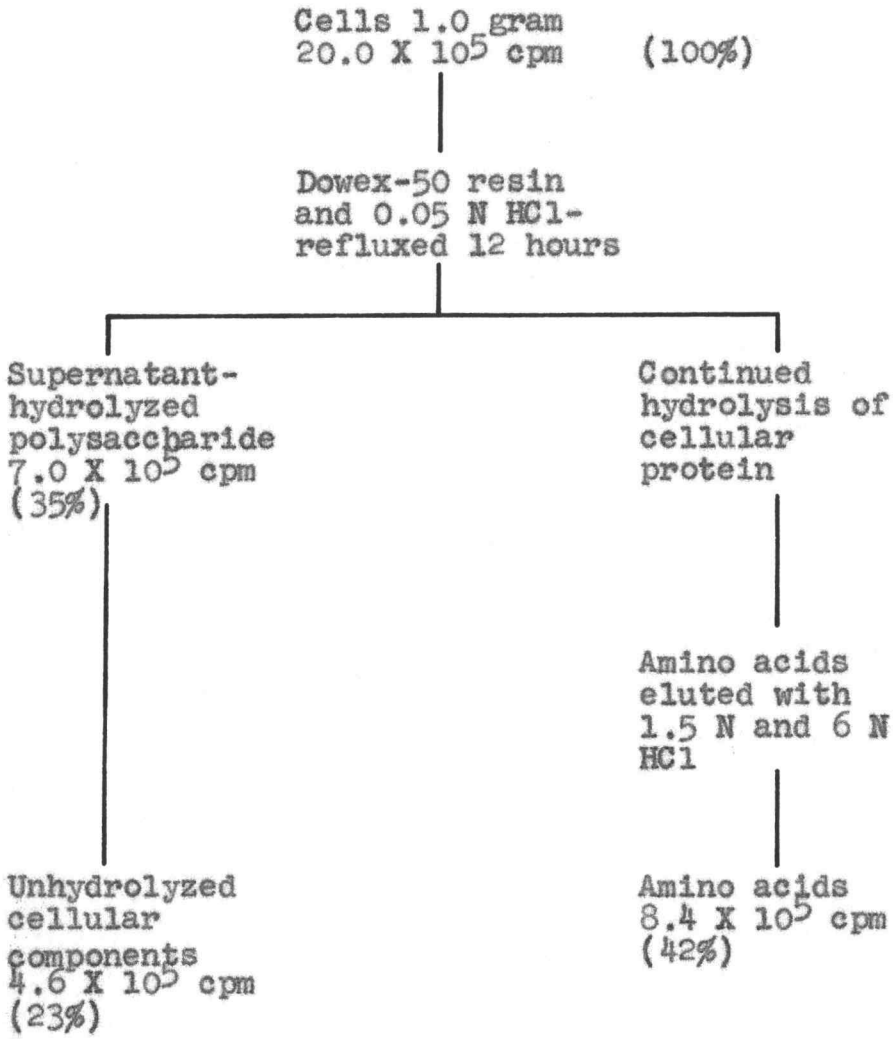


DIAGRAM 2

DISTRIBUTION OF C^{14} ACTIVITY IN VARIOUS CELLULAR CONSTITUENTS OF *PENICILLIUM DIGITATUM* GROWN ON GLUCOSE-6-C^{14}

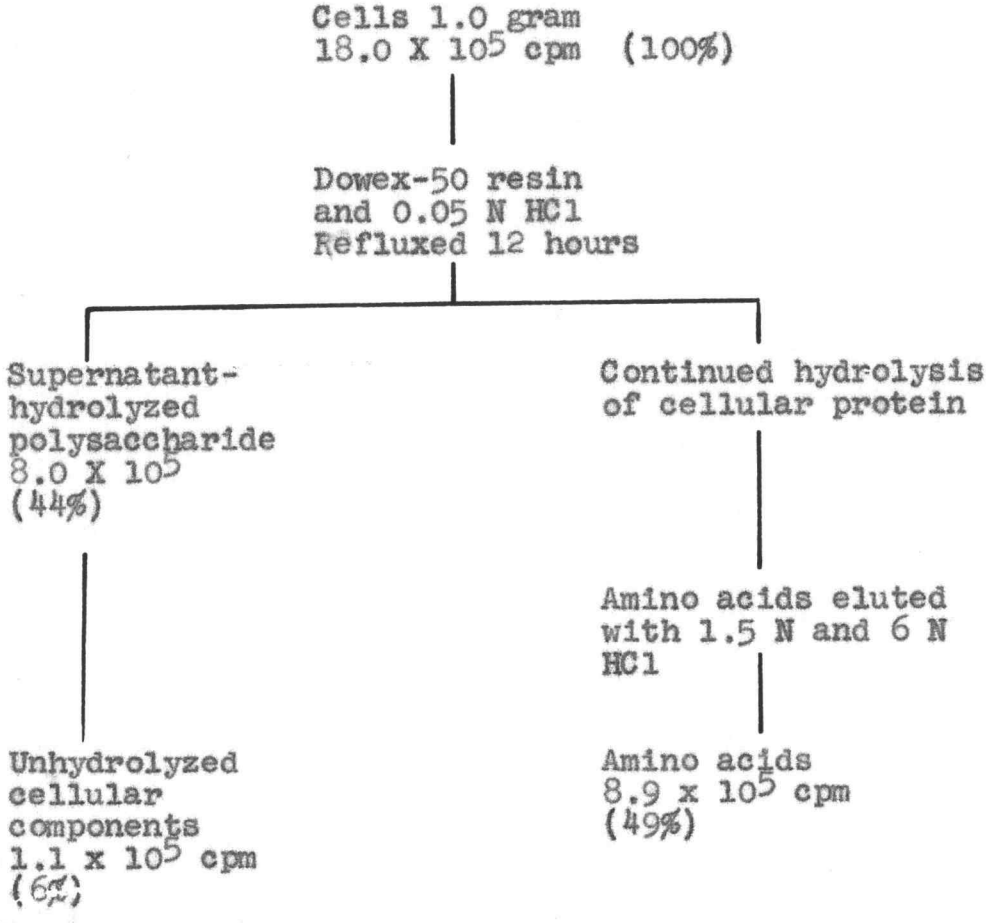
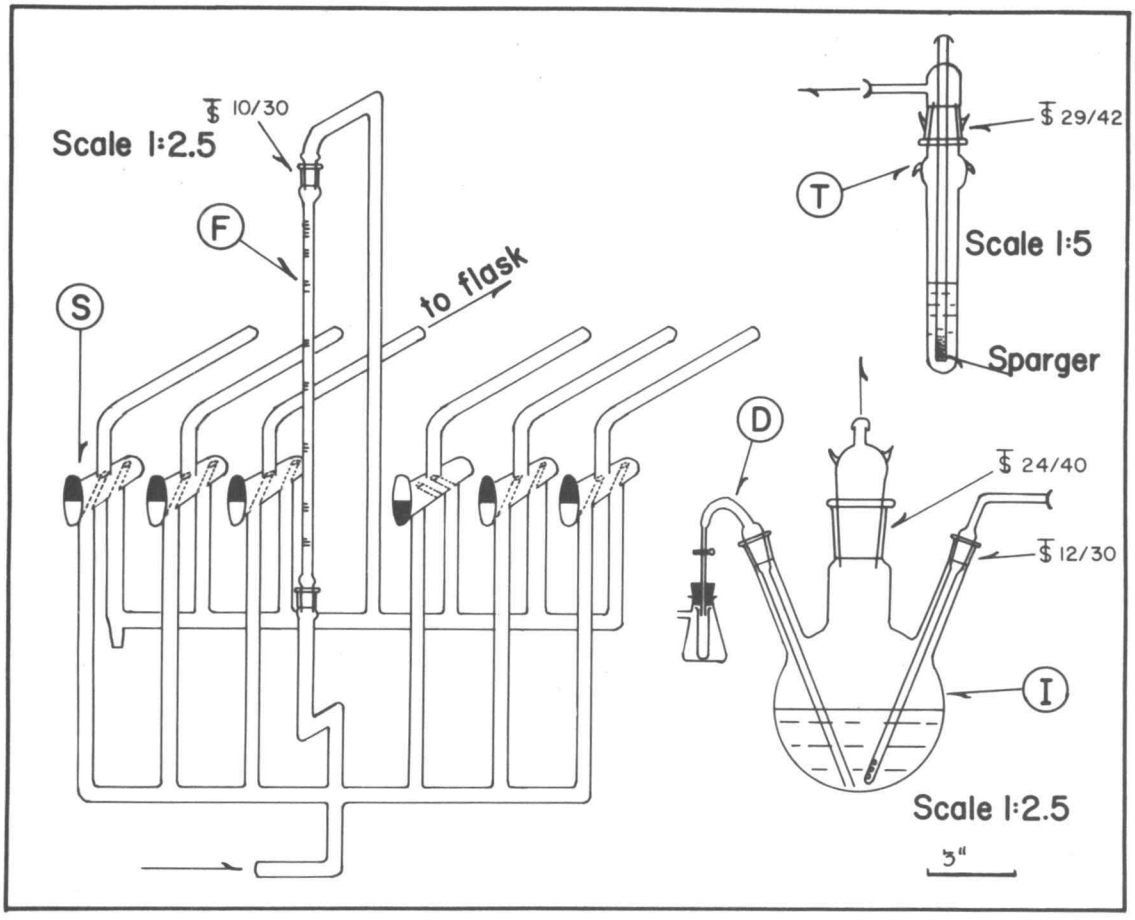
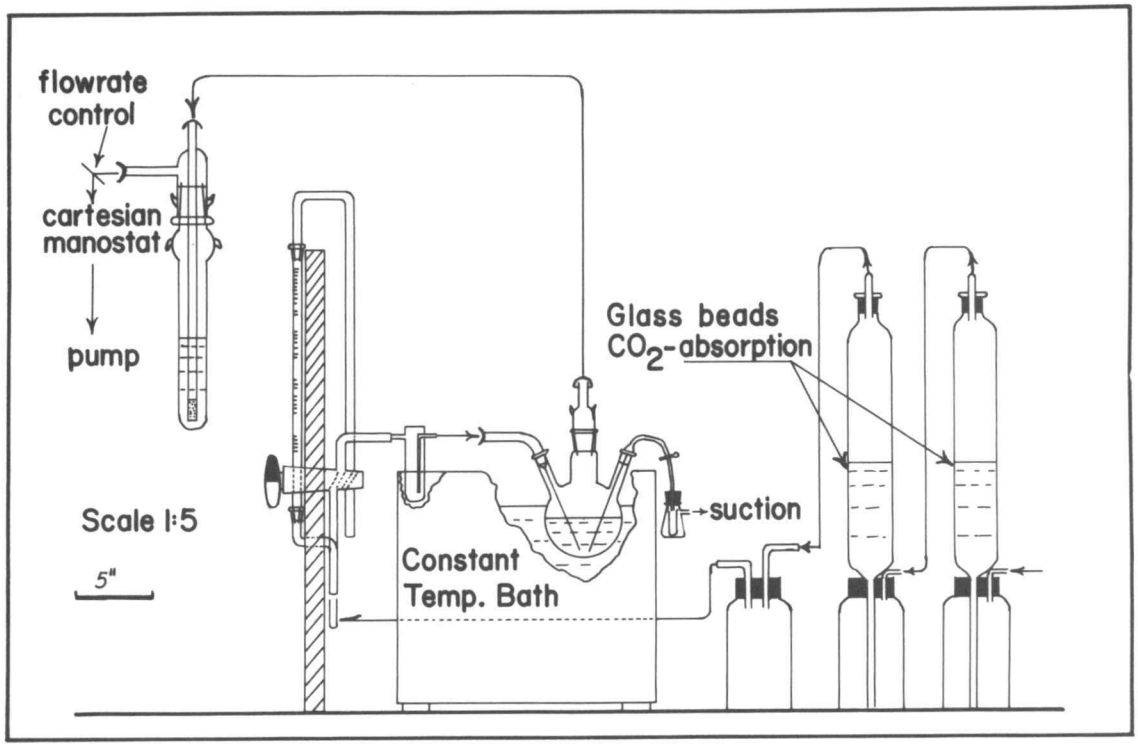


