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Title: CARBOHYDRATE AND NITROGEN METABOLISM OF SPHAEROTILUS NATANS

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The filamentous, sheathed bacterium, Sphaerotilus natans produces copious, objectionable slime-material in waters enriched with suitable carbon and nitrogen sources. Because of the relation between the presence of carbon and nitrogen sources and the subsequent occurrence of S. natans in polluted waters, the growth characteristics of this organism have been studied in some detail. In spite of substantial information concerning isolation and growth of S. natans, there has been no sustained effort to determine the metabolic pathways used by the organism. The primary objective of this investigation was the study of metabolic pathways functioning in S. natans in order to better understand the growth of S. natans in nature.

Several of the experiments were conducted with pure and mixed cultures of S. natans in order to determine whether the metabolism

of the organism was the same in pure culture and in nature.

The radiorespirometric method was used to determine which of the various primary pathways were functioning in S. natans. The results of experiments with specifically labelled glucose indicated that the Entner-Doudoroff and pentose phosphate pathways functioned in the strains of S. natans tested. In addition, the $^{14}CO_2$ patterns and isotope recoveries from C-2, C-6, and C-3,4 of glucose suggested that secondary pathways such as the TCA cycle or glyoxalate probably also were functioning. Radiorespirometric experiments using a natural slime largely composed of Sphaerotilus cells indicated that the same primary pathways were operating in S. natans in nature.

The storage product, poly-β-hydroxybutyric acid was found to contribute significantly to overall carbohydrate metabolism in S. natans. The polymer was used in preference to other cellular carbon sources during endogenous respiration. It is suggested that the polymer probably contributes to the survival of S. natans in nature. Cell-free enzyme studies confirmed the presence of D-(-)-β-hydroxybutyrate dehydrogenase, an enzyme required for the catabolism of poly-β-hydroxybutyric acid.

Growth studies with TCA cycle intermediates and radiorespirometric studies with acetate indicated the participation of the TCA cycle and glyoxalate cycle. Further evidence for operation of the

TCA cycle was obtained by radiorespirometric studies with specifically labelled glutamic acid. During this study it was found that S. natans not only is unable to use the D-isomer of glutamic acid but also is inhibited by its presence.

The fixation of CO_2 was demonstrated in S. natans by the use of $^{14}CO_2$. The relative importance of this reaction was not established. Glucose was found to be a better source of condensing partners for CO_2 than pyruvate, possibly because of a permeability barrier to pyruvate.

The conversion of nitrate to nitrite with the accompanying $DPNH_2 \longrightarrow DPN$ reaction was suggested as a mechanism for the growth of S. natans at low oxygen levels.

CARBOHYDRATE AND NITROGEN METABOLISM OF SPHAEROTILUS NATANS

by

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INTRODUCTION

Sphaerotilus natans is a member of a diverse group of microorganisms known generally as sheath-forming, iron bacteria. Although related genera are thought to derive certain amounts of their total energy requirements from the oxidation of ferrous iron or reduced manganese, most researchers feel that S. natans probably must use heterotropic processes in order to produce metabolic energy. The deposition of varying quantities of iron or manganese in sheath material of S. natans is thought to be an incidental process.

It has been shown that S. natans is capable of growing with a wide variety of carbon and nitrogen sources under diverse growth conditions. However, due to strain variability or possible differences in experimental methods used by different investigators, published reports on this organism have not been in close agreement. In consequence, it would appear that a study of the carbohydrate dissimilitory pathways of several different strains of S. natans would possibly aid in clarifying the basic metabolism of this bacterial species. Correlation of experimental results with pure cultures of S. natans with data obtained for mixed population of S. natans growing in natural conditions would also seem to be a fruitful avenue

of research. The present study has been designed to provide data pertaining to the above points.

The radiorespirometric method of Wang et al. was used with pure and mixed culture. Additional studies included growth experiments, continuous culture studies, electrolytic respirometer experiments, enzyme assays, poly-β-hydroxybutyric acid determinations, CO_2 fixation, and a study of the utilization of nitrate (N) and (O).

HISTORICAL REVIEW

Taxonomic and Nutritional Studies of S. natans

The filamentous, sheathed bacterium, Sphaerotilus natans was described and first named by Kützing (1833). Sixty years later Büsgen (1894) obtained pure cultures of this organism by streaking filaments from polluted waters on meat extract gelatin plates. The pleomorphic nature and physiological variability of members of the Sphaerotilus-Leptothrix group have caused considerable difficulty in their classification. Although the taxonomic works of Zopf (as cited in Stokes, 1954, p. 288), Zikes (1915), Cholodny (1926), Cataldi (as cited in Pringsheim, 1949b, p. 454-455), Pringsheim (1949a) (1949b), Stokes (1954), Dondero (1961) and Mulder (1964) have been useful in establishing criteria for isolation, identification and classification of members of this group, there remain many areas where information is controversial or incomplete (Breed, R.S., E.G.D. Murray, and N.R. Smith, 1957).

The growth of S. natans with various carbon and nitrogen sources has been studied by several investigators: Büsgen (1894), Linde (1913), Zikes (1915), Lackey and Wattie (1940), Wuhrman (1946), Wuhrman and Köstler (1950), Stokes (1954), Höhnl (1955) Scheuring (1957), Dondero, Phillips and Heukelekian (1961), Mulder and Van Veen (1963), and Mulder (1964). Suitable carbon sources

for S. natans included many sugars, organic acids, alcohols and amino acids. Organic sources of nitrogen such as amino acids, peptides and peptone were found to be excellent for growth of S. natans while ammonia and nitrates were found by some authors to support moderate growth: Linde (1913), Lackie and Wattie (1940), Stokes (1954), Höhnl (1955). Mulder and Van Veen (1963) reported that most of their strains of S. natans grew well with ammonia-nitrogen or nitrate-nitrogen if a small amount of vitamin B_{12} (cyanocobalamin) was present in the medium. It was suggested by these investigators that cyanocobalamin is required by the sheath-forming bacteria for the synthesis of methionine because small amounts of this amino acid could replace the vitamin.

Metabolic Pathways

Although several authors have performed growth and respiration studies of S. natans using different carbohydrates as carbon sources, no investigator has presented complete, definitive data on the pathways of carbohydrate dissimilation which exist in this organism. It seems logical to regard knowledge of the mechanisms whereby carbohydrates are utilized for biosynthesis and energy production in S. natans prerequisite for understanding and controlling its life processes. While there has been a lack of information concerning metabolic pathways in S. natans, an extensive study of

carbohydrate dissimilation has been carried out for several Pseudomonas species which may be rather closely related phylogenetically to S. natans. The morphological similarity between cells of S. natans and various Pseudomonas species has been observed by Stokes (1954). It is interesting that Mandel, Johnson and Stokes (1966) found several strains of S. natans to have a guanine-cytosine content which is near the upper limit of the GC range proposed by Mandel (1966) for the genus Pseudomonas. Since little information is available concerning catabolic pathways in S. natans, a review of the pathways found in the related genus, Pseudomonas and other organisms will be presented.

The classical Entner-Doudoroff (ED) pathway of glucose catabolism was first discovered in Pseudomonas saccharophila by Entner and Doudoroff (1951). In this pathway, glucose is converted to 6-phosphogluconic acid and then cleaved to form one pyruvic molecule and one 3-phosphoglyceraldehyde molecule. The 3-phosphoglyceraldehyde is subsequently converted to pyruvic acid with the net result that one molecule of glucose yields two molecules of pyruvate. Later work by MacGee and Doudoroff (1954) using Pseudomonas fluorescens established the presence of 2-keto-3-deoxy-6-phosphogluconic acid as an intermediate in this reaction. The Embden-Meyerhof-Parnas (EMP) pathway of glucose catabolism also results in the formation of two pyruvic acid molecules from glucose but the

mechanisms and most of the intermediates involved in the two pathways are quite different. In the ED pathway, one of the two pyruvate molecules derives its carboxyl group from the C-1 of glucose while the second derives its carboxyl group from the C-4 of glucose. In contrast, the two pyruvate molecules formed by the EMP pathway derive their carboxyl groups from the C-3 and C-4 of glucose, respectively. The pentose phosphate (PP) pathway as outlined by Axelrod (1960) involves the oxidative catabolism of glucose in a cyclic process which has phosphates of hexose, pentose, tetrose, triose and sedoheptulose as intermediates. This pathway is a little more involved than the ED or EMP pathways because of its cyclic nature and the various possible condensation or cleavage products formed. The end result of the PP pathway is that the C-1 is first oxidized to CO_2 and the remaining carbons are then randomly distributed in the various intermediates.

The use of ^{14}C-labelled carbohydrates has been a valuable tool in determining which primary catabolic pathways for glucose are operating in various species of Pseudomonas and also in members of other genera. By investigating the specific activity of acetate produced from specifically labelled glucose, Lewis et al. (1955) concluded that Pseudomonas fluorescens dissimilated glucose by using the ED pathway, concurrently with the PP pathway. They calculated that the ED pathway accounted for approximately one-third to

one-half of the total glucose dissimilated while the remainder was dissimilated via the PP pathway.

A comparative study of the carbohydrate metabolism of several species of Pseudomonas was made by Stern, Wang and Gilmour (1960). The radiorespirometric technique of Wang et al. (1958) was employed in this study using P. saccharophila, P. reptilivora, P. aeruginosa and a related organism, Zymomonas mobilis. These workers found that glucose was catabolized via the ED pathway alone in Z. mobilis while P. saccharophila used the ED pathway in conjunction with the tricarboxylic acid (TCA) cycle. P. reptilivora and P. aeruginosa were found to utilize the PP pathway concurrently with the ED pathway and the TCA cycle.

The denitrifier, Pseudomonas stutzeri was shown by Spangler and Gilmour (1966) to dissimilate glucose via the ED and PP pathways in conjunction with the tricarboxylic acid cycle and glyoxalate bypass. The same pathways were found to be operating with either oxygen or nitrate-oxygen as the terminal hydrogen acceptor.

The glucose catabolism of eight species of the genus Xanthomonas, a group closely related to Pseudomonas, was recently investigated by Zagallo and Wang (1967). The results indicated that the ED pathway accounted for eighty-one to ninety-three percent of the total glucose dissimilated while the remainder was used via the PP pathway. These findings support the previously recognized close

phylogenetic relationship between the xanthomonads and pseudomonads.

At the present time, nearly all of the Pseudomonas species and related organisms investigated have been shown to utilize the ED and/or PP pathways as primary routes of glucose catabolism. One exception was found by Eagon and Wang (1962) who discovered that Pseudomonas natriegens utilized the EMP pathway as the primary pathway with a minor PP pathway. The ED pathway, which was not detected in glucose-grown cells, was induced in gluconate-grown cells. Later work by Cho and Eagon (1967) suggested a tricarboxylic acid cycle was also present but functioned slowly because of an accumulation of $NADPH_2$ and repressed levels of key enzymes.

The utilization of gluconate by Escherichia coli via an induced ED pathway has been reported by Eisenberg and Dobroyosz (1967). These authors found a minor PP pathway also operating in this organism. With gluconate induced cultures, glucose and gluconate were degraded simultaneously and independently by both the ED and EMP pathways.

The tricarboxylic acid cycle is known to exist as an intermediary pathway for the oxidation of carbohydrates in many bacteria. One criterion for establishing the presence of the TCA cycle in an organism is the ability of the organism to use all of the TCA cycle intermediates as oxidizible substrates and energy sources. It was

found by Campbell and Stokes (1950) that P. aeruginosa was able to oxidize only a few of the TCA cycle intermediates without an adaptive period. It was later found that the cells were impermeable to most of the TCA cycle intermediates and could use them only when fresh resting cells had been dried thus making them more permeable to the intermediates.

Several investigators have used certain TCA cycle intermediates in growth and Warburg experiments with various strains of S. natans. The TCA cycle intermediates citric acid, fumaric acid, malic acid and succinic acid were used by Hohnl (1955) in respiration and growth experiment with S. natans. He obtained fair to good responses to these substrates in the form of oxygen uptake but reported that while malic acid resulted in good growth, fumaric and succinic acids produced only weak growth and citric acid gave no growth. Mulder and Van Veen (1963) found that one of their strains of S. natans grew well with citrate but a second strain was unable to use citrate for growth. These data may indicate the presence of a permeability barrier to some of the TCA cycle intermediates in certain strains of S. natans.

A convincing isotopic study of the TCA cycle in nonproliferating cells of Saccharomyces cerevisiae was reported by Demoss and Swim (1957). A suspension of baker's yeast was permitted to oxidize acetate-2-^{14}C in the absence of additional substrates. The

cells were separated into several fractions and the compounds containing ^{14}C were separated from each fraction by chromatographic procedures. All of the isotope was located in acetate, citrate, alpha-ketoglutarate, succinate, fumarate, malate, glutamate, aspartate, alanine and glycine. The citrate, alpha-ketoglutarate, succinate, fumarate and malate were highly labelled and in isotopic equilibrium with each other. This is strong evidence that the TCA cycle is a major pathway of acetate oxidation in nonproliferating suspensions of baker's yeast.

Another study of the TCA cycle in baker's yeast was made by Wang (1961) using specifically labelled glutamic acid. Since some of the TCA cycle acids could not be used by Saccaromyces cerevisiae due to poor permeability the more readily assimilated glutamic acid was used. In an organism with an active TCA cycle, specifically labelled glutamic acid can be converted into an equilibrium mixture with alpha-ketoglutarate of the TCA cycle by deamination or transamination. The subsequent cycling of specifically labelled alpha-ketoglutarate in the TCA cycle resulted in the following order of $^{14}CO_2$ yields: C-1 > C-2 = C-5 > C-3(4). This radiorespirometric method has also been used by Spangler and Gilmour (1966) to furnish strong evidence for the presence of the TCA cycle in Pseudomonas stutzeri.

Polymer Studies (Poly-β-hydroxybutyric Acid)

The storage product, poly-β-hydroxybutyric acid was first isolated from cells of Bacillus megaterium by Lemoigne (1927) who determined its emperical formula to be $(C_4H_6O_2)_n$. Since this early discovery this polymer has been found in a variety of other bacteria. Poly-β-hydroxybutyric acid has been found to accumulate in species of Azotobacter (Lemoigne and Gerard, 1948), Pseudomonas (Doudoroff and Stanier, 1959), Vibrio (Hayward et al., 1959), Rhizobium (Forsyth, Hayward and Roberts, 1958), Rhodospirillum (Hayward et al., 1959) and Spirillum (Hayward et al., 1959). It has also been demonstrated in a Hydrogenomonas sp. (Schlegel, Gottschalk, and Von Bartha, 1961) Chromatium violaceum (Forsyth et al., 1958) and Sphaerotilus natans (Rouf and Stokes, 1962), (Mulder and Van Veen, 1964).

The nature of the role played by the above polymer in bacteria has been investigated by several researchers. Doudoroff and Stanier (1959) examined the products of oxidative assimilation from ^{14}C-labelled glucose, acetate, and butyrate in Pseudomonas saccharophila and of photosynthetic assimilation from acetate and butyrate in Rhodospirillum rubrum. In all cases a major fraction of the assimilated carbon (60-90%) initially accumulated within the cells as poly-β-hydroxybutryic acid. When a starved, washed suspension of

R. rubrum was incubated with ^{14}C acetate, 70% of the initial ^{14}C was incorporated into polymer. During incubation of these cells in the absence of an exogenous carbon substrate but in the presence of a nitrogen source and carbon dioxide, more than 90% of the polymer disappeared with most of the ^{14}C being redistributed into other cellular materials. Further studies with R. rubrum by Stanier et al., (1959) showed that, in the presence of CO_2 but with no nitrogen source, the polymer content fell by about 50% and the carbohydrate level showed a corresponding gain. When both nitrogen and carbon dioxide were furnished, the polymer disappeared almost completely with concomitant increases in both carbohydrate and nitrogen content. The authors concluded that in this case the polymer serves as a store of carbon and reducing power for further carbon dioxide assimilation, which is essential for polymer utilization. Similar experiments with P. saccharophila showed no net transfer of polymer to other cell constituents.

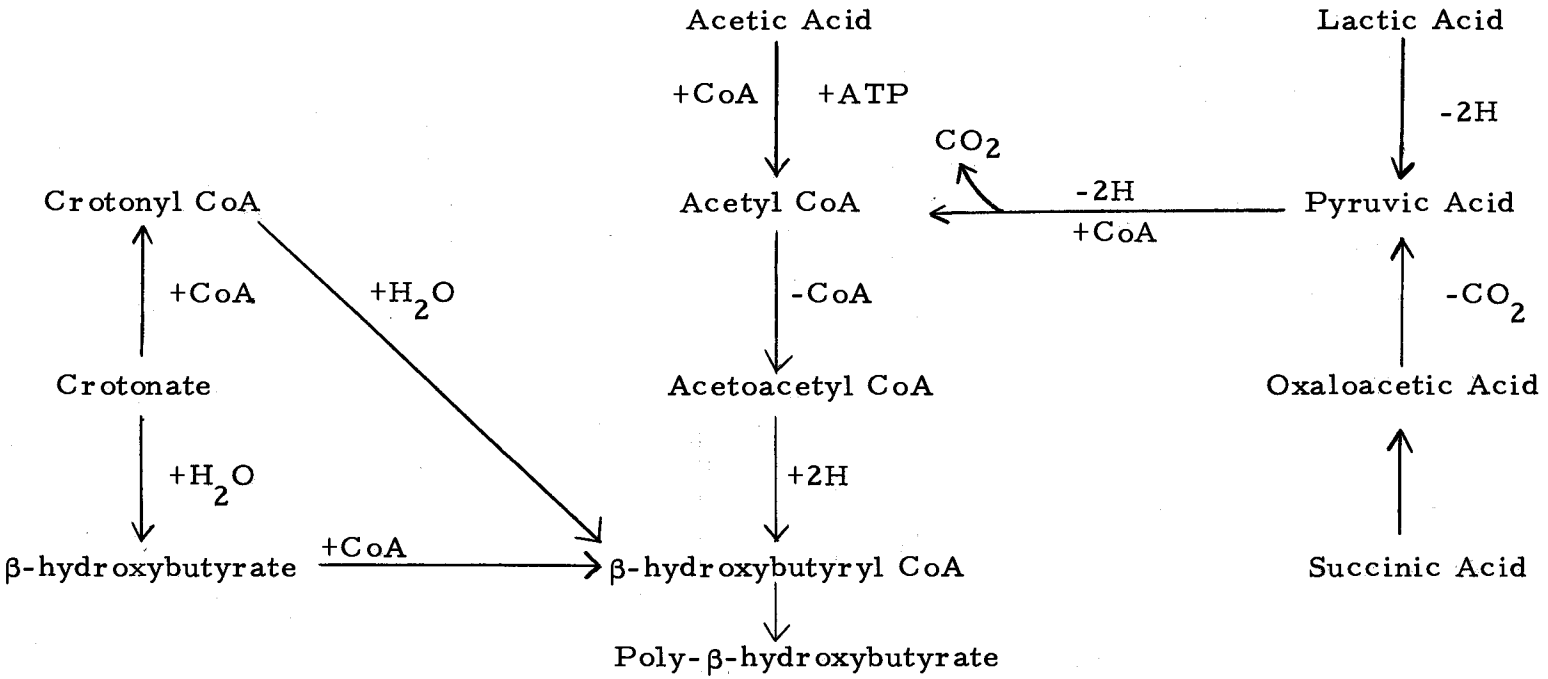
Species of the genus Hydrogenomonas are capable of producing large quantities of polymer from CO_2 fixed during chemolithotropic growth. Schlegel et al., (1961) found that in an atmosphere of hydrogen, oxygen and carbon dioxide when deprived of a nitrogen source these species continue to increase in size with the increase in dry weight due entirely to polymer accumulation. In the absence of carbon dioxide it was found that stored polymer can serve as a

carbon and energy source and will support protein synthesis in the presence of a suitable nitrogen source such as ammonium chloride.

