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ENDOGENOUS METABOLISM OF <u>POLYPORUS</u> <u>SULFUREUS</u>. UTILIZATION OF EXOGENOUS CARBOHYDRATES AND FATTY ACIDS

BY

SHU-MEI WANG, 1942-

A

THESIS

submitted to the faculty of

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ABSTRACT

A strain of Polyporus sulfureus from the collection at the University of Missouri at Rolla was used in this investigation. Organisms of this genus have been reported to produce eburicoic acid in good yield and this was confirmed for the organism used in this study by the isolation and identification of eburicoic acid from the mycelium.

Growth studies of this organism showed that 4% malt extract is a better medium than synthetic media containing asparagine, nitrate or nitrate plus 0.5% yeast extract as nitrogen sources. An examination of the pH of the culture medium led to the conclusion that regardless of whether or not the medium was buffered good growth of the organism caused a decrease in the pH of the medium. Eburicoic acid present in the mycelium of this organism did not account for this decrease in the pH of the medium.

Conventional manometric techniques were used for the respiratory studies. Substrates labeled with ^{14}C were used as a supplement to this technique. Results from these experiments indicated that this organism cannot utilize glucose directly as an energy source. Acetate and succinate stimulated oxygen uptake of the mycelium of this organism whereas pyruvate inhibited it. The reason for pyruvate inhibition of respiration in this organism is not known but certain possible causes are discussed.

The R.Q. value for P. sulfureus was about 0.8. This suggested that an endogenous storage material of the oxidation state of lipid was being utilized rather than carbohydrates.

Results from work with ^{14}C labeled glucose indicated that the pentose cycle in this organism had preference over the glycolytic pathway.

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I. INTRODUCTION

Polyporus sulfureus has the unusual property that almost thirty per cent of the dry weight of the mycelium grown either in surface or submerged culture is a mixture of triterpenoid acids, of which three fourths is eburicoic acid (Gascoigne et al 1950; Pan et al, 1961; and Fried et al, 1964).

Since there is an inverse relationship between storage lipid and carbohydrate of cells, i.e., individual classes of organisms have a tendency to synthesize either but not both, it is of interest to determine the type of control exercised by the organism for selecting the synthesis of its storage material.

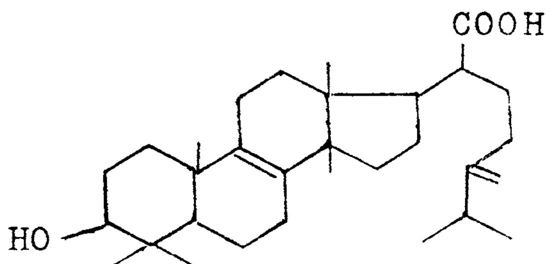
P. sulfureus is an example of an organism which stores lipids rather than carbohydrates. A study of its metabolism was thought to be of interest since such a study might shed light on the control of the "shunt" between carbohydrate and lipid metabolism in this organism.

The determination of actual metabolic controls is a long and difficult task. Therefore one aspect of *P. sulfureus* metabolism was selected for the present investigation, namely, the general aspects of growth of *P. sulfureus* and its endogenous and exogenous respiration.

II. LITERATURE

A. GENERAL SURVEY OF POLYPORUS SULFUREUS AND EBURICOIC ACID

Polyporus sulfureus is a fungus within the class of basidiomycetes. This filamentous fungus has been known to produce in good yield eburicoic acid, a triterpenoid compound with the structure below: (Gascoigne et al, 1950, 1951, 1953)



Eburicoic acid was first isolated from the fruiting bodies of Fomes officinalis Fr., by Kurono and Kariyone who gave the melting point as 283°C and reported an empirical formula of $C_{30}H_{48}O_3$ for the material they isolated. Through a series of studies (Gascoigne et al 1950, 1951, 1953; Lahey and Strasser, 1951; and Holker et al 1953), it was recognized that this acid has an empirical formula of $C_{31}H_{50}O_3$ which corresponds to the structure shown above. The single carbon unit attached to the side chain was found to originate from formate, one of the members of the one-carbon metabolic pool (Dauben and Richards, 1956; Dauben, Ban and Richards, 1957; Dauben, Fonken and Boswell, 1957).

In addition to F. officinalis Fr., eburicoic acid has also been isolated from the mycelium of several Polyporus species; P. anthracophilus, P. eucalyptorum, P. hispidus and P. sulfureus. Poria species such as P. cocos and Lentinus species such as L. dactyloides also contain eburicoic acid.

A patent (Pan et al, 1961) indicates that organisms producing eburicoic acid have been investigated for use as a commercial source of steroids.

Efimenko (1960) reported that eburicoic acid extracted from *P. betulinus Karst* stimulated yeast fermentation by 17-46% and also stimulated mycelial growth of fungi by 20-26%. Recently there has been interest in this acid because of its selective anti-androgenic activity. It selectively inhibits the androgenic effect on ventral prostate glands, seminal vesicles and coagulating glands but does not have the androgenic effect on total body growth. This acid also is reported as an antibiotic (Bates, 1967). It is one of the two anti-fungal steroid antibiotics (another is Viridan produced by *Trichoderma iride*) reported by Harvey et al (1967).

Through an extensive study on the biosynthesis of steroid compounds (Dauben and Richards, 1956; Bloch, 1965; Richards and Henderickson, 1964; Gascoigne et al, 1950), it has been shown that acetate in its active form, acetyl CoA, serves as a precursor for the biosynthesis of eburicoic acid through squalene to lanosterol. This is the same mechanism as that for the biosynthesis of steroids (Dauben and Richards, 1956; Lawrie et al, 1965).

Another triterpenoid, 15α-hydroxyeburicoic acid also has been found in the triterpenoid acids extracted from *P. sulfureus* and because of its occurrence this new acid has been named sulphurenic acid. Thirty per cent of the dry weight of the mycelium of this fungus grown either in surface

or submerged culture is triterpenoid acids, three fourths of which was accounted for as eburicoic acid and the remainder as sulphurenic acid (Fried et al, 1964).

The mycelium of this fungus also has been reported to produce volatile amines such as methylamine, dimethylamine, n-propylamine, iso-amylamine, colamine and phenylethylamine as a result of the decarboxylation of various amino acids (Rainbow and Rose, 1963).

B. BIOCHEMISTRY OF THE BASIDIOMYCETE GENUS, POLYPORUS

The biochemistry of basidiomycetes has been studied very little to date. The following discussion contains information about the basidiomycetes genus, Polyporus, to which the organism dealt with in this thesis belongs. The literature search was not exhaustive but it is felt that the information recorded here is nearly complete in so far as it describes the biochemical activities that have been examined and reported in the literature.

The intracellular enzyme, phenolase, from the mycelium of P. sulfureus was studied by Roesch (1965). He reported a rapid enzymic oxidation of tyrosine and ρ-cresol and a slow attack on pyrocatechol at pH 4-6 from an extract of the mycelium of this fungus. This first demonstration of such an activity in this species is of importance for the detoxication of heartwood toxins.

Some other investigations have been made of the metabolism of the polyporus species, but not necessarily P. sulfureus. The enzymes of the Embden-Meyerhof and pentose phosphate

pathways in *P. brumalis* extracts have been investigated by Casselton (1966). In his work, cell-free extracts were prepared from the mycelium of *P. brumalis* by grinding first with sand for five minutes followed by centrifuging at 26,000 x g for twenty minutes at a temperature between 2-6°C. From the measurement of the oxidation or reduction of pyridine nucleotides in the presence or absence of different substrates, the activities of the enzymes were assayed. From this investigation, Casselton was able to show that the cell-free extracts of *P. brumalis* contained all the enzymes required for the metabolism of glucose by both the Embden-Meyerhof pathway and the hexose monophosphate pathway.

The respiration of non-proliferating cells of *P. palustris* was studied by Newcomb and Jennison (1962). The oxygen and carbon dioxide evolution were measured by conventional manometric techniques in their study. They found that the respiration of *P. palustris* mycelium was sensitive to mechanical disintegration. The changes in respiration with cell age, the effect of starvation on endogenous respiration and on glucose oxidation as well as the effect of buffer systems and pH on respiration were investigated. They found that maximum endogenous and exogenous respiration occurred in 12-hour old pellets from shake cultures, and was associated with the lag phase and early phase of accelerated growth. They found that there was a decrease in exogenous and endogenous Q_{O_2} with starvation and also that the respiring mycelium was relatively insensitive to changes in pH between 4 and 6.

The Q_{O_2} was maximum at pH 5.0 - 5.5, which is the range of the optimum for the growth of most of the wood-rotting basidiomycetes.

The carbohydrate metabolism of *P. circinatus* was studied by Avigad et al (1961). They reported that this fungus possesses a galactose oxidase that oxidizes the carbon-6 position of galactose, yielding galactodialdose, instead of oxidation at the carbon-1 position as with glucose oxidase. This oxidation of a hexose at carbon-6 was thought to occur through a glucuronate-xylulose pathway. The reaction scheme, which uses part of the hexose monophosphate pathway, is as follows:

D-glucose ⟶ D-glucuronate ⟶ L-gulonate ⟶ xylitol ⟶ D-xylulose ⟶ D-xylulose phosphate ⟶ hexose monophosphate cycle ⟶ glucose. The quantitative significance of the cycle is unknown, but it appears to be minor (Blumenthal, 1965).

The isolation of two new polysaccharides, a glucan and a xylomannan from the mycelium of *P. tumulosus* was reported by Ralph and Bender (1965). These two polysaccharides were isolated by means of copper complexing of the alkali-soluble fraction obtained by extracting the mechanically isolated cell wall material with cold 1N alkali under an atmosphere of nitrogen.

C. TECHNIQUES FOR METABOLIC STUDIES

A number of techniques have been developed for following the course of metabolic reactions in microorganisms. Some of

these have been mentioned in the preceding paragraphs. A number of others are discussed below.

Carbon and oxidation-reduction balances have been used to determine the stoichiometry of substrate conversion to products in anaerobic and aerobic fermentations. Although the measurement of carbon and of oxidation-reduction balances by themselves have little use in determining glycolytic pathways, it is a valuable method for ascertaining whether or not the appropriate products, in terms of degree of oxidation, have been recovered (Blumenthal, 1965).

Metabolic inhibitors also have been used to study metabolic pathways. Due to the lack of suitable specific inhibitors, this method alone cannot be used satisfactorily. Cells usually possess more than one glycolytic pathway, and many enzymes are common to all such pathways. Furthermore, even if appropriate inhibitors were developed, it is questionable whether their use would be warranted, since it cannot be determined whether the unphysiological conditions that would result from the accumulation of metabolites might not lead to spurious results.

Another technique for the detection of metabolic pathways is based upon detecting and measuring specific enzymic reactions. The presence of a glycolytic enzyme in a cell extract indicates that the cell has the potential to use it. Thus the pathway in which this enzyme is involved may be evaluated.