FIGURE 1



APPARATUS FOR THE TIME COURSE STUDY OF CARBOHYDRATE METABOLISM IN MICROORGANISMS

FIGURE 2



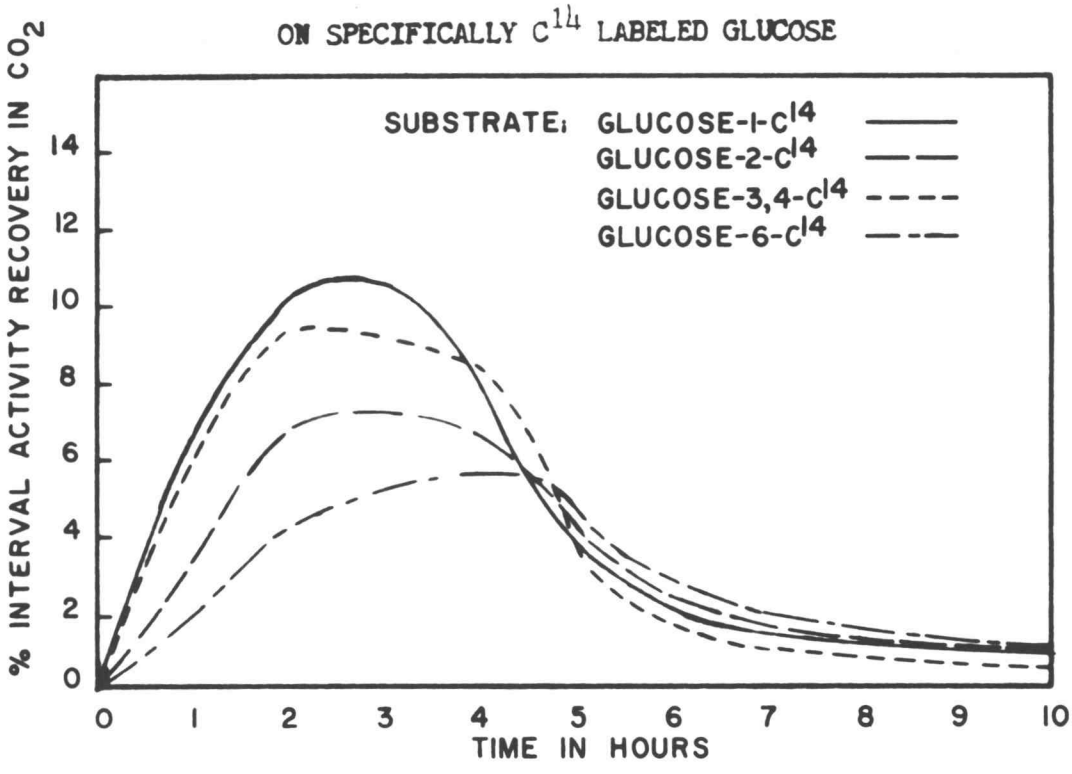
ASSEMBLY FOR THE TIME COURSE STUDY OF CARBOHYDRATE METABOLISM IN MICROORGANISMS

FIGURE 3

INTERVAL RADIOCHEMICAL RECOVERY IN RESPIRATORY CO_2

FROM 44 HOURS OLD CULTURE OF P. DIGITATUM GROWN

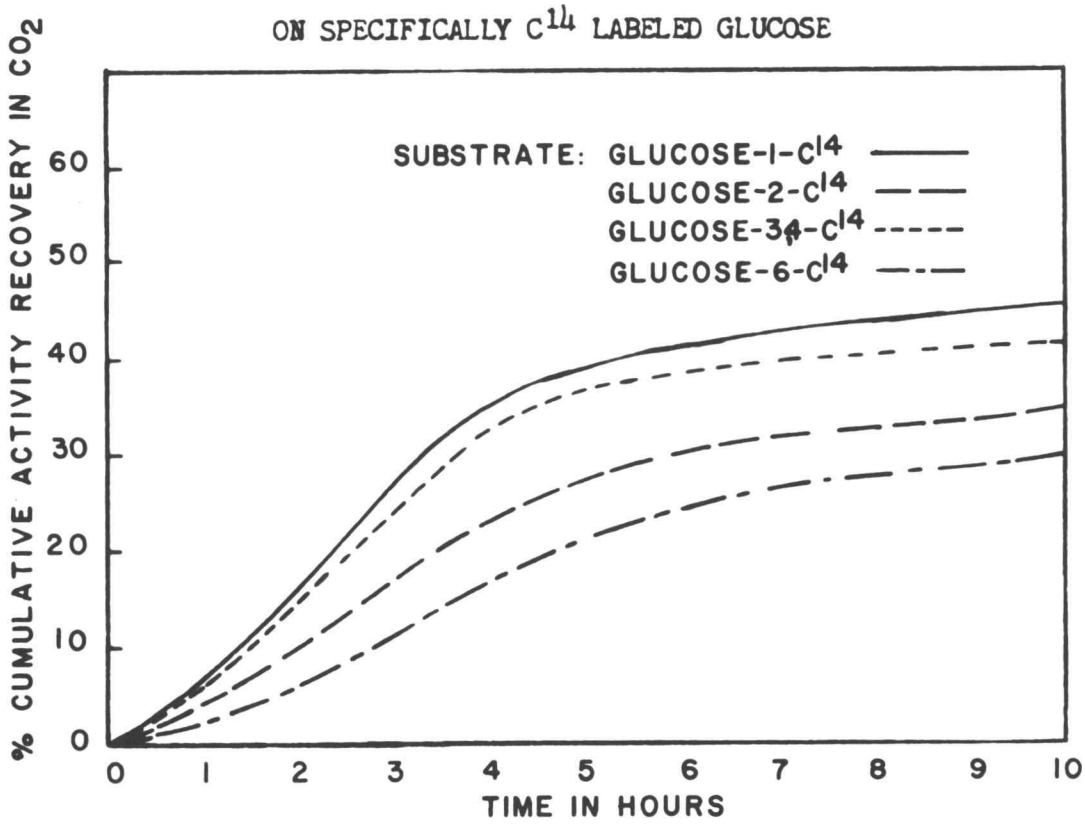
ON SPECIFICALLY C^{14} LABELED GLUCOSE



Time for medium glucose disappearance: 3-4 hours.
Weight of cells per flask: 250 milligrams.
Air-flow rate per flask: 400 milliliters per minute.
Level of substrate per flask: 90 milligrams.
Level of radioactivity per flask: 0.5 microcurie.
Temperature: 30° C.