The pathways and intermediates involved in poly-β-hydroxybutyric acid synthesis in Hydrogenomonas sp. have been determined by Gottschalk (1964a, b, c). The intermediates were determined by following the incorporation of ^{14}C-labelled acetate, β-hydroxybutyrate, crotonate, lactate, and succinate into polymer. A diagram of the various steps involved in polymer synthesis is shown on the next page. The precursors of PHB are intermediates in several catabolic pathways for glucose. The latter include the EMP pathway, the ED pathway, the PP pathway and the TCA cycle.

The pathway for the catabolism of PHB has been studied extensively by several investigators. A depolymerase (involved in polymer breakdown) in extracts of R. rubum was demonstrated by Merrick and Doudoroff (1961). They also found a specific DPN linked D-(-)-β-hydroxybutyrate dehydrogenase which resulted in the formation of acetoacetate. Sierra and Gibbons (1962b) discovered that ATP, CoA, Mg and oxalacetate are required for the conversion of the acetoacetate to acetyl CoA. A diagram of the pathway for polymer catabolism is shown on page 16.

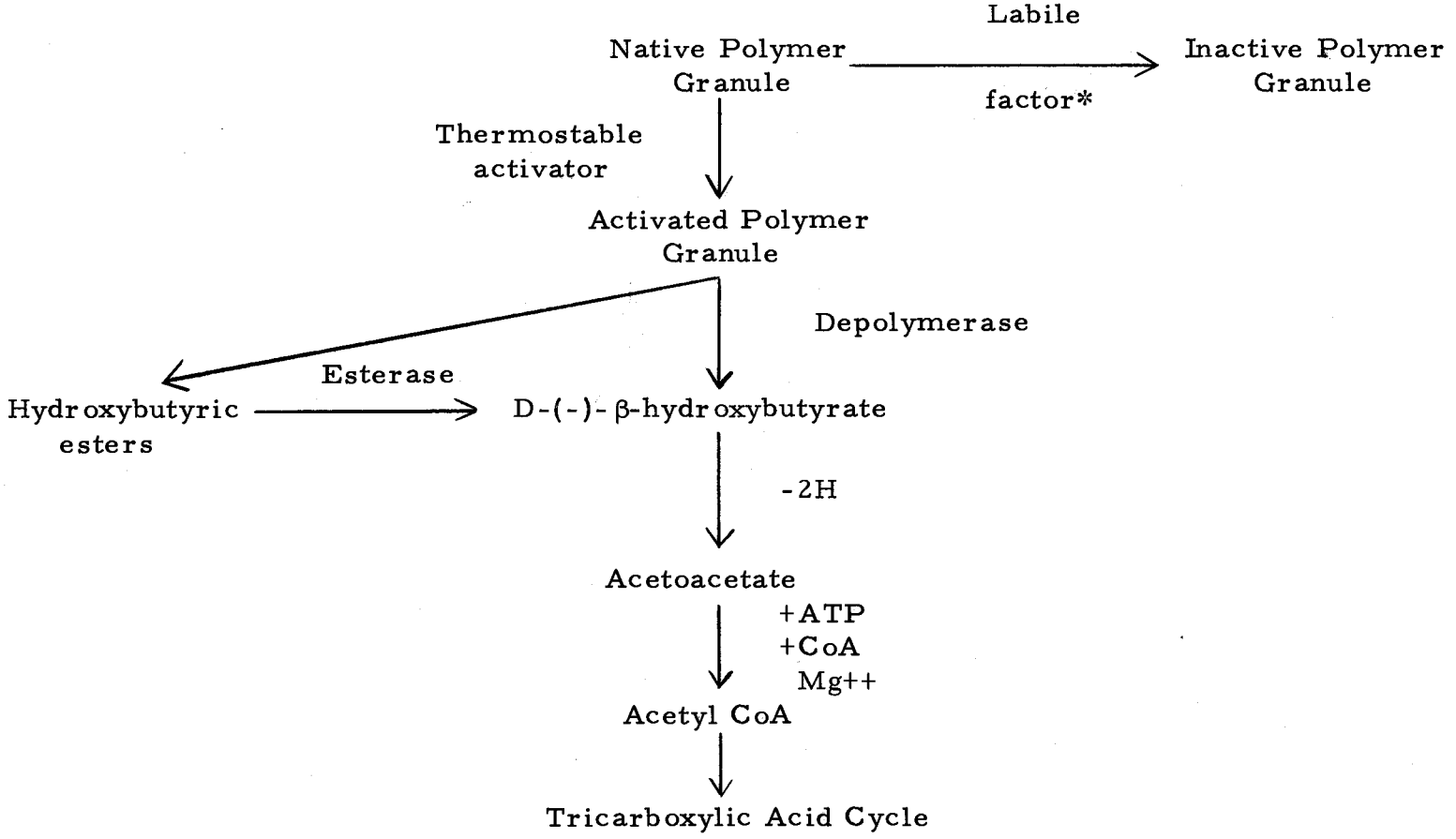
It would appear from the diagram that an organism which synthesizes PHB as a storage product probably has an active TCA cycle in order to efficiently utilize the acetyl CoA. This assumption is


Acetic Acid
+CoA
+ATP
Lactic Acid
-2H
CO_2
Crotonyl CoA
Acetyl CoA
-2H
+CoA
Pyruvic Acid
+CoA
+H_2O
-CoA
-CO_2
Crotonate
Acetoacetyl CoA
Oxaloacetic Acid
+H_2O
+2H
β-hydroxybutyrate
+CoA
β-hydroxybutyryl CoA
Succinic Acid
Poly-β-hydroxybutyrate

supported by considerable evidence that the TCA cycle is functioning in PHB synthesizing organisms which have been previously tested for its presence.

Although no enzyme or pathway work has been done with PHB in S. natans, it is probable that the same pathways of polymer synthesis and degradation previously outlined for other organisms also exist in S. natans. Mulder (1964) has investigated the effect of C/N ratios and carbon-nitrogen sources on polymer synthesis. He found that when S. natans was grown in a medium containing 1 g of peptone and either 1, 2, 5, 10, or 15 g of glucose, all of the cultures accumulated large amounts of polymer in three days. After two days of further incubation, the poly-β-hydroxybutyrate content of the cells grown at the lowest glucose level had decreased sharply, whereas the content of cells grown in higher concentrations of glucose was unchanged. Five days later the low-glucose cells were almost free of polymer which had apparently been utilized in respiration. This was confirmed by a large drop in dry weight of cells from these cultures after incubation for 10 days as contrasted with the cultures grown at high glucose concentrations which had not decreased in dry weight and had not lost their globules of poly-β-hydroxybutyric acid.

The effect of varying the nitrogen supply on the formation of PHB was observed by growing S. natans in a basal nutrient solution



*Labile factor inherent in polymer granule and subject to inactivation by various physical and chemical agents.

enriched with: (a) Casamino acids (Difco), 125 mg of nitrogen/liter of medium; (b) Casamino acids (Difco), 125 mg of nitrogen/liter and glucose, 250 mg/liter; (c) Ammonium nitrate, 125 mg of nitrogen/liter and glucose, 250 mg/liter. In the first medium the cells formed no globules of poly-β-hydroxybutyrate; in the second medium about 5 percent of the cells contained globules, whereas in the third medium the cells were packed with globules. The high concentration of polymer in the latter cells was probably due to the fact that ammonium nitrate was less readily assimilated than amino acids and consequently an accumulation of glucose or other carbon compounds occurred within the cells which promoted the synthesis of the polymer.

The accumulation of PHB by pure cultures of the floc-forming organism Zoogloea ramigera has been associated with the floculation of this organism by Crabtree, et al., (1966). These investigators found that an accumulation of PHB preceded the floc formation. In addition, PHB-rich cells and the isolated native polymer as well as the purified polymer demonstrated an adhesive property. Floc formation was prevented by metabolic blocking of PHB synthesis. Endogenous dissimilation of PHB resulted in deflocculation.

Oxygen Requirements

The relation of oxygen level to growth of S. natans has been

investigated by several authors. Ruchoft and Kachmar (1941) found that S. natans grew best at aeration rates providing at least 6.0 ppm of dissolved oxygen at the end of the aeration period. However, they also found that S. natans can grow and develop appreciably in substrates containing very low quantities, 0.1 to 2.0 ppm of dissolved oxygen. It was considered significant that in a good medium the S. natans could produce up to 598 ppm of solids and utilize as much as 600 to 700 ppm of glucose at the low rates of aeration that were required to maintain the low dissolved oxygen values. These investigators also showed that S. natans was unable to grow in synthetic sewage medium in the absence of oxygen.

S. natans was grown in both liquid and solid media with low oxygen levels by Stokes (1954). He found that good growth was obtained even when stab cultures were layered with parafilm or when cotton plugs were saturated with pyrogallic acid and alkali and then sealed off from the atmosphere with parafilmed rubber stoppers. When extreme precautions were taken to insure complete anaerobiosis, however, no growth occurred. No growth was obtained in anaerobic bottle cultures with media containing 0.2 percent potassium nitrate. Stokes concluded that nitrate cannot replace oxygen in the metabolism of S. natans. This was also the conclusion of Linde (1913). However, all of the strains of S. natans used by Stokes did reduce nitrate to nitrite.

Mulder (1964) investigated the role of S. natans in bulking activated sludge. He suggested that the development of large flocs of S. natans under relatively low oxygen conditions is a primary cause of bulking and leads to even greater oxygen deficiency. This hypothesis was demonstrated in an experiment in which S. natans and Arthrobacter globiformis, (an aerobic corynebacterium isolated from activated sludge), were grown separately in 50 ml portions of basal nutrient solution supplemented with 3 g of peptone and 5 g of glucose/l in 200 ml Erlenmeyer flasks. A number of the cultures were heavily aerated on a vibrating shaker, the others not agitated, which resulted in a poor oxygen supply. Growth was measured by estimating cell yields after different periods of time. The growth rate as well as the maximal cell yield of well aerated S. natans was only slightly higher than that of poorly aerated cultures. This was in strong contrast with A. globiformis which grew more slowly under stationary conditions than with agitation. Another striking phenomenon was the sharp decline in cell yield of aerated S. natans, presumably owing to respiration of reserve materials, shortly after reaching maximal values for cell yield. It was postulated that poor aeration of mixtures of S. natans and A. globiformis would bring about an accumulation of the former organism.

MATERIALS AND METHODS

Organisms Studied

Three strains of Sphaeratilus natans were used as test organisms in this study. The first strain was isolated from an enriched section of the Berry Creek Experimental Stream and will be referred to as OSU strain BC-IV. A culture of this organism was sent to Dr. J. L. Stokes who confirmed our identification. The OSU strain was studied in pure culture in the laboratory and experiments were also conducted with mixed populations of this organism in the form of slime-masses taken directly from Berry Creek. Other strains used in this study were acquired from the American Type Culture Collection in Washington, D. C. They are S. natans ATCC 15291 and ATCC 13338. Strain 15291 has recently been designated the type species for the genus Sphaerotilus.

Isolation Media and Techniques

Unless otherwise stated all chemicals used were reagent grade and the amounts of all ingredients used are expressed as percent (weight per unit volume). Where feasible, stock solutions of carbon sources were prepared with glass distilled water and autoclaved separately. When required, the final pH of a medium was adjusted

with autoclaved solutions of 1 N HCl or NaOH.

(1953) Isolation Medium (Stokes)

Glucose	0.1
Peptone	0.1
$MgSO_4 . 7H_2O$	0.02
$CaCl_2$	0.005
$FeCl_3 . 6H_2O$	0.001
Agar (if desired) final pH 7.2	1.5
Tap water	

Alfalfa Straw Medium

Fresh extracted alfalfa straw*	1.0
K_2HPO_4	0.1
KH_2PO_4	0.1
Tap water	

*Obtained by boiling chopped straw for four hours.

(1961) Isolation Medium (Dondero)

Casein Hydrolysate	0.15
Agar	1.5
Tap water	

(1961) Enriched Medium (Dondero)

Casein Hydrolysate	0.5
Glycerol	1.0
Yeast autolysate	0.1
Agar	1.5
Distilled or tap water	

0.05 Percent Meat Extract Agar

Meat extract	0.05
Agar	1.5
Tap water	

Difco Sphaerotilus Agar

Difco Laboratories Inc., Detroit, Michigan

Several methods used by other investigators for isolation of Sphaerotilus were attempted before a consistently successful procedure was found. Some preliminary methods attempted were:

1. Small clumps of Sphaerotilus were streaked directly on Difco Sphaerotilus agar and Stokes' isolation agar. The cultures were incubated at 20 and 28 C.
2. Water samples containing filaments of Sphaerotilus were added to culture flasks containing 50 ml quantities of 1 percent extracted alfalfa straw. The samples ranged

in volume from 0.1 ml to 5 ml. Incubation temperatures were 20 and 28 C.

3. Small amounts of Sphaerotilus were streaked on Dondero isolation agar and 0.05 percent meat extract agar. Filaments which had been previously washed twice in tap water were also streaked on these agar media and then placed on Dondero and Stokes' liquid isolation media. These were incubated at 10, 15, 20, and 28 C.

The above listed methods have been successfully used in the past by other researchers and did result in the growth of Sphaerotilus-like organisms in most of the various media. It was noted microscopically that many filaments of Sphaerotilus were growing from attached positions on pieces of alfalfa straw. The principal difficulty in the isolation procedures was due to the presence of a great number of small, motile, Gram-negative rods growing in the Sphaerotilus flocs. Some of these contaminants were capable of completely covering an agar surface with a thin, bluish, translucent colony in 24 hours. When this condition existed, the Sphaerotilus filaments, although apparently present in great numbers, simply refused to spread from the initial steak and were rapidly overgrown and crowded out.

The following method proved to be satisfactory for isolation of strains of S. natans from Berry Creek. First, fresh flocs with as

little foreign material as possible were collected and placed in a battery jar with a fine mesh screen over the top. The battery jar was placed under a stream of rapidly-flowing tap water, and the filaments were washed for 20 minutes. It was found that blending the washed flocs only tended to make contaminating bacteria cling more tightly to the sheath material. The washed flocs were then transferred directly to a petri dish containing about 15 ml of sterile 0.01 M phosphate buffer at pH 7. Tiny filaments of Sphaerotilus were agitated in a series of four of these petri dishes. After the fourth washing in phosphate buffer, single filaments were transferred, using a capillary pipette, to a sterile slide where the filaments were examined for contaminating bacteria. Subsequently, the filaments were introduced into Stokes' liquid isolation medium (0.01 percent yeast extract added) and also streaked on Dondero isolation medium. The best isolation temperature was 10 C. At this temperature, the Sphaerotilus filaments attached to sides of the flasks and the growth closely resembled forms growing in Berry Creek. In three days to one week, the organism, if not contaminated, resembled small tufts of cotton in the clear medium. Any contamination resulted in a turbid, cloud medium in which few, if any, cells of Sphaerotilus were observed. Final tests for contamination were made by streaking on Dondero enriched agar and by examining Gram and nigrosin-stained slides. The organism was then streaked on

Stokes' isolation agar enriched with 0.005 percent $MnSO_4$ to determine whether the culture was S. natans or S. discophorus. S. discophorus produces dark brown colonies on this agar as a result of its ability to oxidize manganese and deposit it in sheath material, while Sphaerotilus natans is incapable of this deposition.

Culture Media and Conditions

All stock cultures were maintained on the following medium:

Ingredient	Percent (w/v)
Glucose (autoclaved separately)	0.25
Tryptone	0.25
K_2HPO_4	0.01
$MgSO_4$	0.01
Tris Buffer	0.03
$MgCl_2$	0.008
$CaCl_2$	0.005
NaEDTA	0.001
$FeCl_3 \cdot 6H_2O$	0.001
$MnCl_2 \cdot 4H_2O$	0.00014
$ZnCl_2$	0.000011
$Na_2M_oO_4$	0.000005
$CoCl_2$	0.0000005
$CuCl_2$	0.000000004
Agar	1.5
Distilled water	

This medium is referred to as ASMTT medium and has a final pH of 7.4. Modifications of this medium were made in certain investigations as follows:

1. In radiorespirometric and carbohydrate studies, 0.1 percent potassium nitrate was substituted for tryptone in order to make glucose or some other carbohydrate the sole carbon source. Five μgm of vitamin B_{12} was added aseptically to each liter of medium. The vitamin B_{12} had been previously sterilized by passage through membrane filters with a median pore size of 0.22 μ. Stock solution of sterilized vitamin B_{12} were kept frozen in tiny screw cap vials holding 1 ml each of a 100 μgm/ml solution. When needed, the 1 ml vials were added aseptically to sterile 99 ml water blanks to make a final concentration of 1 μg/ml. Ordinarily, glucose was the sole carbon source. However, in tests for the utilization of tricarboxylic acid cycle substrates, each intermediate was used as the sole carbon source. The synthetic medium with glucose as the carbon source was designated ASMTM medium and has a final pH of 7.4.
2. A second synthetic medium (ASMTB) was devised by modifying the ASMTM medium. This medium contained 0.1 percent potassium nitrate or 0.055 percent ammonium

chloride as the nitrogen source. Tris buffer was excluded from this medium and 0.02 M potassium phosphate buffer at pH 7.4 was substituted. The extra buffering capacity of the phosphate buffer was necessary to control the pH in experiments conducted at low oxygen levels in which an excess of nitrite was formed from nitrate. The medium containing ammonium chloride also needed additional buffering to prevent a drop in pH as the ammonia was used by the cells as a source of nitrogen. The final pH of this medium was 7.4

Cells for metabolic studies were grown on a New Brunswick Gyrorotory, water bath shaker, Model G76 at 28 C. Three transfers in liquid medium were routinely made before inoculating the final test culture to insure an actively growing culture.

The starter culture was inoculated from a three day agar slant or plate. This was accomplished asceptically by cutting out a small piece of agar containing a mass of cells and transferring the cells to 100 ml of liquid medium in a 250 ml Erlenmeyer flask. This was necessary because Sphaerotilus filaments actually grow into agar and are not easily removed unless some agar is also removed. Final cultures to be used for experiments were harvested in the upper portion of the logarithmic growth phase. The periods of growth necessary to attain this phase varied considerably for the

three strains used. Strain BC-IV required 72 hours, strain 13338, 36 hours and strain 15291, 18 to 24 hours to reach the upper-logarithmic growth phase. In cases where equal aliquots of cells were to be used in experiments, the initial liquid growth medium was ASMTT since this medium gave less sheath formation and a more homogenous cell suspension.

