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A Comparison of Glucose and Acetate Metabolism during Spore Germination in PHYCOMYCES BLAKESLEEANUS

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A COMPARISON OF GLUCOSE AND ACETATE METABOLISM DURING SPORE GERMINATION IN PHYCOMYCES BLAKESLEEANUS

by

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Each person whose signature appears below certifies that he has read this dissertation and that in his opinion it is adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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CHAPTER I

INTRODUCTION

SPORE GERMINATION IN FUNGI

The extensive literature pertaining to the germination of fungal spores has been reviewed by a number of authors. Recent reviews of this subject include those of Wolf and Wolf (1947), Hawker (1950), Gottlieb (1950, 1964), Lilly and Barnett (1951), Cochrane (1958, 1960), Allen (1965), Sussman (1965), Sussman and Halvorson (1966), and several papers in Madelin (1966). Two recent reviews on germination of uredospores are also of interest (Staples and Wynn, 1965; Shaw, 1964). Other reviews of older work or of less general interest are cited in Sussman's paper (Sussman, 1965). Only selected aspects relevant to the problem under investigation will be presented here.

A spore is defined, in a general sense, as any one-celled reproductive structure in the cryptogams (Sussman, 1965). However, in fungi, spores usually are defined as being uni- or multicellular propagules containing one or more nuclei (Hickman, 1965; Alexopoulos, 1962).

After sporulation occurs, the cells of the fungus which become spores no longer engage in active synthesis

of cell material and usually show much reduced metabolic activity. Dormancy is the name given this resting stage.

Germination comprises the processes and changes occurring during the resumption of the metabolism and growth of a spore and its transformation to a morphologically different structure, a hypha. The appearance of this hypha or germ tube is the common criterion by which germination is defined (Allen, 1965; Sussman, 1965). This convention will be followed in the present communication. During this transition a shift of metabolic pattern and activity from that characeteristic of spores to that characteristic of mycelia occurs (Yanagita, 1957; V. W. Cochrane, et al., 1963 b; Caltrider and Gottlieb, 1963; Gottlieb, 1964; Allen, 1965). In most spores respiratory metabolism is very low and treatments which stimulate germination also increase the rate of respiration. Wetting most spores leads to little or no increase in oxygen uptake unless germination ensues. For example, when the spores of Aspergillus niger van Tieghem (Yanagita, 1957), Rhizopus arrhizus Fischer (Weber and Ogawa, 1965), or several other fungi requiring external substrates for germination, are suspended in water, there is little increase in the rate of oxygen uptake and no germination (Allen, 1965). Conversely, exposing the spores of obligate parasites, which germinate at the expense of their reserves, to a properly humid atmosphere or to free water

leads to a rapid increase in the rate of respiration (Williams and Allen, quoted in Allen, 1965).

Stimulation of germination

A number of environmental factors have been shown to affect the rate of germination of fungal spores. The effects of these factors, which include low temperature, high temperature, light, alternate wetting and drying, hydrogen ion concentration, oxygen, carbon dioxide, self-inhibitors, carbon and nitrogen sources and others, have been adequately discussed in the references cited above. I will treat only factors directly related to my studies; namely, high temperatures, self-inhibitors, nutrients, and carbon dioxide.

These factors have been discussed in several of the most recent reviews (Sussman, 1965; Allen, 1965; Sussman and Halvorson, 1966).

Heat shock

For a history of the use of heat shock in germination studies see Sussman (1965: 939-940). Observations that spores of fungi germinated best after the passage through the intestines of warm blooded animals were the basis for guessing that relatively high temperatures of the body might be responsible for stimulating germination. These ideas were tested (Welsford, 1907; Fraser, 1907) by germinating ascospores at 38°C. Soon after this Dodge (1912) succeeded in stimulating the germination of ascospores of several species by short (5-30 minutes)

exposure to temperatures well above body temperature (55°C-75°C). Since that time a number of Ascomycetes have been shown to produce ascospores which are able to be heat activated. This heat-activability appears to be of general significance among coprophilous Ascomycetes but rare outside that group. Recently, however, several papers (Sommer and Halbsguth, 1957; Halbsguth and Rudolph, 1959; Rudolph, 1960 a; Lilly, Barnett, and Krause, 1962) suggest that the sporangiospores of Phycomyces blakesleeanus Burgeff "respond to genuine heat activation" (Sussman, 1965).

The breaking of the dormancy of Neurospora tetrasperma Shear and Dodge ascospores results in a 20- to 30- fold increase in their respiratory rate (Goddard, 1935; 1939). Since the ascospores germinate on endogenous metabolites (Sussman, 1954), a change in permeability to nutrients is probably not the explanation of the effect of heat. Furthermore, permeability to an increase in oxygen (Goddard, 1939) or water (Lingappa and Sussman, 1959) can be ruled out. Sussman (1965) indicates that permeability differences between dormant and activated ascospores do occur but are probably of secondary importance. "They follow, rather than induce, the initial steps in the activation process" (Sussman, 1965).

Several metabolic explanations for heat activation of ascospores have been proposed. Goddard and Smith (1938) suggested that a metabolic block caused by the lack of pyruvic carboxylase was responsible for the dormant state.

But Sussman, Distler, and Krakow (1956) determined that pyruvic carboxylase was present in dormant, activated, and germinating spores and concluded that the activity of the enzyme in dormant spores was sufficient to account for the observed rates of carbon dioxide production in activated spores.

Fifteen per cent of the dry weight of an ascospore is trehalose, a non-reducing disaccharide, but lipids are the sole energy source during the dormant period. During heat activation and before protrusion of the germ tube, trehalose appears to be the principal energy source (Lingappa and Sussman, 1959; Sussman and Lingappa, 1959). It has been proposed that heat activation may involve the bringing together of trehalase and its substrate (Hill and Sussman, 1964) but "the restraint upon the breakdown of trehalose in the dormant ascospore still awaits elucidation" (Budd, Sussman, and Eilers, 1966).

Removal of self-inhibitors

Self-inhibition can be suspected when spores at high concentrations will not germinate but will do so under identical conditions at a lesser concentration (Cochrane, 1958; Allen, 1965). Spores of several saprophytic and parasitic species have been shown to carry substances which will inhibit the germination of spores of the same species (Shepherd and Mandryk, 1962; Richardson and Thorn, 1962), related species (Carlile and Sellin,

1963), and even unrelated genera (Hayer, H., 1962, cited by Allen, 1965).

Inhibitors appear to be widespread in the uredospores of the rust fungi and to be present in many other groups (Allen, 1965), but "endogenous chemicals having the properties of the biological inhibitor have yet to be identified" (Staples and Wynn, 1965).

Self-inhibitors can readily be washed from the spores and this may allow germination to occur (Bell and Daly, 1962; Park and Robinson, 1964; Allen, 1965). Self-inhibition may also be overcome without removal of inhibitors by low concentrations of certain chemical substances which thereby act as germination stimulants. The higher straight chain aldehydes and alcohols have been shown to be effective as germination stimulants in uredospores of the rust fungi. Germination stimulants have been isolated from the spores and include coumarins, phenols, and other substances which seem to be distinct from nutrients since they include substances which are metabolic poisons (Allen, 1965). Endogenous stimulators of germination exist in at least some spores that produce self-inhibitors (Van Sumere, et al., 1957).

Nutrients

Some spores are able to germinate with nothing more than a renewal of water supply. Uredospores are an example. Other spores such as the ascospores of Neurospora do not require an exogenous source of nutrients but will

germinate in deionized water after being heat shocked (Sussman, 1954).

The spores of several species of fungi will not germinate in distilled water but seem to require only an exogenous source of nutrients for maximum germination. Rhizopus arrhizus sporangiospores germinate maximally in a medium containing phosphate and potassium or sodium plus sugars such as glucose or fructose (Ekundayo and Carlile, 1964). The spores of Rhizopus arrhizus (Weber and Ogawa, 1965) and Rhizopus stolonifer Lind (Weber, 1962) are strongly stimulated by L-proline. When phosphate is present several other amino acids become stimulatory (Weber and Ogawa, 1965). The conidia of Aspergillus niger are also strongly stimulated by L-proline but this requirement can be replaced by alanine (Yanagita, 1957; Miller, 1962).

Macroconidia of Fusarium solani f. phaseoli (Buck.) Snyder and Hanson require for germination a carbon source in the form of sugars, a nitrogen source, and a factor present in yeast extract. The yeast extract factor was fully replaced by ethanol or acetoin, partially replaced by acetaldehyde or partially replaced by any of several amino acids. Acetate did not replace the factor (J. C. Cochrane, et al., 1963; V. W. Cochrane, et al., 1963 a, c).

Presence of carbon dioxide

With several types of spore the presence of carbon

dioxide is an important factor in the ability to germinate (Hafiz and Niederpruem, 1963; Staples and Weinstein, 1959; Vakil, Raghavendra, and Bhattacharyya, 1961). The evidence that carbon dioxide is necessary for the germination of some fungus spores has been reviewed (Lilly and Barnett, 1951, chapter 16; Sussman, 1965). Yanagita has demonstrated the need for carbon dioxide in the germination of the conidia of Aspergillus niger (Yanagita, 1957; 1964) and has noted the difficulty in removing this gas. It is possible that a requirement is general but the complete removal of the gas is difficult to accomplish since it is a respiratory product (Sussman, 1965).

THE BIOLOGY OF PHYCOMYCES BLAKESLEEANUS

Natural history

The natural habitat for Phycomyces blakesleeanus seems to be dung. Of the 50 strains of P. blakesleeanus assembled by Benjamin and Hesseltine (1959) there were only 12 for which the isolation data were known. Eleven of these originated from dung, at least one of which was from rabbit dung, and one was a contaminant.

It is a heterothallic species and reproduces basically like the black bread mold, Rhizopus stolonifer. During sexual reproduction the caliper-like suspensors, provided with characteristic black, horn-like projections, hold the zygospore between them.

The asexual spores, or sporangiospores, are produced in a sporangium at the tip of a positively phototrophic sporangiophore which is larger and longer than those of any other known fungi (Benjamin and Hesseltine, 1959).

Classification

Phycomyces belongs in the family Mucoraceae, and although asexually more similar to Mucor than any other genus, is thought to be "a genus without any known close relatives in the family" (Benjamin and Hesseltine, 1959). The species Phycomyces blakesleeanus Burgeff was described in 1925.

Cytology

Recent cytological studies are those of Robinow (1957) and Harm (1957). The nuclei of Phycomyces are very small and resemble those of other Mucorales. Germination is accompanied by closer packing of the chromatin and the emergence of a clear cut nucleolus (Robinow, 1957).

Nutrition

Phycomyces blakesleeanus is one of several species of fungi which require thiamine for growth (Lilly and Barnett, 1951; Cochrane, 1958). Because of this total deficiency in ability to synthesize thiamine the members of this genus have been used as thiamine assay organisms (Lilly and Barnett, 1951; Benjamin and Hesseltine, 1959). Sufficient thiamine is said to be stored by the spores to permit germination (Hawker, 1950: 228).

Several papers have been concerned with nitrogen and carbon nutrition of Phycomyces blakesleeanus. An early paper on nitrogen metabolism was that of Schopfer (1937). Burkholder and McVeigh (1940) directed their attention toward obtaining optimum conditions for the growth of Phycomyces blakesleeanus. Leonian and Lilly (1940) studied the growth requirements of several thiamine requiring fungi. They indicate that acetic, lactic, succinic, glutaric, fumaric, tartaric, and citric acids increase the growth of Phycomyces blakesleeanus when added to a nutrient solution containing 2.5% dextrose, 0.25% ammonium nitrate as the only source of nitrogen, thiamine and agar. Two views are entertained concerning the function of such organic acids: (1) The acids are valuable in maintaining the pH of the medium at favorable levels, and (2) the acids are precursors of various keto-acids from which fungi form primary amino acids (Lilly, 1959). Although there is evidence for both points of view (Lilly, 1959), the work of Friend, Goodwin, and Griffiths (1955) strongly suggests that for Phycomyces blakesleeanus the role of the organic acids is other than that of buffers.

Several papers have been concerned with the effect of acetate on the growth of Phycomyces. Robbins, Kavanagh, and Kavanagh (1942) indicate that although acetate markedly affected germination of sporangiospores of Phycomyces blakesleeanus its effect on growth was slight.

Robbins used asparagine at 2 g/l as the sole nitrogen source. As has been indicated above acetate will cause an increase in growth if an ammonium salt is present (Leonian and Lilly, 1940; Lilly, et al., 1960). Phycomyces has been shown to be able to use acetate as the sole source of carbon (Schopfer and Grob, 1952; Lilly, Barnett, and Krause, 1960; Lilly and Barnett, 1961). However, the rate of growth and the weight of the mycelium produced on acetate-carbon were less than on glucose-carbon (Lilly and Barnett, 1961).