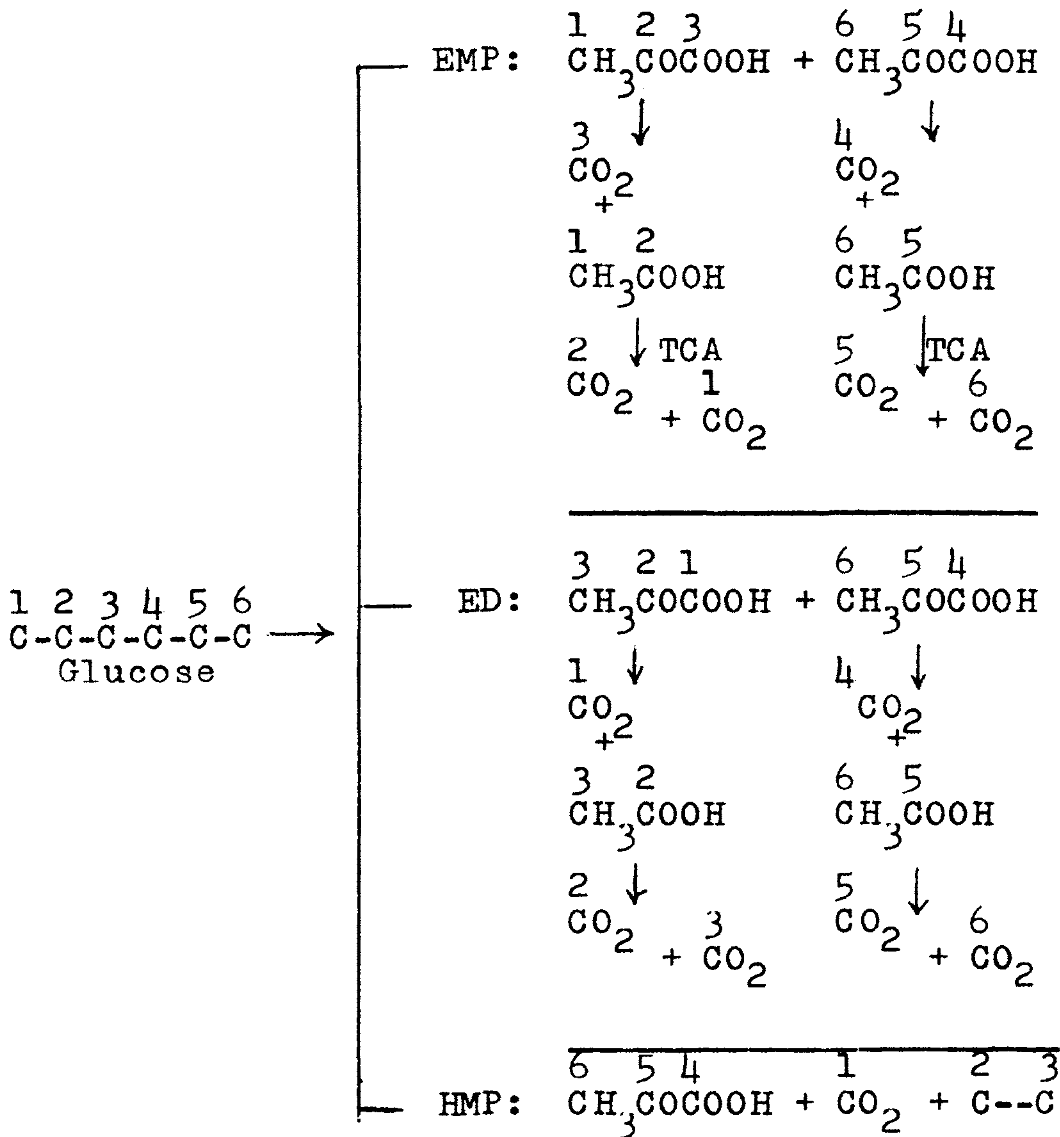
Measuring the respiration of a microorganism by manometric techniques has been used successfully to determine whether certain substrates can be utilized. However, in the filamentous fungi the rate of the endogenous respiration usually is high as compared to the respiration in the presence of an exogenous compound. The high endogenous respiration tends to mask the respiration due to the oxidation of exogenous substrate.

A question of great concern in these experiments with fungi is: Does exogenous substrate repress endogenous respiration or does exogenous and endogenous respiration continue at their normal rates simultaneously? The term "normal rates" is taken to mean the rate of endogenous respiration in the absence of external substrate. From the work done by Kuo and Blumenthal (1961) as well as by Ramachandran and Gottlieb (1963), it has been shown that this problem can be partially solved when manometry is used as a supplementary technique to studies employing metabolic inhibitors and isotopic substrates.

The fact that endogenous substrate is more readily oxidized than exogenous substrate suggests that the exogenous substrate is first converted to endogenous material and that the respiration of the organism is controlled by the availability of the endogenous material. If the endogenous material is different from the exogenous substrate, which appears likely, then what is the pathway by which the exogenous carbohydrate is converted to endogenous substrate? Which

of the several pathways by which glucose can be metabolized is the one preferred by a particular organism?

In principle, a distinction among the pathways should be easy with isotopic tracers. A brief scheme which shows the different fate of individual carbon atoms is as follows (Cheldelin, 1960).



EMP: Embden-Meyerhof-Parnas

ED : Entner-Doudoroff

HMP: Hexose monophosphate pathway

From the figure on page 9, it can be seen that if a molecule of glucose tagged in carbon-1 is metabolized by the EMP (Embden-Meyerhof-Parnas) pathway, the carbon chain is split in half and hence the specific activity of the respiratory $^{14}CO_2$ should be identical whether glucose-1-^{14}C or glucose-6-^{14}C is administered to the test system. If, on the other hand, HMP (hexose monophosphate pathway) prevails, the CO_2 released from glucose-1-^{14}C will be substantially enriched with ^{14}C, whereas if glucose-6-^{14}C is used instead, the CO_2 will have little or no activity at first, and will become active only as the pentose cycle operates to relocate active carbon atoms into the oxidizable position of the glucose molecule. In case ED (Entner-Doudoroff) pathway is operative, then the yield of CO_2 will be identical from carbons 1 and 4 of glucose, from carbons 2 and 5, and from 3 and 6. Administration of glucose labeled specifically in these positions should reveal the presence of this route.

The answer to the question, "What is the nature of the endogenous substrate?" is not easy to answer. One can infer from the respiratory quotient that carbohydrate, lipid or protein is being utilized. Also if the organism stores a particular metabolite in significant quantities it is assumed that the storage material is serving as substrate in the cells. It is strange though that a substance as complex as eburicoic acid would be formed from exogenous substances and then this complex molecule used as an endogenous substrate.

III. EXPERIMENTAL

A. MATERIALS

D-glucose (anhydrous) and sodium pyruvate were obtained from Sigma Co., St. Louis, Missouri.

Disodium succinate was obtained from Eastman Organic Chemicals, Rochester, New Jersey.

Liquid puruvic acid was obtained from Fisher Scientific Co., Fair Lawn, New Jersey, and converted to its sodium salt in this laboratory.

Malt extract and malt agar were obtained from Difco Laboratories, Detroit, Michigan, and from Oxoid Division, Consolidated Laboratories, Inc., Chicago Heights, Illinois.

D-glucose-1-^{14}C (3.95mc/mMole), D-glucose-6-^{14}C (3.83mc/mMole) and sodium acetate-1-^{14}C (2.0mc/mMole) were obtained from New England Nuclear Corp., Boston, Massachusetts.

D-glucose-U-^{14}C (2.7mc/mMole) was obtained from Nuclear Chicago, Chicago, Illinois.

Authentic eburicoic acid was kindly supplied by Dr. Siehr of the University of Missouri at Rolla.

All of these chemicals were used without further purification.

All inorganic salts and solvents were reagent grade and were used without purification.

The original culture of *Poluporus sulfureus* was collected by Dr. Siehr of the University of Missouri at Rolla.

B. METHODS

1. Cultivation of Polyporus sulfureus

The stock culture of P. sulfureus was maintained on malt-agar slants which contained 4% malt extract and 1.5% agar and were stored in the cold room at about 5°C.

Small pieces of the mycelium were transferred from the slants into 300 ml Erlenmeyer flasks containing 60 ml of 4% malt extract. After approximately one month's surface growth at room temperature the contents of the flask was homogenized under sterile conditions in a Waring Blendor (Winsted Hardware Mfg. Co., Winsted, Conn., Model No. PB-5A).

Shake cultures were initiated by transferring 5 ml of the blended mycelium into 300 ml flasks containing 60 ml of 4% malt medium. Cultures were kept on a rotary shaker (New Brunswick Scientific Co., New Brunswick, N. J., Model CS-62630) and routinely transferred to fresh 4% malt medium.

The growth of P. sulfureus in different types of media was also studied. The compositions of these media are given in Table 1 (page 13).

After six days of growth the mycelia grown in 60 ml of the various media in 300 ml Erlenmeyer flasks were transferred quantitatively to Gooch crucibles previously dried to a constant weight in an oven at 100-110°C. The cells and Gooch crucibles were then dried overnight in the oven at 100-110°C and the dry weight of cells was determined.

The pH of the broth at various times throughout the incubation of the cells was measured by means of a pH meter (Leeds and Northrup Co., Philadelphia, Penn.).

TABLE 1

COMPOSITIONS OF MEDIA

Medium	Composition	
Asparagine	glucose	10 grams
	asparagine	2 "
	KH_2PO_4	0.5 "
	$Na_2HPO_4.2H_2O$	0.5 "
	$Mg_2SO_4.7H_2O$	1.0 "
	thiamine.HCl	100µM/liter
	deionized water	1 liter
Nitrate	glucose	30 grams
	$NaNO_3$	3 "
	K_2HPO_4	1 "
	$MgSO_4.7H_2O$	0.5 "
	KCl	0.5 "
	$FeSO_4$	0.01 "
	thiamine.HCl	100µM/liter
	deionized water	1 liter
Nitrate + Yeast	nitrate medium plus 0.5% yeast extract	
Malt	4% malt extract	

2. Extraction and Detection of Eburicoic Acid

For the isolation of eburicoic acid the mycelium was harvested by filtration and dried in an evaporating dish. The collected mycelium was extracted in a Soxhlet apparatus for 24 hours using ether as the solvent. Evaporation of the ether and recrystallization of the crude residue from ethanol gave colorless, crystalline needles. The melting point of this substance was measured on a Nalge melting-point apparatus (Nalge Co., Rochester, N. Y.). A melting point of 272°C was obtained which was comparable to the literature value of 274°C (Pan et al, 1961).

Solvent systems suitable for the paper chromatography of eburicoic acid have been developed by Pan et al (1962). Two of these Bush type solvent systems were used in this work.

Solvent system A: n-hexane, tert-butanol and 4N NH_4OH in the volume ratio of 10 : 3 : 10 were shaken together to give a two phase solvent. Whatman No. 1 paper (22½" x 8½") spotted with the samples was thoroughly equilibrated with the vapor from the lower aqueous phase overnight in a glass tank sealed with a glass cover. The papergram was developed with the upper hexane phase in the descending manner. Solvent system B was prepared by using equal volumes of chloroform, methanol and 1N NH_4OH. This gave a two phase solvent. The papergram was equilibrated overnight with the lower aqueous phase and then developed with the upper organic phase as was done with solvent system A.

After the papergrams were developed and air dried, they were dipped in phosphomolybdic acid reagent (prepared by dissolving 10 grams of phosphomolybdic acid in sufficient 95% ethanol to make a total volume of 100 ml). The wet papergrams were heated at 100°C for 5 minutes in an oven (Precision Scientific Co., Model 16) immediately after the application of the detecting reagent.

3. Measurement of Total Organic Solids and Reducing Sugar

Johnson's method modified by Halliwell (1960) was used for the determination of total organic solids. Two reagents were used in this method.

Reagent A: Two and one half grams of $K_2Cr_2O_7$ was dissolved with heating in 20 ml of H_2O. The solution was cooled and diluted to one liter with 98% H_2SO_4.

Reagent B: Four grams of $Na_2SO_3 \cdot 7\ H_2O$ was dissolved in 20 ml of H_2O. The solution was kept at 1°C.

For each assay, two blanks were required. One blank was completely reduced by the addition of reagent B and served as a sample which contained zero mg of total organic solids and had zero absorbancy. The unreduced blank served as a sample which had the highest absorbancy.

For each determination, medium from a flask was filtered through a Gelman 2 micron multipore filter. One tenth milliliter of the filtrate was diluted to a total volume of 10 ml. This diluted medium was used as a sample for the determination of total organic solids.

To 0.9 ml of sample, 2 ml of reagent A was added and the solution was heated in a boiling water bath for 20 minutes and then cooled. To eliminate dilution of the test solution with steam the test tube opening was covered with a marble before heating. After the solution was cooled, 6 ml of H_2O was added and mixed thoroughly. One tenth of a milliliter of reagent B was then added to completely reduce the $K_2Cr_2O_7$ in the first of the two blanks which contained only deionized water and reagent A. All other tubes including a unreduced blank were read at 440 mμ in a Spectronic 20 colorimeter (Bausch and Lomb, Inc., Rochester, N. Y.) against the reduced blank set to zero optical density. A standard curve (Figure 1, page 17) was made by using a solution containing no more than 0.7 mg of D-glucose in 0.9 ml of water.