Continuous Culture Techniques

A modification of the device used by Novick and Szilard (1951) was used to grow S. natans over extended time periods to study growth kinetics and filament attachment. A diagram of the apparatus is shown in Figure 1. The flow of medium into the culture vessel could be varied from 50 ml per day to many liters per day. The culture vessel contained 175 ml of medium. The temperature could be maintained in the range of 8 to 30 C by manipulation of the water bath temperature and room temperature. The magnetic stirrer was used to simulate current velocity and was capable of speeds from 0 to 500 RPM. The air supply furnished oxygen to the system and was also used to flush excess medium. Pure cultures of S. natans were maintained for periods of over 30 days by using rigid asceptic techniques in replacing empty medium jugs. Cell growth was stopped in the effluent jug by the addition of 0.001 M potassium cyanide, adjusted to the full volume of the jug. Cell export was measured by

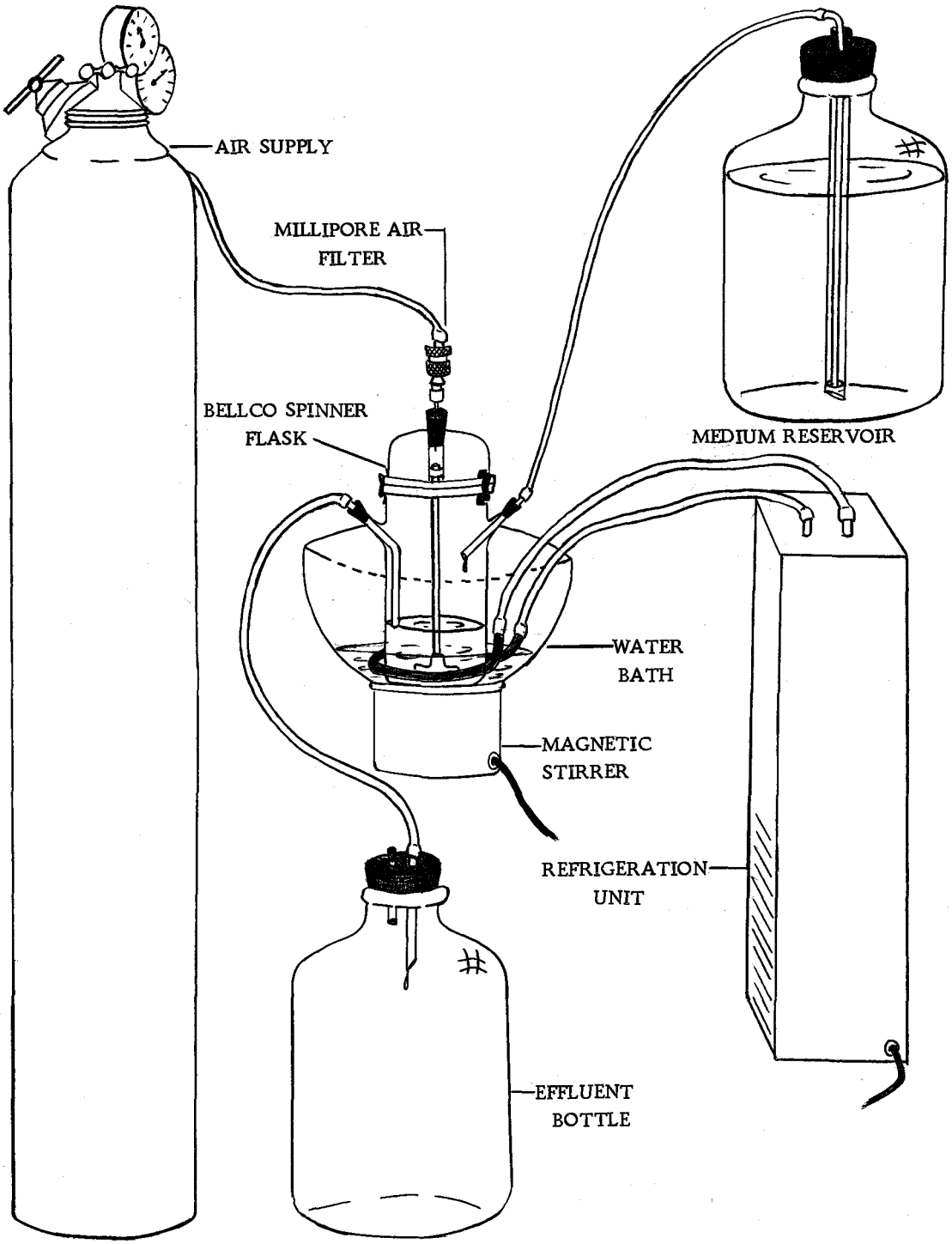


Figure 1. A continuous culture device for growth studies of S. natans.

centrifuging the effluent medium and obtaining dry weights of washed cells. In general, experiments were performed at 10 or 15 C with a flow rate of 1000 to 2000 ml per day.

Radiorespirometric Studies

The primary catabolic pathways functioning in S. natans were investigated by the radiorespirometric method of Wang et al. (1958). This method was also used to study the dissimilation of acetate, pyruvate and glutamic acid in the study of secondary pathways. The endogenous metabolism of S. natans was studied by using a modification of the procedure of Wang et al. (1958).

Cells used in these experiments were grown in ASMTM medium and harvested by centrifugation. The cells were then washed twice in ASMTM medium which contained no glucose or other carbon source. After the cells had been resuspended in carbohydrate-free ASMTM medium, aliquots were taken and washed in distilled water by centrifugation. The samples were resuspended in distilled water, placed in tared planchets and dried under an infrared heat lamp at 80 to 85 C. They were then placed in a desiccator and weighed. The dry weights obtained were used to adjust the final concentration of cells to 20 mg dry weight per 30 ml of substrate-free ASMTM medium. When S. natans BC-IV or Berry Creek slime was used as a test culture, the heavily ensheathed cells were washed

as described above and blended for 15 seconds in a prechilled Waring microblender to insure a homogenous cell suspension. A longer blending time resulted in decreased metabolic activity of the cells. Cell nitrogen determinations were made using the micro-Kjeldahl method (Ballentine, 1957) on equal aliquots of blended cells to determine the accuracy of weighed cell aliquots.

With the exception of endogenous metabolism and polymer studies, all radiorespirometric experiments were carried out by the same procedure and differed only in the kind and amount of specific labels used. The type of incubation flask and CO_2 trap assembly used in this series of experiments is shown in Figure 2. A series of nine incubation flasks could be placed in the Warburg apparatus at one time. Each flask contained 20 mg of cells per 30 ml of substrate-free medium. The sidearms contained specifically labelled substrate (usually about 0.25 μc) and a predetermined amount of carrier substrate. The amount of carrier substrate used was determined by prior experiments as an amount that would be completely exhausted in 6 to 8 hours and would yield a definite peak of hourly CO_2 evolution some time after the first hour. All experiments were carried out at 28 to 30 C with the exception of one experiment involving a mixed population of bacteria from Berry Creek. The latter experiment was run at 15 C.

The flasks were shaken and flushed with air at a rate of 50 cc/

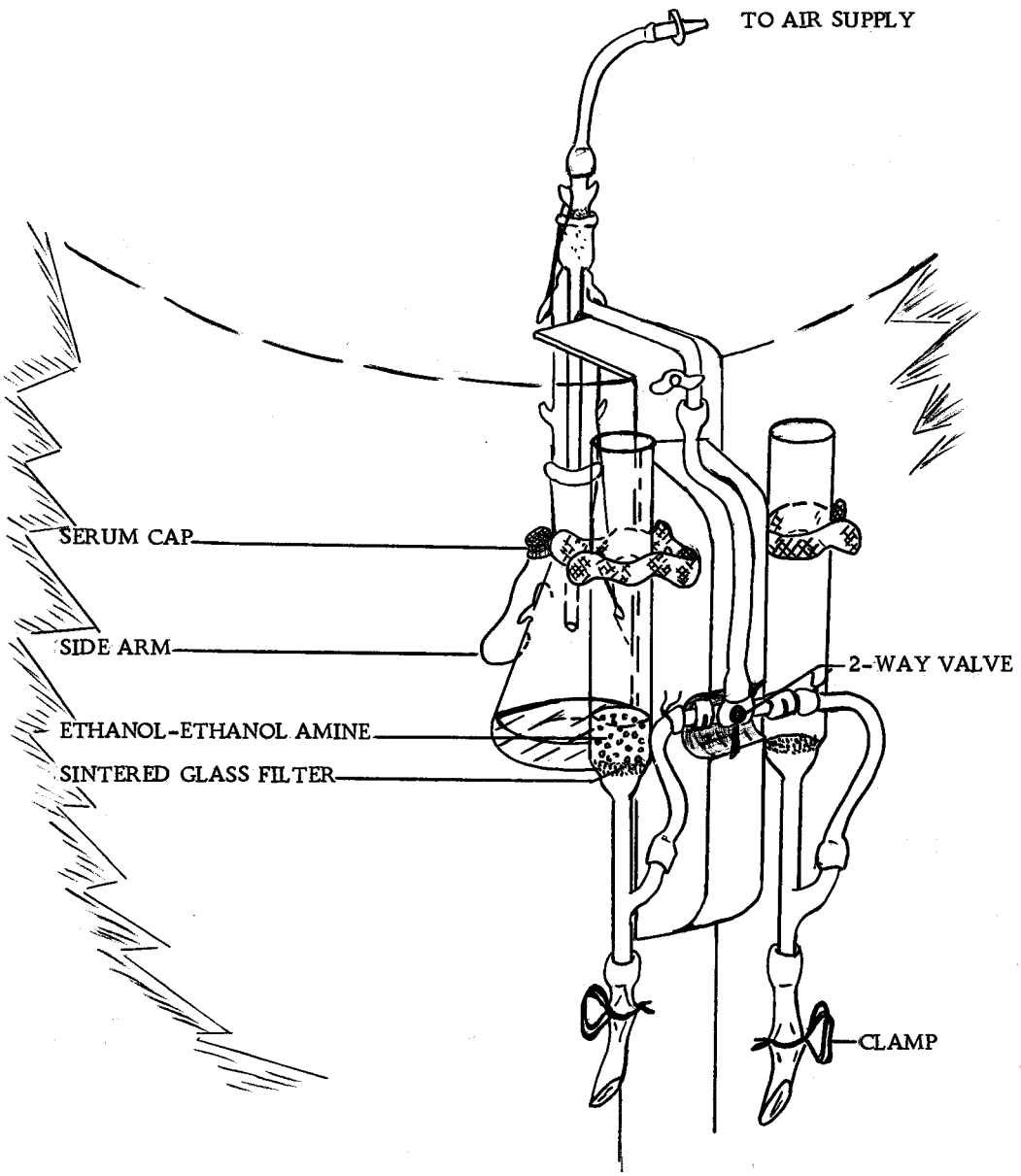


Figure 2. Radiorespirometer assembly.

minute. Following a 15 minute equilibration period, the substrates were tipped in and metabolic $^{14}CO_2$ from each flask was trapped in 10 ml of absolute ethanol-ethanolamine (2:1, v/v). The trapping solutions were replaced at hourly intervals and diluted to 15 ml with absolute ethanol. A 5 ml portion of each hourly sample was placed in 19 ml of counting solution in a 20 ml glass counting vial. The counting solution consisted of 6 g/liter of 2, 5-diphenyloxazole (PPO) and 100 mg/liter of 1, 4-bis-2-(5-phenyloxazolyl-benzene (POPOP) in scintillation grade toluene. The samples were counted in a Packard Tri-Carb Liquid Scintillation Spectrometer, Model 3214.

At the termination of each experiment the cells and medium were separated by centrifugation at 4 C and portions of each were counted in thixotropic gel consisting of: "Thixcin" (Baker Castor Oil Co., Los Angeles, California), 20 g; Tween-80-Span-80 (1:9, v/v), 4 ml; glycerol, 5 ml; and toluene PPO-POPOP. Gel samples were counted under the same counting conditions as liquid $^{14}CO_2$ samples. The efficiency of counting for each sample was determined by use of appropriate internal standards. Counting was carried out over a sufficient period of time to give a relative standard deviation no greater than 1 percent.

The substrate levels used for radiorespirometric studies were as follows (in mg/flask) glucose, 10; ribose, 5; gluconate, 5; L-glutamic acid, 5; acetate, 5; and pyruvate, 5. All specifically

labelled compounds were added at a level of 0.25 μc/flask.

The specifically labelled compounds used in the various radiorespirometric experiments were: glucose-1-, -2-, -3,4-, and 6-^{14}C; Na-gluconate-1-, -2-, -3,4- and -6^{14}C; ribose-1- and -u-^{14}C; DL-glutamic acid -1-, -2-, -3,4- and -5-^{14}C; D-glutamic acid -1-^{14}C; Na-pyruvate -1-, -2-, and -3-^{14}C and Na-acetate -1- and -2-^{14}C. The specifically labelled glucose and glutamic acid were purchased from New England Nuclear, Boston, Massachusetts. The specifically labelled acetate and pyruvate were purchased from Nuclear Chicago Corporation, Des Plaines, Illinois and the specifically labelled Na-gluconate and D-glutamic acid-1-^{14}C were supplied through the generosity of Dr. C. H. Wang of Oregon State University.

Poly-β-hydroxybutyric Acid and Endogenous Metabolism Studies

A modification of the previously described radiorespirometric apparatus of Wang et al. (1958) was used in the study of poly-β-hydroxybutyric (PHB) metabolism. The apparatus was composed of a reciprocating shaker capable of holding 4-2800 ml Fernback flasks, each connected to an air inlet and separate CO_2 traps. This macro-radiorespirometer was used in essentially the same manner as the previously discussed micro-apparatus except for the following differences:

1. The carrier substrate cells and specifically labelled

substrate were all introduced asceptically through the mouth of each flask.

2. A large initial mass of washed cells (500 to 600 mg) was used together with a corresponding amount of substrate (250 to 625 mg).
3. Levels of specifically labelled compounds used varied from 5 to 25 μc/flask.
4. Experiments were carried out at room temperature (25 ± 1 C).
5. It was much easier to assay for ^{14}C-labelled products of cellular metabolism in this system than in the micro-apparatus due to the larger quantities of products formed.

For experiments on the incorporations of ^{14}C from specifically labelled glucose, respiratory $^{14}CO_2$ released by the cells was trapped as previously described and the trapping solution replaced at hourly intervals. The cell suspension was separated by centrifugation and samples of cells and medium were counted in thixotropic gel as previously described. The relative amounts of incorporation of ^{14}C from specifically labelled substrates into polymer were determined by first purifying the polymer and then measuring its activity in a liquid scintillation counter. After a small sample of cells had been taken for determination of the total cell count, the remainder was washed twice in distilled water. The cells were then

resuspended in 100 ml of distilled water and 0.5 ml samples were taken for a dry weight determination. The remaining cells were collected by centrifugation and resuspended in 100 ml of 2 N H_2SO_4. This suspension was subsequently placed in a water bath at 100 C for 2 hours to facilitate hydrolysis and removal of water insoluble polysaccharides. The residue was collected by centrifugation, washed three times in distilled water, and digested for 12 to 18 hours at 35 C in 100 ml of the alkaline hypochloride reagent of Williamson and Wilkinson (1958). The residue from the hypochlorite treatment was collected and washed three times by centrifugation. To remove lipids, the residue was placed in 100 ml of a mixture of ether:acetone, (2:1, v/v) for 24 hours; this was followed by extraction with 50 ml of redistilled ether alone for 6 hours. The ether-extracted residue was treated with 30 ml of hot chloroform (62 C) to dissolve poly-β-hydroxybutyrate and the suspension was then centrifuged to remove chloroform-insoluble materials. The supernatant fluid was dried to constant weight in a tared porcelain crucible or evaporating dish at 62 C.

The purity of PHB was ascertained by comparing infrared absorption spectra of the polymer extracts with those found for PHB from other strains of S. natans and Bacillus megaterium by Rouf and Stokes (1961). The polymer was prepared for infrared spectroscopy by air-drying solutions of PHB-chloroform on silver

chloride discs. The samples were dried in tilted position to obtain a gradient of the polymer layer. Since silver chloride discs are light sensitive, the drying was accomplished in the dark. A Beckman IR5 Double Beam Recording Infrared Spectrophotometer was used in this study. The instrument was standardized before use by running the infrared spectrum of a polystyrene film standard and making suitable adjustments of the machine when this spectrum varied from the standard spectrum. Each silver chloride disc was adjusted until a portion of the PHB gradient was obtained which gave 50 percent transmission at a wave length of 8.0 microns. Each sample was positioned in the machine in a similar manner and the absorption spectrum was recorded from 2 to 16 microns. The melting point range of each polymer sample was obtained using a Büchi melting point apparatus. The melting points of all samples tested were within the range found by other investigators.

The polymer which contained ^{14}C from incorporation experiments was counted by dissolving 50 mg of purified PHB in 5 ml of hot chloroform. Fifty lambda of this solution was carefully spotted on 2.5 x 4 cm strips of Whatman Number 1 chromatography paper. The strips were placed in glass counting vials containing 20 ml of scintillation solution consisting of 4 g PPO and 50 mg POPOP per liter of toluene. The polymer samples were counted under flat spectrum conditions. Relative counting efficiencies were determined

by counting 50 lambda samples and 50 lambda spiked samples of polymer on filter paper strips.

Determination of D-(-)-β-Hydroxybutyrate Dehydrogenase

The assay for D-(-)-β-hydroxybutyrate dehydrogenase was performed by using a modification of the method outlined by Carr and Lascelles (1961). Cells were grown in ASMTT medium for 28 hours at 28 C. The cells from 400 ml of medium were harvested by centrifugation at 4 C and resuspended in .02 M tris buffer at pH 8.5. The volume of the cell suspension was adjusted to give a final concentration of 20 mg dry weight of cells per ml.

A 10 ml suspension of cells was broken in a Raytheon 200 watt, 10 k.c. oscillator at 1.5 amps, 115 volts for 25 minutes. The remaining whole organism and debris were removed by centrifugation at 25,000 g for 10 minutes at 4 C. The supernatant fluid was used as the bacterial extract.

The assay is based on the following reaction: D-(-)-β-hydroxybutyrate + DPN^{+} $\rightleftharpoons$ acetoacetate + DPNH + H^{+}. The reaction mixture contained (in μ moles): tris buffer, pH 8.5, 562; DPN, 2.6; sodium β hydroxybutyrate, 7.5; bacterial extract, 0.05 ml; (1050 μg of protein); water to a final volume of 3.0 ml. The reaction was measured by following the formation of DPNH + H^{+} spectrophotometrically at 340 mμ in a Beckman D.U. spectrophotometer. One unit

of enzyme is defined as that amount which, under the given conditions, causes an initial rate of change in optical density of .001 per minute. Specific activity is expressed in units per mg of protein.

Carbon Dioxide Fixation

Warburg experiments performed by Hőhnl (1955) indicated that an atmosphere containing 5 percent CO_2 was inhibitory to respiration in S. natans but at lower CO_2 levels CO_2 fixation possibly occurred with glucose as an energy source. To determine if Hőhnl's hypothesis was correct, a series of experiments were conducted using $^{14}CO_2$ and glucose, acetate and pyruvate as carbon sources with S. natans strain ATCC 15291 as the test organism.

The apparatus used consisted of 250 ml Erlenmeyer flasks, each with a large center well and sidearm with a serum cap through which the substrate could be added as well as acid for generating $^{14}CO_2$ in the center well. The flasks were fitted with ground glass necks and could be sealed with special tops equipped with gasing valves for safety disposing of residual $^{14}CO_2$ at the termination of an experiment.

Each flask contained 50 mg dry weight of cells in 100 ml of medium. Five microcuries of $NaH^{14}CO_3$ were added to the center well. A carrier solution containing 11.9 mg of cold $NaHCO_3$ was added so that an atmosphere containing 1 percent of CO_2 would be

evolved in the flasks after the addition of acid. Glucose, acetate and pyruvate were added to the respective flasks in amounts approximately proportional to their energy output in oxidative reactions (Krebs, H., 1954). The amounts added were: glucose, 80 mg; acetate, 184 mg; and pyruvate, 150 mg. The generation of $^{14}CO_2$ was initiated by using a hypodermic syringe to deliver 1 ml of 6 N H_2SO_4 through the serum cap into the center well. The flasks were incubated with constant shaking in a 29 C water bath.

After an incubation period of 7 hours, the reactions were terminated by the addition of sufficient 6N H_2SO_4 (0.7 ml) to lower the pH to 2. Residual radioactive CO_2 was trapped in a 6 N NaOH solution by sparging the flasks for 30 minutes. After the cells and medium were separated by centrifugation the cells were washed twice in distilled water and once in ethyl alcohol. Aliquots were taken from an aqueous suspension of the washed cells and used for dry weight determinations and also counted in thixotropic gel. The efficiency of gel-scintillation counting was determined for each sample by spiking with an internal standard.

Electrolytic Respirometer Techniques

Respiration experiments were conducted using a modification of the electrolytic respirometer described by McGarity, Gilmour and Bollen (1958). A diagram of a modified unit is shown in

Figure 3. This unit is more compact and much more sensitive to changes in gas pressure due to oxygen uptake than the original apparatus. Increased sensitivity was achieved by miniaturizing the capillary tube-electrode section of the apparatus which completes the electrolytic circuit. A total of twelve units were housed in an insulated cabinet which allowed temperature control in the 15 to 30 C range.