Spore germination and metabolism

A relatively large number of papers have been published on the germination of the sporangiospores of Phycomyces. The majority of these papers are related to the heat activation of the spores. Several, however, are concerned with chemical activation.

Heat activation

Robbins and associates demonstrated that heat would stimulate the germination of the asexual spores of Phycomyces blakesleeanus (Robbins, et al., 1942). Recently a number of German workers have investigated the mechanism of heat activation (Sommer and Halbsguth, 1957; Halbsguth and Rudolph, 1959; Rudolph, 1960 a, b; 1961 a, b).

Rudolph (1960 a) reported that the Q_{O_2} for dormant spores in a medium consisting of glucose, asparagine, thiamine, and inorganic ions was 1.92 ± 0.23 and was

raised to 11.39 $\pm$ 1.25 after heat treatment. Rudolph (1960 b) indicated that variations such as a difference of 2°C in the temperature used in activating spores caused wide variations in the percentage of germination. Other conditions such as variations in the amount of light and density of the spore suspension before the heat treatment affected the ability of the spores to tolerate long exposures to 50°C temperatures.

Another paper (Rudolph, 1961 a) showed that heating spores to 50°C for 3 minutes caused the release of ethanol and acetaldehyde into the nutrient solution. Spore germination was strongly activated by an extract which was obtained in good quantity from heat treated spores (Rudolph, 1961 b).

The use of inhibitors

Inhibitors are substances which "poison" enzymes and thus decrease the rate of biochemical reactions. Many nonspecific enzyme inhibitors such as heavy metals and acids, such as trichloacetic acid, inactivate all proteins and therefore are not useful in delineating metabolic pathways. Useful enzyme inhibitors are specific in their actions; they inhibit only a few enzymes. The ideal inhibitor inhibits only one enzyme. If ideal inhibitors were available one could measure the rate of a biochemical reaction in the presence and absence of an inhibitor specific in its action toward some enzyme. If a slower rate of biochemical reaction was observed in the

presence of the inhibitor than in its absence one could say that a certain enzyme was probably present in the experimental cells and that metabolic pathways in which the enzyme was a member were also present.

Metabolic inhibitors have been used in spore germination and respiration experiments. When spores will not germinate in the presence of certain inhibitors it is evidence that enzymes characteristíc of certain pathways of metabolism are important in the ability of the spore to germinate. If one is interested in knowing which respiratory pathways are active during any stage of development, he can add a specific inhibitor to spores respiring in an appropriate medium and measure the change in rate of oxygen uptake. A decrease in the rate of oxygen uptake in spores with the inhibitor present indicates that a certain enzyme is active in untreated spores.

Two common inhibitors of the glycolytic sequence are iodoacetic acid and sodium fluoride. Iodoacetic acid is know to inhibit many enzymes but glyceraldehyde-3-phosphate dehydrogenase is most sensitive. Enolase is most sensitive to sodium fluoride.

Malonic acid and fluoroacetate are tricarboxylic acid cycle inhibitors and are relatively specific in their actions. Fluoroacetate is apparently similar enough to acetate to substitute for it in the activating and condensing enzyme systems but is not an acceptable

substrate for further reactions and thus jams the cycle. Malonate is the classical inhibitor of succinic dehydrogenase.

Several inhibitors interfere with normal functioning of the respiratory chain. Cyanide and azide are classical inhibitors of cytochrome oxidase. Antimycin A blocks the transfer of electrons between cytochromes b and c. Dinitrophenol is an inhibitor which prevents the coupling between the carrier molecule adenosine diphosphate and the enzymes transporting the high energy phosphate bonds. Consequently, energy liberated in oxidation is lost to the cell. Inhibitors such as dinitrophenol and coumarin are known as uncouplers. This discussion of inhibitors is based on material found in the texts by Mahler and Cordes (1966) and Beevers (1961).

Effect of inhibitors on heat activated spores

2,4-Dinitrophenol has been used to study the effect of heat activation. Rudolph (1960 a) reported that with heat activated spores (3 minutes at 50°C) dinitrophenol between 0.5 and 2.2 x 10^{-5}M increased respiration up to 130% of control values but that higher percentages inhibited it. Germination of spores was decreased slightly by low concentrations (3.3 x 10^{-5}M) but was markedly decreased by concentrations of 5 x $10^{-5}$and higher. Dinitrophenol at 1.25 x 10^{-5} had no effect on anaerobic carbon dioxide production while higher concentrations

(5×10^{-5} and 10^{-4}M) decreased production for the first hour then increased production for the third hour.

Iodoacetate at 2.69×10^{-8} had little effect on carbon dioxide output or oxygen uptake in spores activated by heating to 54°C for 3 minutes but it did inhibit acetaldehyde production (Rudolph, 1961 a).

Rudolph (1960 a) showed that at pH 4.15 sodium fluoride was strongly inhibitory to oxygen uptake by heat treated spores suspended in standard media in the presence of either glucose or ammonium acetate. Addition of sodium fluoride at pH 4.18 inhibited by fifty per cent the rise in respiratory rate initiated by heat treatment. At other pH values different levels of the poison were needed for the same effect. The respiratory quotient (RQ) was greatly increased by the addition of sodium fluoride at 0.01M to heat activated spores respiring in media containing glucose. Sodium fluoride unexpectedly increased the output of carbon dioxide under anaerobic conditions. Heat treatment combined with 0.01M sodium fluoride increased acetaldehyde excretion above the amount excreted after heat treatment alone (Rudolph, 1960 b).

Fluoroacetate at 5×10^{-5}M strongly inhibited germ tube formation of heat activated spores. At 0.01M it strongly inhibited oxygen uptake and increased the excretion of acetaldehyde. Malonic acid at 0.01M inhibited germination completely (Rudolph, 1961 a).

Chemical Activation

Hypoxanthine accelerated both spore germination and early growth of Phycomyces blakesleeanus. Its action could be partially replaced by guanine (Robbins, 1940, 1941, 1943; Robbins and Hamner, 1940; Robbins and Kavanagh, 1942 a, b; Robbins, et al., 1942; Robbins and Schmitt, 1945; Rother, 1954).

Sodium and potassium acetates have been shown to be spore germination factors as well as stimulators of growth (Robbins, et al., 1942; Sommer and Halbsguth, 1957; Lilly, Barnett, and Krause, 1960, 1962). Stimulation of germination has been effected by 0.1N organic acids, surface active materials and by 0.1M ammonium acetate (Sommer and Halbsguth, 1957). Sommer and Halbsguth (1957) noticed that a defined medium containing glucose, potassium phosphate, and asparagine, after being heated to 110°C in an autoclave, had a marked stimulatory effect on germination. Brinckmann (1960) found that although several breakdown products were formed under these conditions the only one with strong germination promoting properties was acetate.

Brinckmann (1960) has noted that some materials stimulated germination only after the spores have been heated (lactic acid, oxaloacetic acid, succinic acid, dihydroxyacetone, pyruvic acid) while others stimulated germination without heat treatment (acetic acid, propionic

acid, butyric acid, glyoxylic acid). Sommer and Halbsguth (1959) reported that acetaldehyde had no effect on germination. Brinckmann (1960) indicated that it had some effect while Rudolph (1960 a, 1961 b) reported a strong stimulation.

Borchert's paper (1962) is relevant to the research reported in this paper. He reported that pretreatment by 0.1M ammonium acetate, 0.01M potassium propionate or 0.001M potassium glyoxalate stimulated germination at a high rate. This activation was dependent upon the temperature and duration of action of the activating substance, and in contrast to heat activation (Halbsguth and Rudolph, 1959), was irreversible. After acetate activation a small vacuole formed within the spore. This "small vacuole" was formed under other conditions such as soaking the spores in water. Acetate activation in contrast to no treatment or heat activation made the spores sensitive to the effect of heavy metal ions. Borchert tested the effect of acetate on respiration by using manometric methods. He reported that after 15 minutes oxygen uptake had increased fifteen times. If the activated spores were removed and placed in water, the rate decreased to the level of unactivated spores. The respiratory quotient during the first few minutes after acetate activation was between 4 and 5 but soon dropped to 1. If the oxygen pressure was low, this high RQ remained longer. Borchert (1962) concluded that

acetate served the spore as a respirable substrate and that it probably also stimulated the catabolism of the reserve materials of the spore. Glyoxylate at a concentration of 0.0025M stimulated the breakdown of spore reserve materials but was toxic at a concentration of 0.005M.

Rudolph (1960 a) showed that sodium fluoride strongly inhibited the respiration induced by pyruvate or ammonium acetate.

Rationale and purpose

The carbon of both acetate and glucose can be oxidized by the citric acid cycle in fungi (e.g. Niederpruem, 1965). Mycelium of P. blakesleeanus grown in a glucose, acetate, ammonium sulfate medium oxidizes acetate by way of the tricaboxylic acid (TCA) cycle (Gangloff, 1962). Since acetyl CoA is an intermediate in the oxidation of both glucose and acetate it is surprising that glucose which is a good source of carbon for most fungi (Cochrane, 1958) is not adequate for the germination of the sporangiospores of P. blakesleeanus.

At least two possibilities are evident: (1) A block may exist in the usual pathways of glucose metabolism thus making glucose unavailable as an adequate energy source or as a source of intermediates for synthesis of macromolecules during germination; (2) Glucose may be oxidized through normal pathways and may be adequate as an energy source and for intermediates but acetate may be

necessary as a stimulator of germination, its function being unrelated to energy metabolism (e.g., as an inactivator of a self-inhibitor).

The purpose of the research reported here was to investigate some aspects of glucose and acetate metabolism in Phycomyces and, in particular, the possibility of a block of glycolytic metabolism in sporangiospores.

Some relevant questions to the problem seemed to be the following:

1) Does acetate, as compared to glucose, stimulate germination in spores of strain ATCC 8743b(-) of P. blakesleeanus?

2) Does acetate stimulate respiration in spores and is acetate itself oxidized rapidly?

3) What affect do respiratory inhibitors have on the rate of oxygen uptake with acetate as substrate as compared with glucose as substrate? Do spores and mycelium react similarily?

4) What effect does the presence of acetate have on the rate of oxidation of glucose and endogenous material?

5) Does glucose have an effect on the rate of acetate oxidation and the rate of oxidation of endogenous material?

6) Does acetate change the C_6/C_1 ratio of glucose metabolism in spores? The answer to this question should indicate something about which pathways of glucose

oxidation are important in the presence of acetate and in its absence.

7) Is the TCA cycle important in the metabolism of glucose in spores and mycelium and does acetate have an effect on the amount of glucose carbon entering the cycle? Several amino acids closely related to the TCA cycle were isolated after incubation of mycelium and spores with uniformly-^{14}C-labeled glucose to see if carbon from glucose were entering the cycle.

The following pages give the results of an attempt to answer the preceding questions.

CHAPTER II

GERMINATION OF SPORES

Materials and Methods

The strain of *Phycomyces blakesleeanus* used was obtained from the American Type Culture Collection (No. 8743 b -). The methods used to culture this organism and measure spore germination were essentially those of Lilly, Barnett, and Krause (1962).

Asexual spores were obtained from cultures grown on 50 ml of a medium consisting of 2.25 g malt agar (Difco), 0.25 g yeast extract (Difco) and 50 ml of water in liter Erlenmeyer flasks. These cultures were incubated at 21 $\pm$ 1°C and received 12 hours of light daily. The spores were collected by adding 50 ml of sterile distilled water to cultures between two and three weeks old. After the water was added the cultures were swirled to dislodge the spores. A sterile "rubber policeman" often was used to scrape spores from the inside walls of the flasks. The spore suspension was poured through four layers of cheese cloth to remove mycelial fragments; the spores were sedimented by centrifugation, washed twice with distilled water, and resuspended in 1 ml of phosphate buffer of the pH and concentration stated below.

For spore germination studies 125 ml screw-capped Erlenmeyer flasks were used. Each contained 20 ml of medium. Media were sterilized by autoclaving 15 minutes at 121°C. Nutrients and inhibitors were sterilized separately from phosphate buffer.