FIGURE 4

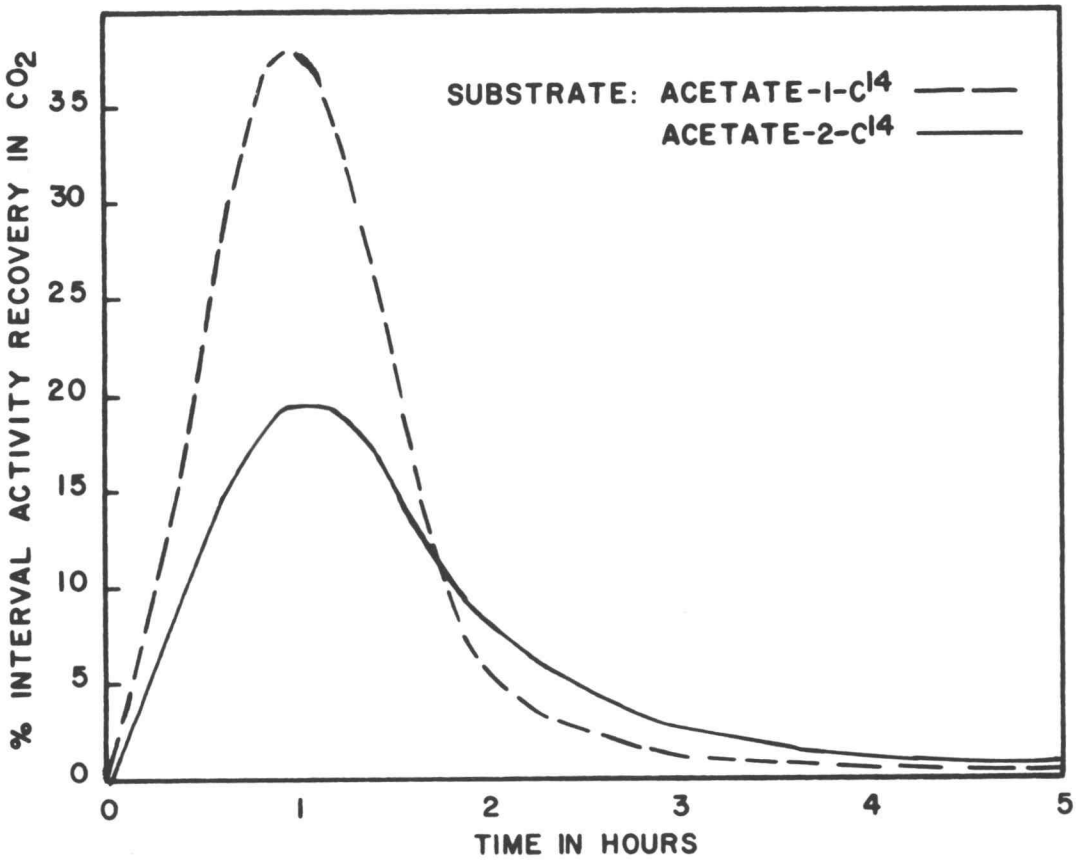
CUMULATIVE RADIOCHEMICAL RECOVERY IN RESPIRATORY CO_2 FROM 44 HOURS OLD CULTURE OF P. DIGITATUM GROWN ON SPECIFICALLY C^{14} LABELED GLUCOSE



Time for medium glucose disappearance: 3-4 hours.
Weight of cells per flask: 250 milligrams.
Air-flow rate per flask: 400 milliliters per minute.
Level of substrate per flask: 90 milligrams
Level of radioactivity per flask: 0.5 microcurie.
Temperature: 30°C.

FIGURE 5

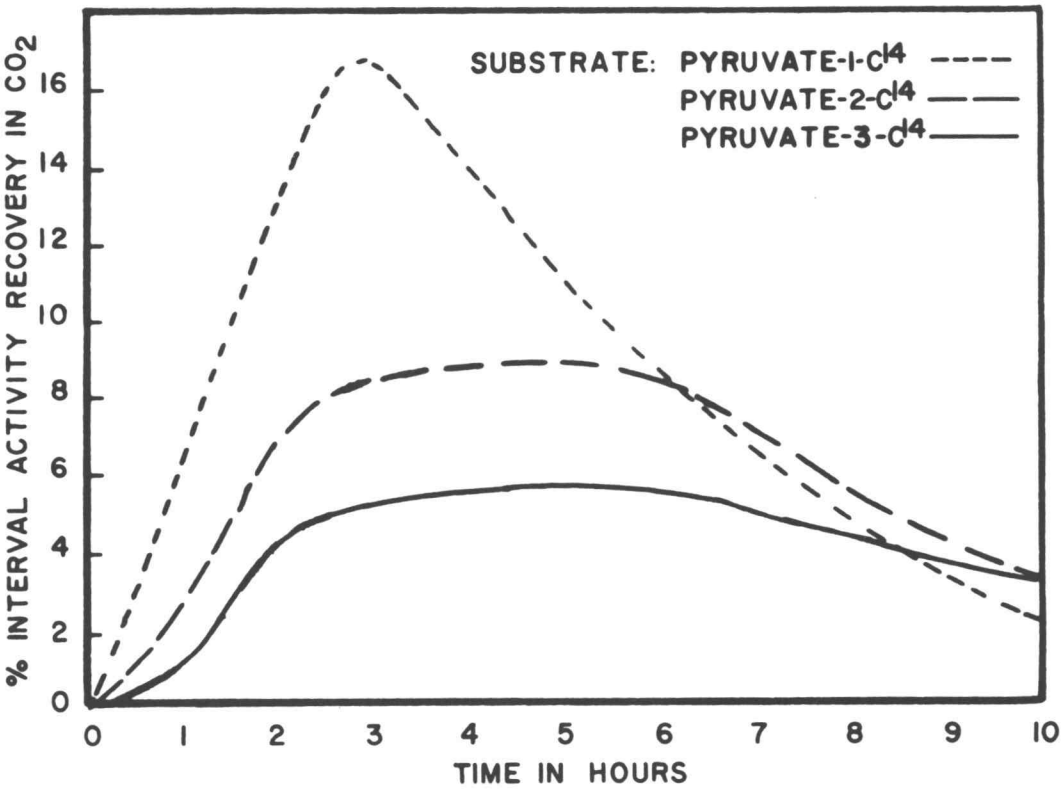
INTERVAL RADIOCHEMICAL RECOVERY IN RESPIRATORY CO_2 FROM 44 HOURS OLD CULTURE OF P. DIGITATUM GROWN ON GLUCOSE IN THE PRESENCE OF TRACER AMOUNT OF SPECIFICALLY C^{14} LABELED ACETATE



Time for medium glucose disappearance: 3-4 hours.
Weight of cells per flask: 250 milligrams.
Air-flow rate per flask: 400 milliliters per minute.
Level of glucose per flask: 90 milligrams.
Level of radioactivity per flask: 1.5 microcuries.
Temperature: 30° C.

FIGURE 6

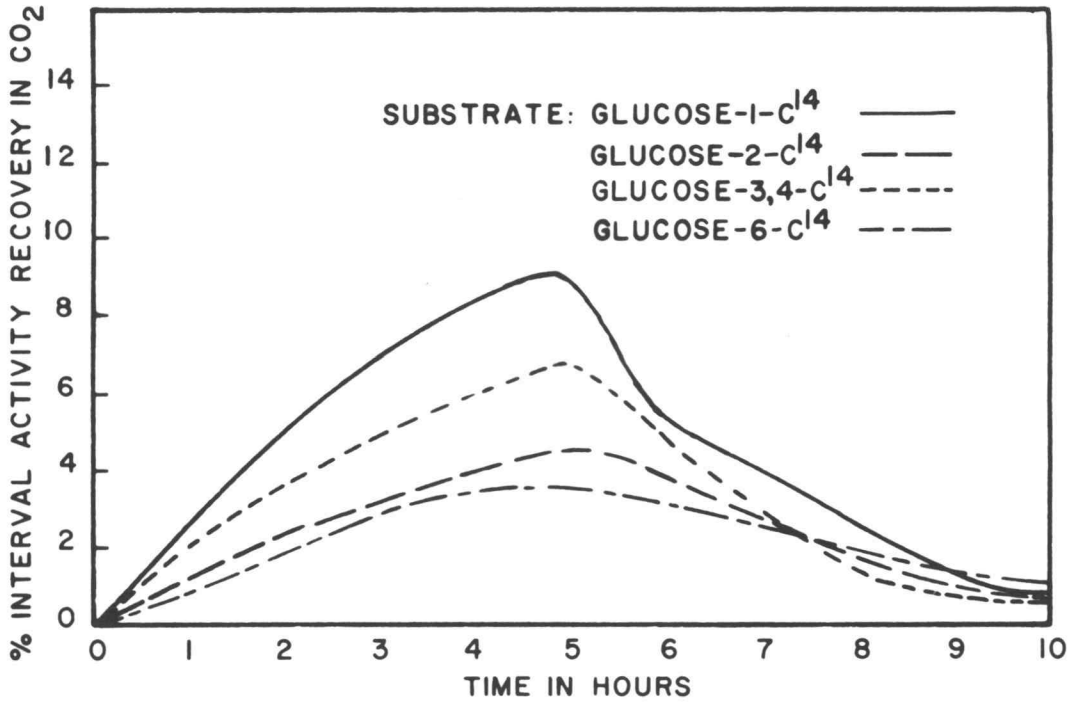
INTERVAL RADIOCHEMICAL RECOVERY IN RESPIRATORY CO_2 FROM 44 HOURS OLD CULTURE OF P. DIGITATUM GROWN ON GLUCOSE IN THE PRESENCE OF TRACER AMOUNT OF SPECIFICALLY C^{14} LABELED PYRUVATE



Time for medium glucose disappearance: 3-4 hours.
Weight of cells per flask: 250 milligrams.
Air-flow rate per flask: 400 milliliters per minute.
Level of glucose per flask: 90 milligrams.
Level of radioactivity per flask: 0.5 microcurie.
Temperature: 30° C.