Mass cell experiments were conducted using 60 mg of washed cells in 100 ml of medium. Experiments with growing cells were conducted by placing a 1 ml inoculum in 100 ml of medium. All experiments were performed with pure cultures of S. natans and sterility checks were made before and after each experiment to insure that no contamination had occurred. All of the components of the flask assembly were sterilized by autoclaving at 121 C for 20 minutes. In experiments where oxygen concentrations less than 20 percent were desired, the proper helium-oxygen gas mixture was flushed through the respirometer assembly after first passing through a sterile cotton filter. The electrolytic respirometer maintained desired oxygen concentrations as low as 5 ± 1 percent, during experiments lasting 40 hours or longer.

The electrolytic respirometers made possible the measurement of oxygen utilization, substrate consumption, and product formation by growing or mass cell suspensions. Magnetic stirrers

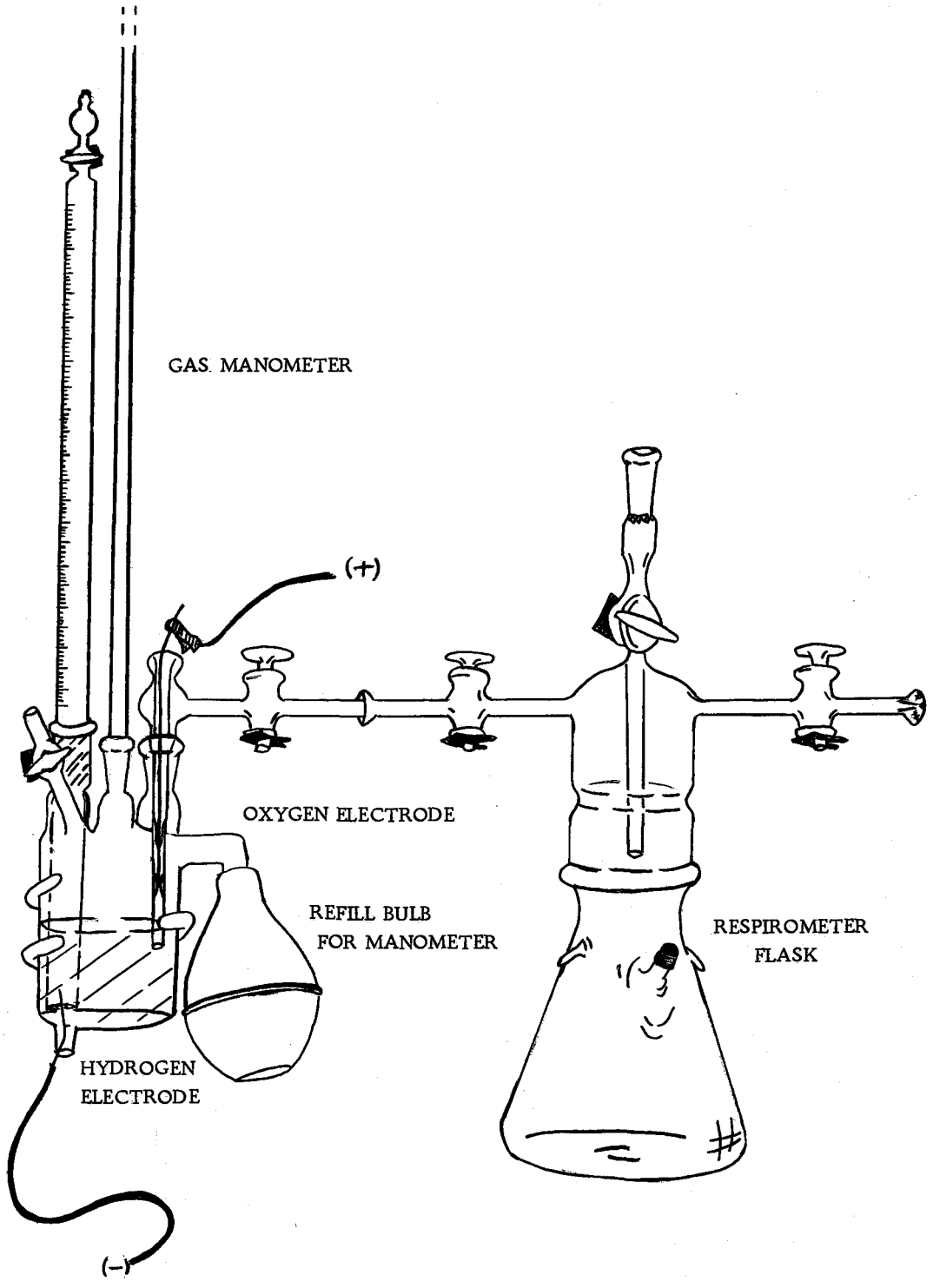


Figure 3. Electrolytic respirometer.

were used to provide adequate gas exchange and agitation. The evolution of nitrogen, oxides of nitrogen and other gases such as hydrogen or methane was checked by a gas chromatograph. At the termination of the experiment, the flasks were attached to a gas manometer which allowed appropriate gas samples to be drawn for analysis. The total carbon dioxide evolved during each experiment was trapped in a glass cylinder containing 10 ml of 40 percent sodium hydroxide. The traps were suspended on strings attached to hooks inside the flask tops. The medium was examined for glucose depletion, nitrate depletion, nitrite formation and pH at the termination of each experiment. Dry weight determinations were also performed with washed cells.

Analytical Procedures

Glucose Analysis

Glucose determinations were conducted according to the procedure of Dubowski (1962). The o-toluidine method was very sensitive and allowed glucose to be measured in concentrations as low as 5 μgm/ml.

Nitrate Analysis

Nitrate-nitrogen determinations were performed according to

the method of Eastoe and Pollard. Reagents were replaced by newly prepared solutions every 30 days.

Nitrite Analysis

Nitrite-nitrogen was determined by the modified N-(1-naphthyl)-ethylene-diamine dihydrochloride technique of Saltzman (1954).

Carbon Dioxide Titration

Carbon dioxide absorbed in sodium hydroxide solution was measured by titration with standardized acid using a Beckman Model K Automatic Titrator.

Gas Analysis

Gas analyses were conducted by the use of Beckman GC-2 Gas Chromatograph. Determinations were conducted at 40 C with helium as the carrier gas. Gas chromatography calibration curves were used for both qualitative and quantitative analyses of carbon dioxide, nitrogen, oxides of nitrogen and other gases. A 12 foot molecular sieve column was used in the analyses for nitrogen, oxygen and nitrogen dioxide, while a 1.5 foot activated charcoal column was used for carbon dioxide and nitric oxide analyses.

RESULTS AND DISCUSSION

Isolation and Identification of *Sphaerotilus natans*

The primary aim of this study was to investigate the metabolism of pure cultures of *S. natans* and to attempt to relate these findings to experimental results obtained for *S. natans* growing in Berry Creek Experimental stream. In order to effectively study *S. natans* in pure culture, it was first necessary to have reliable methods for isolation and identification of this organism. The successful isolation of pure cultures of *S. natans* was contingent on the development of techniques and methods for growing and identifying colonies of *S. natans*. A combination of the methods used by Pringsheim (1949), Stokes (1954) and Dondero (1961) was employed in obtaining pure cultures of *S. natans* from Berry Creek. The use of media with relatively low concentrations of carbon and nitrogen favored the formation of a sheath around cells of *S. natans* which greatly aided in the identification of the organism. Only those colonies which were composed of cells surrounded by a sheath were selected for isolation since a number of *Pseudomonas* species closely resemble non-ensheathed cells of *S. natans*. The poorly enriched medium also served to limit growth of contaminating bacteria which were found in relative abundance in slime masses of

Sphaerotilus in Berry Creek (see Table 1). The counts reflected only numbers of bacteria other than S. natans since this organism produced little or no growth on the counting plates. Counts made from the slime masses were approximately 1000 times greater than those obtained for the unenriched creek water, while water flowing through the enriched sections had counts about 100 times greater than the unenriched water.

During early attempts to isolate pure cultures of S. natans several chain-forming members of the genus Bacillus were isolated which closely resembled trichomes of Sphaerotilus species. Close examination of these organisms revealed, however, that they were Gram-positive while S. natans is Gram-negative. Nigrosin slides and wet mounts examined with a phase microscope were useful in differentiating dead cells in chains of Bacillus species from empty sections of sheath in S. natans.

Figure 4 is a photomicrograph of S. natans negatively stained with nigrosin. The long filaments closely resemble Sphaerotilus species found in natural slime-masses. Although empty sections of sheath appear in abundance, cultures have been examined in which the sheath was virtually obscure and were consequently more difficult to identify. Figure 5 demonstrates the flocculent growth of a pure culture of S. natans strain BC-IV. This strain of S. natans produced more sheath material than the other strains and had to be

Table 1. Plate counts* of bacteria growing in Berry Creek. Counts expressed as number of organisms per milliliter of blended Sphaerotilus slime or creek water.

Date	In slime of Sphaerotilus natans	dry wt of slime mass in mg/ml	In creek water	
			enriched	unenriched
Winter 2/15/65	2×10^6	5.3	1.5×10^3	2.6×10^1
Spring 4/13/65	5×10^7	4.3	4.0×10^4	8.0×10^2
Summer 7/15/65	7×10^7	4.9	6.0×10^4	2.0×10^3
Fall 10/15/65	3×10^7	4.7	7.0×10^4	5.0×10^2

*Plate counts were made according to the procedure outlined in Standard methods for the Analysis of Water and Waste Water, pp. 592-593.

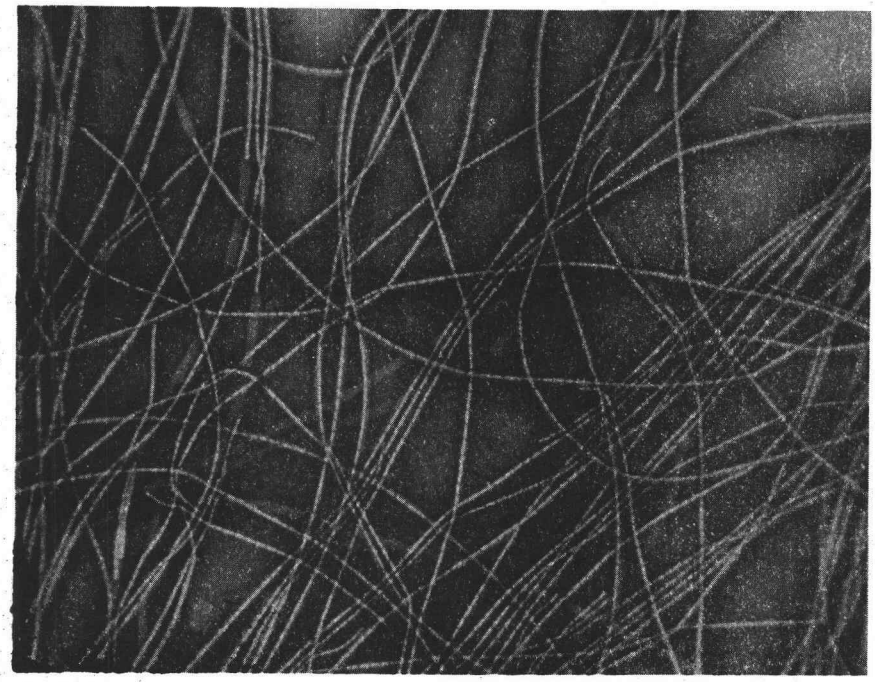


Figure 4. Photomicrograph of filaments of S. natans strain ATCC 15291 x 700.

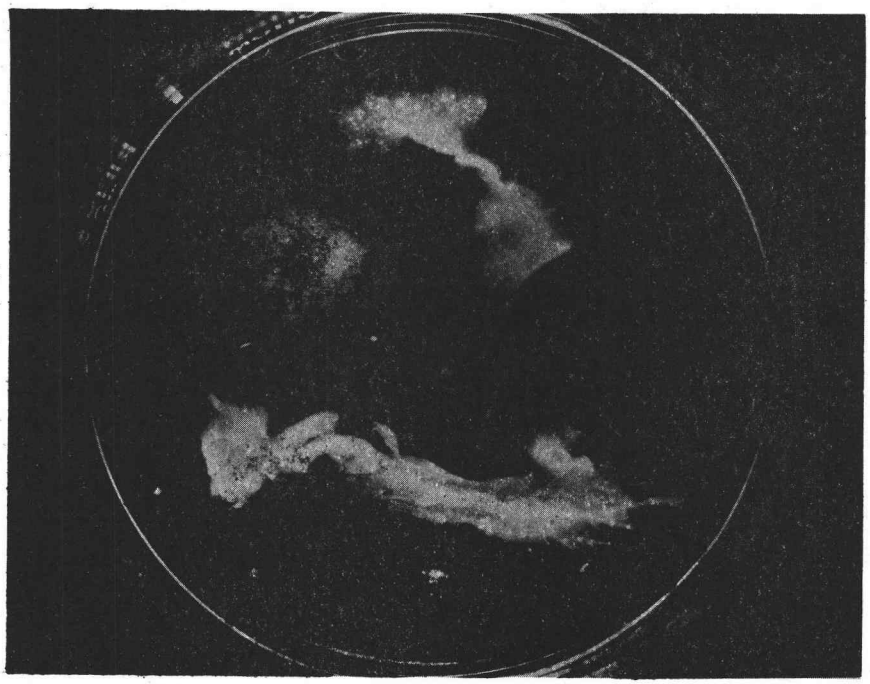


Figure 5. Photograph of flocs from a pure culture of S. natans strain BC-IV x 2.

blended in certain experiments where homogenous suspensions were required. Figure 6 is an electron micrograph of S. natans strain ATCC 15291 showing the cells and sheath in more detail.

The manganese agar of Stokes (1954) was very effective in distinguishing strains of S. natans from S. discophorus due to the deposit of a dark brown precipitate of manganic oxide in the agar around colonies of the latter organism. During the period of isolation of cultures from Berry Creek, one culture was found which caused manganese to be oxidized.

Growth Characteristics of S. natans

An investigation of factors which influence the growth of S. natans was conducted with S. natans strain BC-IV so that relatively optimal conditions for growth could be maintained during metabolic studies. Other investigators have reported that optimum growth conditions vary considerably with different strains of S. natans. The data given in Table 2 indicate the effect of temperature on growth of S. natans strain BC-IV. The Berry Creek strain of S. natans grew in pure culture at temperatures from 3 to 35 C. In general other workers agree with this range for their strains, with the exception that no growth was observed below 5 C. Since excellent growth was observed in Berry Creek during the winter of 1964-65 when temperatures were often as low as 2 C, an

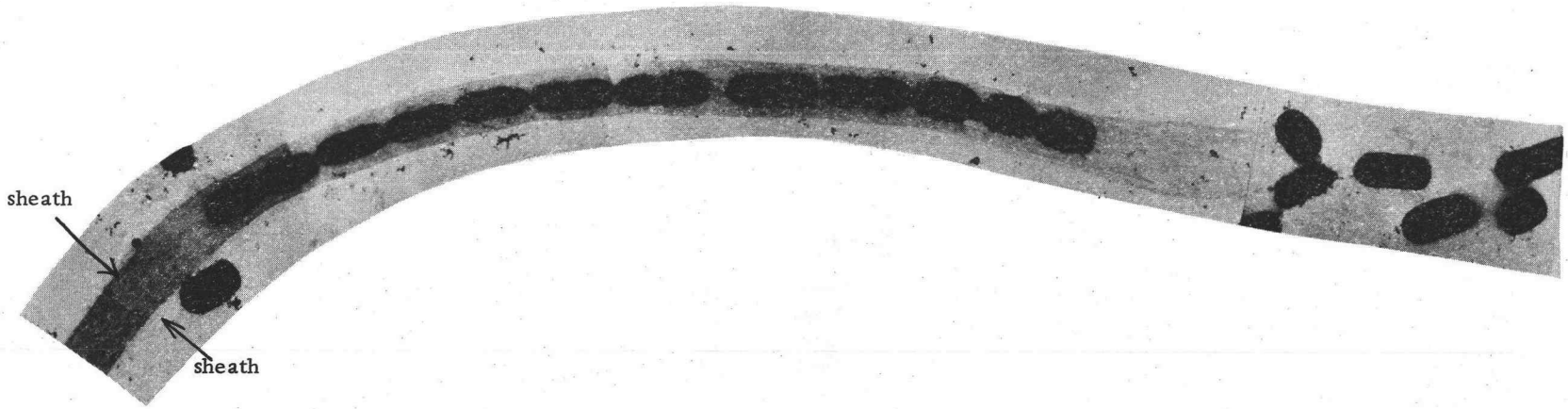


Figure 6. Electron micrograph of S. natans strain ATCC 15291 x 4500.

experiment was designed to attempt to culture this strain at low temperature. A modification of Stokes' medium which contained 1 g of glucose and 1 g of peptone per liter together with 0.1 percent yeast extract was added in 50 ml quantities to 250 ml micro-Fernbach flasks. The flasks were inoculated with 0.5 ml suspensions of S. natans strain BC-IV and shaken in a cold room at a constant temperature of 3 C for thirty days. Although no growth was observed in the first 20 days, good growth occurred by the end of 30 days, and 21 mg of dried material was obtained from 50 ml of culture suspension. In view of these results, it would appear that the ability of S. natans to grow at low temperatures is a selective or adaptive process since little growth occurred during the first 21 days followed by more rapid growth the next 7 days. The aeration by shaking may have also affected the growth of the S. natans since other investigators who obtained no growth at 3 C used unshaken cultures. Microscopic examination of the culture revealed a curious growth characteristic in which two or three cells in a sheath were followed by an empty section of sheath of equivalent length, with this pattern repeating itself throughout the length of the filament. These regular omissions of cells were observed in all of the sheaths examined and cannot be explained at the present time. The optimum growth temperature appeared to be in the range of 25-30 C.

Table 2. Growth of S. natans strain BC-IV at various temperatures expressed as mg dry wt/50 ml of Stokes' medium (all cultures unshaken except at 3 C)

Temp. C	3 days	7 days	14 days	21 days	30 days
3	-	-	-	-	21.0
5	-	-	-	15.2	21.3
10	-	-	14.9	19.7	25.9
15	-	16.8	19.3	21.1	22.8
20	15.4	20.9	22.3	19.5	*
25	18.5	19.7	17.6	*	*
30	21.9	*	*	*	*
35	11.9	*	*	*	*

* Disintegration of cells

The effect of pH on S. natans strain BC-IV was measured using respiration rate as an index of cell activity. The oxygen consumption was measured in a Gilson Differential Respirometer at 20 C. Phosphate buffers ranging from pH 5.7 to 8.0 were used. At first, 0.1 M buffer was used until it was discovered that this concentration of buffer actually inhibited respiration. A 0.01 M buffer was found to have no inhibitory effect on respiration. The results as shown in Table 3 indicated that maximum rate of respiration was obtained at a pH of approximately 7.5. No oxygen consumption was detected at pH 6.0 or lower. The pH in the experimental sections of Berry Creek varied between 7.1 and 7.5,

although slightly higher values were occasionally recorded in the enriched sections. The near optimum pH of the stream was undoubtedly a contributing factor to the luxuriant growths of Sphaerotilus which occurred with a very low-enrichment level (0.5 PPM Nitrogen as urea and 4.0 PPM carbon as sucrose).

Table 3. Effect of pH on respiration of S. natans strain BC-IV. Rates expressed as $\mu l\ O_2/hr/mg$ dry wt.

Conditions	pH					
	5.7	6.0	6.5	7.0	7.5	8.0
Buffer alone (endogenous)	0	0	2.3	9.1	11.1	9.7
Buffer plus 9.5 ml of 1 percent glucose	0	0	6.5	14.3	17.4	15.4

Experiments with different carbon and nitrogen sources revealed that S. natans strain BC-IV can grow well in a variety of media. A subjective evaluation of the growth of the organism in media containing different carbon and nitrogen sources is presented in Table 4. Concentrations of 1 g per liter of carbohydrate nitrogen equivalent to 1 g per liter of peptone (assume peptone to be 16% nitrogen) were used in each medium together with the required minerals. After one week of incubation at 20 C, the flasks were inspected visually and the growth given a rating of fair to excellent.