Spore germination studies were done using a potassium phosphate buffer of Lilly et al. (1962), or a 0.067M Sorensen phosphate buffer of pH 5 (Gortner and Gortner, 1949). Glucose was used at 10 g/l and potassium acetate at 0.72 g/l (Lilly et al., 1962). About 5% of the number of spores in one culture (3×10^7 spores, as estimated by use of a hemocytometer) were inoculated into each flask and were incubated in duplicate at 21 $\pm$ 1°C before being counted. After an incubation period of 48 hours 1 ml of the spore suspension was removed and pipetted into a 50 mm petri dish containing a thin layer of 2% water agar. All the germinated and nongerminated spores seen in 8 to 12 random fields of the compound microscope (100 x) were counted and the percentage of spores that had produced germ tubes was calculated. For most experiments a spore was considered germinated if a germ tube was obvious. During some early work, particularly with heated spores, it was difficult to demonstrate a difference between different treatments by this criterion. However, with some treatments spore swelling was obviously greater than with others. For one experiment spore swelling was used as a parameter showing the effect of different treatments.

A spore was considered swollen if its smallest diameter was as large as, or larger than, the largest diameter of an untreated spore (about 12 μ).

To determine the effect of low carbon dioxide pressure, the usual protocol was followed except that small open vials containing 5 ml of 20% KOH were hung by nichrome wire inside the 125 ml culture vessels.

Heat-treated spores were prepared by placing a tube of freshly harvested spores in a water bath at 50°C for 3 minutes.

Since preliminary experiments showed that well-cleaned glassware was necessary for high germination rates, all glassware was washed, soaked in chromic acid, rinsed repeatedly with tap water and finally with distilled water.

A nitrogen source was not included in any media in which spore germination was tested; the media were thus incomplete and growth beyond germ tube formation did not occur.

Results

Table 1 summarizes the data obtained from an experiment designed to test the effect of acetate, glucose and a low carbon dioxide pressure on spore germination. Media containing only glucose at 10 g/l as experimental variable did not stimulate germination while spores in those containing acetate at 0.72 g/l germinated at a high rate. No difference was noted between the effect of acetate and the effect of a combination of glucose and

TABLE 1

The percentage of germination of Phycomyces blakesleeanus spores occurring in incomplete media after 48 hours incubation with or without carbon dioxide absorption by 20% potassium hydroxide. All media are in duplicate and are buffered at pH 5 with 0.067M potassium phosphate buffer.

Media	Percentage germination ± SD
Buffer	0
Glucose 10 g/l	0
Glucose 10 g/l & KOH	0
Acetate 0.72 g/l	91.0 ± 1.3
Acetate 0.72 g/l & KOH	92.7 ± 3.2
Glucose 10 g/l Acetate 0.72 g/l	89.6 ± 4.3
Glucose 10 g/l Acetate 0.72 g/l & KOH	90.2 ± 1.5

acetate. Low carbon dioxide partial pressure seemed to have no effect on the rate of spore germination regardless of the substrate.

Radin (1952) reported that α-lipoic acid was an acetate replacing factor for a species of Corynebacterium. When I tested α-lipoic acid for its ability to stimulate germination in Phycomyces blakesleeanus spores, however, it showed no effect on nonheated spores incubated with glucose, pyruvate or acetate.

Several other factors were tested for their effect on germination, in experiments of a preliminary nature. Heat treating in buffer alone did not produce germination. Pyruvate at 0.01M seemed to be effective as a germination stimulant with heated spores but not with nonheated ones. Coumarin (10^{-4}M), 2,4-dinitrophenol (10^{-4}M), and sodium fluoride (10^{-3}M) had little or no effect on heated or non-heated spores in the presence of glucose or acetate at pH 5.8. Malonic acid at 0.01M inhibited both swelling and germ tube formation of both heat-treated spores and non-heated spores in the presence of both glucose and acetate. Fluoroacetate at 0.01M was almost as potent as malonic acid as an inhibitor of germination of acetate treated spores but seemed to have little effect with glucose treated spores that had been heated. Sodium cyanide at 0.001M was moderately inhibitory to heat-treated spores in the presence of glucose but was strongly inhibitory to acetate treated spores.

When spore swelling was used as the criterion for germination, treatment with glucose, acetate, and glucose plus acetate gave the results shown in Table 2. Glucose had little or no effect on spore swelling, acetate was highly effective, and glucose plus acetate was even more effective than acetate alone. The difference between the effects of acetate, and glucose plus acetate was significant ($P < .05$).

TABLE 2

Swelling of Phycomyces blakesleeanus spores produced by glucose, acetate, and glucose plus acetate. Spores are suspended in duplicate in media buffered at pH 5.8 with potassium phosphate buffer.

Media	Percentage swelled ± SD
Glucose	0.3 ± 0.8
Acetate	67.0 ± 4.2
Glucose + Acetate	86.5 ± 2.1

CHAPTER III

RESPIRATORY METABOLISM

Since acetate was shown to be effective in stimulating spore germination, it was of interest to determine its effect on the respiratory rate and to compare its effect with that of glucose both on spores and mycelium. Inhibitor studies were also initiated as they seemed to offer promise of showing differences between acetate glucose metabolism. If there are changes in metabolic pathways during development from spore to mycelium, inhibitors should be useful in investigating these changes. Rudolph (1960 a, b; 1961 a) used respiratory inhibitors to study the respiratory behavior of heat-treated spores.

Materials and Methods

Phycomyces spores were grown and collected by essentially the same methods as given in Chapter II. The spores from one culture flask were used for two Warburg flasks. Spores from several culture flasks were pooled before being washed by centrifugation. No special attempt was made to prevent bacterial contamination since the experiments were of relatively short duration.

Respiratory rates were measured at hourly intervals at 25° by standard manometric techniques (Umbreit, Burris,

and Stauffer, 1957) or for intervals of up to 10 minutes by the oxygen polarographic method described by Packer (1964). When manometric techniques were used, 0.2 ml of potassium hydroxide (10%) was in the center well of the vessel except when cyanide or carbon dioxide buffers were being employed. When cyanide was used, 0.2 ml of 1M potassium cyanide was used to absorb carbon dioxide (Laties, 1949). Data are the means of duplicate determinations unless stated otherwise.

Figures 7-10, which illustrate oxygen electrode traces, are copies of original traces with the dimensions reduced by one-half. Each figure illustrates only one trace. The weight of spores varied and was not always determined.

The reproducibility that can be expected from the oxygen electrode is about ± 10% (Packer, 1964). Considering the above limitations, the data obtained with the oxygen electrode must be considered of somewhat limited significance. In most cases, however, experiments were repeated more than once, and the results were qualitatively consistent with those shown in the figures.

Carbon dioxide buffers were prepared and used with modifications of methods of Krebs (1951) and Pardee (1949). I dissolved 0.1 g thiourea in about 30 ml of water, added this to 38.2 ml of diethanolamine and adjusted to 100 ml with water to make 0.001 g thiourea per ml in 4M diethanolamine. Thirty-three ml of this mixture in a 100 ml

graduated cylinder were gassed 30 minutes by passing 100% carbon dioxide through an aquarium stone into it. The 67 ml of ungassed diethanolamine were added and this mixture was stored as a stock solution. Before each experiment 10 ml of the stock buffer were shaken 1-2 hours on a rotary shaker at about 150 RPM in a 125 ml Erlenmeyer flask. Four-tenths of a ml were used in the center well of each Warburg flask (Yanagita, 1957).

Mycelium was used in the form of pellets made by inoculating by aseptic technique one drop of a thick suspension of spores into a 125 ml Erlenmeyer flask containing 25 ml of sterile malt-yeast broth. The broth consisted of 0.75 g malt extract (Difco), 0.125 g yeast extract (Difco), and 25 ml distilled water. The flasks were shaken on a rotary shaker at 125 RPM for 24 hours at room temperature (about 23°C). After 24 hours the medium was filtered off and the pellets washed on a sintered glass filter or Büchner Funnel, resuspended in buffer, and placed in the Warburg vessels.

Since the pellets clumped together, pipetting in uniform quantities was difficult. An alternative method, which I found more reliable than pipetting, was to filter the pellets on a Büchner funnel, remove the paper filter with the pellets adhering as a thin mat to its surface, and cut the filter into uniform size pieces with scissors. The paper was cut into two pieces more than the number of flasks used. The mycelial mat was stripped from the paper

with tweezers, two pieces were placed on filter paper and dried at 100°C for 24 hours. These were used to determine dry weight. The remaining pieces were placed in the Warburg flasks.

Preliminary experiments indicated that the spores of Rhizopus stolonifer metabolized glucose at least as rapidly as acetate. Experiments were done to compare the spores of Rhizopus with those of Phycomyces. Although the comparisons between the two types of spores were not as instructive as was hoped, the results were interesting and are included.

Spores of Rhizopus stolonifer were grown on malt-yeast agar slants and were removed when the cultures were three days old. About 10 ml of distilled water were added to the slants and the spores were poured off after they had been scraped from the culture into the water by a stiff bacteriological loop. The spores were filtered through cheese cloth, washed twice with distilled water, suspended in an appropriate quantity of buffer, and dispensed into the Warburg flasks. The spores from one 50 ml slant culture were more than adequate for an experiment using 10 Warburg flasks. Pellets of Rhizopus were grown as described for Phycomyces, except that the incubation time was 15 hours. Unless stated otherwise duplicate flasks were used for manometric data.

Because of variation between results of experiments, all the curves in any particular figure represent

simultaneous observations so as to facilitate comparison. In some cases several experiments could have been used to illustrate the same point.

Results of Experiments in Which Manometric Methods Were Used

Respiration of Phycomyces Spores With Various Substrates

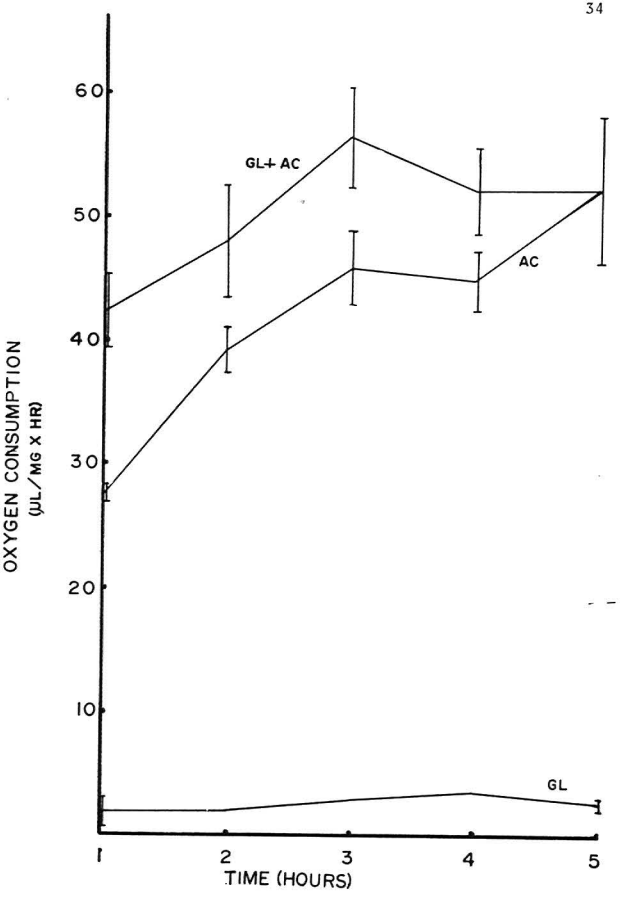
The rate of endogenous respiration of resting Phycomyces spores is so low as to be difficult to measure manometrically. A Q_{O_2} of 1.4 (expressed as μl oxygen uptake per mg dry weight of spores per hour) has been reported (Borchert, 1962).

In my studies the Q_{O_2} of freshly harvested sporangiospores in phosphate buffer of pH 5 and glucose at 10 mg/ml was near two over a five-hour period (Fig. 1). Another experiment showed the rate to remain constant at 2.5 over a three-hour period (Fig. 4).

In comparison to the low rates of respiration of spores in the presence of glucose, spores with acetate at 0.72 mg/ml respired at a rate 16 times higher than the rate with glucose during the first hour and more than 20 times higher during the fifth hour. A combination of glucose and acetate resulted in even higher rates (Fig. 1).

For the five hours of the experiment P values for a t-test for acetate vs glucose plus acetate were: 0.01, 0.09, 0.10, 0.10, 0.47. When the carbon dioxide pressures were kept constant and equal to those of the atmosphere by

Figure 1. The effect of acetate (AC) (0.72 mg/ml) and glucose (GL) (10 mg/ml) individually or in combination on the rate of oxygen uptake of Phycomyces blakesleeanus spores. Points shown are the means of two determinations. Each vertical line represents one standard error of the mean.


OXYGEN CONSUMPTION
(µL/MG X HR)
60
50
40
30
20
10
GL+ AC
AC
GL
1
2
3
4
5
TIME (HOURS)

a carbon dioxide buffer, acetate was at least as effective as glucose plus acetate in stimulating respiration.