FIGURE 7

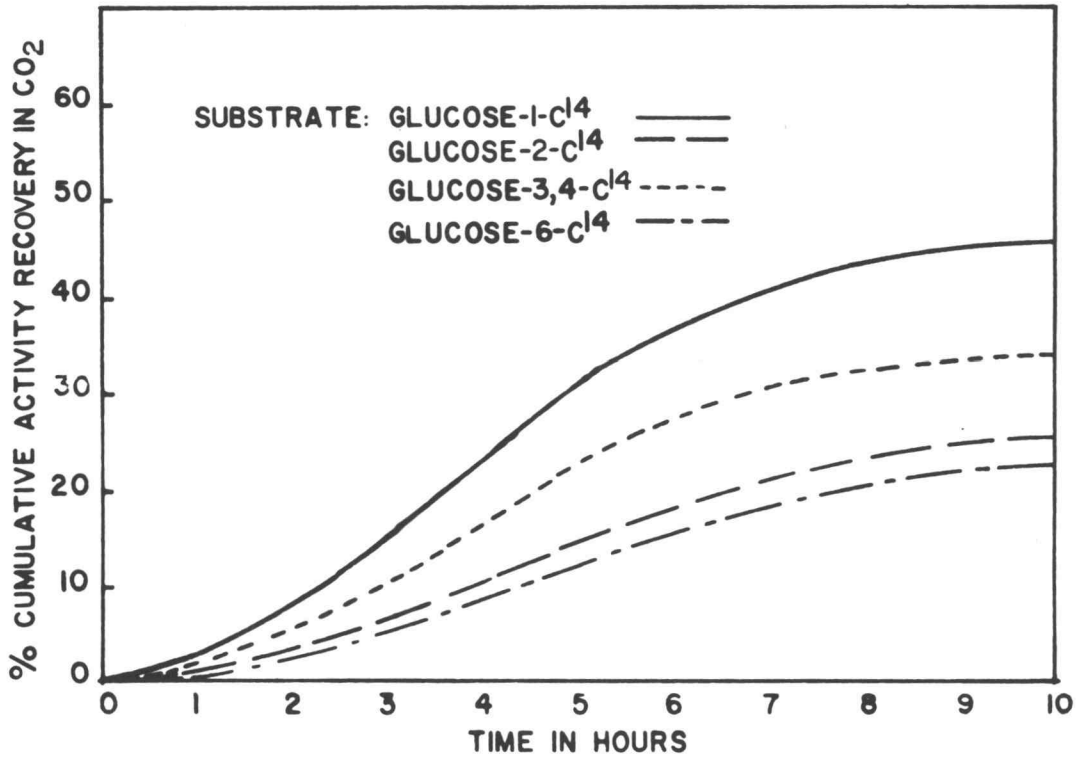
INTERVAL RADIOCHEMICAL RECOVERY IN RESPIRATORY CO_2 FROM 44 HOURS OLD CULTURE OF P. CHRYSOGENUM GROWN ON SPECIFICALLY C^{14} LABELED GLUCOSE



Time for medium glucose disappearance: 3-4 hours.
Weight of cells per flask: 250 milligrams.
Air-flow rate per flask: 400 milliliters per minute.
Level of substrate per flask: 90 milligrams.
Level of radioactivity per flask: 0.5 microcurie.
Temperature: 30°C.

FIGURE 8

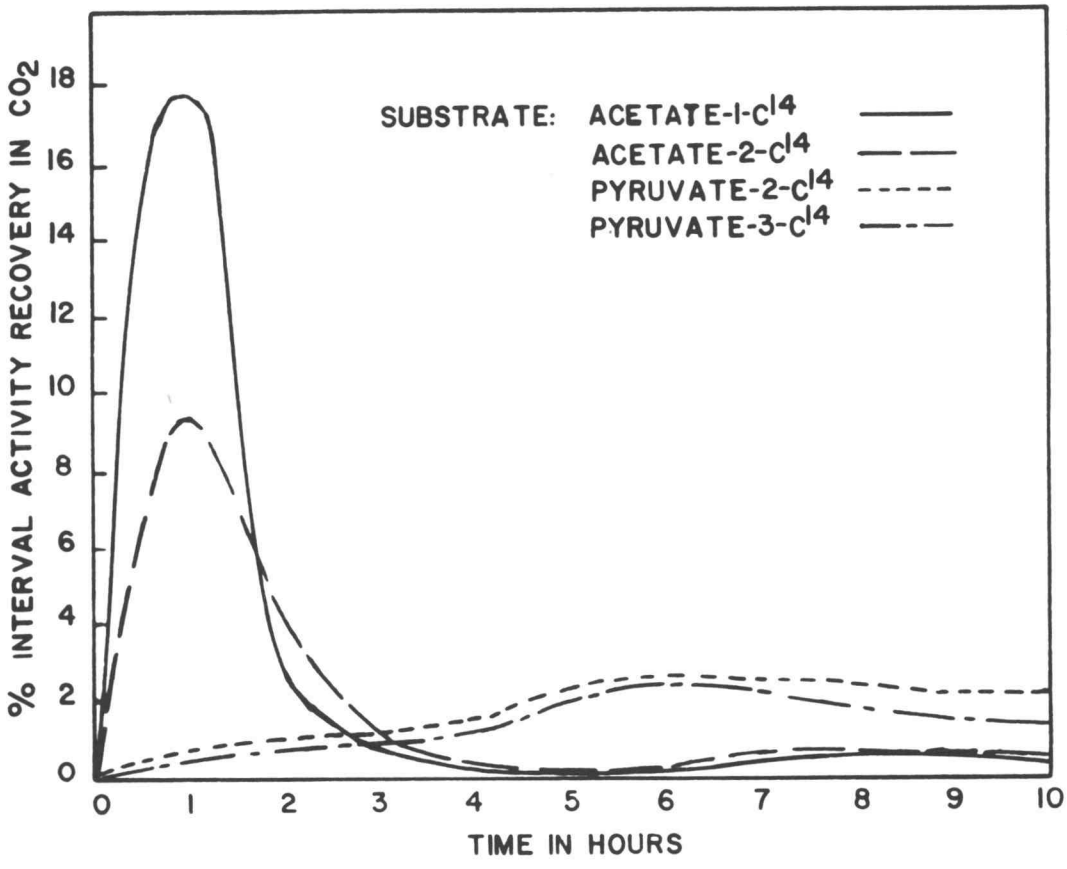
CUMULATIVE RADIOCHEMICAL RECOVERY IN RESPIRATORY CO_2 FROM 44 HOURS OLD CULTURE OF P. CHRYSOGENUM GROWN ON SPECIFICALLY C^{14} LABELED GLUCOSE



Time for medium glucose disappearance: 3-4 hours.
Weight of cells per flask: 250 milligrams.
Air-flow rate per flask: 400 milliliters per minute.
Level of substrate per flask: 90 milligrams.
Level of radioactivity per flask: 0.5 microcurie.
Temperature: 30°C.

FIGURE 9

INTERVAL RADIOCHEMICAL RECOVERY IN RESPIRATORY CO_2 FROM 44 HOURS OLD CULTURE OF P. CHRYSOGENUM GROWN ON GLUCOSE IN THE PRESENCE OF TRACER AMOUNT OF SPECIFICALLY C^{14} LABELED ACETATE AND PYRUVATE



Time for medium glucose disappearance: 3-4 hours.
Weight of cells per flask: 250 milligrams.
Air-flow rate per flask: 400 millliliters per minute.
Level of glucose per flask: 90 milligrams.
Level of radioactivity per flask: acetate 1.5 microcuries.
pyruvate 0.5 microcurie.

Temperature: 30°C.

TABLE 1

FATE OF SUBSTRATES IN PROLIFERATING *P. DIGITATUM*

Substrate	Percentage Recovery			
	Respiratory CO_2	Cells	Fermentation Product (Medium)	Total
Glucose-1-C^{14}	46	50	3	99
Glucose-2-C^{14}	35	59	4	98
Glucose-3,4-C^{14}	41	36	6	83
Glucose-6-C^{14}	30	54	14	98
Pyruvate-1-C^{14}	87	7	8	102
Pyruvate-2-C^{14}	69	29	6	104
Pyruvate-3-C^{14}	46	40	10	96
Acetate-1-C^{14}	48	20	2	70
Acetate-2-C^{14}	35	37	2	74

TABLE 2

FATE OF SUBSTRATES IN PROLIFERATING *P. CHRYSOGENUM*

Substrate	Percentage Recovery			
	Respiratory	Cells	Fermentation Products (Medium)	Total
Glucose-1-C^{14}	46	46	3	95
Glucose-2-C^{14}	24	64	4	92
Glucose-3,4-C^{14}	32	41	5	78
Glucose-6-C^{14}	22	61	3	86

TABLE 3

DISTRIBUTION OF C^{14} ACTIVITY IN *PENICILLIUM DIGITATUM* UTILIZING GLUCOSE-2-C^{14} AND GLUCOSE-6-C^{14}

Fractions	Percent	
	Glucose-2-C^{14}	Glucose-6-C^{14}
Respiratory CO_2	12	6
Cells	41	34
Medium	37	25
Sample aliquots	10	10
Total recovery	100	75

Weight of cells per flask: 1.5 grams.

Air-flow rate per flask: 400-500 ml per minute.

Level of substrate per flask: 750 mg.

Level of radioactivity per flask: 50 uc.

Temperature: 30°C.