Nelson's colorimetric method (Hodge and Hofreiter, 1962) was tried for the determination of the amount of glucose in the medium at different times during the growth of the organism. However, the reproducibility of the results were poor so that it was not used in this investigation. Instead, the 3,5-dinitrosalycylic acid method (Reese and Mandels, 1962) which gave quite satisfactory results was used.

The components of the reagent used in this method were as follow:

Component	Weight
Phenol	2.57 g.
Sodium hydroxide	12.86 "
Sodium sulfite	0.64 "
3,5-dinitrosalycylic acid	12.86 "
Sodium potassium tartrate (Rochelle salts)	257.00 "
Deionized water	1 liter

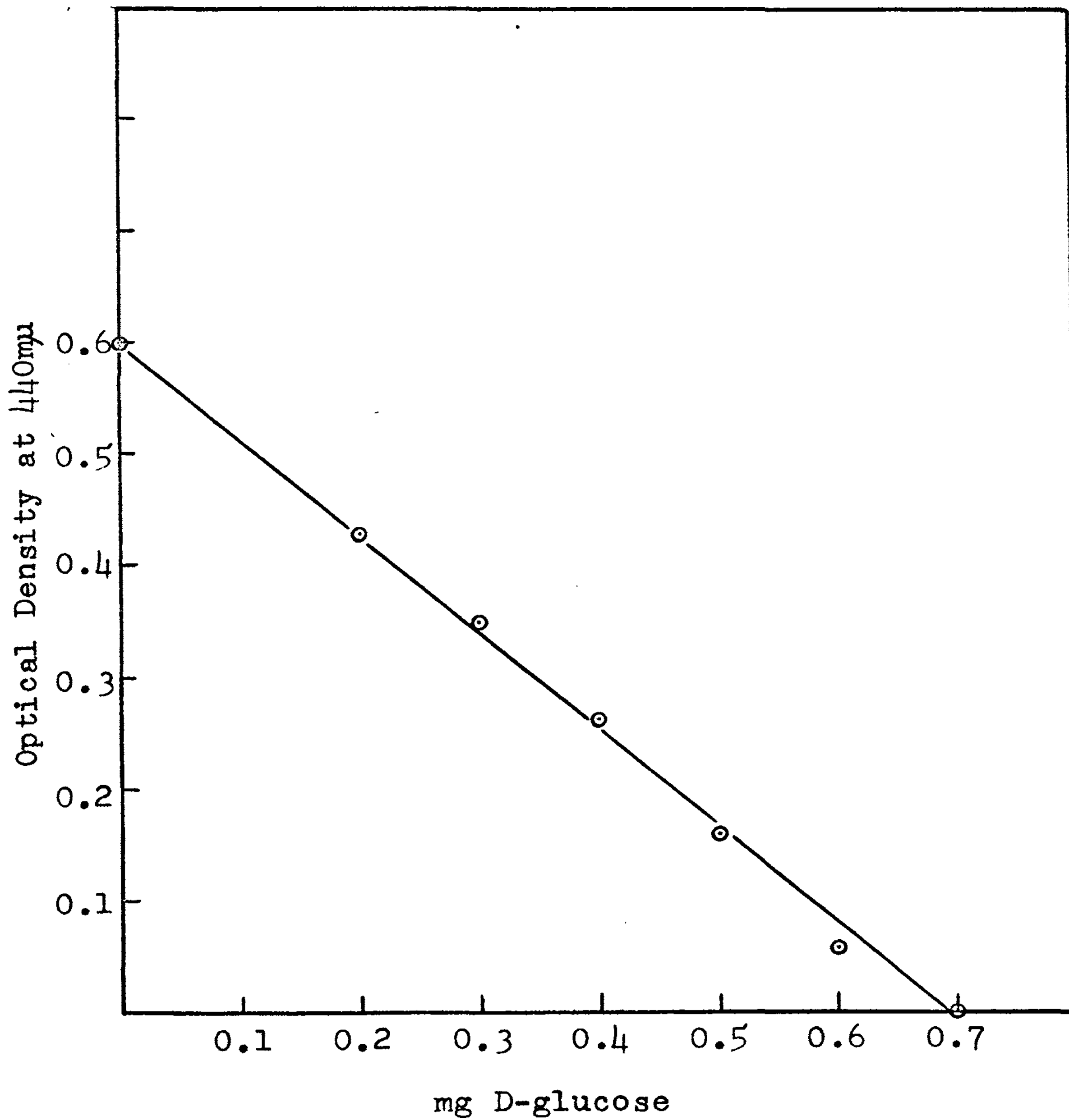


FIGURE 1. STANDARD CURVE FOR TOTAL ORGANIC SOLIDS

To 1 ml of the filtered culture medium containing reducing sugar, 2 ml of the reagent was added and the solution heated in a boiling water bath for 15 minutes. After cooling, it was diluted with 17 ml of water. The absorbancy was read at 550 mμ with a Spectronic 20 colorimeter. A standard curve using a solution of D-glucose was made (Figure 2, page 20). The range of glucose used per tube in the preparation of the standard curve was from 0.0 to 1.0 mg.

4. Respirometry

All manometric techniques used in this investigation were carried out at 30°C with air as the gas phase in the flasks according to the manner described by Umbreit, Burris and Stauffer (1964). The preparation of phosphate buffer solutions of various pH values were as described in Biochemisches Tashenbuch (Rauen, 1956).

The treatment of the mycelium for use in these experiments was as follows. The mycelium from shake cultures was collected by centrifugation, the supernatant liquid was discarded, and the mycelium was resuspended in phosphate buffer (0.067 M, pH 6.0) and again centrifuged. The washed mycelium was resuspended in 20 ml of the phosphate buffer and blended by means of a Waring Blendor for 15 seconds to produce a uniform mycelial suspension that could be pipetted. All the above procedures were done at a temperature of about 5°C. The mycelial suspension was kept cold in an ice container and pipetted just prior to the manometric determinations. The dry weight of mycelia was measured by pipetting 2.5 ml of the

mycelial suspension into a tared beaker and heating the beaker in an oven overnight at 100-110°C and then weighing the beaker and contents.

When non-radioactive materials were tested as substrates, single-side-arm Warburg flasks were used. The center well of the flask contained 0.2 ml of 20% KOH with a small strip of filter paper. The main body of the flask contained 2.5 ml of blended mycelium. Exogenous substrate to be studied was put in the side-arm. The final fluid volume was 3.0 ml.

For the measurement of carbon dioxide evolved, the Warburg direct method was used (Umbreit et al, 1964). In this method the contents of two flasks were exactly the same and treated in the same manner, except that in one the CO_2 was absorbed in KOH whereas in the other it was not.

For each of the substrates studied a Q_{O_2} value was obtained. This value is defined as the oxygen consumption in μl per mg dry weight of cells per hour. This value is obtained by plotting the μl oxygen taken up per mg dry weight of cells versus time in minutes. The slope of the straight line portion of this plot starting at zero time was determined. This slope gave the initial rate in μl oxygen taken up per mg dry weight of cells per minute. From this value, the Q_{O_2} value was calculated.

The respiratory quotient (R.Q.) value is calculated as the value of carbon dioxide produced divided by the volume of oxygen consumed by the respiring organism.

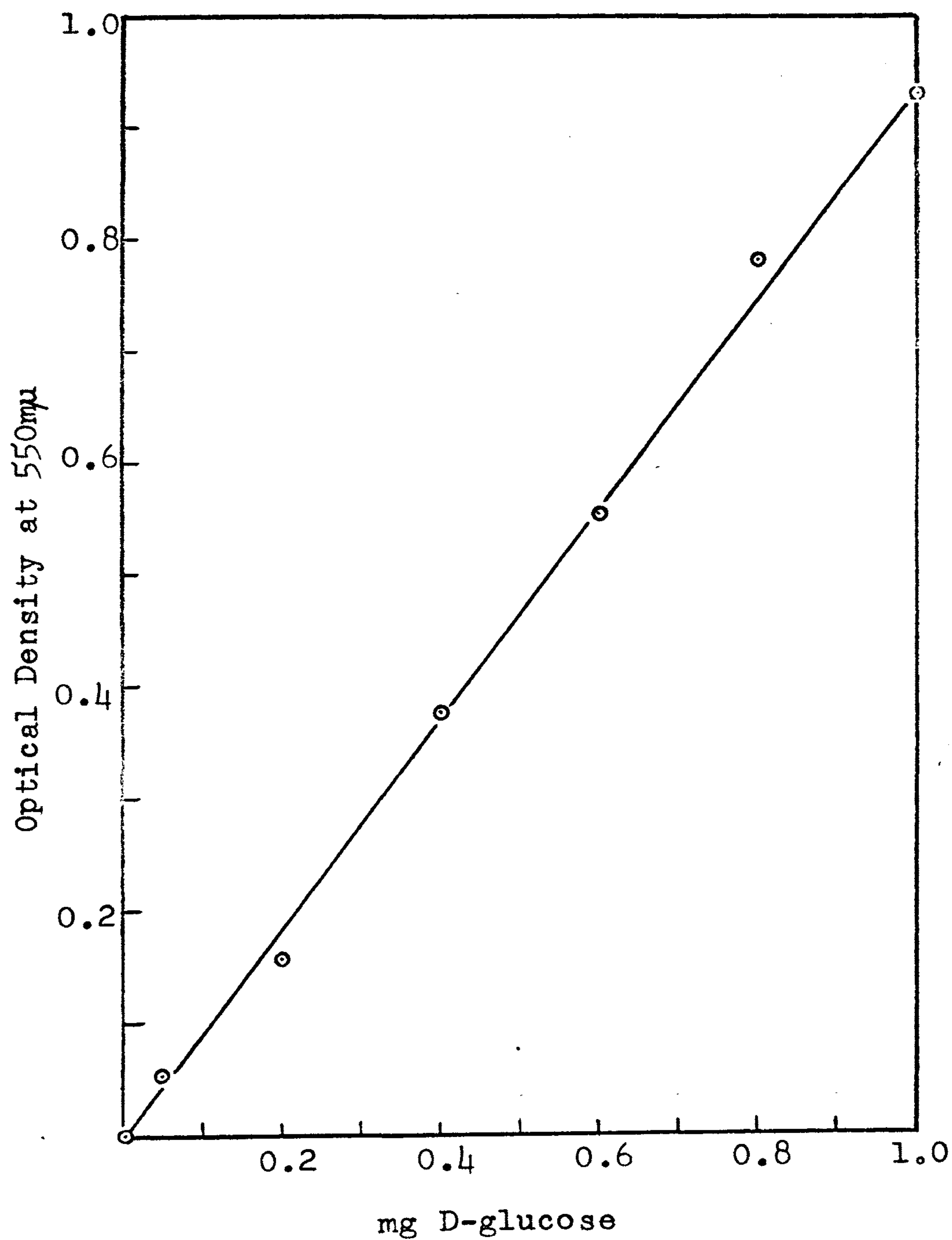


FIGURE 2. STANDARD CURVE FOR REDUCING SUGAR

When ^{14}C was used in the manometric studies, two-side-arm flasks were used. The center well of the flask contained 0.2 ml of 20% carbonate-free NaOH but no paper. The contents of the main flask compartment was the same as in the studies with unlabeled material. In one side-arm, either labeled or unlabeled substrate was added; the other side-arm contained 0.2 ml of 70% perchloric acid. After 20 minutes of equilibration in the water bath at 30°C, the substrate was tipped in the main compartment. At the end of 1, 2, 3, and 4 hours, the perchloric acid was tipped into the various sets of flasks to stop the enzymic reaction. The 1, 2, and 3 hour flasks were left on the Warburg apparatus until the reaction in the 4 hour flask had been stopped and the carbon dioxide evolved in this flask had been allowed to equilibrate with the NaOH for one hour. The flasks were then removed, and the NaOH was pipetted from the center well into a centrifuge tube and 3 ml of 2N barium hydroxide was added. The precipitate was collected by centrifugation, washed twice with ethanol and transferred to a tared metal planchet with a diameter of 2.4 cm. The barium carbonate precipitate was air-dried or dried under a heat lamp, weighed and counted in a gas-flow proportional counter (Riol, Model 40-9B, 2-9 and 49-30). All the prepared samples were stored in covered Petri dishes to minimize loss of radioactivity by way of exchange reactions involving carbon dioxide in the atmosphere.