Effect of Various Inhibitors on Respiration of Phycomyces Spores

Studies dealing with the effects of the uncoupling agent 2,4-dinitrophenol indicated that the respiratory capacity of spores in the presence of glucose was considerably greater than the data reported above indicate. Oxygen consumption amounting to from more than 200% to more than 400% of control levels was evoked by dinitrophenol at 5 x 10^{-5}M during the five hours of incubation (Fig. 2). P values for the five hours for glucose vs glucose plus dinitrophenol were: 0.02, 0.01, 0.06, 0.02, 0.001. These data indicate a tightly coupled respiratory activity of dormant spores.

Classical cytochrome oxidase inhibitors were tested for their effect on the respiratory system. Instead of inhibition, a stimulation of oxygen uptake was observed when potassium cyanide and sodium azide were offered with glucose as the substrate. Figure 2 shows that in the presence of potassium cyanide at 0.01M the rate of respiration of spores with glucose as substrate is close to the rate with both glucose and acetate.

During the first hour sodium azide at 0.01M had no effect on respiration, but as time progressed a stimulatory effect became apparent (for hours 2-5 $P < .01$;

Figure 2. Effects of some inhibitors on the rate of oxygen uptake by Phycomyces blakesleeanus spores. Substrates were glucose (GL) (10 mg/ml), acetate (AC) (0.72 mg/ml), and glucose plus acetate. Inhibitors were sodium azide (AZ) (0.01M), 2,4-dinitrophenol (DNP) (5×10^{-5}M) and potassium cyanide (0.01M). Points shown are the means of two determinations. Each vertical line represents one standard error of the mean.

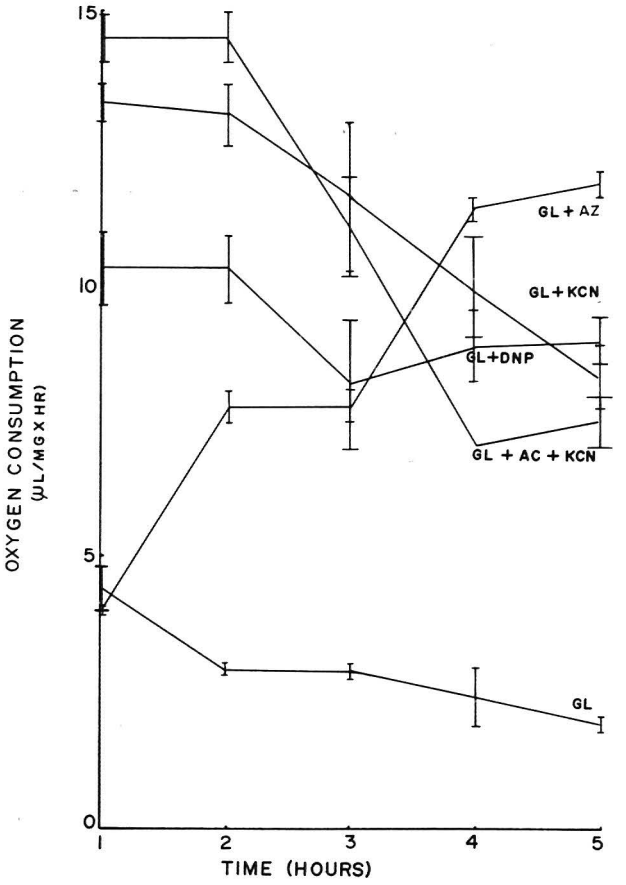

15
10
5
0
OXYGEN CONSUMPTION
(μL/MG X HR)
GL + AZ
GL+KCN
GL+DNP
GL + AC + KCN
GL
1
2
3
4
5
TIME (HOURS)

Fig. 2). At the fifth hour the stimulation caused by sodium azide was significantly higher than for dinitrophenol ($P < .02$).

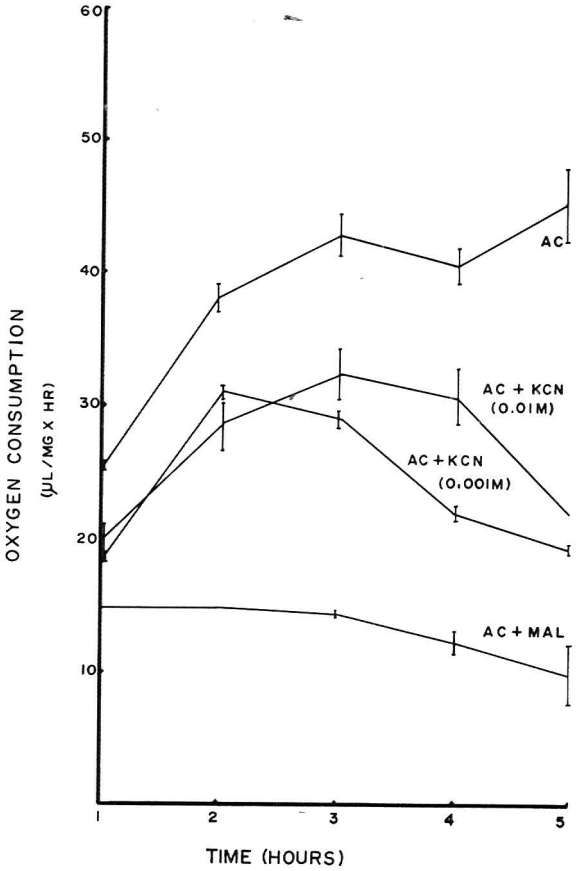
The data of Figure 3 indicate that with acetate as sole substrate potassium cyanide at 0.01 and 0.001M inhibited about equally. The stimulatory effect of acetate is significantly inhibited by cyanide at these relatively high concentrations. For potassium cyanide (0.01M) P values for the five hours were 0.03, 0.06, 0.10, 0.07, and 0.05; for potassium cyanide (0.001M) P values were 0.01, 0.06, 0.03, 0.01, and 0.04. A component of the respiratory system having to do with glucose or possibly endogenous oxidation seems resistant to the effect of cyanide, and, in fact, is stimulated by it (Fig. 2).

Malonate when present with acetate and phosphate buffer at pH 6 had little or no effect on the rate of metabolism, but as can be seen in Figure 3, it is a potent inhibitor of acetate metabolism when present at pH 5 ($P < .01$ for hours 1-4; $P < .05$ for hour 5). Under these same conditions malonate at 0.01M and sodium amytal at 0.01M had little or no effect on glucose respiration.

Effect of Various Nitrogen Sources on Respiration of Phycomyces Spores

The provision of ammonium ion at 0.001M had no effect on the rate of spore respiration in the presence of

Figure 3. Effect of inhibitors on the rate of oxygen uptake by Phycomyces blakesleeanus spores at pH 5 in the presence of acetate. Inhibitors were potassium cyanide (KCN) (0.001M and 0.01M), malonate (MAL) (0.01M). Points shown are the mean of two determinations. Each vertical line represents one standard error of the mean.


60
50
40
30
20
10
OXYGEN CONSUMPTION
(µL/MG X HR)
AC
AC + KCN
(0.01M)
AC + KCN
(0.001M)
AC + MAL
1
2
3
4
5
TIME (HOURS)

glucose and did not significantly increase the rate of oxygen uptake for the first two hours when it was present with acetate (Fig. 4). The values for glucose were arbitrarily chosen from another experiment and were used to facilitate visual comparison. During the third hour of incubation with acetate, a significant difference ($P < .05$) appeared between the rate in the presence of the ion and in its absence. Proline had a similar effect in this respect ($P < .01$ for second and third hours) but did not increase oxygen uptake appreciably when present as the sole carbon source (Fig. 4).

Effect of Various Substrates and Inhibitors on Respiration of Mycelial Pellets of Phycomyces

In contrast to the almost negligible endogenous oxygen uptake with the spores of P. blakesleeanus, the freshly harvested pellets of mycelium showed a high rate of oxygen uptake (Fig. 5). This rate dropped slowly over several hours. Glucose, acetate, and a combination of glucose and acetate appeared to stimulate respiration above endogenous levels but differences between the treatments were not significant. Data obtained from another experiment done under the same conditions showed that pellets incubated with glucose and acetate respired at a higher rate than pellets incubated with acetate only ($P. < .05$ for three of the four hours).

Malonate was an effective inhibitor of pellet respiration but the degree of inhibition depended on the

Figure 4. Effect of ammonium chloride (NH_4) (0.001M) or proline (PRO) (0.01M) on glucose (GL) or acetate (AC) metabolism in Phycomyces blakesleeanus spores as measured by rate of oxygen uptake. Points shown are the means of two determinations. Each vertical line represents one standard error of the mean.

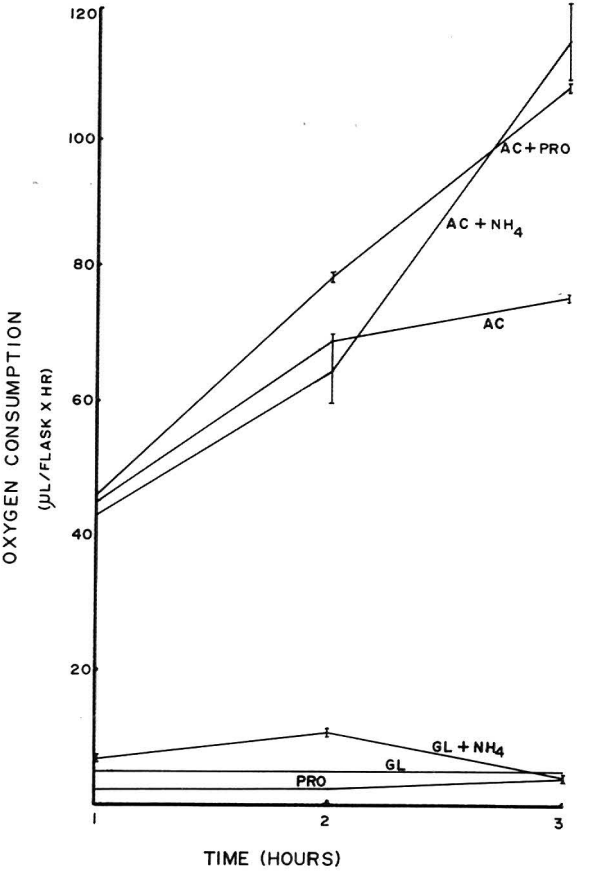
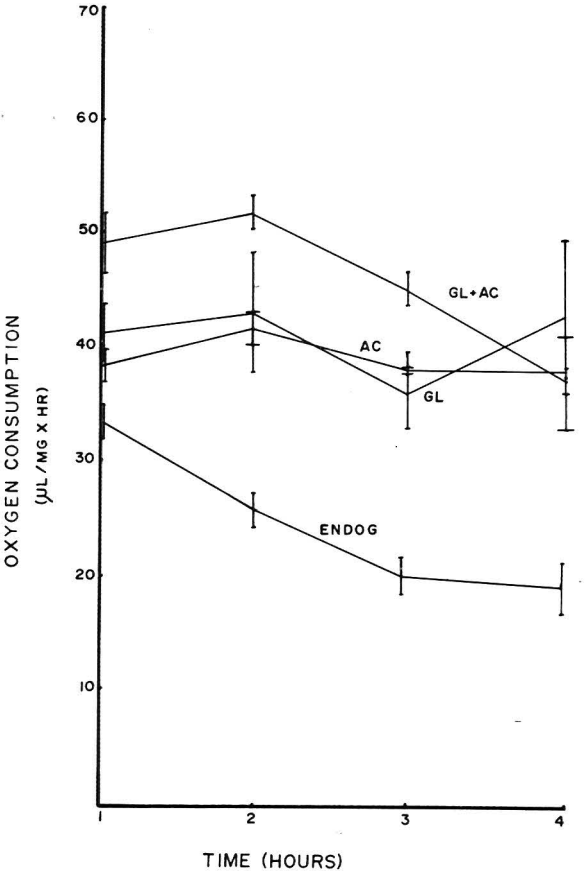

120
100
80
60
40
20
OXYGEN CONSUMPTION
(μL/FLASK X HR)
AC+PRO
AC + NH4
AC
GL + NH4
GL
PRO
1
2
3
TIME (HOURS)

Figure 5. Rate of oxygen uptake of Phycomyces blakesleeanus mycelial pellets with glucose (GL) and acetate (AC) as subtrates vs endogenous rate (ENDOG). Points shown are the means of two determinations. Each vertical line represents one standard error of the mean.