TABLE 4

DISTRIBUTION OF C^{14} IN ASPARTIC ACID FROM PENICILLIUM DIGITATUM UTILIZING C^{14} LABELED SUBSTRATES

		Substrates		
	$C^{14}O_2$*	Acetate-1-C^{14}*	Glucose-2-C^{14}	Glucose-6-C^{14}
Whole Molecule in CPM/mM of Amino Acid			$.76 \times 10^5$	$.95 \times 10^5$
COOH	24%	25%	23%	20%
$CHNH_2$	18	36	32	35
CH_2	0	0	21	42
COOH	58	40	25	4

*Data taken from the Ph.D. thesis of Dr. E. P. Noble (54).

TABLE 5

DISTRIBUTION OF C^{14} IN GLUTAMIC ACID FROM *PENICILLIUM DIGITATUM* UTILIZING C^{14} LABELED SUBSTRATES

	Substrates			
	$C^{14}O_2$*	Acetate-1-C^{14} *	Glucose-2-C^{14}	Glucose-6-C^{14}
Whole Molecule in CPM/mM of Amino Acid			2.35 x 10^5	2.91 x 10^5
COOH	85 %	28%	19 %	8 %
$CHNH_2$	13	38	36	89
CH_2				
CH_2				
COOH	2	33	45	3

* Data taken from the Ph.D. thesis of Dr. E. P. Noble (54).

TABLE 6

DISTRIBUTION OF C^{14} IN ALANINE, GLYCINE AND SERINE FROM *PENICILLIUM DIGITATUM* UTILIZING C^{14} LABELED SUBSTRATES

Amino Acid		Percent Distribution	
Whole molecule in CPM/mM of amino acid		Substrates Glucose-2-C^{14}	Glucose-6-C^{14}
Alanine	Total	1.62×10^5	1.62×10^5
	COOH	11 %	5 %
	$CHNH_2$	78	8
	CH_3	11	87
Glycine	Total	$.39 \times 10^5$	$.12 \times 10^5$
	COOH	17 %	31 %
	CH_2NH_2	83	69
Serine	Total	$.46 \times 10^5$	$.80 \times 10^5$
	COOH	7 %	4 %
	$CHNH_2$	41	6
	CH_2OH	52	90

DISCUSSION

TIME COURSE STUDIES

Nature of the pathways for glucose utilization in Penicillium digitatum

The nature of the catabolic pathways for glucose utilization by P. digitatum was studied by a series of time course experiments. The relative radiochemical recovery in the respiratory CO_2 from proliferating cells metabolizing specifically labeled glucoses (Figure 3) allowed the identification of the nature of the pathways involved in the catabolism of glucose by this organism.

The preferential conversion of C-1 of glucose to respiratory CO_2, as compared to that of C-6, undoubtedly reflects the operation of an alternate pathway other than classical EMP glycolysis. Currently, there are two known pathways which could be responsible for this observation. Interestingly, there is no evidence in the literature for the extensive operation of one of these alternate pathways (the Entner-Doudoroff pathway) in an organism which is known to utilize the glycolytic pathway. In the case of the operation of the Entner-Doudoroff pathway, it is expected that eventually 2 moles of pyruvate will be formed from one mole of

glucose, with the methyl carbon being formed from C-3 or 6, carbonyl carbon from C-2 or 5, and carboxyl carbon from C-1 or 4 of glucose. Inasmuch as glucose-3,4-C^{14} was used in the present experiments, one would expect the radiochemical recovery of C-3 and 4 of glucose in the respiratory CO_2 to be no more than the average of that of C-1 and C-6 from glucose-1-C^{14} and glucose-6-C^{14} respectively. This is under the assumption that pyruvate would be decarboxylated, followed by the metabolism of the resulting acetate by way of citric acid cycle processes. That the radiochemical recovery from glucose-3,4-C^{14} in the respiratory CO_2 is greater than that from the average of glucose-1-C^{14} and glucose-6-C^{14} respiratory CO_2 is shown in Figure 3 and Table 1. Consequently, it appears that the observed preferential conversion of C-1 of glucose to CO_2 is the result of the operation of a 1-5 split pathway of glucose, probably of the phosphogluconate decarboxylation type, rather than the Entner-Doudoroff pathway. Further evidence against the Entner-Doudoroff pathway occurring in this organism is the extent of the cellular incorporation from the various substrates employed in these experiments. Cellular incorporation of C-6 of glucose should be much greater than C-1; however, Table 1 shows that this is not the case. In addition, C-2 of

glucose should be incorporated into the cells to a lesser degree than C-6, but C-2 of glucose actually incorporates to a slightly greater extent than C-6, again referring to Table 1. In regards to C-3,4 incorporation into the cells, it is evident that the incorporation from these carbons is much less than would be expected if this pathway were operating.

Evidence presented above indicates that for the glucose-3,4-C^{14} curve to have as high an interval activity recovery as it does requires that both carbons 3 and 4 be contributing to the respiratory CO_2 to a major extent. This is strong evidence for the EMP glycolytic pathway being a major pathway of glucose utilization in P. digitatum. The rapid oxidation of carbons 3 and 4 of glucose to respiratory CO_2 via glycolysis would be through the formation of two trioses, the conversion of these trioses to two molecules of pyruvate, which in turn would be decarboxylated to two molecules of CO_2, carbons 3 and 4, and the formation of two molecules of acetate.

It is true that if all the pyruvate formed via glycolysis were decarboxylated, the glucose-3,4-C^{14} interval activity recovery in the respiratory CO_2 would be much higher than the observed value and would be comparable to that found in the case of bakers' yeast

(76). An explanation for this difference may well be in the amount of glucose-3,4-C^{14} activity incorporated into the cellular constituents of P. digitatum (Table 1) as compared to yeast (70). This consequently reflects the important role played by C_3 units in the biosynthetic schemes of this organism.

The percent interval $C^{14}O_2$ recovery from glucose-2-C^{14} is considerably lower than that of glucose-1-C^{14}, as shown in Figure 3. This finding may indicate that the newly formed pentose was not metabolized extensively to respiratory CO_2 via the pentose cycle pathway. In fact, evidence provided by Noble (54) and from this work, give strong support for the conservation of the C-2 of glucose via two possible mechanisms. First, the C_3 derived from glycolysis may condense with CO_2 to form C_4 intermediates which would conserve both the C-6 and C-2 of glucose to the same extent, and second, an active C_2 unit being formed from the first two carbons of the pentose formed from phosphogluconate decarboxylation can undergo several reactions. Noble (54) has proposed an active C_2 unit undergoing a double CO_2 fixation by this organism to form C_4 intermediates. In addition to this mechanism, Wong and Ajl (77) have isolated an enzyme from acetate-grown Escherichia coli which is responsible for the condensation

of acetate and glyoxylate to malic acid. Such a conversion may well involve the active C_2 unit found in this organism. Other possible reactions which would allow the preferential incorporation of C-2 of glucose compared to C-6 into cellular constituents have been shown in a review article on carbohydrate metabolism by Gunsalus *et al* (36).

The extent to which C-6 of glucose appears in the respiratory CO_2 (Figure 3) is probably due to conventional glycolysis in combination with an active citric acid cycle. However, it is entirely possible that by recombination of trioses through reverse aldolase activity C-6 of glucose would become C-1 of glucose and then a portion rapidly lost to CO_2 by phosphogluconate decarboxylation. The above process does not appear to occur to any great extent in this case, because, if this mechanism were operative, C-6 would undoubtedly be converted to respiratory CO_2 to a greater extent than C-2 of glucose, which is not observed in the present work (Figures 3 and 4).

Effects of endogenous respiration upon respiratory CO_2 patterns derived from specifically labeled substrates

It is known that microorganisms vary considerably in their ability to assimilate and dissimilate carbon sources. The ability of an organism to assimilate any

appreciable amount of a carbon source is probably directly related to both its endogenous capacity and substrate metabolism. And in turn the amount of endogenous respiration which a microorganism displays could well be related to its cellular protein content. Interestingly enough, a microorganism with a "high" cellular protein content has a low endogenous respiration, as in the case of yeast (70), whereas, a microorganism with a "low" cellular protein content will have an appreciable to extensive endogenous respiration, as in the case of microorganisms such as P. digitatum (70), P. chrysogenum (66), Streptomyces griseus (6), and Aspergillus niger (70).

The lack of extensive endogenous respiration in a microorganism, such as yeast, is shown by its depletion curves of respiratory CO_2 substrate after C^{14} incorporation into the cells. Essentially, a reversal of the citric acid cycle is observed in the respiratory patterns from such organisms as extensive depletion is brought about. In the case of microorganisms with a high endogenous respiration, depletion does not bring about reverse citric acid cycle activity nearly as rapidly, if at all.

Stout and Koffler (66) reported extensive endogenous respiration in P. chrysogenum during the

utilization of substrate glucose. Further evidence of high endogenous respiration in this organism is indicated by the slow rate of conversion of substrate activity to CO_2, as shown in Figure 7. In fact, the peak in the $C^{14}O_2$ interval activity recovery curve was realized after the exhaustion of glucose in the medium. The endogenous respiration pattern in P. digitatum, is similar to that for P. chrysogenum, however, endogenous respiration does not appear to be as extensive in this organism.

In the calculations used for determination of the relative contribution of pathways, based on the method of Wang et al (76), a correction can be made for the incorporation of glucose per se into cellular polysaccharide. With P. digitatum, the amount of incorporation of glucose into the cells during the time course experiments was determined to be approximately 20% of the total activity administered. It should be mentioned that it has been assumed glucose per se was incorporated into the cellular polysaccharide, since it is possible that the incorporation occurred by reforming hexose from metabolic intermediates.