The plateau voltage for ^{14}C for this counter was determined using a sample of ^{14}C-benzoic acid. On the basis

of this determination an operating voltage of 2310 volts was selected.

All counts were corrected for background radiation first, then corrected for the counting efficiency of the counter. For the determination of counting efficiency, a standard ^{14}C sample with a radioactivity of 0.167μc (New Engalnd Nuclear Corp.) was used. Calculated counts per minute for this standard were 0.167μc x 2.22 x 10^6CPM/μc = 370740 CPM. However the measured counts per minute were 95175. Hence the counting efficiency for this counter with its geometry was 95175/370740 x 100 = 25.67%.

Since in the proportional detector the resolving times are so extremely short that they are not the cause of significant coincidence losses and since the counting rates in this study never exceeded several hundred thousand counts per minute, no correction was made for coincidence loss (Wang and Willis, 1965).

A self-absorption correction curve was made by using the $Ba^{14}CO_3$ obtained from the respirometry experiments. Since from the manometric experiments, it was known that acetate-1-^{14}C would give a higher percentage of $^{14}CO_2$ recovery, hence acetate-1-^{14}C was chosen for the purpose of making a self-absorption curve. The collected $Ba^{14}CO_3$ precipitate was suspended in ethanol and this suspension in different amounts dispensed onto ten tared metal planchets each with a diameter of 2.4 cm. The planchets were dried under a heat lamp and then weighed. The net sample weight was calculated and the

radioactivity counted. The respective apparent specific activities (in CPM/mg $BaCO_3$) were plotted against the sample thickness (in mg $BaCO_3/cm^2$). This curve was extrapolated to zero thickness and the value of apparent specific activity at zero thickness considered as the actual specific activity without any reduction by self-absorption. Using the value of the actual specific activity as 100 per cent, the percentage relative specific activity observed at each sample thickness were plotted against thickness. This gave a self-absorption correction curve as shown in Figure 3, (page 24). (Also see Appendix F, page 66 for the data in detail). From this figure, the saturation thickness value was determined as about 30 mg/cm^2.

In this investigation, the counting samples prepared from the manometry experiments using radioactive materials as substrates were all above the saturation thickness, i.e., they were thicker than 30 mg/cm^2 (See Appendix E, page 65), thus the observed count rate would be directly proportional to the specific activity of the sample, regardless of the sample thickness. Therefore the radioactivity in all samples was compared directly by their observed total counts per minute without any correction for self-absorption.

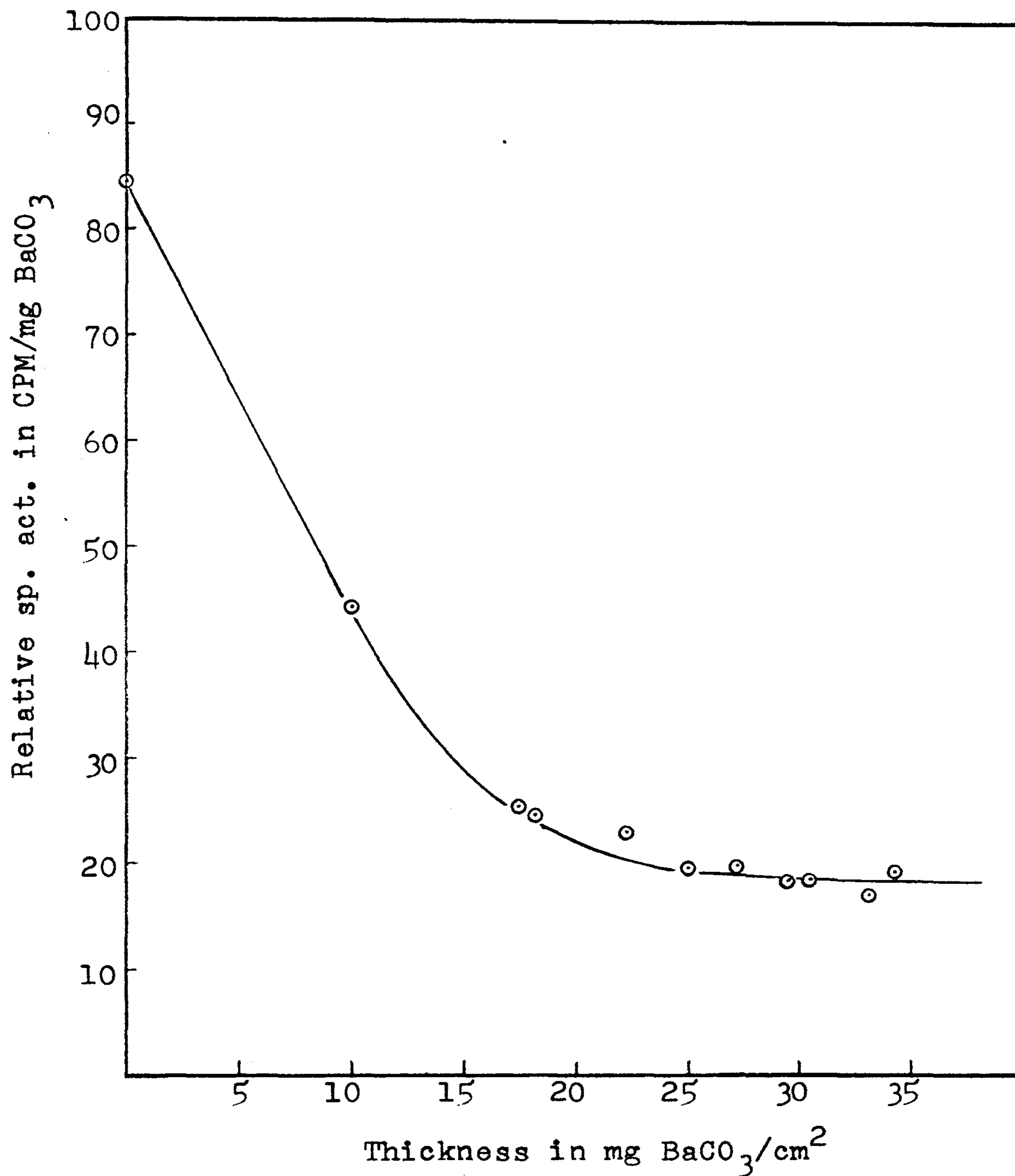


FIGURE 3. SELF-ABSORPTION CORRECTION CURVE FOR ^{14}C IN THE GAS FLOW PROPORTIONAL COUNTER (RIOL, MODEL 40-9B, 2-9 and 49-30)

C. RESULTS

The influence of the media compositions on the growth of P. sulfureus as well as on the final pH of the broth are given in Table 2 (page 26).

It is obvious that the 4% malt extract medium is a better medium for the growth of P. sulfureus than the others. Asparagine and nitrate media are poor nutrient media for the development of mycelium of P. sulfureus.

The change in the pH of the broth as a function of the growth time was observed and is shown in Table 3 (page 27). From these data it can be seen that if the nutrient is free of buffering agent, the pH of the cultivated medium decreased with age and growth.

Since this organism was reported to produce eburicoic acid in good yield, it was thought advisable to confirm this fact for the culture used in these investigations. An attempt was therefore made to extract eburicoic acid and identify it by paper chromatography.

An attempt to chromatograph eburicoic acid using solvent system A (page 14) was not successful. However, when solvent system B (page 14) was used, eburicoic acid extracted from P. sulfureus gave an R_f value of 0.87 which is identical with that for an authentic sample. The R_f value of each spot was calculated by measuring the distance the spot moved in relationship to the distance the solvent moved. Pan et al (1962) had reported an R_f value of 0.93 for eburicoic acid in this solvent system.

TABLE 2

GROWTH OF *P. SULFUREUS* ON DIFFERENT MEDIA

Medium	Dry wt. of cell after six days (mg)	Average wt.	Initial pH at 26°C	Final pH at 26°C
Asparagine	9.3 9.8 10.5 10.6	10.1	6.6	5.7
Nitrate	4.3 4.6 3.8 6.7	4.9	6.1	5.7
Nitrate + 0.5% yeast extract	50.8 49.0 63.4 ----	54.4	5.7	3.7
4% malt extract	93.8 94.5 92.6 89.4	92.6	4.3	2.2

TABLE 3

CHANGE OF MEDIUM pH WITH GROWTH OF
P. sulfureus ON 4% MALT MEDIUM

Days of growth	pH at 26°C	Days of growth	pH at 26°C
0	4.6	35	2.1
7	2.3	47	1.8
10	1.8	53	1.8
14	2.2	60	1.8
17	2.0	73	1.8
21	1.8	80	1.9
28	1.9		

In order to find the time when the depletion of reducing sugars in the spent medium occurred as well as to determine if there was an excretion of organic acids from the cells into the medium, the total organic solids and reducing sugar in the medium was followed with age of the mycelium.

The results for the determination of total organic solids and reducing sugar in the spent medium is listed in Table 4 (also see Figure 4).

The unwashed and unblended cells of P. sulfureus from the surface growth culture were used for respirometry studies at the beginning of this investigation. However, the whole cell pad which floated on the buffer solution gave less than 10 µl of oxygen uptake after 5 hr. Due to the difficulties of obtaining the same weight of cell pad in each Warburg flask and due to the slowness of gas exchange, this method was not used for any of the latter experiments. Instead, a homogeneous cell suspension was made from mycelium grown in submerged culture.

It was observed that the acidity of the suspending medium had some influence on the oxidation of glucose by P. sulfureus over the range of pH 5 to 8. This is shown in Figure 5 (page 31).

The R.Q. values calculated for P. sulfureus utilizing endogenous substrate in the presence of exogenous glucose and its absence are given in Table 5 (page 32).

The endogenous respiration and the change in mass of this organism was followed with age of the culture. The

TABLE 4

TOTAL ORGANIC SOLIDS AND REDUCING SUGAR AT VARIOUS TIMES DURING THE GROWTH OF *P. SULFUREUS* ON 4% MALT MEDIUM

Growth (days)	T.O.S.* (mg/ml broth)	Red. Sugar# (mg/ml broth)	T.O.S. less Red. Sugar
0	52.2	32.5	19.7
1	45.4	31.5	13.9
2	55.6	37.0	18.6
5	49.4	31.8	17.6
7	47.6	28.5	19.1
8	45.8	31.0	14.8
32	25.3	9.0	16.3
58	15.0	2.5	12.5
69	13.7	3.9	9.8