70
60
50
40
30
20
10
OXYGEN CONSUMPTION
(μL/MG X HR)
GL+AC
AC
GL
ENDOG
1
2
3
4
TIME (HOURS)

substrate present (Table 3). Malonate at 0.01M decreased the rate of pellet respiration by at least 50% below control levels when glucose was present, decreased it by more than 90% when both glucose and acetate were present, and essentially eliminated it when acetate alone was the carbon source.

Potassium cyanide at 0.01M was a powerful inhibitor irrespective of the substrate present. No detectable amount of oxygen uptake was observed when acetate was the sole carbon source; from 2-8% of control levels were observed with glucose or glucose plus acetate as substrate (Table 3).

Spore respiration appears relatively resistant to the inhibitory effects of cyanide with acetate as substrate. When Figure 3 is compared with Table 3 (acetate as substrate) it can be seen that cyanide inhibited mycelial respiration much more strongly than spore respiration.

Comparison of Respiration in Phycomyces with Rhizopus

When the respiratory behavior of spores of Phycomyces and Rhizopus was compared, differences were apparent. Glucose had little effect on the rate of respiration of Phycomyces spores but acetate was highly stimulatory. With Rhizopusspores glucose and acetate had about equal effects but both together stimulated respiration significantly ($P < .05$) over either separately. Malonate inhibited oxygen uptake with acetate as substrate

TABLE 3

Effect of metabolic inhibitors on oxygen uptake of Phycomyces blakesleeanus mycelial pellets. Values are the means of two determinations and indicate rates of oxygen uptake in the presence of inhibitors as per cent of values with no inhibitor present.

	Substrate														
	Glucose					Acetate					Glucose + Acetate				
	Time (hours)														
Inhibitor	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
KCN 0.01M	8	6	5	6	4	0	0	0	0	-	8	4	0	4	2
KCN 0.001M						0	0	0	0	-					
Malonate 0.01M	*50	46	46	55	-	9	1	0	0	-	10	7	5	5	-

* Values obtained by an extrapolation of the results of two experiments.

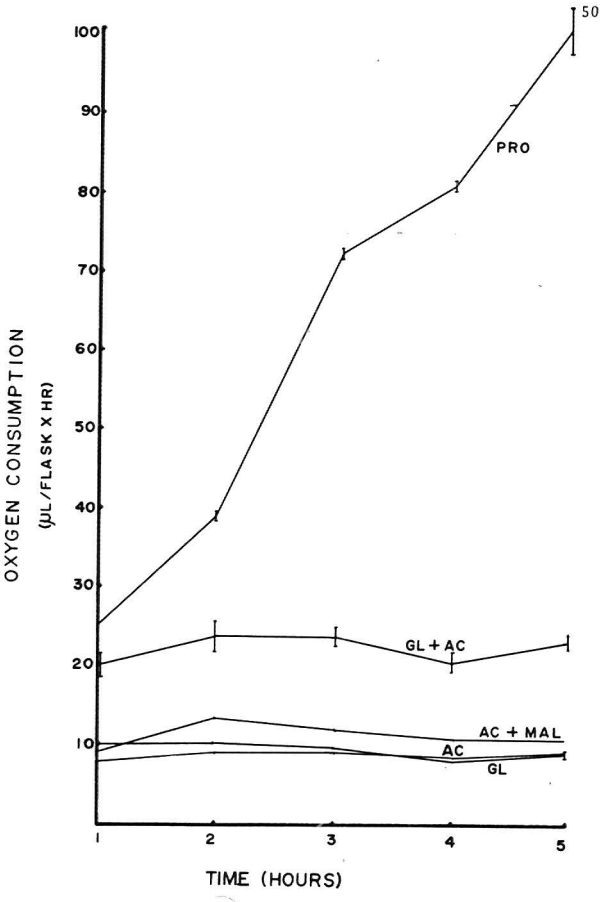
in Phycomyces spores but significantly stimulated respiration in Rhizopus spores from the second to the fifth hour ($P < .05$; Fig. 6).

Proline stimulates germination of Rhizopus stolonifer sporangiospores (Weber, 1962) and the germination and respiration of R. arrhizus spores (Weber and Ogawa, 1965). Proline had a highly stimulatory effect on Rhizopus stolonifer spore respiration (Fig. 6) but was without effect on Phycomyces spores (Fig. 4).

As with Phycomyces pellets, the effect of malonate was dependent upon the substrates present with Rhizopus pellets. All tests were done in duplicate. Malonate (0.01M, pH 5, three hours) had no effect on pellets with glucose as substrate but inhibited pellet respiration about 70% with glucose and acetate present ($P < .01$). It inhibited respiration nearly 100% with acetate as sole substrate.

Inconsistent results between experiments were observed when the effect of glucose and acetate was tested on pellet respiratory rates. In some experiments a stimulation was observed, in others, no effect was noticed. Potassium cyanide (0.01M, pH 5, five hours) inhibited oxygen uptake nearly 100% with acetate as substrate; it inhibited oxygen uptake significantly ($P < .01$) with glucose as substrate during two hours of incubation. Later values (two hours) were not significantly different from controls.

Figure 6. Rate of oxygen uptake of Rhizopus stolonifer spores in the presence of acetate (AC) (0.72 mg/ml), glucose (GL) (10 mg/ml), proline (PRO) (0.01M), acetate plus malonate (MAL) (0.02M), and glucose plus acetate. Points shown are the means of two determinations. Each vertical line represents one standard error of the mean.


50
100
90
80
70
60
50
40
30
20
10
PRO
GL + AC
AC + MAL
AC
GL
1
2
3
4
5
OXYGEN CONSUMPTION
(µL/FLASK X HR)
TIME (HOURS)

Results of Experiments in Which Polarographic Methods Were Used

The endogenous rate of oxygen uptake by freshly harvested Phycomyces spores with a potassium phosphate buffer of pH 6 as measured by the Clark oxygen electrode was 1.1 µl per mg dry weight per hour (Q_{O_2}). When acetate was present for four minutes and glucose for one minute, the Q_{O_2} increased to 24.2. 2,4-Dinitrophenol at 5 x 10^{-5}M increased the rate slightly to 25.9 while azide at 0.001M added seven minutes from the start of the experiment increased the rate to 38.4 (Fig. 7). An experiment in which acetate, glucose, and dinitrophenol were used, followed by potassium cyanide rather than azide, showed potassium cyanide to produce a marked inhibition rather than a stimulation. This result was not confirmed manometrically or otherwise.

As can be seen in Figure 8, no stimulation in rate of oxygen uptake occurred until after glucose was added.

The addition of glucose to freshly harvested spores in phosphate buffer was stimulatory to the respiratory rate. The data obtained from the experiment shown in Figure 9 indicated a Q_{O_2} of 8.5 for the one minute immediately after the addition of glucose. For three short-time experiments in which the oxygen electrode was used, this stimulatory effect of glucose was consistently observed. Acetate increased the rate of oxygen uptake in the experiment shown in Figure 9 to a Q_{O_2} value of 21.1 which

Figure 7. Clark electrode trace of oxygen uptake by Phycomyces blakesleeanus spores showing effects of acetate (0.72 mg/ml), glucose (0.72 mg/ml), 2,4-dinitrophenol (DNP) (5×10^{-5}M), and sodium azide (0.001M). Reaction mixture prepared in phosphate buffer (pH 6, 0.067M), spores (4.8 mg dry weight) in a total volume of 3.0 ml.

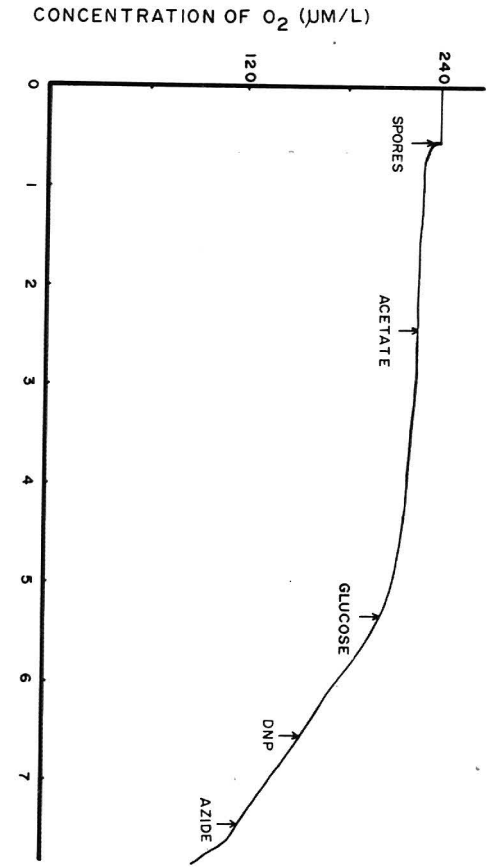

CONCENTRATION OF O_2 (μM/L)
240
120
0
SPORES
ACETATE
GLUCOSE
DNP
AZIDE
0
1
2
3
4
5
6
7
TIME (MINUTES)

Figure 8. Clark electrode trace of oxygen uptake by Phycomyces blakesleeanus spores showing effect of sodium azide (0.001M), glucose (10 mg/ml), and potassium cyanide (KCN) (0.001M). Reaction mixture prepared in phosphate buffer (pH 6, 0.067M, spores (weight not determined) in a total volume of 3.0 ml. Spores had been soaked two hours in phosphate buffer pH 6 before beginning of experiment.

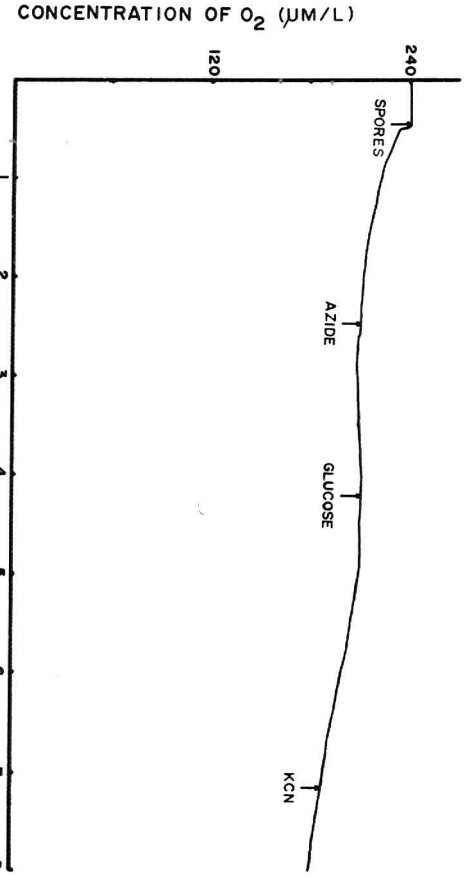
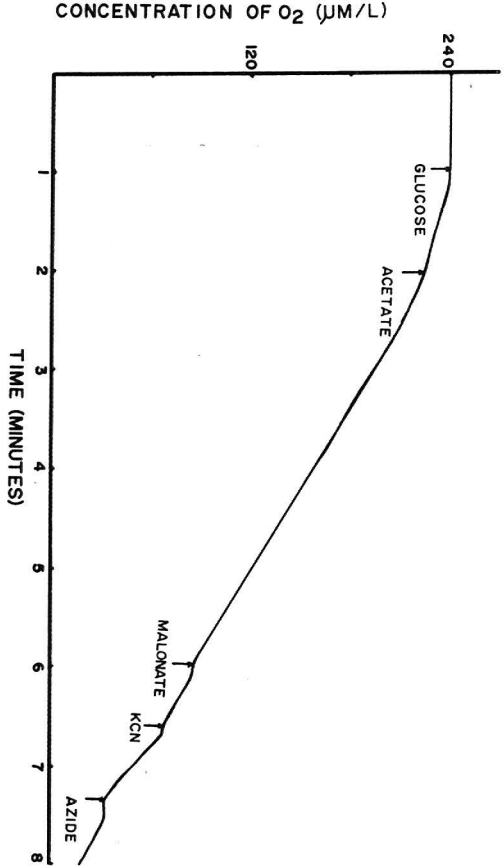

CONCENTRATION OF O_2 (μM/L)
240
120
SPORES
AZIDE
GLUCOSE
KCN
1
2
3
4
5
6
7
8
TIME (MINUTES)

Figure 9. Clark electrode trace of oxygen uptake by Phycomyces blakesleeanus spores showing effect of glucose (10 mg/ml), acetate (0.72 mg/ml), malonate (0.017M), potassium cyanide (KCN) (0.001M), sodium azide (0.001M). Reaction mixture prepared in phosphate buffer (pH 5, 0.067M), spores (5.3 mg dry weight) in a total volume of 3 ml. Chemicals were added dissolved in 0.1 ml buffer except for glucose which was added dry.