Utilization of tracer amounts of acetate and pyruvate in the presence of glucose by P. digitatum

In order to understand better the fate of the

possible intermediates of glucose metabolism in this organism, the present series of experiments were carried out. The utilization of tracer amounts of acetate and pyruvate, in the presence of glucose, as shown in Figures 5 and 6, is indicative that these compounds are intermediates in the metabolism of glucose by P. digitatum. The theoretical maximum ratio of

$$\frac{C^{14} \text{ from acetate-1-}C^{14}}{C^{14} \text{ from acetate-2-}C^{14}} = 2.0$$

may be realized if there is an equal dilution of both carbons of acetate by endogenous material in the course of its metabolism to CO_2 via typical citric acid cycle processes. Using tracer amounts of specifically labeled acetates, the ratio at the end of 4 hours was found to be 1.5. Very similar ratios were found by Noble (54) in the growth of this organism on labeled acetates as the sole source of carbon. The lower ratio, as compared to the theoretical maximum, is undoubtedly due to the drainage of citric acid cycle intermediates for biosynthetic purposes.

Tracer amounts of labeled pyruvates in the presence of a glucose substrate to P. digitatum, were practically exhausted from the incubation medium as is shown in Table 1. Pyruvate-1-C^{14} data show that approximately 90% of the pyruvate metabolized was decarboxylated to CO_2 and a C_2 unit. However, in lieu of the knowledge

on the mechanism of CO_2 fixation in this organism, it is entirely possible that trioses derived from glucose may not be decarboxylated to the same extent. The above C_2 unit was then metabolized via a citric acid cycle as indicated by the ratio of

$$\frac{C^{14}O_2 \text{ from pyruvate-2-}C^{14}}{C^{14}O_2 \text{ from pyruvate-3-}C^{14}} = 1.6$$

This ratio is based on the percent-cumulative recoveries in the respiratory CO_2 at the end of the fourth hour. The value of 1.6 for this ratio is in good agreement with that of acetate which was shown to be 1.5.

In summary, the major pathways for glucose catabolism in P. digitatum, under aerobic and proliferating conditions, have been established. The major pathway is EMP glycolysis in combination with an active citric acid cycle. An oxidative pathway, which involves the preferential loss of C-1 of glucose, is contributing to an appreciable extent.

Estimation of pathways

The quantitative estimation of the relative contributions made by the EMP glycolysis pathway and the oxidative pathway in the utilization of glucose by P. digitatum has been made by the use of two methods employing respiratory CO_2 data.

The method of Wang et al, which has been applied to the metabolism of bakers' yeast (76) and Streptomyces griseus (6), was employed in these experiments. The point of exhaustion of glucose within the cells was determined as previously described and was found to occur at approximately 4 hours. The following assumptions have been used for the calculations which are based upon the cumulative radiochemical recoveries of metabolic CO_2 from each labeled glucose at the end of 4 hours: (1) glucose is metabolized to CO_2 either by a 1-5 cleavage of glucose, or the EMP glycolytic pathway in combination with the citric acid cycle, (2) any triose formed via the EMP pathway has an equal opportunity to be derived from glucose carbons 1 to 3 or 4 to 6 and these trioses are equivalent to each other in respect to further metabolic reactions, (3) carbons 3 and 4 of glucose are rapidly converted to respiratory CO_2, (4) preferential decarboxylation of carbon 1 of glucose to respiratory CO_2 is also a rapid process, (5) in the course of the rapid utilization of glucose by a biological system, there cannot be appreciable reverse aldolase activity.

The calculation of the distribution of the metabolism of administered glucose between individual pathways is made in the following manner: Let G_1, G_2, $G_{3,4}$ and G_6

be the cumulative radiochemical recoveries of the respective labeled glucose in respiratory CO_2 at the time of substrate exhaustion within the cell. The fraction of glucose catabolized via phosphogluconate decarboxylation, G_p, is

$$G_p = \frac{G_1 - G_6}{T - T'} \qquad (1)$$

where T is the total activity of each labeled substrate administered to the medium, and T' is the amount of glucose substrate activity which has been incorporated into the polysaccharide *per se* and thus does not involve either pathway.

Since it has been assumed that only two pathways exist, the fraction of the glucose utilized by the EMP glycolytic pathway, G_e, is:

$$G_e = 1 - G_p \qquad (2)$$

Fate of pyruvate

The fate of pyruvate derived from administered glucose can be calculated by the method of Wang *et al* (76). Two factors must be considered which affect the amount of pyruvate available on a radiochemical basis for further metabolism: (1) in the preparation of glucose-3,4-C^{14} by biological methods, Woods *et al* have shown, by chemical degradation of the glucose molecule, that randomization has occurred to the

extent of 3% into carbons 1,2,5, and 6; (2) some glucose-3,4-C^{14} activity was remaining in the medium after the termination of the experiment and can be assumed to be derived essentially from the amino acids such as aspartic and glutamic acids as was reported by Noble (54). It can be assumed that the majority of this activity is derived from glucose by the glycolytic pathway and may or may not involve a C_3 plus C_1 condensation.

The theoretical amount of pyruvate which can be formed from glucose via glycolysis is 2 moles per mole of glucose. In addition, for each mole of pyruvate decarboxylated to acetate, one should obtain one mole of CO_2 from C-3 or C-4. Thus, the fraction of pyruvate converted to acetate should equal the fraction of pyruvate decarboxylated, P_d:

$$P_d = \frac{2\ G_{3,4}}{2\ G_e \times .97} = \frac{G_{3,4}}{.97\ G_e \times T} \qquad (3)$$

The factor, 0.97, is a correction factor applied for the partial randomization in glucose-3,4-C^{14}. By difference, the fraction of pyruvate which remains intact, due to C_3 plus C_1 condensation or any other process which utilizes the pyruvate molecule

as such, will be:

$$P_i = G_e - m - P_d \qquad (4)$$

In the above equation, m, represents the glucose-3,4-C^{14} activity remaining in the medium, regardless of its origin.

Fate of acetate

Assuming acetate is formed exclusively from glycolysis, it is possible to calculate the efficiency of the conversion of the carboxyl carbon of acetate to respiratory CO_2, R_c, with the following equation:

$$R_c = \frac{G'_2}{G'_{3,4}} \qquad (5)$$

The efficiency of utilization of the carboxyl carbon of acetate in biosynthesis is calculated by difference:

$$S_c = 1 - R_c \qquad (6)$$

The efficiency of the methyl carbon being oxidized to respiratory CO_2, R_m, is given by:

$$R_m = \frac{G'_6}{G'_{3,4}} \qquad (7)$$

The efficiency of utilization of the methyl carbon of acetate, S_m, in the biosynthesis is obtained by difference:

$$S_m = 1 - R_m \qquad (8)$$

The estimations of the metabolic pathways of glucose utilization by P. digitatum according to the methods discussed above are given below.

G_1	31,017 CPM
G_2	20,711 CPM
$G_{3,4}$	28,473 CPM
G_6	14,896 CPM
T	86,545 CPM
T'	17,309 CPM
Relative contribution by glycolysis, G_e (Corrected for polysaccharide incorporation)	77%
Relative contribution of phosphogluconate decarboxylation, G_p	23%
Extent of pyruvate decarboxylated oxidatively, P_d	29%
Extent of pyruvate remaining intact at the specified time, P_i	49%
Fraction of acetate carboxyl respired as CO_2, R_c	75%
Fraction of the carboxyl carbon of acetate utilized in biosynthesis, S_c	25%
Fraction of the methyl carbon of acetate respired as CO_2, R_m	49%
Fraction of methyl carbon of acetate utilized in biosynthesis, S_m	51%

The late Dr. Korkes, author of a recent review on carbohydrate metabolism (47), presented several equations for the calculation of pathway participation

with respect to the origin of respiratory CO_2 from glucose. The method is primarily based on the data of specific activities of respiratory CO_2 as derived from glucose-1-C^{14} and glucose-6-C^{14}. The method assumes that all carbon atoms of glucose are converted to respiratory CO_2 at a rapid rate and equal rate in the glycolytic pathway. When the oxidative pathway is operative, the assumption is made that the C-1 of glucose is rapidly and exclusively converted to respiratory CO_2.

In the case of P. digitatum, it is evident that the rate of conversion of the carbons of glucose via glycolysis, in combination with citric acid cycle processes, is by no means uniform and hence an expression, N, has been derived to correct for this difference in rates. In developing the following equations, another factor, P, has also been included which represents the average combustion efficiency of the carbon atoms of glucose to CO_2 via EMP-TCA processes and thus allows for the estimation of the amount of glucose metabolized via glycolysis rather than the fraction of respiratory CO_2 which has been derived via glycolysis and the citric acid cycle.

The expressions derived are as follows:

$$R = \frac{X_6}{X_1} = \frac{G''_6}{G''_1} = \frac{\frac{AE}{N}}{\frac{AS}{1} + \frac{AE}{N}}$$

$$S = \frac{1 - R}{1 + (N - 1) R} \qquad E = 1 - S$$

$$G'_p = \frac{S}{S + E/6P} \qquad G'_e = 1 - G'_p \quad (9)$$

X_6, X_1 equals the specific activity of CO_2 from glucose-1-C^{14} or glucose-6-C^{14}

G''_6, G''_1 equals the interval accumulated activity from glucose-1-C^{14} or glucose-6-C^{14}

A equals the specific activity of the labeled glucose (CPM/mM)

E equals the fraction of respiratory CO_2 derived via EMP-TCA activity

S equals the fraction of respiratory CO_2 derived via the phosphogluconate decarboxylation

G'_e equals the fraction of administered glucose metabolized via EMP-TCA processes

G'_p equals the fraction of administered glucose metabolized via phosphogluconate decarboxylation

N equals the relative rate of conversion of C-1 or C-6 of glucose to respiratory CO_2 via EMP-TCA

P equals the combustion efficiency of glucose to respiratory CO_2 via EMP-TCA

The fundamental assumptions of these calculations are identical to those for the expression of Wang *et al* (76).