* Total Organic Solids

Reducing Sugar

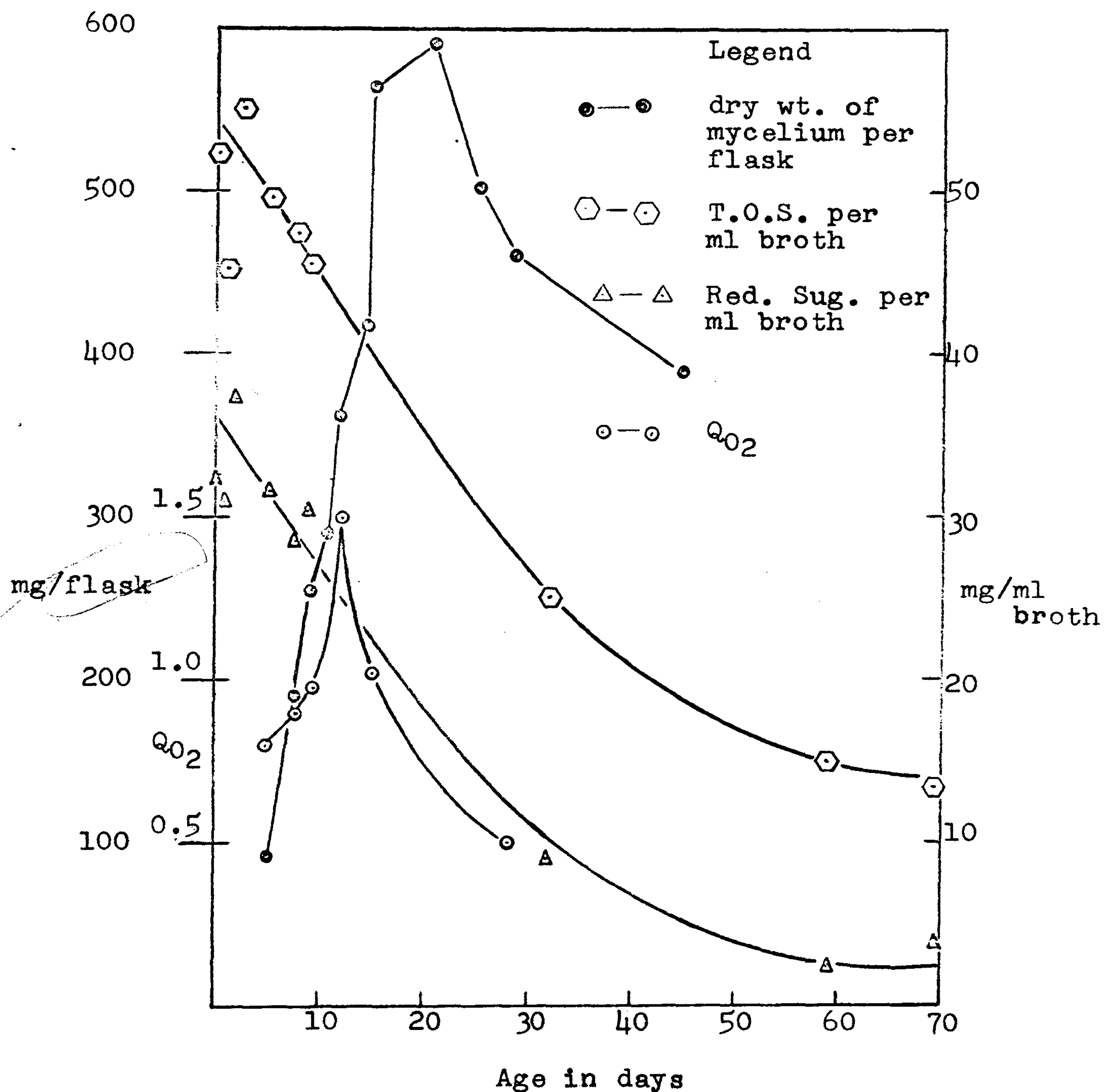


FIGURE 4. VARIATION IN MYCELIAL MASS, RESPIRATION OF MYCELIUM AND TOTAL ORGANIC SOLIDS AND REDUCING SUGAR IN THE MEDIUM WITH AGE OF MYCELIUM

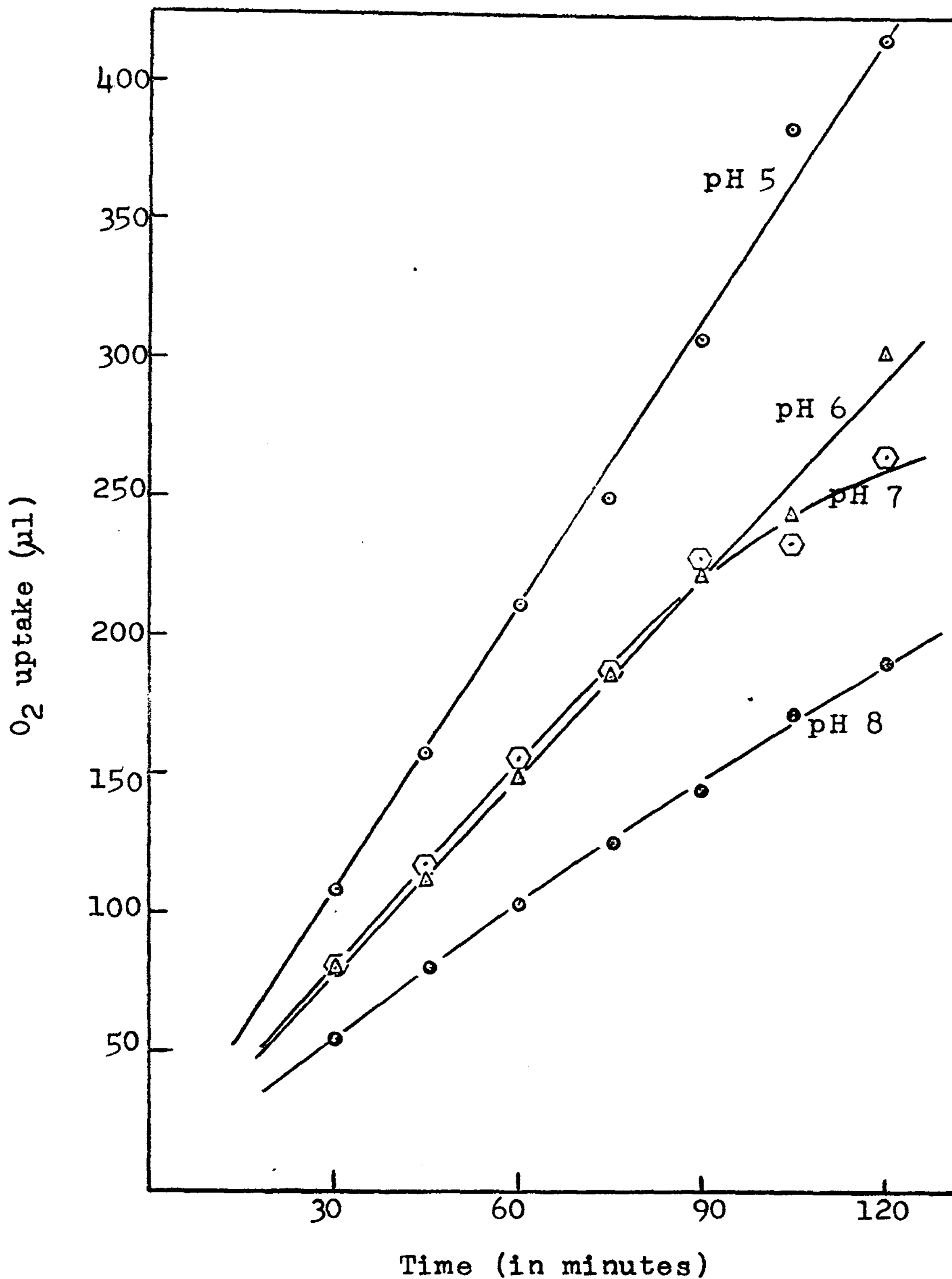


FIGURE 5. THE INFLUENCE OF pH ON GLUCOSE OXIDATION BY 7 DAY OLD CELLS OF *P. SULFUREUS*

TABLE 5

RESPIRATORY QUOTIENT FOR P. SULFUREUS WITH AGE

Days of growth	Endogenous R.Q.*	Exogenous R.Q.#
5	0.70	0.69
7	0.76	0.77
9	0.80	0.83
11	0.87	0.84
	Av. 0.78 ± 0.03	Av. 0.79 ± 0.04

* No substrate added

\# With 30 µM glucose as substrate

results of these measurements are shown in Figure 4 (page 30).

Xylose, glucose and maltose were tested as possible exogenous substrates for P. sulfureus. Results of these experiments showed that glucose and xylose stimulate oxygen uptake very little. Maltose is a slightly better substrate than the other two carbohydrates. The results are recorded in Table 6.

TABLE 6

INFLUENCE OF CARBOHYDRATES ON THE RESPIRATION OF P. SULFUREUS

Age in days	No substrate (control)	Glucose* added		Xylose* added		Maltose* added	
	Q_{O_2}	Q_{O_2}	% of control	Q_{O_2}	% of control	Q_{O_2}	% of control
9	1.0	1.0	100	0.02	82	1.10	110
9	0.97	0.97	100	----	----	----	----
5	0.97	0.97	100	----	----	----	----

* The concentrations of all substrates are 30µM

Intermediates in the Krebs cycle and pyruvic acid which occurs in several metabolic pathways and occupies a central position in carbohydrate metabolism were also chosen as exogenous substrates. Either the commercial sodium salt of pyruvic acid or the salt of pyruvic acid prepared in this laboratory was used. Regardless of the source, sodium

pyruvate gave very similar results. Results show that acetate and succinate stimulate oxygen uptake to some extent, but pyruvate does not. Repeated experiments indicate that oxygen uptake is inhibited by the addition of pyruvate to the cell suspension (See Figure 6, page 35). The results from these experiments are given in Table 7. The effect of age of the culture on the utilization of these substrates was also determined.

TABLE 7

Q_{O_2} VALUES IN THE PRESENCE OF ORGANIC ACIDS

Age in days	Substrate added*						
	No substrate (control)	Acetate		Succinate		Pyruvate	
		Q_{O_2}	% of control	Q_{O_2}	% of control	Q_{O_2}	% of control
5	0.77	1.17	152	1.52	198	----	---
7	0.90	1.10	122	1.42	158	0.51	57
9	0.97	1.30	134	----	---	0.68	70
12	1.50	2.06	137	1.66	111	1.00	67
15	1.06	1.67	157	1.21	114	0.73	69

* The concentrations of all substrates are 30µM

Even though glucose did not stimulate the respiration of P. sulfureus, it was of interest to determine if glucose was being utilized by the cells. To this end glucose labeled with ^{14}C was added to cell suspensions in the Warburg flasks.

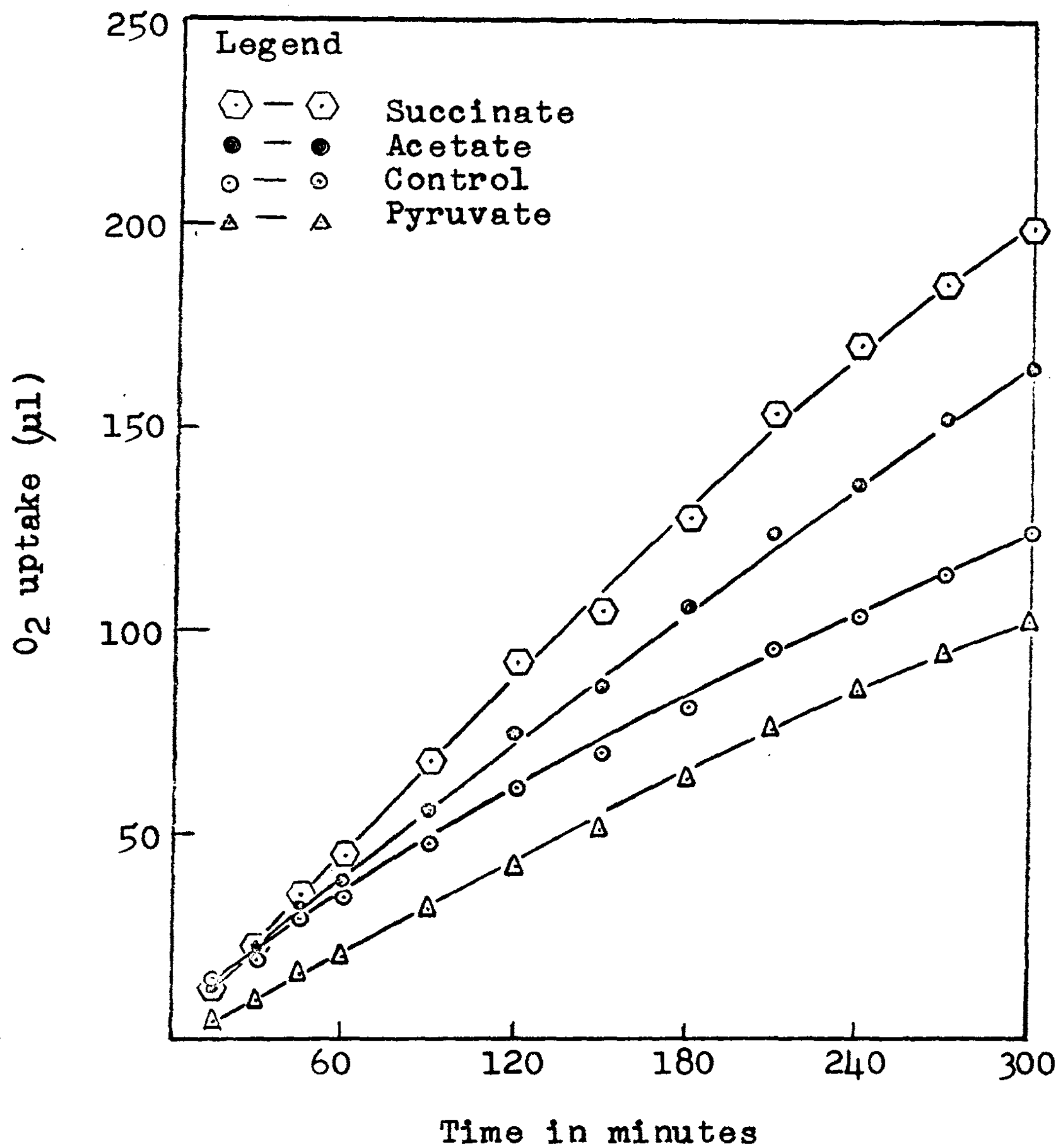


FIGURE 6. EFFECT OF EXOGENOUS SUBSTRATE ON THE RESPIRATION OF 7 DAY OLD <u>P. SULFUREUS</u>

The results of one such experiment are given in Table 8. The fact that radioactive CO_2 is recovered indicates glucose is being metabolized by the organism even though the percent utilized is low.