Table 4. Effects of various carbon and nitrogen sources on the growth of S. natans strain BC-IV. (Inorganic nitrogen sources supplemented with 2.5 μg of vitamin B_{12} per liter)

Carbon Source	Nitrogen Source	Evaluation of Growth
glucose	peptone	++++
glucose	urea	++
glucose	$CaNO_3$	++
glucose	$NH_4(NO_3)$	++
glucose	KNO_3	++
glucose	casein hydrolysate	+++
sucrose	peptone	+++
sucrose	urea	+++
sucrose	$CaNO_3$	++
sucrose	casein hydrolysate	+++
sucrose	$NH_4(NO_3)$	++
sucrose	KNO_3	++
lactose	peptone	+++
fructose	peptone	+++
fructose	urea	++
fructose	KNO_3	++
meat extract	meat extract	++

++++ excellent
+++ good
++ fair

It was necessary to add the equivalent of 2.5μg of vitamin B_{12} per liter to cultures containing an inorganic nitrogen source since this cofactor seems to be required in the synthesis of some of the sulfur amino acids (Mulder and Van Veen,(1963). When vitamin B_{12} was omitted from media containing an inorganic nitrogen source, the growth was greatly reduced and in some cases no growth occurred. The Berry Creek strain of S. natans grew better when supplied with preformed amino acid sources such as peptone or casein hydrolysate. Glucose was the best carbon source of the carbohydrates tested. The meat extract did not produce as much growth as some of the other carbon sources although it did furnish an ample supply of preformed amino acids. The probable explanation for this occurrence is that meat extract was a poorer energy source for the cells than the carbohydrates used.

Results of experiments with S. natans strain BC-IV grown in different concentrations of peptone and glucose revealed that aeration by shaking enhanced growth, and that the organisms could utilize relatively high concentrations of organic substrates with subsequent production of good yields of cells and sheath material (Table 5). At the higher concentrations, microscopic examination revealed that the sheath was much wider (4-8μg) than that formed at lower concentrations (2-3μg). In some sheaths, two and three filaments of cells were observed growing side by side. This

Table 5. Growth of S. natans strain BC-IV with different concentrations of peptone and glucose (supplemented with basal minerals).

Glucose (g/liter)	Peptone (g/liter)	Yield of Cells (mg dry weight/50 ml at 20 C)	
		Shaken	Not shaken
0.25	0.25	4.3	2.7
0.05	0.5	10.4	5.0
1.0	1.0	23.5	17.6
2.0	2.0	51.6	44.3
4.0	4.0	70.5	61.8
8.0	8.0	187.6	121.9
16.0	16.0	91.5	51.6

condition probably occurs during a period of very rapid growth and may indicate that the sheath material is less firm during this period. There were also greater numbers of free conidia and less sheath material in the more concentrated media. Less attachment was observed at concentrations of glucose and peptone above 4 g per liter, indicating that the attachment mechanism could be affected by nutritional conditions.

Experiments were carried out in the continuous culture apparatus to determine the effect of enrichment and current velocity on growth of S. natans. It was found that autoclaved water from Berry Creek sustained a satisfactory state of growth at 10 C when enriched with 100 PPM carbon as sucrose and 50 ppm nitrogen as urea, if the medium was exchanged at a rate of 1 liter per day (Table 6). Below this level of enrichment, growth was slow and an accumulation of biomass could not be observed visually. When the culture flask was not stirred, the filaments grew as a film at the surface of the medium. A slow stirring action (60 to 100 rpm) resulted in the formation of relatively long plumes (2 to 5 cm) loosely attached to the sides and bottom of the culture vessel. As stirring was increased to 200 to 300 rpm, the plumes became shorter (1 to 2 cm) and more firmly attached; and at a stirring rate of approximately 400 rpm, growth was restricted to a densely packed layer coating the vessel walls. Maximum biomass accumulation occurred at

stirring rates between 150 and 200 rpm. The results obtained with the continuous culture device agree closely with growth of S. natans observed in nature in Berry Creek (Reese 1966). The ability of pure cultures of the organism to form typical growth with fairly low concentrations of sucrose and urea in autoclaved Berry Creek water seems to indicate that these carbon and nitrogen sources were used directly by S. natans growing in Berry Creek and did not require conversion to other metabolic intermediates by other bacteria before their utilization by S. natans.

Table 6. Growth of S. natans strain BC-IV in a continuous culture apparatus with different concentrations of sucrose and urea. (Stirring rate = 200-300 rpm)

Sucrose ppm	Urea ppm	Yield from effluent mg/day (Ave)
10	5	2
100	50	20
200	100	45

Primary Routes of Glucose Catabolism in Sphaerotilus natans

Because of the lack of agreement among various investigators regarding carbohydrate metabolism of various strains of S. natans and the apparent existence of strain variability in this organism,

three strains of S. natans from different sources were used in primary pathway studies.

Most researchers agree that S. natans is a highly oxidative organism and produces little or no fermentation products. One strain of S. natans has been found which produces lactic acid as an end product (Ruchoft and Kachmar, 1941); however, this organism cannot be called a true facultative anaerobe since it required oxygen even when producing lactic acid. It was, therefore, of interest to determine by means of the radiorespirometric method of Wang et al. (1958), which primary pathways of glucose catabolism were functioning in the strains tested.

Fate of Metabolic ^{14}C from the Dissimilation of Specifically Labelled Glucose

Figure 7 shows the radiorespirometric patterns for the utilization of glucose by S. natans strain BC-IV. The data are presented in Table 7.

The radiorespirometric patterns for glucose are similar to patterns obtained by Stern, Wang and Gilmour (1960) for an organism with a major ED pathway and a very minor PP pathway in conjunction with the secondary TCA cycle.

When the ED pathway is the sole primary pathway for glucose catabolism functioning in an organism and the secondary TCA cycle

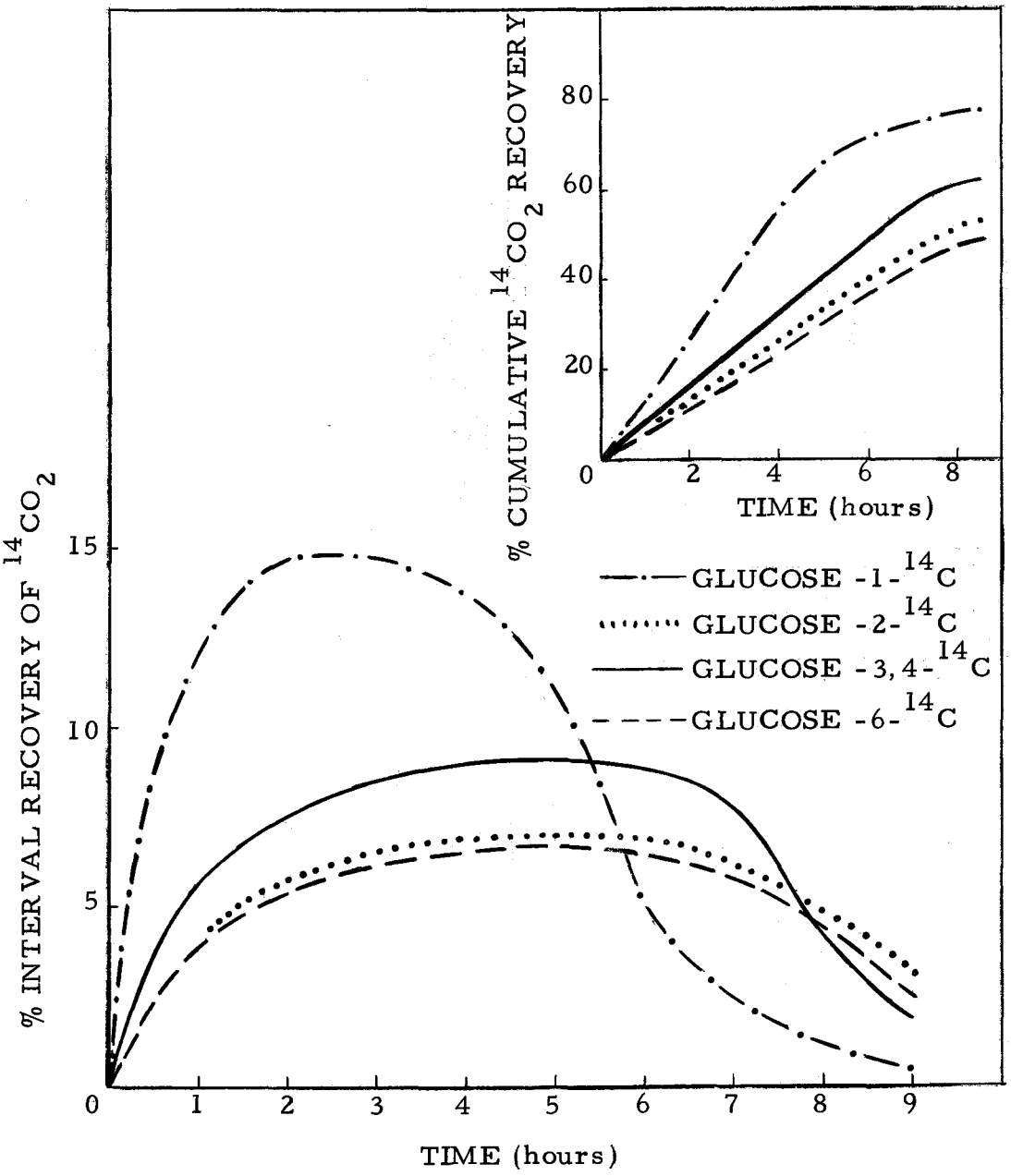


Figure 7. Radiorespirometric patterns for glucose utilization by S. natans strain BC-IV.

is also operative, a definite pattern of $^{14}CO_2$ evolution may be expected. Pseudomonas saccharophila is such an organism and has produced the following $^{14}CO_2$ recoveries in radiorespirometric experiments: C-1 = C-4 > C-2 = C-5 > C-3 = C-6. This pattern is obtained as the result of the conversion of glucose → glucose-6-phosphate → 6-phosphogluconic acid → 2-keto-3-deoxy-6-phosphogluconate → pyruvic acid → 3-phosphoglyceraldehyde.

Table 7. Isotope recoveries from the catabolism of labelled glucose by S. natans strain BC-IV.

Substrate	Isotope recoveries			
	CO_2*	Cells	Medium	Total
	%	%	%	%
Glucose-1-^{14}C	78	8	6	92
Glucose-2-^{14}C	55	19	17	91
Glucose-3,4-^{14}C	63	19	9	91
Glucose-6-^{14}C	49	26	16	91

*Calculated value of CO_2 recovery from C-4 = 77 percent.

The 3-phosphoglyceraldehyde is subsequently converted to a second molecule of pyruvic acid so that the overall result is glucose → 2 pyruvic acid with the carboxyl carbon of the initial pyruvate derived from C-1 of glucose and the carboxyl carbon of the second pyruvate derived from C-4 of glucose. The decarboxylation of the two pyruvic acid molecules then results in the formation of 2 CO_2 + 2

acetyl CoA with the acetyl of Co-A being further degraded to CO_2 in the TCA cycle. The C-2 and C-5 become the carboxyl carbons of acetyl CoA and are evolved as $^{14}CO_2$ much faster than the C-3 and C-6 which are either conserved as cellular material or evolved in $^{14}CO_2$ in subsequent reactions in the TCA cycle. In summation, the C-1 and C-4 from pyruvic acid are evolved as CO_2 first followed by C-2 and C-5, while C-3 and C-6 are conserved and yield the lowest evolution of $^{14}CO_2$.

When radiorespirometric data of the evolution of $^{14}CO_2$ from specifically labelled glucose-3-^{14}C and glucose-3,4-^{14}C is available, the relative amount of $^{14}CO_2$ evolved from C-4 can be calculated by subtracting the $^{14}CO_2$ evolved from C-3 from twice the amount of $^{14}CO_2$ evolved from C-3,4. Unfortunately glucose-3-^{14}C was not available so the evolution of $^{14}CO_2$ from C-4 was calculated by the theoretical formula of Wang et al. (1958). This formula has been found to be reasonably accurate for calculation of the relative CO_2 evolution from C-4 in organisms which possess little or no EMP pathway. Since all the strains of S. natans tested produced a great deal more $^{14}CO_2$ from C-1 than from C-3,4, it was strongly suggestive that the EMP pathway was insignificant in this organism. Data showing the rates of ^{14}C incorporation from specifically labelled glucose into poly-β-hydroxybutyric acid presented in the following section of this thesis lend further support to this hypothesis.

A comparison of the recovery data for $^{14}CO_2$ evolved from specifically labelled glucose by strain BC-IV suggests that the ED pathway is the predominant pathway operating in this strain. $^{14}CO_2$ evolution from C-1 is approximately equal to the value calculated for C-4 and these values are much greater than C-2 which is in turn greater than values for C-6.

The interval recovery patterns for $^{14}CO_2$ from C-2, C-6 and to a lesser extent C-3, 4 are somewhat different from typical radiorespirometric patterns since they do not taper off at approximately the same time as C-1. This phenomenon also resulted in higher total $^{14}CO_2$ recoveries from these carbons and lower total amounts recovered in the cells.

It appears that these carbons were being conserved or cycled through an intermediary pathway in contrast to C-1 which was fairly rapidly decarboxylated and evolved as $^{14}CO_2$.

Although participation of the pentose pathway results in some cycling of carbon, examination of the radiorespirometric patterns from an organism known to possess an ED and contributing PP pathway revealed that the extended time of CO_2 evolution from other labelled carbons over that of C-1 is not typical. A possible explanation for these results is the effect of a higher rate of endogenous metabolism on substrate catabolism.

Endogenous metabolism is affected by the age of bacterial

cells and also by the amount of storage product present. The periods of time required for the three strains of S. natans to reach upper logarithmic growth phase ranged from 72 hours for strain BC-IV to 24 hours for the more rapidly growing strain ATCC 15291. Consequently even though all of the strains were harvested for radiorespirometric experiments at the same stage of growth and development, there was a considerable difference in their ages. Although all three strains were shown to deposit the storage product, poly-β-hydroxybutyric acid, strains BC-IV and ATCC 15291 produced 25 to 33 percent dry weight of polymer while strain ATCC 13338 accumulated only 5 to 8 percent of polymer with ASMTM medium. The role of PHB in carbohydrate metabolism will be discussed further in a later section but it is reasonable to suggest at this point two possible ways in which PHB could affect radiorespirometric results:

1. The amount of PHB a given organism deposits in relation to the amount deposited by another organism could affect the quantities of C-2, C-6 and to a lesser extent C-3, 4 which are conserved in their cells.
2. Radiorespirometric patterns could be affected by the rate of polymer synthesis or catabolism in an organism during a radiorespirometric experiment.

Rouf and Stokes (1962) showed with one strain of S. natans that polymer began accumulating in cells after 24 hours of incubation and increased to a maximum on the third day, after which the polymer content declined rapidly. One can easily envision a radiorespirometric experiment with a three day old culture of S. natans BC-IV in which the cells were depositing polymer during the first few hours and then degrading polymer toward the end of the run as the substrate was used up. The situation just described would result in the $^{14}CO_2$ patterns and recovery data obtained for strain BC-IV. Although the results vary somewhat from typical radiorespirometric data, the participation of the ED and pentose phosphate pathways in conjunction with the TCA cycle is indicated for this organism. The extended $^{14}CO_2$ evolution from C-2, C-3,4 and C-6 did not occur with strains ATCC 15291 and 13338 because polymer degradation probably did not occur in these strains as rapidly as that indicated for strain BC-IV.

The radiorespirometric patterns for glucose utilization by S. natans strain ATCC 13338 shown in Figure 8 suggest that this organism was dissimilating glucose via essentially the same pathways which were functioning in strain BC-IV. The recovery data in Table 8 indicate that the decarboxylation of C-1 was even more pronounced in this organism than in strain BC-IV. The calculated value for C-4 recovery in $^{14}CO_2$ is considerably lower than C-1.

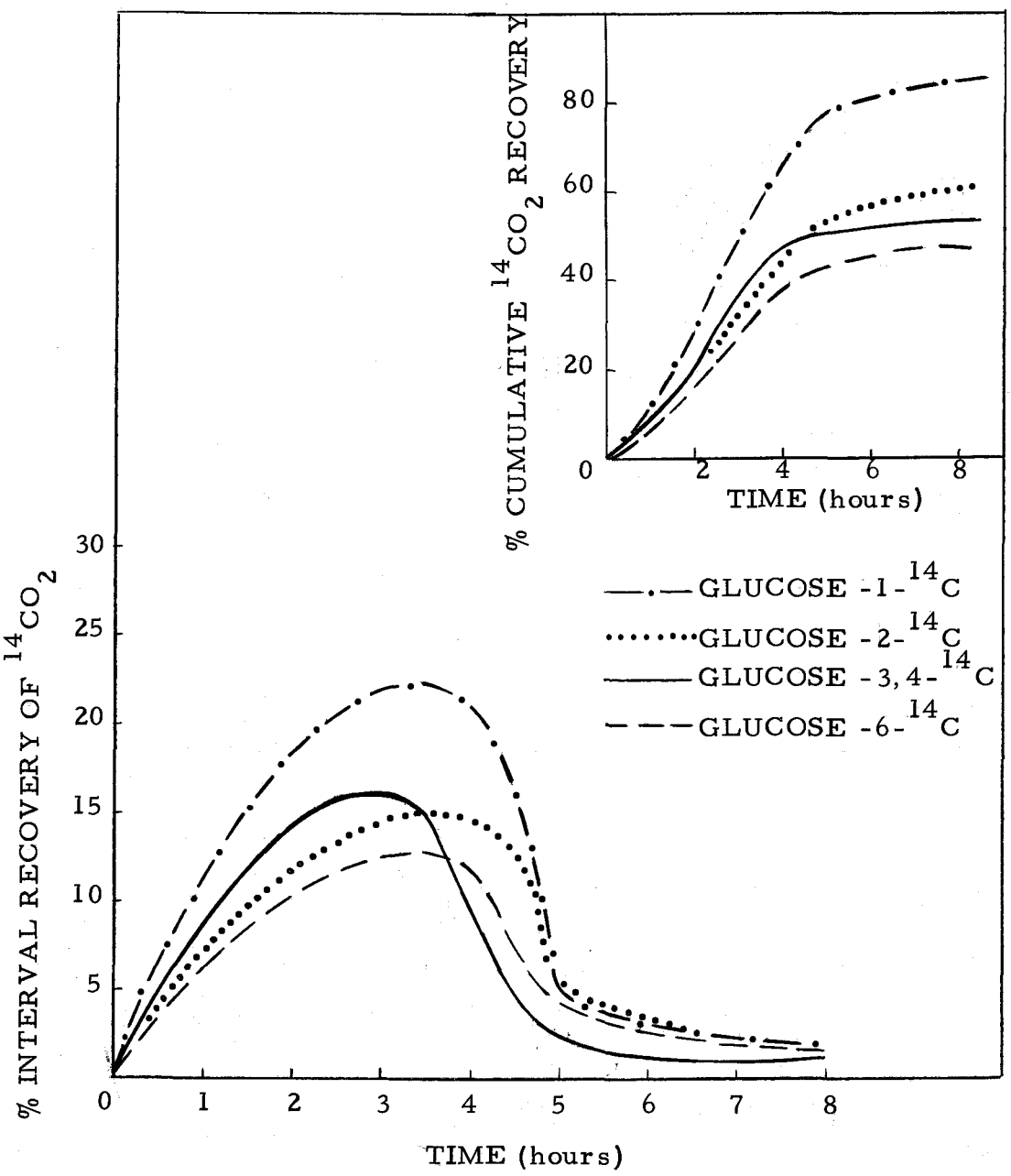


Figure 8. Radiorespirometric patterns for glucose utilization by S. natans strain 13338.

It would appear from this evidence that a second primary pathway is functioning along with the ED pathway in strain ATCC 13338. It is obvious from the much greater yield of metabolic $^{14}CO_2$ from C-1 in relation to that obtained from C-3,4 (85% to 53%) that glycolysis is probably not contributing significantly to glucose catabolism in this strain. What primary pathway for glucose catabolism will combine with the ED pathway with the results just described? The pentose phosphate pathway meets all the requirements for such a pathway and has been shown by Wang et al. (1958) to result in radiorespriometric patterns and $^{14}CO_2$ recoveries from Pseudomonas reptilivora which are virtually identical with those found for S. natans strain ATCC 13338.