The term N is used to designate the combustion efficiency of C-6 of glucose to respiratory CO_2. The theoretical value used by Korkes for N was 6, assuming that the conversion of all carbons of glucose to respiratory CO_2 was at an equal and rapid rate via EMP-TCA. In the present expression, N was derived in the following manner:

$$N = \frac{2\,G_2 + 2\,G_{3,4} + 2\,G_6}{G_6} \qquad (10)$$

The following table indicates that the N values level off as the system approaches a steady state for the C^{14} isotope.

Time in hours	N value
1	11.5
2	10.0
3	9.1
4	8.6
5	7.9
6	7.6
7	7.4
8	7.3
9	7.2
10	7.1

The variation of the N value from the theoretical value of 6 is due mainly to the more rapid rate of conversion of carbons 3 and 4 of glucose to respiratory CO_2 than carbons 2 and 5 or carbons 1 and 6 via the EMP-TCA processes.

By the use of the P term shown in equation 9, it is possible to convert the fraction of CO_2 originating by way of phosphogluconate decarboxylation, S, to the corresponding fraction of glucose metabolized via this pathway, G'_p

$$G'_p = \frac{CO_2 \text{ via PGD pathway} \times 1}{CO_2 \text{ via EMP-TCA/6P} + CO_2 \text{ via PGD} \times 1} \quad (11)$$

$$= \frac{S}{E/6P + S}$$

The term P is derived in the following manner:

$$P = \frac{2\,G_6 + 2\,G_2 + 2\,G_{3,4}}{6 \times (T - T')} \qquad (12)$$

where T is the total activity of each labeled substrate administered to the medium, and T' is the amount of glucose substrate activity which has been incorporated into the polysaccharide <u>per se</u> and thus does not involve either pathway.

The following table shows the relative distribution of pathways as calculated by the methods just presented.

Relative distribution of pathways for P. digitatum
Time of turnover equilibrium was at 4 hours.

G_1	31,017 CPM
G_2	20,711 CPM
$G_{3,4}$	28,473 CPM
G_6	14,896 CPM
T	86,545 CPM
T'	17,309 CPM
R (X_6/X_1)	.480
N (Eq 10)	8.6
P (Eq 12)	.309
G_p	23%
G_e	77%
S (Eq 9)	.112
G_p' (Eq 11)	19%
G_e' (Eq 9)	81%

In comparing the values calculated for the relative contribution of pathways, one should bear in mind that the equations employed have been derived from different concepts. Hence, the values presented for the percent distribution of pathways represent minimum and maximum values. When consideration is given to the basic understandings required for each of these calculations,

it becomes apparent that the results obtained by these two methods agree reasonably well.

In conclusion, the present work indicates that the relative contribution of an oxidative pathway in proliferating cells of P. digitatum is between 19-23% while the contribution from a glycolytic pathway is 77-81%.

Nature of the pathways for glucose utilization in Penicillium chrysogenum

The interval activity recovery vs. time curves of the respiratory CO_2 as shown in Figure 7, for P. chrysogenum, and those for P. digitatum (Figure 3) illustrate the similarity of the catabolic pathways in these two species of Penicillia. One of the more noticeable differences is the effect of endogenous respiration. The delay of the appearance of C^{14} in the respiratory CO_2 for P. chrysogenum provides evidence for its high endogenous respiration as reported by Stout and Koffler (66).

The general pattern of the interval activity recovery curves indicate a similar complement of pathways for glucose metabolism are operating in this organism as were found in P. digitatum. It is noted that the recovery of glucose-3,4-C^{14} activity in the respiratory CO_2 is much lower than that for glucose-1-C^{14} in this organism as compared to the small differences observed in the case of P. digitatum. This suggests, either less extensive EMP-TCA processes are functioning, or more C_3 units formed via the EMP pathway are being utilized in the biosynthesis of cellular constituents. Calculations presented in a later section are in accord with less participation of the EMP pathway and a relatively greater amount of the substrate glucose being metabolized by P. chrysogenum via an oxidative pathway than is the case with P. digitatum.

Utilization of tracer amounts of acetate and pyruvate in the presence of glucose by P. chrysogenum

Experiments similar to those described for P. digitatum were carried with P. chrysogenum to determine the possible roles played by acetate and pyruvate as intermediates in glucose metabolism by this organism.

The utilization of tracer amounts of acetate in the presence of glucose (Figure 9) by this organism gave a value of 1.4, at the end of 5 hours, for the

ratio,

$$\frac{C^{14}O_2 \text{ from acetate-1-}C^{14}}{C^{14}O_2 \text{ from acetate-2-}C^{14}}$$

in the respiratory CO_2. This provided evidence for the operation of a citric acid cycle in the metabolism of acetate by P. chrysogenum and is in agreement with the work reported by Goldschmidt et al (66).

Tracer amounts of labeled pyruvates were metabolized in the presence of glucose by this organism as shown in Figure 9. The rate of utilization of pyruvate from the medium was considerably slower than was found with P. digitatum, however an examination of the ratio

$$\frac{C^{14}O_2 \text{ from pyruvate-2-}C^{14}}{C^{14}O_2 \text{ from pyruvate-3-}C^{14}}$$

in the respiratory CO_2 at the end of 5 hours gives a value of 1.4. This value agrees with the value from the ratio of acetate-1-C^{14} to acetate-2-C^{14} shown above. From these values it is indicated that pyruvate is decarboxylated to CO_2 and a C_2 unit which is then metabolized via citric acid cyclic processes.

The estimation of pathways in P. chrysogenum

The quantitative estimation of the relative contribution of the catabolic pathways for glucose utilization by P. chrysogenum has been calculated by the two methods discussed under a similar section on

P. digitatum.

While the time interval used for glucose exhaustion within the cell for *P. digitatum* was 4 hours, the equivalent point for *P. chrysogenum* was found to be 5 hours. The amount of glucose *per se* incorporated into the polysaccharide of this organism, while it is indicated to be quite extensive, was not determined and therefore the equations employed are not corrected for this factor. As it will be seen later in the calculations presented, using equation 1 without the application of this correction, the calculated percent contribution of EMP pathway in overall glucose catabolism tends to be higher than the corrected value and the reverse is true for that of the 1-5 cleavage pathway. However, in the case of calculation according to equation 9, one finds the calculated value for EMP participation is higher than the value obtained by applying the correction for direct incorporation of glucose into the cells. In all other aspects the calculations and assumptions are the same for both organisms.

The estimation of the metabolic pathways of glucose utilization by P. chrysogenum according to the method of Wang et al (76).

G_1	35,171 CPM
G_2	15,472 CPM
$G_{3,4}$	23,540 CPM
G_6	12,635 CPM
T	86,545 CPM

Relative contribution by glycolysis, G_e (Uncorrected for polysaccharide incorporation)	74%
Relative contribution of phospho-gluconate decarboxylation, G_p (Uncorrected for polysaccharide incorporation)	26%
Extent of pyruvate decarboxylated oxidatively, P_d	38%
Extent of pyruvate remaining intact at the specified time, P_i	31%
Fraction of acetate carboxyl respired as CO_2, R_c	66%
Fraction of the carboxyl carbon of acetate utilized in biosynthesis, S_c	34%
Fraction of the methyl carbon of acetate respired as CO_2, R_m	54%
Fraction of the methyl carbon of acetate utilized in biosynthesis, S_m	46%

Relative distribution of pathways for P. chrysogenum calculated by the method derived from Korkes' review (47). Time of turnover equilibrium was at 5 hours.

G_1	35,171
G_2	15,472
$G_{3,4}$	23,540
G_6	12,635
T	86,545
R (X_6/X_1)	.359
N (Eq 10)	8.18
P (Eq 12)	.199
G_p (Eq 1)	26%
G_e (Eq 2)	74%
S (Eq 9)	.179
G'_p (Eq 11)	21%
G'_e (Eq 9)	79%

The results from the two methods of calculating the relative participation of pathways indicate that the amount of participation by the oxidative pathway in the catabolism of glucose by this organism lies between 21-26%, while that for the EMP-TCA pathway is 74-79%. As previously mentioned, no attempt was made to correct

for the amount of substrate glucose converted *per se* directly into polysaccharide by this organism.

Experiments by Koffler *et al* (46) on the catabolism of glucose by *P. chrysogenum* show, by the use of specifically labeled substrates, a much slower rate of conversion of carbons 2, 3, 4, and 6 to the respiratory CO_2 in comparison to carbon 1 of glucose than we have observed with the same organism (Figure 7). In addition, Koffler and co-workers have reported tracer experiments that indicate during a complete growth period, 0-40 hours, 49% of C-1 of glucose was converted to the respiratory CO_2 and in contrast to this, only 8% of the remaining carbons of glucose, carbons 2-6, were recovered in the CO_2. This implies that on a molar basis, for every mole of glucose converted to CO_2 approximately 5 moles of glucose have been assimilated by the mycelia. This results in an assimilation/dissimilation ratio of 5 to 1. Such a ratio is indeed surprisingly high as it has been shown that bacteria and yeast generally have an assimilation/dissimilation ratio of 1 to 5 and in the case of molds a ratio ranging from 1 to 2 to approximately 2 to 1 is considered to be a maximum value (27). Without further study it is impossible at the present time to explain this observation from an energy standpoint.