TABLE 8

THE UTILIZATION OF GLUCOSE-U-^{14}C
(50µM, 1850CPM/µM carbon)
BY THE INTACT CELLS OF P. SULFUREUS

Time (hour)	Total µl CO_2 evolved (1)	Total µM CO_2 evolved (2)	Radioactivity of $^{14}CO_2$ in CPM (3)	µM $^{14}CO_2$ in terms of (3) (4)	% exogenous*	% endogenous
1	49.2	2.20	320	0.173	7.9	92.1
2	89.8	4.01	644	0.348	8.7	91.3
3	136.5	6.10	1115	0.602	9.9	90.1
4	178.5	7.98	1540	0.833	10.5	89.5

* % of µM $^{14}CO_2$ in the total µM CO_2 evolved, i.e. (4)/(2)

In an experiment where glucose-U-^{14}C with a specific activity of 1850 CPM/µM carbon was used as a substrate for the 4%-malt extract-grown mycelium, there was a total of 1540 CPM in the CO_2 evolved after four hours. Thus it can be calculated that 0.83µM (1540/1850 = 0.83) of carbon from the glucose was oxidized to CO_2 during this experiment. Since a total of 7.98µM CO_2 was actually produced in the flasks containing glucose, the amount of endogenous CO_2 liberated in the presence of glucose can be calculated to be 7.15µM (7.98 - 0.83 = 7.15). The actual endogenous liberation of

CO_2 (in the absence of substrate) was also 7.98µM, so that the inhibition of endogenous CO_2 production during the oxidation of glucose was 10.4% $[(7.98 - 7.15)/7.98 \times 100 = 10.4\%]$. This calculation was in the manner described by Blumenthal (1963).

Table 9 (also Figure 7) shows the results when glucose-1-^{14}C, glucose-6-^{14}C and acetate-1-^{14}C were used as exogenous substrates by washed mycelium of _P. sulfureus_. Though the amount and the radioactivity of the substrate glucose which was added to the test system were the same, the percentage of radioactivity recovered is not identical. This suggests that glycolysis is not the sole pathway of glucose metabolism in this organism. Should glycolysis be the major pathway for the utilization of glucose in _P. sulfureus_, then the specific activity of the respiratory $^{14}CO_2$ would be identical whether glucose-1-^{14}C or glucose-6-^{14}C is used as substrate. The results showed that $^{14}CO_2$ evolved from the respiration of glucose-1-^{14}C is substantially enriched with ^{14}C whereas when the label position was in C-6, the CO_2 had little activity. Thus it seems that the pentose pathway in this organism has preference over the glycolytic pathway.

TABLE 9

UTILIZATION OF ^{14}C-LABELED SUBSTRATES BY CELLS OF *P. SULFUREUS*

Substrate*	Radioactivity (CPM)	Time (hour)	$^{14}CO_2$ evolved (CPM)	% recovery of radioactivity
Glucose-1-^{14}C	5.55×10^4	1	82	0.15
		2	137	0.25
		3	199	0.36
		4	246	0.44
Glucose-6-^{14}C	5.55×10^4	1	23	0.04
		2	55	0.10
		3	70	0.13
		4	59	0.12
Acetate-1-^{14}C	1.11×10^6	1	1780	0.16
		2	4740	0.43
		3	5400	0.49
		4	8390	0.76

* The concentrations are all 50µM

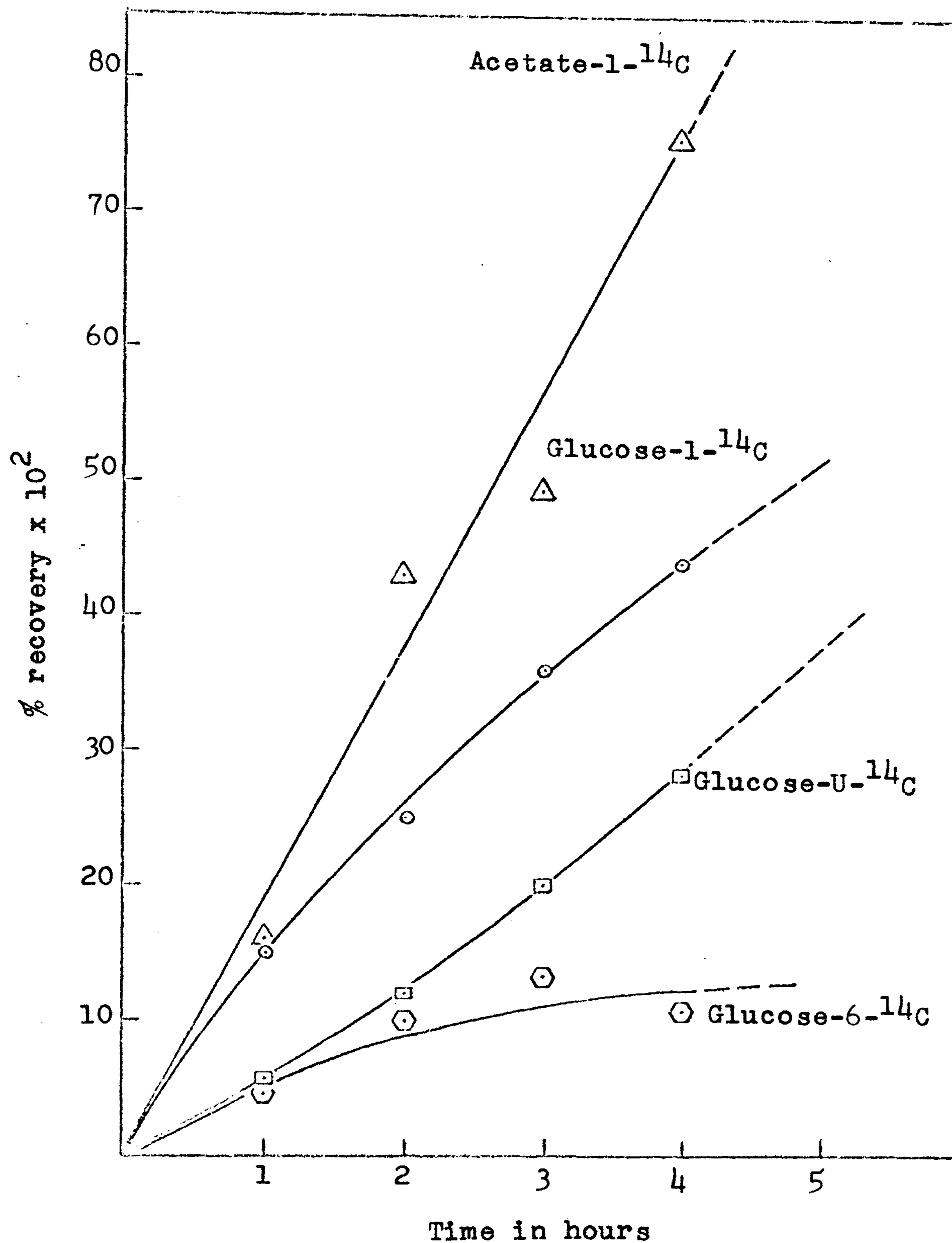


FIGURE 7. RECOVERY OF ^{14}C AS CO_2 WITH VARIOUS ^{14}C LABELED EXOGENOUS SUBSTRATES

IV. DISCUSSION

Fungi can endure acidic environments better than the bacteria and actinomycetes. In culture, the basidiomycetes are often unable to grow at an initial pH above 7.0. However, there are some exceptions such as the species of Coprinus which requires alkaline conditions for best growth (Cochrane, 1958). *P. sulfureus* is an example of a basidiomycete which is restricted to acid environment. The pH of the sterilized 4% malt extract medium was 4.6 at 26°C before inoculation with mycelium. After one week's growth, the pH of *P. sulfureus* had dropped to 2.2.

The pH of the medium can be raised by the absorption of anions or the production of ammonia from nitrogenous compounds or lowered by the formation of organic acids or the absorption of cations.

Although about 30% of the dry weight of the cells of this organism are triterpenoid acids, this does not account for the observed decrease in pH, since there is little or no increase in the organic solids in the medium during the growth of the organism (Figure 4, page 30).

It is conceivable that some constituents especially cations in the malt extract were absorbed by the cells during their growth and thus lowered the pH of the medium.

The internal pH of the fungal cell is not known and whether or not the internal pH responds to changes in the acidity of the external medium is uncertain although the

respiration of the organism is affected by changes in the external pH (Figure 5, page 31).

On the basis of the experimental work done it cannot be ascertained whether the buffer-free medium is the most suitable one for the growth of this fungus nor whether its growth is restricted by the presence of buffer. This is in part in agreement with the results reported by Pan et al (1961) that by merely omitting the buffering substance and maintaining the pH below about 6, a fully developed submerged culture which produced eburicoic acid in high yields was obtained. Asparagine and nitrate media, which contained phosphate buffer and would be expected to show little change of pH, were poor nutrient sources for the growth of this organism. Nitrate plus 0.5% yeast extract medium which also was a buffered medium, supported five to ten times the growth of mycelium as did the asparagine and nitrate media. The pH in the nitrate plus yeast extract medium dropped from 5.7 to 3.7 (Table 2, page 26). The conclusion to be drawn from these results is that regardless of whether the medium is buffered or not good growth of the organism will cause a decrease in the pH of the medium.

An examination of the results of the experiments with growth media would suggest that 4% malt extract is the best of the four media tested (Table 2, page 26). It seems that amino nitrogen in the asparagine medium and nitrate nitrogen in the nitrate medium are not suitable nitrogen sources for the growth of this organism. Yeast extract and malt extract

each may contain some growth-promoting substances. Robbins (1950) and Robbins and Hervey (1958) have reported growth factors for basidiomycetes in both malt and yeast extracts.

Manometric methods for estimating exchange of gases have been used in the study of biological reactions for years. The respiration of whole cells is usually measurable in terms of gas exchange - oxygen uptake or carbon dioxide formation - by means of a Warburg constant volume respirometer. Since this technique was used throughout this investigation, the following discussion will deal with some aspects of this technique as well as the results obtained with it.

Though the preparation of cells of fungi for respiratory studies has been described by many authors, nevertheless, there are still some problems about selecting the appropriate methods for a particular organism.

Cochrane (1958) reported that discs of growing mycelium from a surface culture can be transferred to a respirometer flask and oxygen uptake measured. This was tried at the beginning of this investigation but was found impractical with this organism. The mycelial pad floated on the surface of the liquid in the reaction flask and gave less than 10 ml of oxygen uptake in 5 hours.