Table 8. Isotope recoveries from the catabolism of labelled glucose by S. natans strain ATCC 13338.

Substrate	Isotope recoveries			
	CO_2*	Cells	Medium	Total
	%	%	%	%
Glucose-1-^{14}C	85	11	5	101
Glucose-2-^{14}C	61	30	11	102
Glucose-3,4-^{14}C	53	36	9	98
Glucose-6-^{14}C	51	42	8	101

*Calculated value of CO_2 recovery from C-4 = 55 percent.

Figure 9 and Table 9 contain the results of radiorespirometric experiments with S. natans strain ATCC 15291. The radiorespirometric patterns appear to be the result of a system for glucose catabolism which is quite similar to that functioning in the two previous strains. It should be noted at this point that although strain ATCC 15291 apparently possesses a predominately oxidative phosphogluconate pathway, the CO_2 evolution rates were somewhat lower from C-2, C-3,4 and C-6 than in the two previous strains. The rates of incorporation of these carbons as cellular material was also higher than corresponding rates in strains BC-IV and ATCC 13338.

Table 9. Isotope recoveries from catabolism of labelled glucose by S. natans strain ATCC 15291.

Substrate	Isotope recoveries			
	CO_2*	Cells	Medium	Total
	%	%	%	%
Glucose-1-^{14}C	84	11	6	101
Glucose-2-^{14}C	44	40	13	97
Glucose-3,4-^{14}C	51	44	8	103
Glucose-6-^{14}C	32	62	8	102

*Calculated value of CO_2 recovery from C-4 = 70 percent.

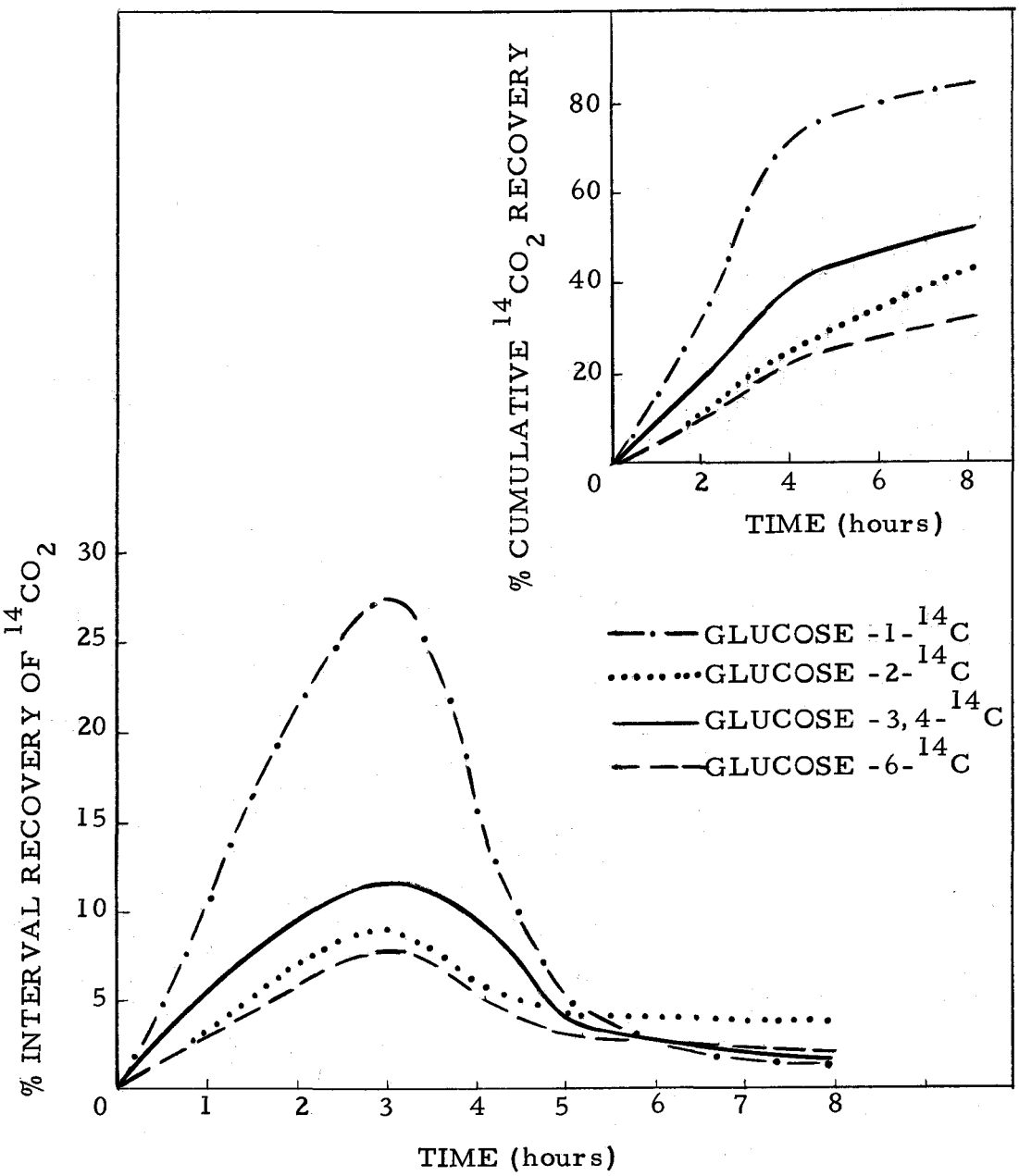


Figure 9. Radiorespirometric patterns for glucose utilization by S. natans strain ATCC 15291.

The relatively high incorporation rates for the ^{14}C label into cells of strain ATCC 15291 over the other two strains tested suggested the possibility that ^{14}C-labelled poly-β-hydroxybutyric acid accumulated in the cells in significant amounts in addition to the normal cell material. To investigate this possibility and also to further clarify the relative participation of the various pathways in cellular metabolism and polymer synthesis, a macroscale radiorespirometer experiment was performed, with strain ATCC 15291. The results of this experiment are shown in Figure 10 and Table 10. A large quantity of 28 hour cells were washed in carbohydrate-free medium and resuspended in synthetic medium containing either glucose-1-^{14}C or glucose-6-^{14}C. The cells were incubated for 8 hours and the hourly metabolic $^{14}CO_2$ evolution was measured. The patterns and recovery data obtained were virtually identical for those obtained for the same two labels in the previously discussed micro-radiorespirometric experiment. The most interesting piece of information revealed by this experiment is shown in the isotope recoveries in polymer given in Table 10. Twenty-eight percent of the total input ^{14}C from C-6 was recovered in polymer while only 0.2 percent of the C-1 was recovered in polymer. This is a 140 fold difference and is very strong evidence that glycolysis (EMP pathway) was not functioning in this organism. If the EMP pathway was the predominant pathway functioning, then the yields of ^{14}C

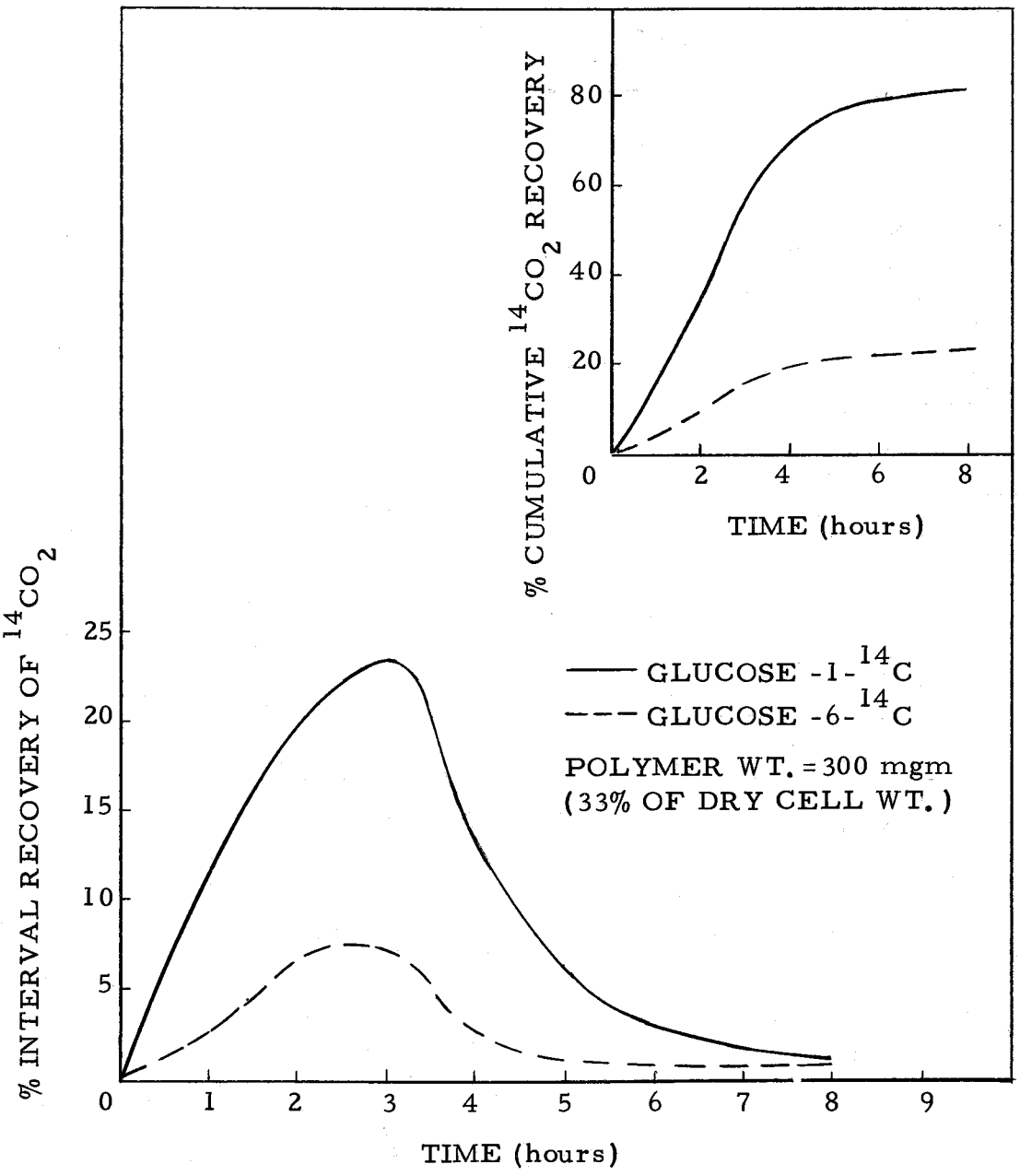


Figure 10. Poly-β-hydroxybutyrate synthesis and $^{14}CO_2$ evolution by S. natans strain ATCC 15291.

label in the polymer from C-1 and C-6 would have been approximately equal.

Table 10. Isotope recoveries during polymer synthesis from specifically labelled glucose by S. natans strain ATCC 15291.

Substrate	Isotope recoveries*				
	CO_2	Cells	Medium	Total	Polymer
	%	%	%	%	%
Glucose-1-^{14}C	81	9	11	101	0.2
Glucose-6-^{14}C	23	68	9	100	28.0

*All recoveries based on total ^{14}C input value.

The purity of the ^{14}C labelled polymer was established by solubility properties, determination of melting points and by comparison of infrared spectra of the polymer with standard spectra. The melting points of purified PHB have been determined by Williamson and Wilkinson (1958) and by Law and Slepecky (1961) to be 160 to 169 C and 173.5 to 175.5 C, respectively. The melting point of PHB from S. natans strain BC-IV was found to be 168 to 173 C, and the melting point of strain ATCC 15291 was 173-176 C. The infrared spectra of PHB from these strains are shown in Figure 11. The two spectra are almost identical and are remarkably similar to IR spectra obtained for PHB from S. natans by Rouf and Stokes (1961). The prominant band at 5.7 μ has shown to be characteristic of this polymer and has been associated with the

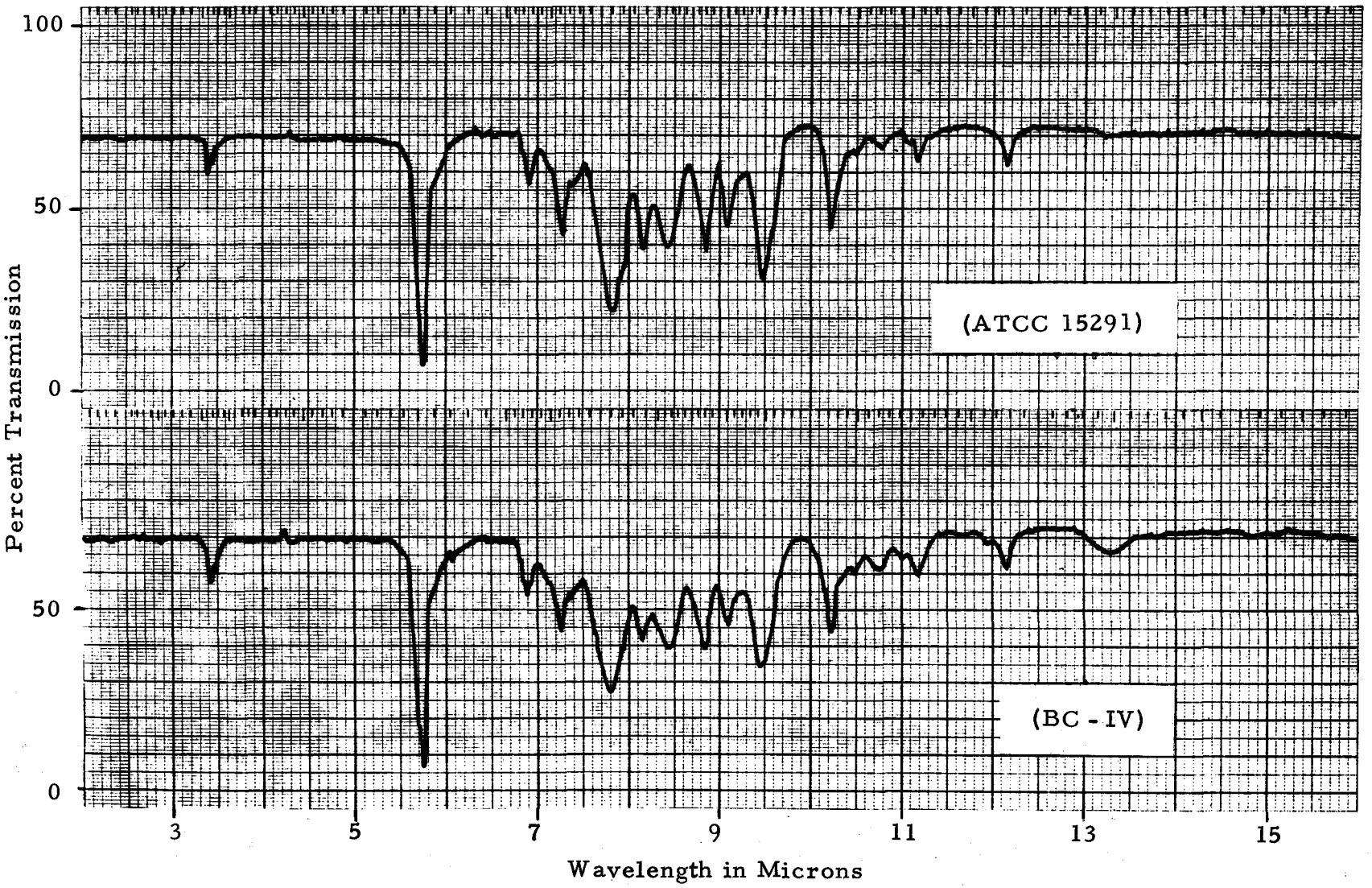


Figure 11. Infrared spectra of purified polymer from strain ATCC 15291 and strain BC-IV.

β-hydroxy group.

Radiorespirometry of a Mixed Population of S. natans

Since slime-masses growing in Berry Creek appeared to be composed largely of cells and sheath material of Sphaerotilus, it was of considerable interest to see if glucose catabolism in Sphaerotilus slime was proceeding via the same pathways previously found for pure cultures of S. natans. Only the rapidly growing tips of the attached slime were used in the experiment and this material was thoroughly washed three times in sterile phosphate buffer to remove as many contaminating bacteria as possible. After the third washing, the cells were collected by centrifugation, resuspended in carbohydrate-free ASMTM medium and blended for 15 seconds in a Waring blender to insure a homogenous suspension. The temperature of the cells was maintained at stream temperature (10 to 15 C) during harvest, washing and the respiration experiment, to make sure the cells were not "shocked" by sudden temperature change. Microscopic examination of the blended slime verified the presence of a population of bacteria which was largely composed of Sphaerotilus cells mostly in short chains of 2 to 6 or more cells. The radiorespirometric data from this experiment are recorded in Figure 12 and Table 11. The radiorespirometric patterns and recovery data closely resemble results obtained for the three pure

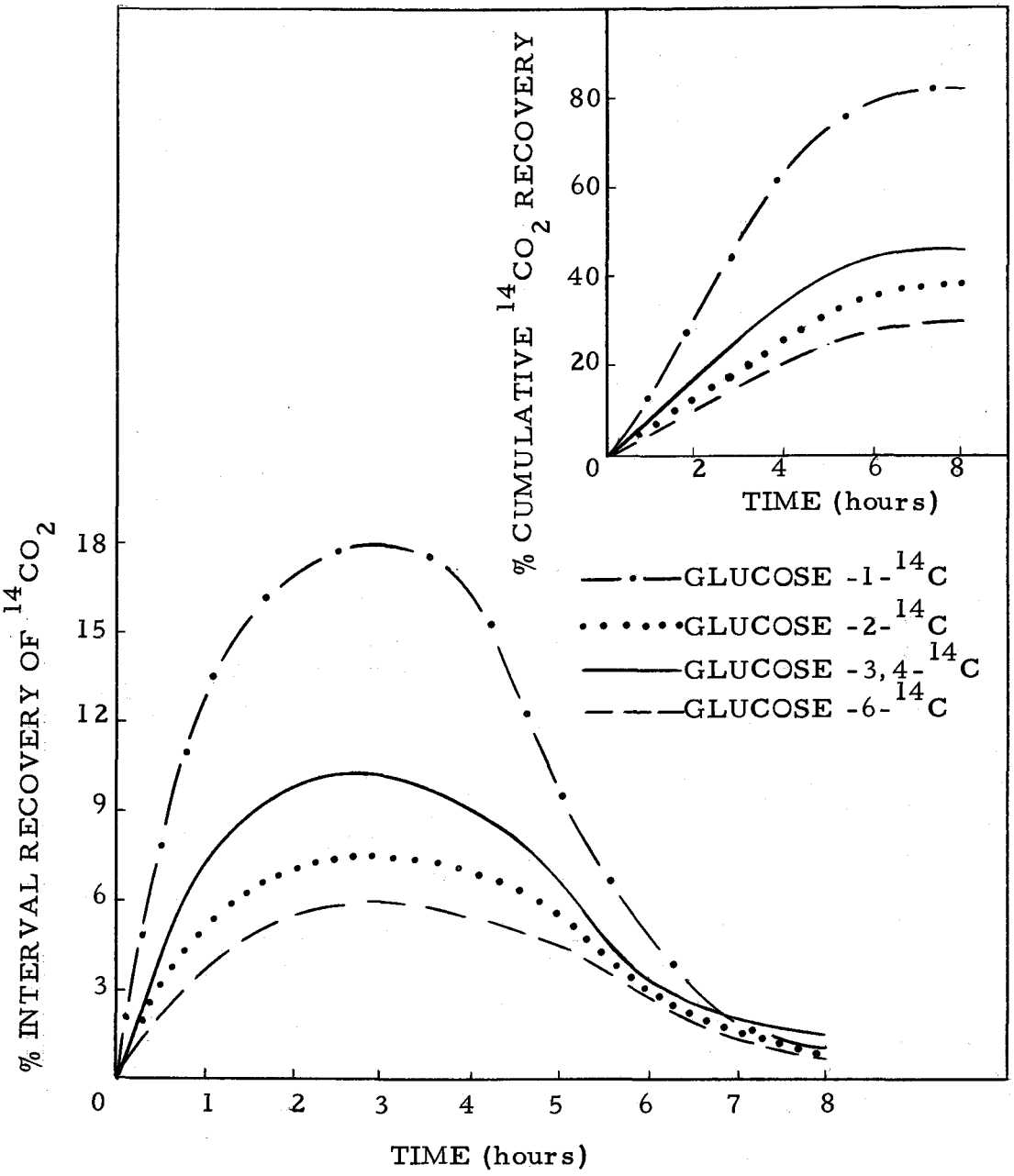


Figure 12. Radiorespirometric patterns for glucose utilization by a mixed population of S. natans.

strains of S. natans. The participation of the ED and pentose pathways in conjunction with the TCA cycle are indicated by the recovery data in Table 11. The ratio of CO_2 evolution from C-1 to C-3,4 is even greater than that found for the pure strains of S. natans from Berry Creek (BC-IV). The recovery data are almost identical to the data obtained for strain ATCC 15291. Although the Sphaerotilus slime contained a number of contaminating bacteria, it was estimated that at least 95 percent of the cells in the washed suspension were Sphaerotilus cells. Since most of the substrate was exhausted in the first four hours of the experiment and the temperature was kept at 15 C, it is very unlikely that the S. natans was overgrown by contaminating bacteria. This was substantiated by microscopic examination of the cells at the end of the experiment. The great majority of cells were still enclosed in a sheath and recognizable as Sphaerotilus species.