CONCENTRATION OF O2 (μM/L)
240
120
GLUCOSE
ACETATE
MALONATE
KCN
AZIDE
1
2
3
4
5
6
7
8
TIME (MINUTES)

was linear after the first half minute, malonate had no effect, potassium cyanide was stimulatory, and azide proved to be inhibitory to this increase.

CHAPTER IV

THE METABOLISM OF CARBON-14-LABELED ACETATE AND GLUCOSE SHOWING SOME EFFECTS OF EACH ON THE METABOLISM OF THE OTHER

Since acetate had a highly stimulatory effect on spore germination and respiration, it seemed important to identify the specific source of carbon dioxide production when acetate, glucose, or acetate together with glucose was the substrate. Uniformly-^{14}C-labeled acetate or glucose was used to assess the importance of acetate, glucose, and spore reserves to carbon dioxide output.

It also seemed that a determination of the so-called "C_6/C_1 ratio" of glucose metabolism with glucose alone as substrate and together with acetate would be instructive in investigating the function of acetate in stimulating spore metabolism. If the ratio is changed when acetate is present, this would be evidence that pathways of glucose metabolism change when acetate is present.

The idea that the C_6/C_1 ratio could be used as a means of demonstrating the participating of the Hexose Monophosphate (HMP) pathway was introduced by Bloom and Stetten (1953). Some of the assumptions recognized and

implied in the use of the ratio have since been shown not to hold universally. "As a means of estimating the pathways of total glucose consumption ... the C_6/C_1 ratio does not give an unequivocal result" (Beevers, 1961). Wood, Katz, and Landanu (1963) have discussed the assumptions and limitations of the method as a means of estimating the participation of the HMP pathway and the Embden-Meyerhof-Parnas (EMP) sequence of carbohydrate metabolism.

The rationale for the use of a C_6/C_1 ratio in estimating metabolic pathways is briefly as follows: For glucose molecules which are broken down exclusively by the EMP sequence, the first carbons to appear in carbon dioxide are those from positions 3 and 4. They constitute the carboxyl groups of pyruvate. Carbons 2 and 5 appear only after a complete circuit of the citric acid cycle by the fragment (acetyl group) remaining after pyruvate decarboxylation. Carbons 1 and 6 appear last after further circuits (Beevers, 1961).

By contrast, C-1 is the first carbon to appear from those glucose molecules entering the pentose phosphate sequence. The rate of appearance of C-6 would depend on whether the trioses produced in the pathway are converted to pyruvate or recycled (Beevers, 1961). In any case, its appearance is delayed in comparison with C-1. In a cell with the pentose phosphate pathway operating exclusive of others, a low C_6/C_1 ratio results, at least over short time intervals.

Materials and Methods

Spores and mycelial pellets were grown as indicated in previous chapters.

Oxygen consumption and carbon dioxide evolution were measured by the techniques described by Umbreit, Burris, and Stauffer (1957) at pH 6 (corrected for dissolved carbon dioxide) and 25°C in air.

Carbon dioxide was trapped in 0.25 ml of 20% KOH in the center well of Warburg flasks (no filter paper wick was used in the center well).

Radioactive compounds were purchased from commercial sources. They consisted of uniformly-^{14}C-labeled glucose, uniformly-^{14}C-labeled acetate, glucose-1-^{14}C, and glucose-6-^{14}C.

Warburg flasks contained 1.95 ml of a cell suspension (either spores or mycelial pellets) in the main compartment, 0.5 ml of a ^{14}C substrate solution in one side arm, and 0.5 ml of 70% perchloric acid in the other side arm. Non-radioactive substrates, when used, were added directly to the main chamber just before equilibration of the flasks began. Radioactive substrate was tipped into the main compartment at 0 time, and oxygen consumption was measured at hourly intervals. The experiment was stopped by the addition of the perchloric acid at the end of the experiment. The flasks were shaken for at least one additional hour to allow for complete absorption of the carbon dioxide.

In some cases the radioactivity of the cells was determined. After an experiment, spores were washed by centrifugation and dispersed into two 0.1 ml samples of water and placed into counting vials. Three ml of absolute ethanol and 12 ml of scintillation solution were added. This solution used toluene as a solvent and 2,5-diphenyloxazole (PPO) and 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP) as primary and secondary fluors.

The radioactivity of carbon dioxide was determined as for spores by adding 0.1 ml samples of the potassium hydroxide from the center well of a Warburg flask to two counting vials. Absolute ethanol and scintillation fluid were then added. Counting was done by liquid scintillation spectrometry. All counts were corrected for background and quenching and are expressed as disintegrations per minute (dpm).

Manometric-isotopic methods (Blumenthal, 1963) were used to estimate the rates of release of carbon dioxide from glucose, acetate, or endogenous sources.

The specific activity of the carbon dioxide released from the ^{12}C-cells during the oxidation of ^{14}C-substrate was calculated on the basis of the total carbon dioxide released in additional duplicate flasks without alkali in the center wells or by calculating the volume of carbon dioxide released from the volume of oxygen taken up when the respiratory quotient was known.

Results

The results of experiment 27 (Table 4) will be used to demonstrate how the contribution of glucose to total carbon dioxide output was calculated. In this experiment the specific activity of the initial uniformly-^{14}C-labeled glucose substrate was 19,459,600 dpm/μM carbon and there was a total of 2,164,100 dpm in the carbon dioxide when glucose was the substrate. Thus it can be calculated that (2,164,100 dpm)/19,459,600 dpm/μM carbon)= 0.111 μM or 111 nM carbon from the glucose was oxidized to carbon dioxide during the experiment. Duplicate flasks without potassium hydroxide in the center wells were used to calculate that the total volume of carbon dioxide evolved during the four hours of the experiment was (18.8 μl)/(22.4 μl/μM)= 0.848 μM or 848 nM. The total amount of carbon dioxide produced during the experiment then was 848 nM and the amount which was produced from glucose was 111 nM. Thus it can be calculated that the amount of carbon dioxide evolved which came from spore material was 848 - 111 = 737 nM. The per cent which came from glucose was 111 nM/848 nM x 100 = 13.0%.

A summary of the results of four experiments concerned with the quantity of glucose oxidized by spores when offered alone and in the presence of acetate is presented in Table 4. When glucose was present at the low concentration of 0.25 μM/flask, nearly 90% of the carbon

TABLE 4

Contribution of uniformly-^{14}C-labeled glucose to total CO_2 output by Phycomyces blakesleeanus spores.

Experiment	Experimental time (hours)	Substrate (μM)	Specific activity dpm/μM C (glucose)	dpm in CO_2	Total nM CO_2 (manometric)	nM CO_2 released from glucose	nM CO_2 released endogenous	% CO_2 from glucose $\bar{x} \pm$ SD
27	4	Glucose (0.25)	19,459,600	2,164,100	848	111	737	13.0 ±0
28	1	Glucose (0.25)	19,459,600	445,110*	178	23	155	12.8
28	4	Glucose (0.25)	19,459,600	1,779,207*	804	91	713	11.4
28	4	Glucose (0.25) K acetate (22)	19,459,600	2,504,076	34,227	129	not determined	0.4 ±0.01
29	4	Glucose (166.9)	20,772	22,521	3,700	1,120	2,580	29.3 ±3.2
29	4	Glucose (166.9) K acetate (22)	20,772	102,538	41,150	5,100	not determined	12.3 ±2.0
36	1	Glucose (166.9)	29,129	6,177	884	307	577	34.7 ±6.2

* Only one flask used.

dioxide evolved came from spore materials (see Exp. 27 and 28, 1 and 4 hours, Table 4). The rate of carbon dioxide evolution did not change appreciably over four hours. When non-labeled acetate was present together with glucose, carbon dioxide output from glucose was stimulated about 42% over the level produced with glucose alone (Exp. 28, 4 hours, Table 4); the percentage contributed to the total carbon dioxide output was reduced to less than one-half of one percent. Increasing the glucose concentration effected an increase in the amount of carbon dioxide coming from glucose. When the concentration of glucose was the same as in spore germination studies (166.9 μM/flask) and glucose was the only carbon source, 30% of the carbon dioxide produced came from glucose over a four hour period (Exp. 29, Table 4). With the addition of acetate, the amount of carbon dioxide coming from glucose increased 455% but the relative importance of glucose-derived carbon dioxide decreased to about 12% of the total carbon dioxide output (Exp. 29. Table 4). Thus, an increase in glucose concentration increased the amount of carbon dioxide coming from glucose, and the addition of acetate increased the output still more.

Simultaneous studies of assimilation are summarized in Table 5. With 0.25 μM glucose per flask over a one hour period, only 6 nM of carbon were assimilated into spore material. The rate of assimilation increased slightly over a four hour interval; after four hours, the amount

TABLE 5

Assimilation of uniformly-^{14}C-labeled glucose carbon into spores.

Experiment	Experimental time (hours)	Substrate μM	Specific activity (dpm/μM carbon)	dpm in spores	nM carbon in spores from glucose $\bar{x} \pm SD$
28	1	Glucose (0.25)	19,459,600	116,620	6 ± 0.6
28	4	Glucose (0.25)	19,459,600	627,520	32 ± 2.2
28	4	Glucose (0.25) K acetate (22)	19,459,600	3,281,860	168 ± 3.0
29	4	Glucose (166.9)	20,772	9,578	475 ±74
29	4	Glucose (166.9) K acetate (22)	20,772	214,356	10,650 ±80

assimilated amounted to 32 nM (Table 5). The addition of acetate to the reaction vessel resulted in a more than five-fold increase. The higher glucose concentration of 166.9 μM per flask was even more stimulatory. However, an increase in glucose concentration plus the addition of acetate resulted in a spectacular increase in assimilation (Table 5). Acetate, although effective in stimulating carbon dioxide release from glucose, functions even more effectively to increase the conversion of carbon from glucose into cell material. Since perchloric acid was added at the end of each experiment before the spores were analyzed for radioactivity, it can be expected that much of the low molecular weight materials could be lost from the spores. The radioactivity in the spores might then represent high molecular weight materials such as protein and wall material.

Table 6 summarizes the results of experiments designed to test the effect of glucose on spore carbon dioxide output from acetate. The data are difficult to interpret because of variations between duplicate flasks and some inconsistencies between experiments. Manometric data of experiments 37 and 39 (Table 6) indicate that glucose plus acetate was about twice as effective in stimulating carbon dioxide output as was acetate alone.

Results from experiment 39 show that the percentage of carbon dioxide which came from acetate remained nearly constant when acetate was present alone or when glucose

TABLE 6

Contribution of uniformly-^{14}C-labeled acetate to CO_2 output by spores during one hour of incubation. Values represent the means of two determinations plus or minus one standard deviation of the mean (SD).

Experiment	Substrate (μM)	S.A. dpm/μM C (acetate)	dpm in CO_2	Total nM CO_2 (manometric) $\bar{x} \pm$ SD	nM CO_2 released from acetate $\bar{x} \pm$ SD	nM CO_2 released endogenous	% CO_2 from acetate $\bar{x} \pm$ SD
37	Acetate (22)	126,357	97,056	2,022 $\pm$ 160	766 $\pm$ 52	1,256	37.9 $\pm$ 0.4
37	Acetate (22) Glucose (166.9)	126,357	315,527	4,732 $\pm$ 178	2,497 $\pm$ 970	not determined	52.9 $\pm$22.4
38	Acetate (22) Glucose (166.9)	63,179	41,072	3,031 $\pm$ 384	950 $\pm$ 40	not determined	21.5 $\pm$ 0.7
39	Acetate (22)	63,179	39,986	2,151 $\pm$ 240	622 $\pm$ 22	1,529	28.9 $\pm$ 2.0
39	Acetate (22) Glucose (166.9)	63,179	78,876	4,364 $\pm$ 246	1,248 $\pm$ 240	not determined	28.5 $\pm$ 2.0

was added while experiment 37 shows an apparent increase in the acetate component of carbon dioxide output when glucose was present. Data from both experiments, however, show that the presence of glucose was highly stimulatory to carbon dioxide release from acetate. The increase in carbon dioxide output from acetate seems to be in proportion to the total increase in carbon dioxide production which resulted when glucose was added to the medium.

Although the data are equivocal in some cases, as indicated above, an examination of Tables 4 and 6 shows that glucose stimulated an increase in carbon dioxide output from acetate. When glucose, acetate, and spore materials were all present, each contributed significantly to carbon dioxide release. Possibly the largest contributor to respiratory carbon dioxide is material of the spores itself.

When acetate was presented to spores alone, 60% or more of the carbon dioxide released came from endogenous sources (Table 6). Acetate, then, was metabolized rapidly by the spore but also stimulated a large increase in metabolism of spore material. Glucose may have stimulated some increase in breakdown of spore material, but the effect obviously was much less than that of acetate.