It is of interest that deFiebre and Knight (19) were unable to observe any appreciable amount of glucose-2-C^{14} activity appearing in the respiratory CO_2 (0.2%) from P. chrysogenum cells which have been previously traumatized by blending, yet glucose-1-C^{14} activity recovery in the respiratory CO_2 was 30% of that administered. It can be inferred from this work that experimental conditions, including cell treatment, may have rendered the cells essentially incapable of metabolizing carbons 1-6 of glucose to respiratory CO_2 by any other pathways but did not appreciably effect the preferential loss of C-1 of glucose by this organism. This would then give rise to a rather distorted picture of the relation distribution of catabolic pathways in glucose metabolism by this organism.

INCORPORATION OF GLUCOSE-2-C^{14} AND GLUCOSE-6-C^{14} INTO THE CELLULAR CONSTITUENTS OF P. DIGITATUM

Incorporation experiment

The distribution of glucose-2-C^{14} and glucose-6-C^{14} activity in the various fermentation fractions such as, respiratory CO_2, fermentation products (medium), and the cells is given in Table 3 for these cellular incorporation experiments. It should be noted that the aeration rate was somewhat lower in proportion to the amount of cells utilized in these experiments as compared to the time course experiments. This limitation in aeration rate increased the amount of fermentation products found in the medium as compared to the time course experiments, however, it was felt that no appreciable difference would be inflicted on the biosynthetic mechanisms employed by this organism.

Interestingly, a fair amount of the activity in the medium was found to be in a fraction which is of a volatile nature and neutral to litmus. Since our main interest in this work has been the biosynthesis of the amino acids, no attempts were made to characterize these fermentation products.

Comparison of the specific activity of some of the amino acids isolated from P. digitatum

Glycine, serine and alanine

Of these three amino acids, alanine appears to be derived more directly from the labeled substrates employed, as shown by the specific activity data given in Table 6. However, from a standpoint of specific activity, there does not appear to be a close interrelation between alanine and serine or glycine. Glycine and serine appear to be related to each other somewhat as indicated by their respective specific activities. Glycine appears to have derived the major portion of its C^{14} labeling from glucose-6-C^{14} via randomization, as would be expected if glycine is formed via serine. In addition, however, the specific activities for glycine and serine from glucose-2-C^{14} are about equal indicating an additional pathway is involved in the biosynthesis of glycine.

Glutamic and aspartic acids

The relative specific activities of both aspartic acid and glutamic acid from glucose-2-C^{14} and glucose-6-C^{14} indicate that these amino acids have incorporated slightly more of C-6 of glucose than C-2. This could be an indication of the importance of a citric acid cycle in the biosynthesis of these amino acids. However, the specific activity of aspartic acid is sufficiently lower than that of glutamic acid to suggest that unless

there is a considerable difference in the pool sizes of these amino acids they may be related only indirectly through citric acid cyclic activity.

Origin of the carbon skeletons of glycine, serine and alanine

Alanine appears to be derived directly by transamination of pyruvate formed via the glycolytic pathway from glucose. It is of interest that in the case of alanine, both of the C_3 units formed from a molecule of glucose have contributed equally to provide the C_3 units for transamination. This is shown by the specific activity data given in Table 6. However, this does not appear to have occurred in the case of serine since the specific activity of the serine derived from glucose-2-C^{14} is considerably less and with much more randomization of the isotope than that derived from the glucose-6-C^{14} substrate.

The C^{14} isotope labeling pattern of glycine and serine indicate a limited interconversion of these two amino acids. In fact, it appears quite certain that glycine and serine may have been synthesized via different routes in this organism. The extent of the randomization of the C^{14} labeling in serine derived from glucose-2-C^{14} indicates quite strongly that while some direct transamination could have occurred with a

C_3 intermediate derived from glycolysis, other biosynthetic schemes may be operating in this case. A possible mechanism would be the formation of an active C_2 unit by way of a ketolase cleavage (42) of ribose, which is then followed by a subsequent condensation with a C_1 unit giving rise to the precursor of serine. In this manner it is possible to explain why the majority of the C^{14} labeling is in the hydroxymethyl carbon of serine. In addition, it appears that serine formation from the bottom C_3 of glucose, as shown using glucose-6-C^{14} substrate, was derived directly via the transamination of hydroxypyruvic acid or phosphohydorxypyruvic acid.

Glycine has been shown to be formed via glycolaldehyde from ribose-5-phosphate in a soluble extract from spinach leaves (53). Using 1-C^{14}-ribose-5-phosphate, Weissbach and Horecker (53) found the distribution of C^{14} activity within the glycine molecule to be 8 % in the methylene carbon and 16% in the carboxyl carbon. This is in very close agreement with the labeling pattern derived from glucose-2-C^{14} for glycine in P. digitatum, as shown in Table 6. It can be visualized by a loss of C-1 of glucose-2-C^{14} to give 1-C^{14}-ribose-5-phosphate which then can undergo the sequence of reactions described by Weissbach and

Horecker. In addition to the above scheme, the labeling pattern of glycine from glucose-6-C^{14} suggests the possibility of at least a part of the glycine within the cell can be synthesized via a loss of the beta carbon of serine.

Aspartic acid

The C^{14} distribution patterns found in aspartic acid from various labeled substrates (Table 4) give strong evidence that aspartic acid is synthesized in this organism by more than one pathway. Therefore any consideration given to C^{14} labeling patterns for this amino acid must be in terms of relative contributions of different pathways. The following generalizations can be drawn from the C^{14} patterns shown in Table 4 which gives some insight as to the origin of the carbon atoms of aspartic acid. The beta carboxyl of aspartic acid appears to be closely related to metabolic CO_2. The methylene carbon is most closely related to the methyl carbon of a C_2 unit (such as glycolaldehyde which can be derived by pentose cleavage of the ketolase type ([42])) or a C_3 unit (such as pyruvate derived from glycolytic cleavage of glucose). The amino carbon is probably derived from (1) the carboxyl carbon of a C_2 unit from glucose by way of a 1-5 cleavage followed by

a ketolase cleavage of the resulting pentose and finally the subsequent CO_2 fixation of the C_1 plus C_2 plus C_1 type and (2) the carboxyl carbon of a C_3 fragment of glucose via glycolytic cleavage and a subsequent CO_2 fixation of the C_3 plus C_1 type. The alpha carboxyl of aspartic acid appears to be in equilibrium with CO_2 and the carboxyl groups of both C_2 and C_3 units.

Thus, with the above information one can only speculate at the present time as to the major contributing pathways in the formation of this amino acid. The following schemes could be occurring to limited extents: (1) typical EMP-TCA cycle acitivity, (2) a C_1 plus C_3 condensation followed by limited TCA recycling, (3) double fixation of CO_2 onto a C_2 unit and (4) a C_2 plus C_2 condensation between dissimilar C_2 units of the type reported by Wong and Ajl (77), which involves the condensation of acetate and glyoxylate.

Glutamic acid

The evidence furnished by the partial degradation of glutamic acid derived from various labeled substrates (Figure 5) indicates a rather clear cut picture for the biosynthesis of this amino acid in P. digitatum. Bearing the labeling pattern of aspartic acid (and hence oxalacetate) in mind, it is evident that from

each substrate employed, typical TCA cyclic processes with limited recycling could furnish the C^{14} labeling patterns shown in Figure 5. The fact that TCA recycling may not be so extensive is supported by the observation that some glutamic acid has been shown to accumulate as a fermentation product in the medium during the growth of *P. digitatum*.

In view of the complexity observed in the isotopic distribution patterns of various amino acids discussed above, it is clear that the mechanism for the biosynthesis of these amino acids, which in turn reflects the mechanism of carbohydrate metabolism, in mold is far more involved than that in yeast or *E. coli*. Therefore, considerably more studies are required to elucidate the basic metabolic functions prevailing in this organism.

SUMMARY

Time course experiments utilizing C^{14}-labeled glucoses were employed to quantitatively estimate the nature and extent of the catabolic pathways for glucose utilization by Penicillium digitatum.

1. An oxidative pathway accounts for 19-23% of the glucose oxidized to respiratory CO_2 by this organism.

2. EMP glycolysis in combination with typical citric acid cyclic processes is the metabolic pathway for 77-81% of the total glucose utilized by this organism.

3. A comparative study with P. chrysogenum indicates that 21-26% of the glucose utilized was via an oxidative pathway while 74-79% was metabolized by the EMP-TCA processes.

4. Endogenous cellular carbon sources appear to play an important role in the metabolism of external carbon sources by molds.

5. Specifically labeled pyruvates and acetates were also used in the elucidation of the catabolic pathways of intermediates derived from glucose breakdown.

6. Incorporation of glucose-2-C^{14} and glucose-6-C^{14} into the cellular constituents of P. digitatum

gives further evidence of an important role being played by an "active" C_2 unit in the biosynthetic schemes of this organism.

7. Alanine appears to be derived via transamination of glycolytically formed pyruvate. Glycine and serine biosynthesis appears to involve an active C_2 unit, possibly glycolaldehyde, and limited interconversion of these two amino acids.

8. Aspartic acid is derived by more than one pathway, the possible pathways being: (1) a C_1 plus C_3 condensation involving CO_2 fixation, (2) C_2 plus C_2 condensation of dissimilar C_2 units (3) double CO_2 fixation onto a C_2 unit and (4) via TCA cyclic processes.

9. Glutamic acid C^{14} labeling patterns give evidence for this amino to be formed via TCA processes involving limited recycling.

In conclusion, it has been shown by this work that the metabolic pathways in this organism are much more complex than those found in yeast or E. coli. Some of the metabolic functions of an active C_2 unit have been suggested, however more work needs to be done to determine the exact nature of these schemes.

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