Table 11. Isotope recoveries from the catabolism of labelled glucose by a mixed population of S. natans.

Substrate	Isotope recoveries			
	CO_2*	Cells	Medium	Total
	%	%	%	%
Glucose-1-^{14}C	83	10	6	99
Glucose-2-^{14}C	39	41	15	95
Glucose-3,4-^{14}C	47	40	11	98
Glucose-6-^{14}C	31	60	9	100

*Calculated value of CO_2 recovery from C-4 = 63 percent.

In view of the results obtained, it seems very likely that the pathways found for pure cultures of S. natans are also functioning to a large extent in Sphaerotilus cells in nature.

Radiorespirometry of Specifically Labelled Gluconate and Ribose

Experiments were conducted using specifically labelled gluconate and ribose in order to further clarify the roles of the ED and PP pathways in the metabolism of S. natans. Low CO_2 recoveries indicated a possible permeability barrier to these substrates. Although the strain of S. natans produced fair growth in gluconate and ribose, the level of degradation achieved with these substrates was too low to result in useful radiorespirometric data. $^{14}CO_2$ recoveries from gluconate did, however, suggest that the ED and/or PP pathways were in operation since the C-1 recovery was approximately 3 to 4 times the recoveries from the other carbons.

Endogenous Metabolism of S. natans

The dissimilation of the storage product, poly-β-hydroxybutyric acid has been shown by Sierra and Gibbons (1961) to result in high rates of endogenous respiration in Micrococcus halodenitrificans. The possible effects of PHB storage and dissimilation on radiorespirometric patterns of S. natans has already been discussed. In order to investigate further the amount of endogenous

respiration which resulted from the utilization of PHB as an internal carbon and energy source, uniformly labelled glucose was used as a substrate during PHB synthesis by strain ATCC 15291. The utilization of the uniformly labelled glucose was monitored by following $^{14}CO_2$ evolution in the macro-radiorespirometric apparatus. When the rate of $^{14}CO_2$ evolution indicated that the labelled substrate had been nearly degraded, the cells were harvested and washed by centrifugation, and one-half was sacrificed in order to assay for ^{14}C-labelled PHB while the other half was resuspended in carbon-free synthetic medium for measurement of endogenous metabolism. The results obtained during the incorporation of ^{14}C into polymer are shown in Figure 13 and Table 12.

Table 12. Isotope recoveries during polymer synthesis from uniformly-labelled glucose by S. natans strain ATCC 15291.

Isotope Recoveries*					Specific activity of Polymer
CO_2	Cells	Medium	Total	Polymer	DPM/mg
%	%	%	%	%	
50	35	10	95	10	22, 340

*All recoveries based on total ^{14}C input value.

The curve obtained for $^{14}CO_2$ evolution during polymer synthesis (Figure 13) is a typical respiration curve and indicates by the plateau reached after 6 hours that the major part of the substrate

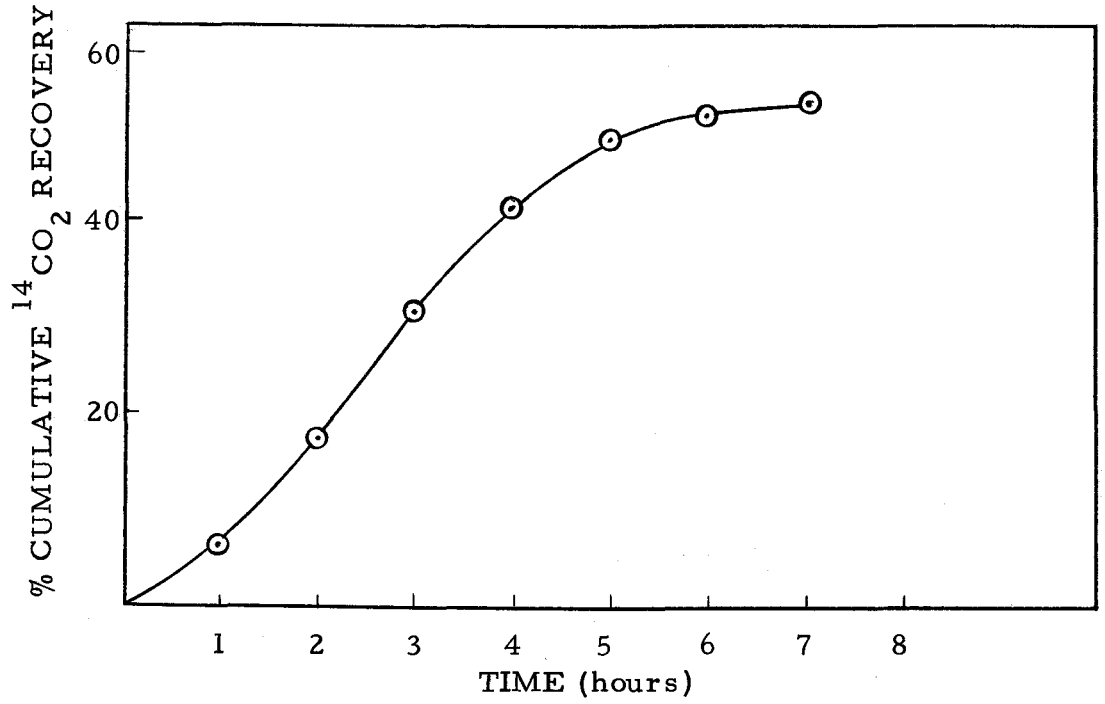


Figure 13. $^{14}CO_2$ evolved during polymer synthesis with glucose-u-^{14}C as substrate.

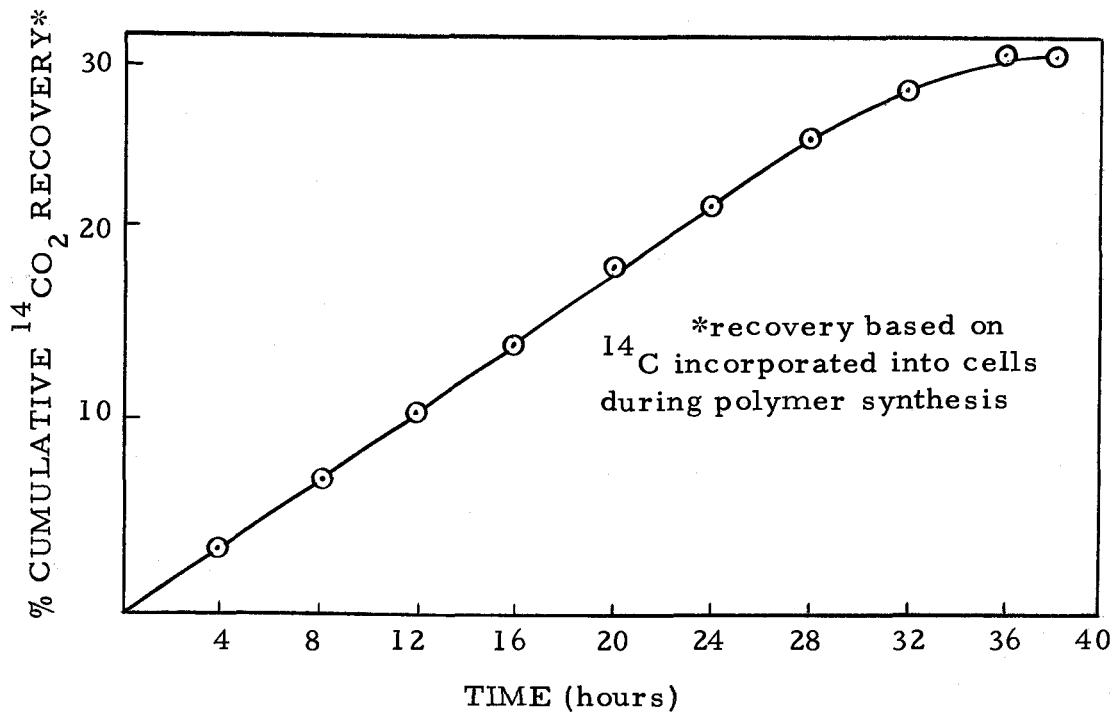


Figure 14. Observed dissimilation of endogenous poly-β-hydroxybutyrate.

had been metabolized. The recovery data in Table 12 also indicate that the ^{14}C from labelled glucose had been largely evolved in $^{14}CO_2$ or incorporated into cellular components. Only 10 percent of the ^{14}C label remained in the medium while 50 percent was evolved as CO_2 and 35 percent incorporated into cellular material.

The curve of observed dissimilation of endogenous polymer (Figure 14) demonstrates the capacity of PHB to serve as a stored source of carbon and energy in S. natans. The cumulative $^{14}CO_2$ recovery remained almost constant for 30 hours before showing a steady rate of decline. This virtual "steady state" of endogenous CO_2 evolution suggests a possible mechanism by which S. natans is able to survive at low nutrient levels in nature for extended periods of time. When Sphaerotilus slime from Berry Creek was examined for the presence of polymer it was found that the material contained 7 to 10 percent dry weight of polymer; thus indicating that S. natans does indeed store PHB under natural conditions.

An interesting aspect of polymer synthesis and degradation is revealed in Tables 12 and 13 by examination of the specific activities for PHB before and after endogenous catabolism. The amount of ^{14}C per mg of polymer was 44 percent lower after polymer catabolism than before polymer catabolism.

It has been suggested by Williamson and Wilkinson (1958) that PHB has an average chain length of about 60 residues. A possible

Table 13. Isotope recoveries during dissimilation of endogenous polymer by S. natans strain ATCC 15291.

Isotope Recoveries*					Specific Activity of Polymer DPM/mg
CO_2	Cells	Medium	Total	Polymer	
%	%	%	%	%	
28	72	2	102	10	12,486

*All recoveries based on ^{14}C incorporated into cells during polymer synthesis.

Table 14. Losses of cellular-^{14}C, polymer-^{14}C and polymer during endogenous dissimilation.

Cell-^{14}C	Polymer-^{14}C	Polymer Dry Weight
% loss	% loss	% loss
28	65	42

explanation for the reduction of ^{14}C content in PHB during catabolism would be that the polymer chain is synthesized by the addition of β-hydroxybutyric acid units one at a time to the ends of the chains. The chains are probably degraded in a similar manner in which single units or small segments are hydrolyzed from the ends of the chains. Since some PHB was synthesized by the growing cell mass prior to addition of glucose-u-^{14}C, the initial sections were unlabelled while the terminal ends of the chains were highly labelled. Subsequent hydrolysis of the ends of the polymer chains would then result in a lowering of specific activity of the polymer as the more highly labelled ends were hydrolyzed. This hypothesis is supported by the data for specific activity of PHB prior to and following endogenous respiration. Additional supporting evidence is shown in Table 14. The utilization of 42 percent of the polymer in endogenous catabolism resulted in the loss of 65 percent of the ^{14}C-label in the polymer. A comparison of the cell-^{14}C loss and polymer dry weight loss data in Table 14 indicates that the polymer was used as an endogenous carbon source in preference to other cellular components such as protein. The polymer decreased by 42 percent while the cellular-^{14}C decreased by only 28 percent. Schlegel, et al. (1961) have shown that PHB can act as a carbon source for protein synthesis in Hydrogenomonas species in the presence of CO_2 and a suitable nitrogen source.

It therefore appears that PHB is a useable storage product and

is especially valuable in the conservation of cellular protein in organisms which store it.

D-(-)-β-Hydroxybutyrate Dehydrogenase Activity in S. natans

D-(-)-β-hydroxybutyrate dehydrogenase has been shown to be a key enzyme in the pathway for PHB catabolism in other genera of bacteria by Hayward et al. (1960) and Merrick and Doudoroff (1961). Although the presence of this enzyme in PHB producing strains of S. natans was most probable, it was considered of interest to confirm its presence and to estimate its activity in strain ATCC 15291 which produced up to 33 percent dry weight of polymer. The presence of this enzyme would indicate that PHB was being converted to acetoacetate and then converted to acetyl CoA. The acetyl CoA could then be subsequently degraded in the TCA cycle. Radiorespirometric data for previous experiments strongly indicated that the catabolism of PHB in S. natans was indeed proceeding via the above route. Figure 15 shows that the enzyme D-(-)-β-hydroxybutyrate dehydrogenase was present and the data in Table 15 indicate the activity of the enzyme was quite high. The cells of S. natans were harvested a few hours after the glucose substrate was determined to be exhausted. This enhanced the probability of obtaining high enzyme activity since the polymer is degraded more rapidly in the absence of an external

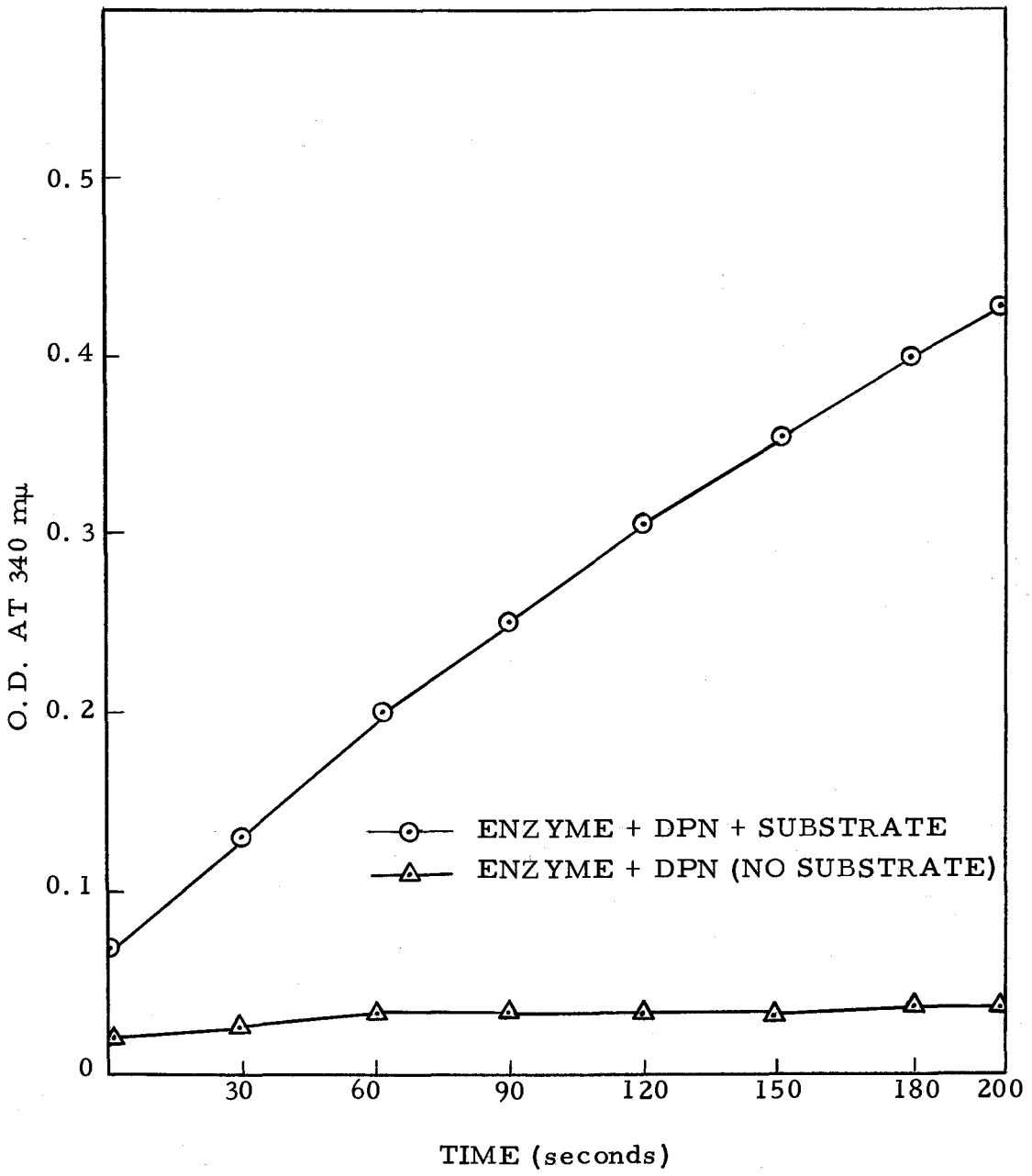


Figure 15. D-(-)-β-hydroxybutyrate dehydrogenase activity of a cell-free preparation from S. natans 15291.

carbon source. The curve obtained for the reaction mixture minus substrate shown in Figure 15 indicates that the reaction was specific for D-(-)-β-hydroxybutyrate and that there was almost no substrate present in the enzyme extract.

Table 15. Assay of D-(-)-β-hydroxybutyric acid dehydrogenase from S. natans strain ATCC 15291.

Protein mg/ml	ml Used	mg Protein Used	Δ O. D. at 340 mμ/min
10.5	0.5	0.503	0.12

Secondary Metabolic Pathways in Sphaerotilus natans

The results previously obtained in radiorespirometric studies and polymer studies with labelled glucose strongly suggested that the TCA and/or glyoxalate cycles were operating in the test organisms. To obtain further evidence for the presence or absence of one or both of these secondary pathways, a series of growth and radiorespirometric experiments were conducted with appropriate substrates.

Utilization of TCA Cycle Intermediates

The ability of an organism to utilize each of the TCA cycle intermediates as a sole carbon source in a synthetic medium is one

indication that the TCA cycle is probably operative.

However, the absence of growth does not necessarily preclude the presence of a functioning TCA cycle since low permeability to certain TCA intermediates such as citrate is quite common. Table 16 contains the results of experiments in which all three strains of S. natans were grown in ASMTM containing 2.5 μg of vitamin B_{12} per liter plus the appropriate substrate. Strain ATCC 15291 showed growth with all of the substrates tested but growth was poor with citrate and pyruvate, probably because of low permeability factors. Strains BC-IV and ATCC 13338 would not grow in citrate even after several transfers thus indicating a permeability difficulty.

Table 16. Growth of S. natans with TCA intermediates.