The data of Table 7 show that the C_6/C_1 ratio in spores incubated with glucose-6-^{14}C or glucose-1-^{14}C was very low but that the contribution of both C-1 and C-6 was

TABLE 7

Effect of potassium acetate on C_6/C_1 ratio of glucose in spores and mycelium. The specific activity for glucose-6-^{14}C was 67,581 dpm/μM carbon and for glucose-1-^{14}C, 68,247 dpm/μM carbon. Glucose was used at 166.9 μM and potassium acetate at 22 μM per flask. Values are the means of two determinations.

Experiment	Tissue	Experimental time (hours)	Substrate	dpm in CO_2 ±SD	Total nM CO_2 (manometric) ±SD	nM CO_2 released from C_6 of glucose ±SD	nM CO_2 released from C_1 of glucose ±SD	% of total carbon from glucose	C_6/C_1 ratio
35	Spores	1	Glu-6-^{14}C	312 ± 30	1,160 ± 4	4.6 ± 0.4	---	*1.1	
35	Spores	1	Glu-1-^{14}C	2,266	1,243	---	33 ± 0.4	*7.7	0.14
33	Spores	4	Glu-6-^{14}C K acetate	37,351 ± 1,502	42,031 ± 402	553 ±22	---	+10.6	
33	Spores	4	Glu-1-^{14}C K acetate	127,164 ± 3,990	44,285 ± 543	---	1,863 ± 58	+33.9	0.30
40	Pellets	1	Glu-6-^{14}C	10,687 ± 2,074	3,705 ± 258	158 ±30	---	---	
40	Pellets	1	Glu-1-^{14}C	41,308 ±15,542	3,977 ± 668	---	605 ±228	---	0.26

* Used 34.7% as per cent of CO_2 from glucose (Experiment 36, Table 4).
\+ Used 12.3% as per cent of CO_2 from glucose (Experiment 29, Table 4).

less than 10% of the carbon released from glucose. Apparently, much of the carbon released as carbon dioxide arose from carbon atoms other than C-1 and C-6. When acetate was added to a medium in which spores were metabolizing glucose-1-^{14}C or glucose-6-^{14}C, the C_6/C_1 ratio doubled as compared to the ratio when no acetate was present (Table 7).

Although the results of experiments 33 and 35 (Table 7) are not strictly comparable because of the difference in time periods, the discussion given by Blumenthal (1966) suggests that recycling of pentose phosphate in most species is limited. The results are probably a reflection of the true conditions.

Discussion

Since carbons 1 and 6 contributed less than 10% of the carbon dioxide released from glucose in absence of acetate, one can probably assume that carbons 3 and 4 contributed most of the carbon dioxide. If carbons 3 and 4 did indeed contribute most of the carbon dioxide, the suggestion is that most of the glucose oxidation was through the EMP pathway. But if the two-carbon fragments, resulting from decarboxylation through the EMP pathway, were catabolized carbons 1 and 6 would appear at equal rates. A larger percentage of the total carbon dioxide output would be from carbons 1 and 6, and the C_6/C_1 ratio would be greater than what was observed. A possible

explanation is that the two-carbon fragments are not catabolized but are stored in the spores or excreted into the medium.

The rise in the relative importance of C-6 to respiratory carbon dioxide and the increased importance of both carbons 1 and 6 to respiratory carbon dioxide, when acetate was added, are facts which seem consistent with the idea that acetate stimulates catabolism of the two-carbon fragments resulting from decarboxylation of glucose through the EMP pathway. The relatively low C_6/C_1 ratio in both the presence and absence of acetate indicate that the HMP pathway may be important in both activated and unactivated spores.

With the data at hand, no definite conclusions can be made as to the quantitative importance of pathways of glucose catabolism in spores with or without the presence of acetate. The HMP pathway may be an important means of oxidation of glucose throughout the life cycle of the fungus. The low C_6/C_1 ratio in mycelium (Table 7) tends to confirm this idea.

CHAPTER V

THE INCORPORATION OF GLUCOSE AND ACETATE CARBON INTO AMINO ACIDS

The amino acids, aspartate, glutamate, and alanine are thought to be synthesized from the keto acids oxaloacetate, α-ketoglutarate and pyruvate by amination or transamination reactions in fungi as with other organisms (Nicholas, 1966). Serine is synthesized from D-3-phosphoglyceric acid (Mahler and Cordes, 1966).

When glucose is catabolized through the EMP pathway and the TCA cycle, the carbon of glucose becomes incorporated into pyruvate, α-ketoglutarate and oxaloacetate (Beevers, 1961) and thus, if amino acid synthesis is occurring, into alanine, glutamate, and aspartate. Incorporation into serine could mean metabolism of glucose carbon into phosphoglyceric acid.

Since free amino acids are relatively easy to extract from tissues and identify, it was decided to extract and determine the amount of radioactivity present in the four amino acids mentioned above after incubating Phycomyces mycelium and spores with uniformly-^{14}C-labeled glucose or uniformly-^{14}C-labeled acetate. If glutamate and aspartate were found to be labeled as heavily after

incubation with radioactive glucose as with radioactive acetate, this would be consistent with the hypothesis that glucose was metabolized through similar pathways as acetate. If alanine or serine were heavily labeled, this would constitute evidence that glucose is readily metabolized to phosphoglyceric acid or pyruvate in the tissue examined. Any difference between spores and mycelium in pattern of labeling of amino acids after incubation with uniformly-^{14}C-labeled glucose might indicate a difference in metabolic pathways between the two tissues. Little or no labeling of amino acids would indicate pathways of glucose catabolism not leading to phosphoglycerate or pyruvate.

Materials and Methods

Pellets and spores were grown as described in Chapter 2. Pellets were 47 hours old when incubation with labeled glucose began and 24 hours old when incubation with labeled acetate began.

The incubation solution for pellets consisted of 50 μM $(NH_4)_2HPO_4$, 1667 μM uniformly-^{14}C-labeled glucose with a specific activity of 6.6 x 10^3 dpm/μM carbon or 220 μM uniformly-^{14}C-labeled acetate with a specific activity of 4.3 x 10^4 dpm/μM carbon in 30 ml of a 0.067M potassium phosphate buffer pH 6.

Before incubation, pellets were washed twice with distilled water by filtration. Incubation was for five hours in a large flask (about 150 ml) on a Warburg

respirometer at 25°C. After incubation with glucose, pellets were removed from the incubation flask and washed three times with distilled water. They were then placed in 100 ml of 1% picric acid and blended one minute in a food blender at high speed. The homogenate was filtered, the blender blades were washed with water, and the washings were combined with the filtrate. This was passed through a 2 x 5 cm column of Dowex 1 (Cl^-form) resin to remove the picric acid. The column was washed with water, and the effluent evaporated by used of a rotary vacuum evaporator to a volume of 5 ml. Two ml of this were analyzed on a Beckman Model 120 Amino Acid Analyzer. The chart obtained from this analysis was used to identify and quantitatively determine the amino acids present in the sample. Another 2 ml sample was then passed through the long column of the analyzer but was not analyzed. Instead, 5 ml fractions were collected at about ten minute intervals by a fraction collector from the bottom of the column. The peaks of the chart obtained from the analysis of the first sample were correlated with the fractions collected from the second sample and tubes containing fractions corresponding to aspartate, glutamate, alanine, and serine were removed from the fraction collector and their contents analyzed for the presence of radioactivity. One ml quantities were removed from each tube, and the radioactivity determined by liquid scintillation spectometry.

After incubation with acetate, amino acids were extracted, identified, quantitatively determined, and their radioactivity measured by using the same methods as described above except that in preparing the extract for analysis the amino acids were adsorbed on a 1 x 5 cm column of Dowex 50 (200 to 400 mesh) ion exchange resin and eluted with 4N ammonium hydroxide. The eluate was evaporated to 5 ml for use on the amino acid analyzer.

Methods to test the incorporation of ^{14}C from uniformly-^{14}C-labeled glucose into the free amino acids of spores were those of Tweedy and Turner (1966) with slight modifications. The spores from seven cultures (About 50 mg dry weight) were removed, washed, and dispersed into a medium as described for pellet incubation. The specific activity of the glucose was 2.2×10^3 dpm/ μM carbon. The spores were shaken 1.5 hours at room temperature (about 23°C) on a rotary shaker, removed from the medium by centrifugation, suspended in 105 ml of 80% ethanol, and refluxed for three hours in a Soxhlet extractor. The residue was washed twice with 20 ml of hot 50% ethanol. The alcoholic solution was extracted with petroleum ether and evaporated to approximately 15 ml. The amino acids were retained on a Dowex 50 (200 to 400 mesh) column and eluted with 4N ammonium hydroxide. The eluate was evaporated to 1 ml, 0.2 ml was removed for thin layer chromatography, and 0.8 ml of

concentrated hydrochloric acid added. The acidic solution was heated at 100°C for one hour and then evaporated to dryness. The residue was washed three times with water and the solution evaporated to dryness after each washing. The residue was dissolved in 1 ml of sodium citrate buffer (pH 2.8) and the individual amino acids were separated and quantitatively determined by use of a Technicon Autoanalyzer.

Thin layer chromatography was done on Eastman chromotogram sheets (Type K301R) by use of a solvent system consisting of chloroform-methanol-5N aqueous ammonia (50:30:2, V/V/V).

The petroleum ether fraction was evaporated to 1 ml, transferred to a counting tube, and evaporated to dryness. Fifteen ml of liquid scintillation fluid were added and the radioactivity determined.

Results

After the quantities of each of the four amino acids extracted from the mycelium were determined, a comparison of the amounts of amino acids from pellets incubated with glucose with those incubated with acetate showed that mycelium incubated with acetate contained considerably more of each amino acid than did mycelium incubated with glucose (Table 8). These data, however, may not be comparable because the pellets incubated with glucose were nearly twice as old. An unlabeled sample of mycelium 24 hours old (the same age as the sample incubated with acetate) incubated five hours with glucose and

TABLE 8

Radioactivity of four amino acids extracted from mycelial pellets or spores after incubating five hours with uniformly-^{14}C-labeled glucose or uniformly-^{14}C-labeled acetate. These data are the result of only one determination.

I. Pellets labeled with radioactive glucose (10 mg/ml; 6.6 x 10^3 dpm/uM carbon).

Amino acid	Quantity of a.a. extracted (μM/g wet wt)	Specific Activity dpm/g wet wt ± SD	Specific Activity dpm/μM amino acid ± SD	Quantity of carbon from glucose incorporated into a.a. from 1 g wet wt mycelium (μM x 10^{-3})
Asp	0.30	803 ± 12	2,677 ± 40	120 ± 2
Ser	0.25	658 ± 12	2,632 ± 48	100 ± 2
Glu	0.55	1,161 ± 18	2,110 ± 33	170 ± 3
Ala	0.95	1,158 ± 18	1,640 ± 19	170 ± 3

II. Pellets labeled with radioactive acetate (0.72 mg/ml; 4.3 x 10^4 dpm/μM carbon).

Amino acid	Quantity of a.a extracted (μM/g wet wt)	Specific Activity dpm/g wet wt ± SD	Specific Activity dpm/μM amino acid ± SD	Quantity of carbon from acetate incorporated into a.a. from 1 g wet wt mycelium (uM x 10^{-3})
Asp	0.85	800 ± 225	941 ± 265	19 ± 5
Ser	1.35	875 ± 225	648 ± 167	20 ± 5
Glu	1.65	738 ± 225	447 ± 136	20 ± 5
Ala	1.60	162 ± 225	102 ± 141	4 ± 5

TABLE 8 (continued)

III. Spores labeled with radioactive glucose (10 mg/ml; 629.1 dpm/μM carbon).

Amino acid	Quantity of a.a. extracted (μM/g dry wt)	Specific Activity dpm/g dry wt	dpm/μM amino acid
Asp	10.8	Not determined	
Ser	none detectable	quantitatively.	
Glu	6.6	Some acids	
Ala	0.3	heavily labeled.	

analyzed contained 0.20 μM/g aspartate, 0.28 μM/g serine, 0.77 μM/g glutamate, and 2.28 μM/g alanine. Although these results are closer to the values obtained after extracting amino acids from mycelium 47 hours old and incubating with glucose than from mycelium 24 hours old and incubating with acetate, they do not compare closely with either, and the amount of alanine is considerably higher than either.

If one could assume that the values for 24 hours old cultures are true values, then it could be seen that the quantities of amino acids from acetate incubated mycelium (Table 8) are considerably more than from glucose incubated mycelium except for alanine, which appears to be more concentrated after glucose incubation. Further experimentation would be necessary to confirm the data but it does give some indication of the amounts of free amino acids which can be expected to be found in mycelium grown under the conditions described.