Since most workers have preferred to use, when possible, the more homogeneous mycelium obtained from shake or aerated cultures (Cochrane, 1953), the whole pellets from the submerged culture were then tried for the respiratory studies. However, since a dispersed culture could not be obtained in submerged

culture and since the pellets were too large to be pipetted, the mycelium was blended for 15 seconds in a Waring Blendor to make homogeneous suspensions which could be pipetted easily. This was done in spite of the risk of lowering the respiration of the organism.

deFiebre et al (1953) and Newcomb et al (1962) have reported that mechanical injury may reduce respiratory capacity and the latter workers suggested that whole mycelial pellets were to be preferred for respiration studies over the more homogeneous but partly disintegrated mycelium prepared by treating the pellets in a blendor for short periods of from 5 to 30 seconds. This was found to be the case for *P. sulfureus* also. The whole pellets had higher respiratory activity than did the blended preparation (Appendix C, page 58). If the cultivation of a uniformly dispersed mycelium can be achieved, it should be used directly for the respiratory studies instead of the blended cells.

Figure 4 (page 30) shows a typical curve for the growth of the culture; a phase of no apparent growth initially followed by a phase of rapid growth and finally autolysis of cells and decline in dry weight. The highest respiratory activity of most microorganisms is usually found during the period of most rapid growth and it then falls off with increasing age similar to this activity in *P. sulfureus* as shown in Figure 4 (page 30). The decrease in respiratory activity may reflect the fact that more and more dead cells accumulate in the older mycelium. The content of total organic solids in

the medium decreased with the growth of the culture and then reached a minimum and constant level as shown in Figure 4 (page 30). The decrease of reducing sugars paralleled that of total organic solids. Total organic solids and reducing sugars were still utilized by the organism even in the phase of decline. This suggests that cell division still occurs but is overcome by the death or autolysis of cell.

Figure 5 (page 31) shows the influence of the pH on the respiration when glucose was added to the suspending medium as exogenous substrate. However, since glucose apparently does not stimulate exogenous respiration, it might be assumed that the pH effect was in truth an effect upon endogenous respiration.

The respiratory quotient (R.Q.) value which is defined as the volume of carbon dioxide produced divided by the volume of oxygen consumed by the respiring organism, usually serves to indicate the nature of the substrate being metabolized. The R.Q. value for the complete oxidation of glucose and hence most carbohydrates, is one.

$$C_6H_{12}O_6 + 6\ O_2 \longrightarrow 6\ CO_2 + 6\ H_2O$$

The R.Q. values for most lipids is 0.8 and 0.9 for most proteins. Though the finding of a certain R.Q. value does not prove that the organism is utilizing either carbohydrate, protein or lipid exclusively, it is an index of the primary process occurring. Eburicoic acid, with a formula of $C_{31}H_{50}O_3$ would have a theoretical R.Q. value of 0.74 if it were completely oxidized.

$$C_{31}H_{50}O_3 + 42\ O_2 \longrightarrow 31\ CO_2 + 25\ H_2O,$$

$31/42 = 0.74$

Studies in this laboratory showed that the R.Q. value of P. sulfureus was about 0.8 (Table 5, page 32) whether or not glucose was added as substrate. Though this value does not quite approach 0.74 (the value calculated for eburicoic acid), the fact that this value is less than one suggests that an endogenous substrate of the oxidation state of lipid was being utilized in the starved mycelium of P. sulfureus.

It is difficult to distinguish between intrinsic inability of a cell to respire an exogenous substrate and failure of the compound to penetrate to the interior of the cell. Results from this investigation (Table 6, page 33) showed that P. sulfureus did not utilize carbohydrates well. Though failure of exogenous substrate to be utilized cannot always be ascribed to membrane inpermeability (Foulkes, 1954), there is a possibility that the permeability barrier may have some effects.

The fact that exogenous sugars are not utilized by this organism also may be due to the saturation of some terminal systems and an addition of substrate will not cause any further increase in oxygen uptake. In respirometric studies, the absence of an increase in the rate of respiration upon addition of a substrate, may not mean that the substrate is not being oxidized. The Crabtree effect, i.e. the inhibition of respiration upon the addition of hexoses, is not uncommon in animal tissues. Perhaps this effect may also be found in microorganisms (Blumenthal, 1965).

Though the gas-exchange from respirometry was the same whether or not glucose was used as exogenous substrate for the mycelial suspension of *P. sulfureus*, it does not mean that glucose is not utilized by this organism. From the experiments with ^{14}C-labeled glucose (Table 8, page 36), it is shown that about 10% of exogenous glucose was being metabolized by this organism.

The method of calculating the C-6 : C-1 ratio of carbons of glucose utilized by an organism as suggested by Bloom and Stetten (1953) as giving directly the maximal fraction of the glucose respired by the glycolysis was used. This gave for this organism the value of 0.27. It indicates that 27% of glucose was metabolized via glycolysis whereas 73% via the pentose pathway. But this technique has been criticized because it over estimates the extent of the pentose pathway. The amount of ^{14}C converted to CO_2 from the 1-position of glucose through the pentose pathway after only a few enzymatic steps, is compared to that coming from the 1-position of glucose which had to go through the entire glycolysis sequence followed by several passages through the Krebs cycle. If there is a block in the Krebs cycle, the C-1 via the glycolysis pathway would not yield CO_2 whereas it would via the pentose pathway (Blumenthal, 1965).

A plot of the total per cent recovery of $^{14}CO_2$ versus time (Figure 7, page 39) indicated that the rate of evolution of $^{14}CO_2$ from glucose-1-^{14}C was much faster than that from glucose-6-^{14}C. Therefore even though the C-6 : C-1

ratio might not give an accurate quantitative estimation of the metabolic pathways, this plot did indicate that the pentose cycle in this organism had preference over the glycolysis pathway.

A tabulation of the major and minor pathways for glucose catabolism in fungi and related microbes using only estimates made with intact cells was summarized by Blumenthal (1965). Glycolysis was the major route used in fifteen organisms, the pentose cycle in five, and Entner-Doudoroff pathway in two. In all five instances where the pentose cycle was the major one, the glycolysis pathway was the minor.

Though it was found from this work with ^{14}C-labeled glucose that this organism most likely utilized the pentose cycle, xylose is not metabolized (Table 6, page 33). An examination of the pentose cycle indicated that the phosphates of ribose, ribulose and xylulose are all intermediates in this cycle but not xylose. Xylose and xylulose have the same formula $C_5H_{10}O_5$ but the former is an aldose while the latter is a ketose.

Theoretically, oxygen uptake is increased by the addition of an intermediate in the energy producing system of an organism. The lack of increased oxygen uptake upon the addition of xylose may indicate that the interconversion of xylose and xylulose does not occur in this organism. The fact that this organism cannot utilize any of the added carbohydrates directly (Table 6, page 33) shows that the endogenous reserves in this organism were utilized preferentially to the exogenous

carbohydrates for energy production. An experiment using ^{14}C-labeled xylulose would indicate whether this pentose was more readily utilized than glucose.

Acetate and succinate which are intermediates in the Krebs cycle stimulated oxygen uptake of this organism whereas pyruvate inhibited it as shown in Table 7 (page 34) and Figure 6 (page 35). The increase in oxygen uptake by acetate and succinate indicated that these two substrates were oxidized by this organism and hence are involved in the energy producing system of P. sulfureus. Manometry with acetate-1-^{14}C showed that the CO_2 evolved had radioactivity and that the per cent radioactivity recovery was higher than when either glucose-1-^{14}C or glucose-6-^{14}C was metabolized (Table 9, page 38).

If the assumption that the oxidation of exogenous glucose is prevented by the saturation of some terminal system is correct then this terminal system must lie somewhere between the glycolytic system and the Krebs cycle since exogenous members of the Krebs cycle are readily used. The observation that exogenous pyruvate inhibits respiration is interesting from the standpoint that it is possible that the terminal system whose saturation prevents exogenous glucose utilization may be at the point of pyruvate utilization.

Acetate with its structural formula of CH_3COOH is a 2-carbon compound, pyruvate $CH_3COCOOH$, a 3-carbon compound and succinate $COOH\text{-}CH_2CH_2COOH$, a 4-carbon compound. All of them have a structural similarity, i.e. they all have a

carboxyl group. From the fact that acetate and succinate stimulate while pyruvate inhibits the oxygen uptake, it may be speculated that the two readily utilized substrates may have properties close to that of the endogenous reserves or that the endogenous reserves are broken down into acetate in this organism.

The fact that pyruvate inhibits the respiration in this organism might also be looked upon as a "sparing" effect of pyruvate. The high endogenous respiratory rate of fungi might be looked upon as being wasteful since the rate of endogenous substrate utilization must be well above that required for the so-called "maintenance energy". Once organisms, which are usually finely tuned to maintain and conserve a very constant environment within the cell, begin to be wasteful of their resources they are thought of as having lost one of their control mechanisms. Fungi on this basis then could be regarded as a whole class of organisms which have lost their ability to control the utilization of their endogenous storage material. Pyruvate may help to restore part of this control in some manner and thus act to "spare" endogenous substrate.

The "sparing" effect of pyruvate and the earlier speculation of pyruvate being involved in saturating a terminal system in the utilization of glucose could be identical processes.

If ^{14}C-labeled glucose were added to cells whose respiration was partly inhibited by pyruvate, the percentage

of ^{14}C in the CO_2 evolved should be greater if pyruvate was playing a role of simply sparing the utilizating of endogenous substrate. If pyruvate were saturating a terminal system common to both the utilization of endogenous substrate (which is apparently of the oxidation level of lipids) and carbohydrate, then the previous experiment would show no increase in ^{14}C in the expired CO_2. If pyruvate is acting in both ways then the experiment tells us little.

It is also possible but not too likely that pyruvate is a toxic material in this organism and blocks some reaction steps in the metabolic sequence thus decreasing the oxygen uptake. Another possibility, which is not too likely either, is that the pyruvate used in this experiment might contain some impurities thus causing a decrease in the utilization of this compound.

Foulkes (1955) studied the cell permeability in yeast and showed that the undissociated pyruvic acid could get into the inner region of the cell whereas pyruvate ion was confined to the outer region of the cell. However, the pyruvate ion can get into the inner region of the cell under those conditions which allow active metabolism to take place. In the study described, only sodium pyruvate was used for the respiratory studies and it showed inhibition of the respiration of *P. sulfureus*. This indicated that pyruvate ion did get into the cells and cause an inhibitory effect. It would be of interest to use free pyruvic acid and see if it still inhibits the respiration.

V. CONCLUSIONS

A 4% malt extract was the best nutrient source among the four kinds tested. Asparagine, nitrate and nitrate plus 0.5% yeast extract were poor media for the development of the mycelium of this organism. It was found that good growth of this organism caused a decrease in the pH of the cultivated medium.

Eburicoic acid was extracted from the mycelium of this organism and was identified by comparison with an authentic sample by means of paper chromatography.

An R.Q. value of 0.8 was obtained for this organism whether or not glucose was added as substrate. This indicated that endogenous substrate of the oxidation level of lipid was being utilized as an energy source.

Xylose, glucose and maltose did not stimulate oxygen uptake although radioisotope experiments using ^{14}C-labeled glucose showed that glucose was being utilized to some extent for energy production even though perhaps indirectly. This indicated that the endogenous reserves in this organism were utilized preferentially to exogenous carbohydrates.

Sodium salts of acetic and succinic acids stimulated oxygen uptake whereas pyruvate inhibited it. It was not asertained whether the inhibition of oxygen uptake by pyruvate was caused by the permeability barrier of cell membrane, saturation of some terminal system essential for the production of energy, the impurities in the pyruvate used, the toxicity

of pyruvate in this organism or the sparing effect of pyruvate on the utilization of endogenous substrates.

Manometric experiments using glucose-1-^{14}C and glucose-6-^{14}C showed that the pentose cycle in P. sulfureus had preference over the glycolytic pathway.