In Table 8 the data shown in the first column under "specific activity" indicate the amount of radioactivity incorporated into amino acids during the five hours of the experiment and are probably a more meaningful measurement of incorporation than is the data of the second column because the quantities of amino acids were not known before the experiment was begun and are only a measure of amino acid synthesis during the five hours of the experiment and under the experimental conditions used.

The specific activities shown in Table 8 (Part I) were calculated from the activity of the one 5 ml fraction which was most active. For some of the amino acids most of the activity was found in one tube (aspartate, serine); in the others (glutamate, alanine) two tubes showed high levels of radioactivity. A bias then was introduced into the table in favor of a higher specific activity of some amino acids over others. Since the amino acids present in lesser quantities are more likely to be found in one tube than those of greater quantities, a bias probably was introduced in favor of higher specific activities for amino acids found in smaller quantities. The radioactivity from only one tube from each amino acid was included for amino acids extracted from pellets incubated with glucose because the radioactivity from only one tube of each amino acid from pellets incubated with acetate was counted. It was thought that including the data from only one tube would make a comparison of the results (acetate vs. glucose) more valid.

The results of Table 9 (final column), show that little or no carbon from acetate was incorporated into alanine. Other amino acids were labeled from 5 to 8.5 times more heavily after glucose incubation than after acetate incubation. Although there is room for a considerable amount of uncertainty about the results because of the limited amount of experimentation, there can be little doubt

that under the conditions used more carbon from glucose is incorporated into the four amino acids tested than is carbon from acetate. It seems inescapable that in pellets the carbon of glucose rapidly enters amino acids which are known to be intimately associated with the TCA cycle. It has not been established that the TCA cycle is, in fact, important in glucose metabolism in Phycomyces nor, if it is, how carbon from glucose enters it. The facts, however, are consistent with the idea that glucose carbon rapidly enters the TCA cycle.

Aspartic and glutamic acids were found to be present in high concentrations in spores incubated with glucose (Table 8, III). Only a trace of some other acids were found. Thin layer chromotography of amino acids did not successfully separate them so that the radioactivity of individual acids could be determined. It was established, however, that most, if not all, of the radioactivity was found on the chromatography plates in positions corresponding to amino acids with low mobility in the solvent system used. Analysis of the petroleum ether soluble fraction showed that more than 0.5 μM of carbon from glucose was incorporated into this fraction.

CHAPTER VI

GENERAL DISCUSSION

Acetate stimulated both germination and respiration of spores in contrast to the effects of glucose (Table 1, Fig. 1). Borchert (1962) indicates that Phycomyces spores after being activated 15 minutes in acetate and stored in distilled water eight days will germinate at a high rate after being transferred to a medium which contains glucose but no acetate. Acetate, therefore, seems not to be necessary as an energy source for germination but only as a "trigger" which sets the germination process going.

The effects on spores of cyanide were different depending on whether acetate or glucose was the substrate. Cyanide stimulated oxygen uptake with glucose as substrate but strongly inhibited respiration with acetate as substrate (Figs. 2,3). Cochrane, et al. (1963 b,c) working with Fusarium solani, noted that the action of cyanide on acetate respiration was quite different from its effect on endogenous oxygen uptake. The effect of cyanide and azide on Phycomyces spores may, therefore, have been on the endogenous respiration rather than that of glucose. But since no stimulatory effect was noticed until after glucose was added (Fig. 8),

glucose metabolism may have been involved. Additional work involving the effect of cyanide and azide on spores without substrate is indicated.

Potassium cyanide was a strong inhibitor of mycelial respiration regardless of the substrate (Table 3). This is in contrast with the situation in spores where a stimulation was noted when spores were incubated with glucose and cyanide (Fig. 2). This differential effect of cyanide, which depends on whether spores or mycelium was tested, indicates a shift in metabolic pattern of glucose metabolism during germination.

The fact that malonate inhibited spore metabolism with acetate as substrate suggests that acetate metabolism and possibly the endogenous metabolism stimulated by acetate (Table 6) are through the TCA cycle.

It has been suggested that the TCA cycle is lacking in dormant spores of Phycomyces and that the cycle is induced when the spores are stimulated to germinate (Rudolph, 1961 a; Sussman and Halvorson, 1966). Since acetate stimulates oxygen uptake in spores (Fig. 1), is rapidly catabolized in spores (Table 6), and since malonate inhibits respiration of spores with acetate as substrate (Fig. 3), it seems likely that acetate releases the cycle from inhibition rather than inducing it. Furthermore, the fact that acetate acts rapidly to increase respiration (Figs. 7 and 9) tends to rule out any

extensive metabolic adjustments as being involved in its stimulatory effect on oxygen uptake.

With acetate as substrate, the provision of ammonium ion or proline to spores had a stimulatory effect on oxygen uptake (Fig. 4). This fact suggests that synthesis requiring nitrogen and ATP occurred after the first hour of incubation. Since no increase in oxygen uptake was noted when spores were incubated with glucose instead of acetate, it is reasonable to assume that major synthesis did not occur with glucose as substrate. Acetate seems to stimulate synthetic reactions, but whether its primary action is that of supplying necessary energy is conjectural.

Malonate was a strong inhibitor of Phycomyces mycelial respiration with glucose as substrate, but was much more effective with acetate (p. 41, 46). This suggests that in mycelia, as well as in spores, glucose or endogenous respiration is at least partly through pathways other than the TCA cycle. Malonate, being a weak acid, is often prevented from penetrating cell membranes (Beevers, 1961). It is sometimes difficult, therefore, to assess the significance of experiments in which malonate is used as an inhibitor. Malonate was completely ineffective as an inhibitor for spore respiration when the oxygen electrode was used. This can probably be accounted for, in some cases, by assuming that the inhibitor did not penetrate the cells to

produce an inhibitory concentration during the few minutes it was applied.

Glucose assimilation in spores is increased when acetate is present (Table 5). Two observations militate against the idea that the function of acetate is to increase the permeability of the spore membrane to glucose. Oxygen electrode studies show that after the addition of glucose to spores, oxygen uptake was immediately stimulated (Fig. 9). Glucose produced 30% of the respiratory carbon dioxide from spores when it was the sole carbon source (Table 4). Acetate may function in spore germination to increase the synthesis of glucose carbon into the cell material.

Most of the carbon dioxide produced after acetate activation is from the spore (Table 6). This suggests that acetate releases from inhibition some enzyme system which functions to catabolize spore reserves.

The data suggest that although the EMP pathway may be important in the oxidation of glucose, the complete breakdown of glucose may not occur in dormant spores (Table 7 and discussion on pages 71 and 72). The addition of acetate appears to increase the importance of carbons 1 and 6 of glucose to carbon dioxide production. This fact is consistent with the view that acetate may activate the TCA cycle and that carbon fragments not

metabolized completely in non-activated spores may enter the cycle after acetate activation. The C_6/C_1 ratio remains low and it seems that the HMP pathway may be at least as important in activated spores as in non-activated ones, and, in fact, may remain so throughout the life cycle of the fungus.

The oxidation of NADPH is often the limiting factor in operation of the HMP pathway (Butt and Beevers, 1961; Holzer, 1959). If this were the case in spores of Phycomyces, one might speculate that after the stimulation of the TCA cycle, carbon fragments and energy might be available for synthesis. The NADPH produced in the HMP pathway could then be oxidized in synthetic events in which reducing power was necessary (e.g. production of glutamate from α-ketoglutarate). This possibly could result in a stimulation of glucose oxidation through the HMP pathway.

Radioactive carbon appeared in amino acids in more abundance after pellets were incubated with radioactive glucose than after they were incubated with radioactive acetate (Table 8). Since one would assume more carbon of acetate to flow through the TCA cycle than carbon of glucose, it could be expected that the amino acids aspartate and glutamate would contain more carbon from acetate than from glucose. A repetition of this experiment is indicated. But one can reconcile these data with the hypothesis that acetate is rapidly catabolized

through the TCA cycle. One could assume that when acetate was present, more synthetic events were occurring and the free amino acids formed were used, or that the carbon of acetate was oxidized rapidly and appeared as carbon dioxide with little carbon entering the amino acids. Both of these events could, of course, be occurring simultaneously.

In spores, after incubation with radioactive glucose, the only amino acids to appear in quantity were aspartate and glutamate. Others were either absent or present only in minute quantities. Some radioactivity was present in the amino acids indicating that possibly the carbon of glucose can enter the TCA cycle with glucose as sole substrate. Further work to locate the specific carbon atoms labeled in the amino acids would be necessary to enable one to decide whether the carbon enters the cycle by way of acetyl Co A or as the result of carbon dioxide fixation.

Borchert (1962) showed that acetate serves as a respirable substrate for spores and hypothesizes that it stimulates the catabolism of reserve material of the spore. The results of my research show that acetate does indeed stimulate the catabolism of spore reserves, and, when it is available, the oxidation of glucose. The mechanism whereby this action is accomplished is unclear. Lingappa and Lingappa (1965) have shown that spores of Glomerella cingulata (Stoneman) Spaulding and von Schrenk

germinate well at low spore density in distilled water, but at high spore densities germinate only if large concentrations of peptone or phenylalanine are available. These data suggest that nutrients operate to reduce self-inhibition. There is no strict relationship between the ability of a chemical to increase respiration and its ability to produce germination. In some spores, acetate is rapidly metabolized but does not support germination (J. C. Cochrane, et al., 1963). Perhaps in P. blakesleeanus acetate serves both as a respirable substrate, thus supplying energy, and a substance which reduces self-inhibition. The identity of this hypothesized self-inhibitor is, of course, unknown but evidence suggests that if such a self-inhibitor does exist it may function to inhibit the action of the TCA cycle.

The fact that the spores of Phycomyces and Rhizopus were widely different in respiratory behavior must reflect differences in ecology. Rhizopus stolonifer seems almost ubiquitous in its habitat while Phycomyces seems confined to dung. Since both produce large numbers of non-motile spores of relatively the same size, wind dispersal should not be dissimilar. The nutritional requirements are evidently different enough to influence the ecological niche of these two fungi.

The long sporangiophores of Phycomyces may assist in the transfer of sporangiospores to vegetation likely to be eaten by herbivores. The effects of heat,

enzymes, surface-active substances, and acids in the digestive tract and of organic acids produced by bacteria in dung no doubt stimulate germination, but which is most important in nature is not known.

Some coprophilous fungi are specialized in their habitat. For example, one species of coprophilous Ascomycete has been found only on the dung of porcupine and another only in the tundra on the dung of lemming (Cain, 1956). The reasons for these specializations are unknown. It should be interesting to correlate nutritional requirements with ecology of several coprophilous fungi, including Phycomyces blakesleeanus.

CHAPTER VII

SUMMARY AND CONCLUSION

The sporangiospores of Phycomyces blakesleeanus Burgeff (ATCC 8743 b-) did not germinate in a medium consisting of phosphate buffer and glucose (10 g/l). When acetate (0.72 g/l) was used instead of glucose, spore germinated at a rate of about 90%. This confirmed the results of previous investigators.

Manometric techniques were used to show that respiratory rates were low (about 2 μl oxygen uptake per mg dry wt of spores per hour, over a five hour period) with glucose (10 g/l) as the sole carbon source. When acetate (0.72 g/l) was used instead of glucose the rate of oxygen uptake increased more than 15 fold.

Manometric-isotope methods were used to assess the individual contributions of glucose, acetate, and spore (endogenous) material to carbon dioxide production. Results obtained showed that acetate was rapidly catabolized by spores, stimulated the catabolism of spore material and exogenously supplied glucose, and the assimilation of glucose into spores. Glucose appeared to stimulate some increase in carbon dioxide production from acetate when both were supplied together.

When spores were incubated with glucose-1-^{14}C or glucose-6-^{14}C alone, or together with unlabeled acetate, the low C_6/C_1 ratio which resulted suggested that the Hexose Monophosphate pathway was important in glucose catabolism. The ratio was also low in mycelium.

Potassium cyanide and sodium azide seemed to stimulate the oxidation of glucose in spores but cyanide strongly inhibited oxygen uptake in mycelium with glucose as substrate.

In contrast to its effects on spore respiration with glucose as substrate, cyanide significantly inhibited oxygen uptake with acetate as substrate. But with acetate as substrate, spores, when compared to mycelium, were relatively resistant to the effects of cyanide.

It is hypothesized that before spore activation by acetate, glucose catabolism is incomplete and that acetate acts to stimulate spore germination by releasing the Tricarboxylic Acid cycle from self-inhibition thus allowing a complete oxidation of glucose.

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