VI. RECOMMENDATIONS

It would be advisable to follow the quantity of eburicoic acid formed in the mycelium of *P. sulfureus* with the culture age. The mycelium of this organism produced eburicoic acid in high yield and manometry experiments showed that an endogenous substrate of the oxidation state of lipid was being utilized in the starved mycelium of this organism. Studies on the change of the amount of acid formed with culture age, and the effect of age on the activity of the endogenous and exogenous respiration might shed light on what kind of reserve material in this organism was being utilized as endogenous energy source.

Results from this investigation indicated that the intact cells of this organism did not utilize carbohydrates and pyruvate as sources of exogenous substrates. It was thought that the permeability might have some effect. It is recommended that cell-free extracts of this organism be prepared and used to study the respiration to see if the cell-free preparation can utilize substrates not used by the intact cells. Also free pyruvic acid should be used instead of pyruvate in the intact cell of this organism to see if it still inhibited the respiration of this organism. Though the inhibition of oxygen uptake by the addition of pyruvate might be accounted for by some other reasons, it would be of interest to know if there was a difference in the penetration ability of the dissociated pyruvate ion and the undissociated form.

The presence of a glycolytic enzyme in a cell extract indicates that the cell has the potential to use it. The glycolytic pathways are known in fungi, namely, the EMP, HMP and ED pathways. By detection of the presence or absence of enzymes involved in these three pathways, one might gain a truer picture of the glucose catabolism in this organism. The study on pathways of glucose catabolism in Caldariomyces fumago by Ramachandran and Gottlieb (1963) and the study on the enzymes of the EMP and HMP pathways in Polyporus brumalis by Casselton (1966) were examples of this type of work.

Two isotopic techniques have been used to determine the status of the endogenous respiration during concurrent utilization of externally furnished substrates. One involves the use of unlabeled cells and a uniformly-^{14}C-labeled substrate. The other involves the use of uniformly-^{14}C-labeled cells and an unlabeled substrate. The former one was used in this investigation but not the latter one. An attempt to grow uniformly-^{14}C-labeled cells from the culture of P. sulfureus failed during this research. The culture was contaminated each time. The reason for its being contaminated is thought to be the type of growth vessel used. A better designed vessel for the growth of P. sulfureus on ^{14}C-labeled materials should be tried. If a carbon balance was performed by measuring the radioactivity of $^{14}CO_2$ evolved during endogenous respiration and by assaying the ^{14}C-labeled intermediates presented in the ^{14}C-labeled cells, one might have obtained a better understanding of the status of endogenous metabolism in this organism.

When the ^{14}C-labeled materials were added to the Warburg flasks as exogenous substrate for the respiration of the unlabeled cells of this organism, only the radioactivity evolved as $^{14}CO_2$ was counted. It would be of considerable value if one analyzed the distribution of radioactivity in the CO_2 evolved, the cells and the culture medium so that one may have more information as to the utilization of exogenous substrate in this organism.

APPENDIX A

DATA FOR THE PREPARATION OF STANDARD CURVE FOR TOTAL ORGANIC SOLIDS*

mg D-glucose	Optical Density (440 mμ)
0.0	0.00 (reduced with $Na_2SO_3.7H_2O$)
0.0	0.60
0.2	0.42
0.2	0.43
0.3	0.35
0.3	0.35
0.4	0.27
0.4	0.25
0.5	0.17
0.5	0.15
0.6	0.06
0.6	0.04
0.7	0.00
0.7	0.00

* For complete description of the method of preparation of the standard curve see page 16, these data are plotted in Figure 1 (page 17).

APPENDIX B

DATA FOR THE PREPARATION OF STANDARD CURVE FOR REDUCING SUGARS*

mg D-glucose	Optical Density (550 mμ)
0.0	0.00
0.0	0.00
0.1	0.05
0.1	0.06
0.2	0.16
0.2	0.18
0.4	0.39
0.4	0.37
0.6	0.57
0.6	0.59
0.8	0.76
0.8	0.76
1.0	0.93
1.0	0.94

*For complete description of the method of preparation of the standard curve see page 18; these data are plotted in Figure 2 (page 20).

APPENDIX C

VARIATION IN MYCELIAL MASS AND RESPIRATORY ACTIVITY WITH AGE#

Days of Growth	Q_{O_2}	mg Dry Wt. per Flask
5	0.77	85.8
6	---- (3.04)*	92.6
7	0.90	191.0
9	0.97	256.0
11	----	294.0
12	1.50	362.0
14	----	415.2
15	1.06	565.0
21	----	592.0
26	----	505.0
28	0.50	460.0
45	0.31	389.0

See Figure 4 (page 30).

* Unblended Cells

APPENDIX D

OXYGEN UPTAKE OF 7 DAY OLD P. SULFUREUS IN THE PRESENCE OF DIFFERENT SUBSTRATES*

Assay medium: 2.5 ml of cell suspension in pH 6 and 0.067 M Phosphate buffer (dry weight 33.1 mg) + 0.3 ml 0.1M Substrate

Time in Minutes	µl Oxygen Uptake			
	No Substrate	Acetate	Pyruvate	Succinate
5	6.0	0.0	0.0	3.5
10	9.7	6.3	0.7	8.5
15	15.0	12.7	3.5	12.8
20	15.7	14.8	4.2	14.2
25	19.4	19.7	7.8	20.6
30	19.4	21.1	8.8	22.0
35	25.4	24.6	13.4	29.1
45	29.9	32.4	16.2	34.7
60	32.9	38.7	20.4	45.4
75	44.1	50.0	26.1	57.5
90	47.8	57.0	31.1	68.0
105	56.8	67.6	38.1	80.8
120	61.2	75.3	42.3	92.2
150	70.2	86.5	50.8	105.0
180	81.4	105.5	63.5	127.5
210	96.4	123.8	76.2	152.5
240	102.0	137.0	86.8	170.0
270	113.0	152.0	95.3	186.0
300	125.0	164.7	101.5	198.5

* For complete description of the assay methods see page 19; these data are plotted in Figure 6 (page 35).

APPENDIX E

THE UTILIZATION OF ^{14}C-LABELED SUBSTRATES BY CELLS OF *P. SULFUREUS**

Apparatus used:
Warburg Respirometer

Concentration of Substrates Used:
50 µM

Radioactivity of Substrates:

Glucose-1-^{14}C	5.55×10^4 CPM/50 µM Glucose
Glucose-6-^{14}C	5.55×10^4 CPM/50 µM Glucose
Glucose-U-^{14}C	5.55×10^5 CPM/50 µM Glucose = 1850 CPM/µM Carbon
Acetate-1-^{14}C	1.11×10^6 CPM/50 µM Acetate

* For complete description of the assay methods see page 21; these data are summarized in Table 8 and Table 9 (pages 36 and 38). Also see Figure 7 (page 39).

Data for the Utilization of Glucose-1-^{14}C

Assay Medium: 2 ml of cell suspension in pH 6 and 0.067 M phosphate buffer + 0.5 ml of 0.1 M glucose-1-^{14}C (5.55×10^4 CPM/50 µM Glucose).

Dry weight of cell suspension used in Run II are 23.6 mg and 23.7 mg in Run III.

	Run II			Run III		
Time (Min.)	µl O_2	CPM in CO_2 evolved	% recovery of radio-activity	µl O_2	CPM in CO_2 evolved	% recovery of radio-activity
5	7			8		
10	13			11		
20	27			26		
30	36			41		
40	42			51		
50	64			64		
60	74	82	0.15	76	78	0.14
70	92			90		
80	102			102		
90	121			118		
100	133			132		
110	---			145		
120	167	137	0.25	159	144	0.26
150	224			199		
180	276	199	0.36	240	273	0.49
210	355			280		
240	387	246	0.44	321	367	0.66

Data for the Utilization of Acetate-1-^{14}C

Assay Medium: 2 ml of cell suspension in pH 6 and 0.067 M phosphate buffer + 0.5 ml of 0.1 M acetate-1-^{14}C (1.11 X 10^6 CPM/50 μM Acetate).

Dry weight of cell suspension used in Run IV are 25.4 mg and 23.6 mg in Run V.

	Run IV			Run V		
Time (Min.)	μl O_2	CPM in CO_2 evolved	% recovery of radio-activity	μl O_2	CPM in CO_2 evolved	% recovery of radio-activity
5	4			4		
10	8			13		
20	20			25		
30	31			43		
40	39			47		
50	50			56		
60	55	1780	0.16	66	1965	0.18
70	67			69		
80	74			76		
90	86			104		
100	88			105		
110	100			106		
120	108	4740	0.43	121	4510	0.41
155	135			158		
180	176	5400	0.48	174	5440	0.49
210	198			203		
240	240	8390	0.76	232	8000	0.72

Data for the Utilization of Glucose-U-^{14}C

Assay Medium: 2 ml of cell suspension in pH 6 and 0.067 M phosphate buffer + 0.5 ml of 0.1 M glucose-U-^{14}C (5.55×10^5 CPM/50 μM Glucose).

Dry weight of cell suspension used in Run VI are 24.1 mg and 25.5 mg in Run VII.

	Run VI			Run VII		
Time (Min.)	μl O_2	CPM in CO_2 evolved	% recovery of radio-activity	μl O_2	CPM in CO_2 evolved	% recovery of radio-activity
5	---			8		
10	2			13		
20	8			25		
30	11			31		
40	16			38		
50	---			52		
60	36	324	0.06	63	320	0.06
70	41			69		
80	46			78		
90	50			82		
100	60			92		
110	66			103		
120	75	527	0.10	115	644	0.12
150	96			143		
180	124	900	0.16	175	1115	0.20
210	151			192		
240	175	1240	0.22	229	1540	0.28

Data for the Utilization of Glucose-6-^{14}C

Assay Medium: 2 ml of cell suspension in pH 6 and 0.067 M phosphate buffer + 0.5 ml of 0.1 M glucose-6-^{14}C (5.55 X 10^4 CPM/50 µM Glucose).

Dry weight of cell suspension used in Run VIII are 23.5 mg and 23.4 mg in Run IX.

	Run VIII			Run IX		
Time (Min.)	µl O_2	CPM in CO_2 evolved	% recovery of radio-activity	µl O_2	CPM in CO_2 evolved	% recovery of radio-activity
5	12			6		
10	14			14		
20	20			26		
30	36			29		
40	---			47		
50	---			57		
60	40	31	0.06	70	23	0.04
70	42			75		
80	46			88		
90	53			97		
100	55			105		
110	57			116		
120	62	43	0.08	128	55	0.10
150	78			177		
180	102	66	0.12	202	70	0.13
210	114			215		
240	133	43	0.08	242	59	0.12

Weitht of $BaCO_3$ per Square Centimeter of Planchet in Determination of Radioactivity in Evolved CO_2*

Substrate	Warburg Run. Time (Hour)	Weight of $BaCO_3$
Glucose-1-^{14}C	1	34.5
	2	45.0
	3	48.0
	4	50.0
Glucose-6-^{14}C	1	53.2
	2	55.4
	3	51.6
	4	50.3
Glucose-U-^{14}C	1	51.0
	2	53.0
	3	54 6
	4	54.9
Acetate-1-^{14}C	1	68.2
	2	60.8
	3	68.3
	4	73.7

* For complete description of the method of determination See pages 22-23.

APPENDIX F

DATA FOR THE PREPARATION OF SELF-ABSORPTION CORRECTION CURVE#

Thickness (mg $BaCO_3/cm^2$)	App. Sp. Act. (CPM/mg $BaCO_3$)	% of Relative* Sp. Act.
10.1	146.0	44.7
16.7	85.3	26.1
18.2	80.2	24.6
21.8	75.5	23.1
24.6	62.0	19.0
26.6	64.6	19.8
28.6	58.4	17.9
31.0	58.8	18.0
33.2	55.7	17.0
34.2	63.5	19.0

For complete description of the method of preparation of the curve see page 22; these data are plotted in Figure 3 (page 24).

* The respective apparent specific activities were plotted against the sample thickness, this curve was extrapolated to zero thickness and this value (327.0 CPM/mg $BaCO_3$) considered as the actual specific activity. The percentage of relative specific activity at each sample thickness was obtained by using the value of the actual specific activity as 100 per cent.

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VITA

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