Substrate	ATCC 15291	Strain BC-IV	Strain ATCC 13338
Pyruvate	+	+	+
Acetate	+	+	+
Citrate	+	-	-
-ketoglutarate	+	+	+
Succinate	+	+	+
Fumarate	+	+	+
Malate	+	+	+
Glucose	+	+	+
Minus Substrate	-	-	-

Radiorespirometry of Specifically Labelled Pyruvate

Since pyruvate supported a fair amount of growth in the test organisms an attempt was made to obtain further evidence for the operation of the TCA cycle through the use of ^{14}C-labelled pyruvate. When specifically labelled pyruvate is oxidized via the TCA cycle, rather definite radiorespirometric patterns are obtained. C-1 of pyruvate is rapidly converted to CO_2 and evolved from the cells while C-2 and C-3 of pyruvate become C-1 and C-2 of acetyl CoA, respectively. The conversion of C-2 to $^{14}CO_2$ proceeds more rapidly than C-3 which requires an extra "turn" of the TCA cycle before being evolved in CO_2. Therefore, the overall recovery pattern for $^{14}CO_2$ should be C-1 > C-2 > C-3, and the amounts of ^{14}C recovered in the cells should be in reverse of the above due to the conservation of C-3 and C-2 in cellular material.

Unfortunately, only low levels of $^{14}CO_2$ were evolved from the specifically labelled pyruvate even when low levels of carrier were used. Although pyruvate was capable of supporting growth when furnished in relatively high concentrations, only low concentrations of substrate were suitable for most radiorespirometric experiments. Therefore most of the pyruvate probably remained outside the cells because of a permeability effect. The low levels of $^{14}CO_2$ evolved did result in recoveries for C-1 as CO_2 which were much greater

than C-2 and C-3. However, this data was not considered to be acceptable since the recoveries were only about 10 percent of the expected values.

Radiorespirometry of Specifically Labelled Acetate

An organism capable of utilizing acetate as a sole carbon source is almost certain to possess a cyclic pathway known as the glyoxylate cycle or glyoxylate bypass in which two C_2 units may be combined to form a C_4 compound. If pyruvate and CO_2 are present, some organisms are capable of synthesizing the C_4 compound, malate, by condensation of these two moeities. The ability of an organism to synthesize C_4 compounds is essential to the organisms' survival unless a constant supply of C_4 compounds is present in its environment. This is because C_4 compounds are important precursors for certain amino acids and other essential cell constituents.

Since the glyoxylate cycle produces no $^{14}CO_2$ directly, the rapid evolution of CO_2 from acetate by an organism which is capable of utilizing acetate as a sole carbon source is indicative of the concurrent operation of both pathways. As previously discussed, the C-1 of acetate may be derived from C-2 of pyruvate and the C-2 of acetate may be derived from the C-3 of pyruvate. Sine the $^{14}CO_2$ recoveries for pyruvate are C-1 > C-2 > C-3, the recoveries of $^{14}CO_2$ of acetate being utilized via the glyoxylate cycle and TCA cycle

or TCA cycle alone should be C-1 > C-2. Examination of Figure 16 shows that S. natans strain ATCC 15291 utilized specifically labelled acetate giving results similar to those predicted for an organism possessing active TCA and glyoxylate cycles. Recovery data for ^{14}C for acetate in Table 17 also agree with the predicted results. The $^{14}CO_2$ recovery from C-1 of acetate was 87 percent whereas the $^{14}CO_2$ recovery from C-2 was only 63 percent. Almost three times as much carbon from C-2 of acetate was recovered in the cells as that from C-1 acetate. These data also agree with recoveries expected from an organism possessing the two cyclic pathways.

Table 17. Isotope recoveries from the catabolism of labelled glutamic acid and acetate by S. natans strain ATCC 15291.

Substrate	Isotope recoveries			
	CO_2	Cells	Medium	Total
	%	%	%	%
Glutamic Acid-1-^{14}C	38	37	23	98
Glutamic Acid-2-^{14}C	30	37	25	92
Glutamic Acid-3,4-^{14}C	12	67	20	99
Glutamic Acid-5-^{14}C	30	39	27	96
Acetate-1-^{14}C	87	11	3	101
Acetate-2-^{14}C	63	31	6	100

Radiorespirometry of Specifically Labelled Glutamatic Acid

The use of specifically labelled glutamic acid as the sole or

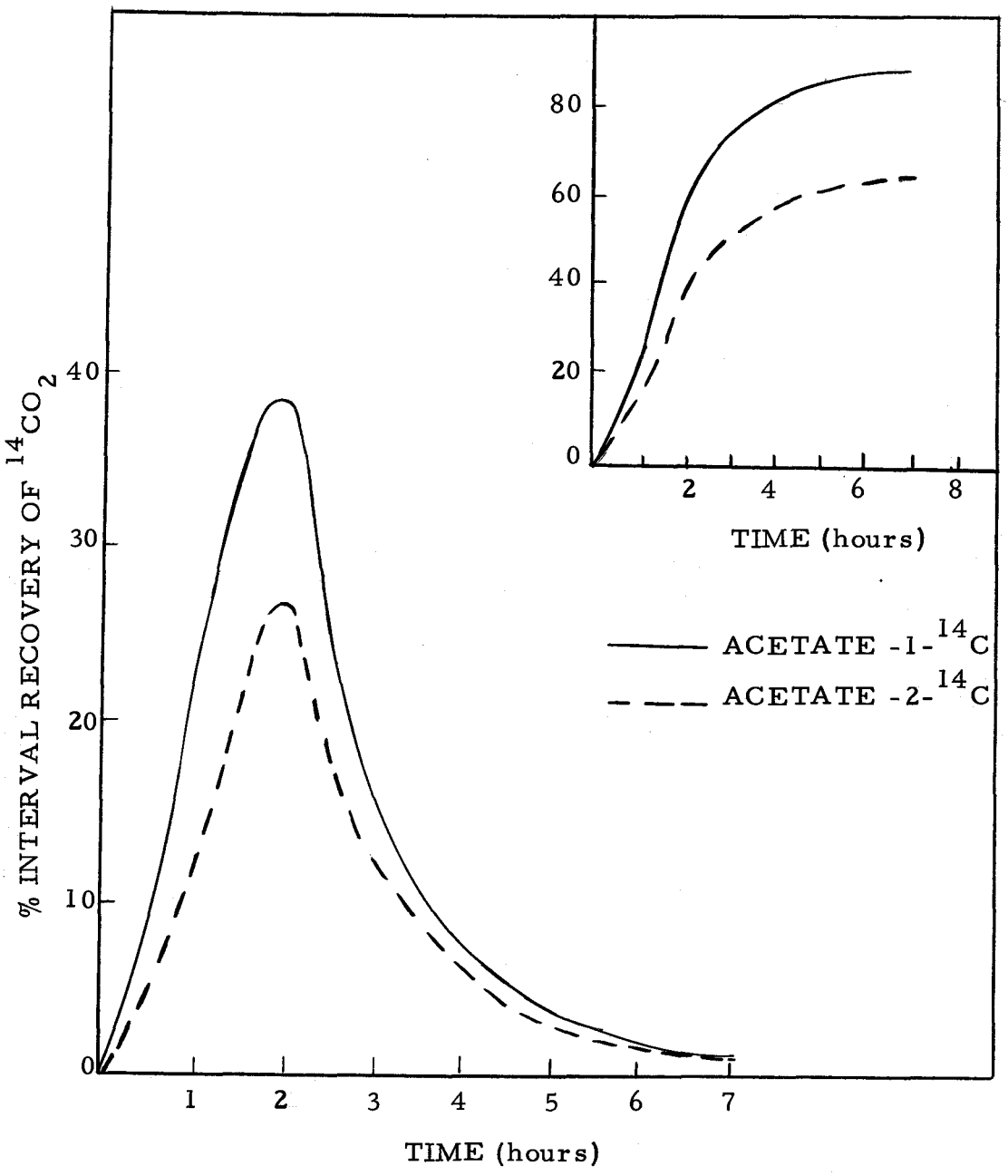


Figure 16. Radiorespirometric patterns for the utilization of acetate by S. natans strain ATCC 15291.

major carbon source for an organism during radiorespirometry has been offered by Wang (1961) as a method for indicating the operation of the TCA cycle. Glutamic acid is taken into the cell with less difficulty than some of the TCA cycle intermediates and once inside the cell it rapidly reaches equilibrium with the TCA cycle intermediate alpha-ketoglutaric acid through the action of glutamic dehydrogenase or by transamination. It is relatively easy to calculate that the rate of $^{14}CO_2$ evolution from specifically labelled alpha-ketoglutarate participating in the TCA cycle should be C-1 > C-2 = C-5 > C-3 = C-4. This pattern would be expected since the C-1 is decarboxylated and evolved first as $^{14}CO_2$ during succinyl CoA formation. The C-2 and C-5 then become terminal carboxyl carbons of the symmetrical succinic acid molecule and the C-3 and C-4 become the C-2 and C-3 of succinic acid. The carboxyl carbons are removed next as $^{14}CO_2$ while the C-3 and C-4 from the original glutamic acid are evolved as $^{14}CO_2$ last and to a lesser extent than the other carbons. The only problem which is readily apparent in the process just described is: What is the origin of the condensing partner acetyl CoA when glutamic acid is the only carbon source? The answer is readily apparent in the case of S. natans since the acetyl CoA may be easily acquired from the degradation of PHB in this organism. Acetyl CoA in organisms lacking this polymer would most probably come from transamination of alanine to pyruvate or glycine to acetate. However, this

would be detrimental to the cell since useful protein building blocks would be used up.

It was found that S. natans grew poorly or not at all in a DL-mixture of glutamic acid. Good growth was obtained however in L-glutamic acid by first growing cultures in the presence of L-glutamic acid and a small amount of glucose and then sub-culturing with L-glutamic acid as the sole carbon source. The apparent inhibition by D-glutamic acid may have possibly been due to the irreversible formation of an enzyme-substrate complex which tied up all of the enzyme necessary for glutamic acid catabolism. No growth was obtained when D-glutamic acid was the sole carbon source. This phenomenon is possibly the reason that Lackey and Wattie (1941) and others found no growth for S. natans with a DL mixture of glutamic acid. Vitamin B_{12} also increased growth when L-glutamic acid was used as a carbon source.

In order to investigate the effect of D-glutamic acid on S. natans strain ATCC 15291 a radiorespirometric experiment was performed using D-glutamic acid -1-^{14}C as the sole carbon source. Only 1 to 2 percent of the C-1 was evolved as $^{14}CO_2$ indicating that the organism was able to catabolize only very small quantities of D-glutamic acid. It is also possible that a slight impurity in the labelled glutamic acid could have resulted in the evolution of a small amount of $^{14}CO_2$. The participation of the D-isomer of glutamic acid was therefore

considered to be insignificant in the metabolism of S. natans strain ATCC 15291.

It was impossible to obtain specifically-labelled L-glutamic acid, therefore, a very small amount of specifically-labelled Dl-glutamic acid was used in radiorespirometric experiments along with a substantial amount of L-glutamic acid carrier. The results are shown in Figure 17 and Table 17. The inability of the organism to utilize D-glutamic acid is demonstrated by the low yield of $^{14}CO_2$ from the various labels in Table 17. The $^{14}CO_2$ recoveries are approximately one-half of those found in other organisms tested, Wang (1961). The relatively high rates of cellular ^{14}C recovery suggests that the specifically labelled D-glutamic acid was being bound to cellular enzymes. The amount of the D-isomer was apparently low enough in relation to quantity of cells, and labelled and unlabelled L-glutamic acid to allow degradation of the L-isomer. In other words, the turnover number of the unbound enzymes for L-glutamic acid was probably sufficiently high to allow normal degradation of the L-isomer. This is supported by the fact that the radiorespirometric patterns in Figure 17 rapidly reach a peak of activity and then rapidly decline to low levels indicating that the substrate (L-glutamic acid) had been nearly exhausted.

A careful examination of the radiorespirometric patterns in Figure 17 and the recovery data in Table 17 shows that the $^{14}CO_2$

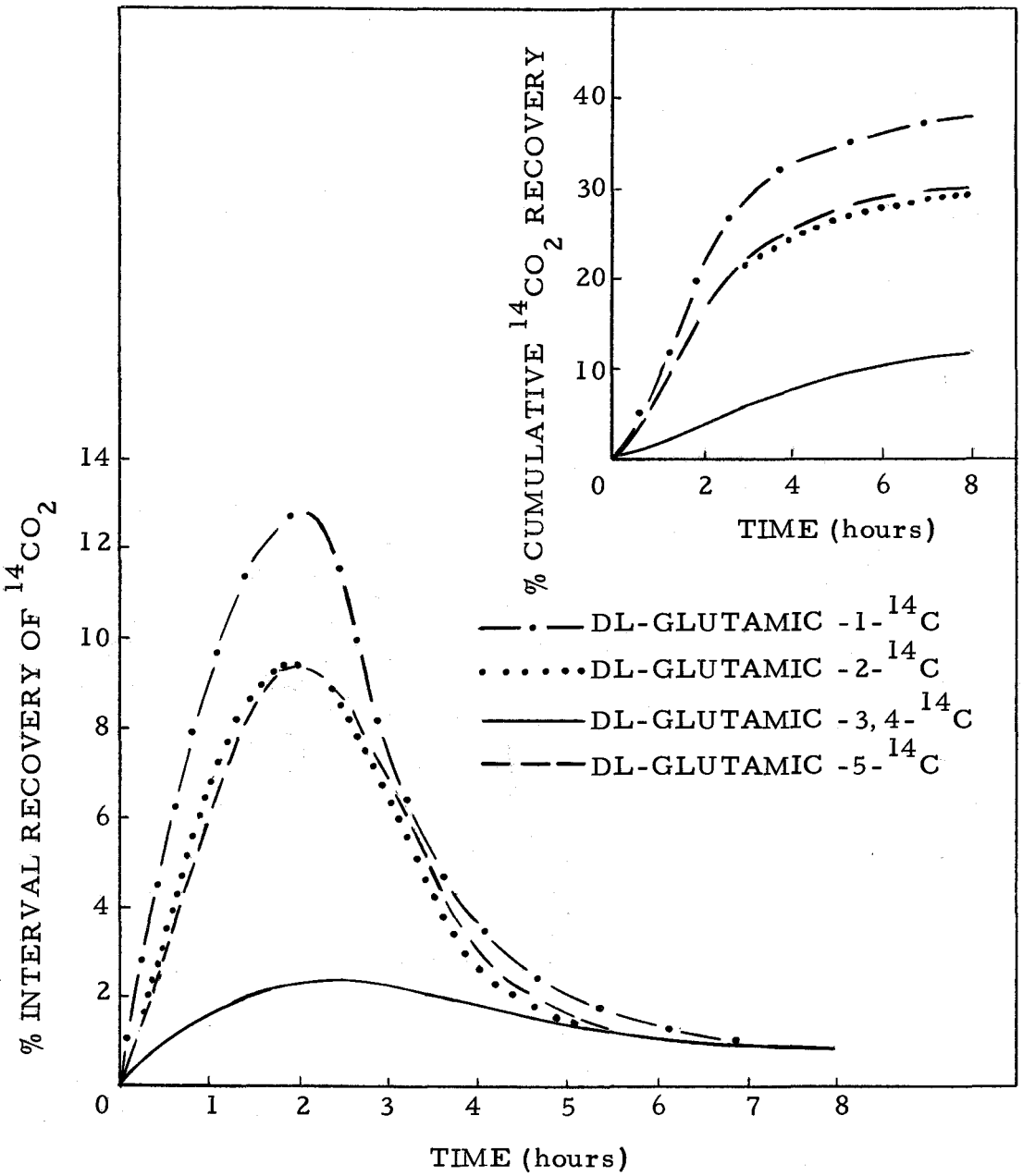


Figure 17. Radiorespirometric patterns for the utilization of glutamic acid by S. natans strain ATCC 15291.

recoveries were in the proper sequence one would expect in the presence of an active TCA cycle with C-1 > C-2 = C-5 > C-3 = C-4. This evidence lends further support to previous data which suggested that an active TCA cycle was functioning in S. natans. It is probable that virtually all organisms which store and degrade PHB have an active TCA cycle or glyoxylate cycle in order to effectively utilize the acetyl coA produced.

CO_2 Fixation by S. natans

Results of Warburg experiments with S. natans by Höhnl (1955) suggested that some fixation was occurring in the presence of low levels of CO_2; however, Höhnl was unable to confirm fixation due to limitations in experimental methods. Thus, a series of experiments was conducted to investigate the possibility that S. natans is capable of CO_2 fixation.

Glucose, acetate and pyruvate were used in amounts approximately equal to their energy output in oxidative reactions (Krebs, 1954). The results as shown in Table 18, represent the fixation of a moderate amount of $^{14}CO_2$ when glucose was present and a considerably smaller amount when pyruvate was present. $^{14}CO_2$ fixation was almost negligible when acetate was the carbon source, probably because acetate does not normally combine with CO_2 in non-photosynthetic organisms.

Table 18 $^{14}CO_2$ fixation by S. natans strain ATCC 15291.

Substrate	Total DPM Fixed	Percent $^{14}CO_2$ Fixed*
Acetate	37,160	0.3
Pyruvate	294,500	2.7
Glucose	477,180	4.4

*Based on a total input of 5 μc of NaH $^{14}CO_3$.

The fixation with pyruvate was significantly lower than with glucose because the pyruvate probably did not enter the cells as easily as glucose. The significance, if any, of CO_2 fixation by S. natans would be difficult to ascertain since the cells are able to furnish, to a large extent, their own supply of CO_2 from respiration. This mechanism is undoubtedly useful to growing cells since it makes available a supply of C_4 compounds to the cells.

Oxygen and Nitrate Utilization by S. natans

The ability of S. natans to grow reasonably well with only a limited amount of oxygen has been reported by several investigators. A series of experiments was conducted in order to determine, if possible, the mechanism(s) which enable S. natans to grow well at low oxygen levels.

Preliminary experiments were carried out in special

respirometer flasks capable of being filled with any desired gas atmosphere. At the start, a pure helium gas was used in the flasks to see if any growth or denitrification took place in synthetic medium containing nitrate as the sole nitrogen source. Little or no growth occurred and there was no evolution of nitrogen or any of its gaseous oxides. There was, however, considerable conversion of nitrate to nitrite in the medium. In a subsequent experiment to test for the production of nitrite from nitrate, five duplicate pairs of flasks were filled with a 95 percent helium-5 percent oxygen atmosphere and shaken at 30 C. The results of analyses taken from duplicate flasks during a time-course experiment are shown in Table 19. Examination of the gases in the flasks showed that no molecular oxygen was present after 24 hours. It is apparent that, as the oxygen was depleted with time, the amount of nitrite increased. Viability tests from each flask indicated that the massive accumulation of nitrite resulted in death of all the cells in 36 hours.

Table 19. Utilization of nitrate by S. natans strain ATCC 15291.

Flask*	Time (Hrs)	NO_2^--N μgm	% Conversion of NO_3^- to NO_2^-	mgm C as CO_2
1	12	5600	40	13.11
2	24	8100	57.7	13.85
3	36	9200	65.7	16.30
4	40	9280	66.3	16.30
5	60	9280	66.3	15.90

*Flask atmosphere -95% helium, 5% oxygen, 14000/μg-NO_3^--N/100 ml, 250 mg glucose, incubation at 30 C.

In order to further clarify the relation between oxygen level and nitrite production, a series of respirometer flasks were flushed with helium-oxygen mixtures as shown in Table 20. At the end of 48 hours the flask contents were analyzed and the production of NO_2^- was found to be inversely proportional to the amount of oxygen originally present. As shown by the preceding experiment, toxic levels of nitrite were rapidly produced as the oxygen level decreased.

Table 20. Utilization of nitrate by S. natans strain ATCC 15291 at various free oxygen levels.*

O_2 Level at Start of Run	μgm NO_2-N	% Conversion of NO_3^- to NO_2^-	mgm C as CO_2
5%	7850	54	18.5
10%	5250	37.5	44.3
15%	3000	21.4	62.1

*250 mgm glucose, 14,000 μgm NO_3-N/100 ml, 48 hour incubation at 30 C.

Since toxic levels of nitrite were not produced until oxygen levels were quite low, an experiment using electrolytic respirometers was conducted in which the levels of oxygen were maintained at the original concentrations. The results are shown in Figure 18 and Table 21. The concentrations of cells and substrate used were proportional to amounts used in typical Warburg experiments. The results in Table 21 indicate that almost twice as much conversion of

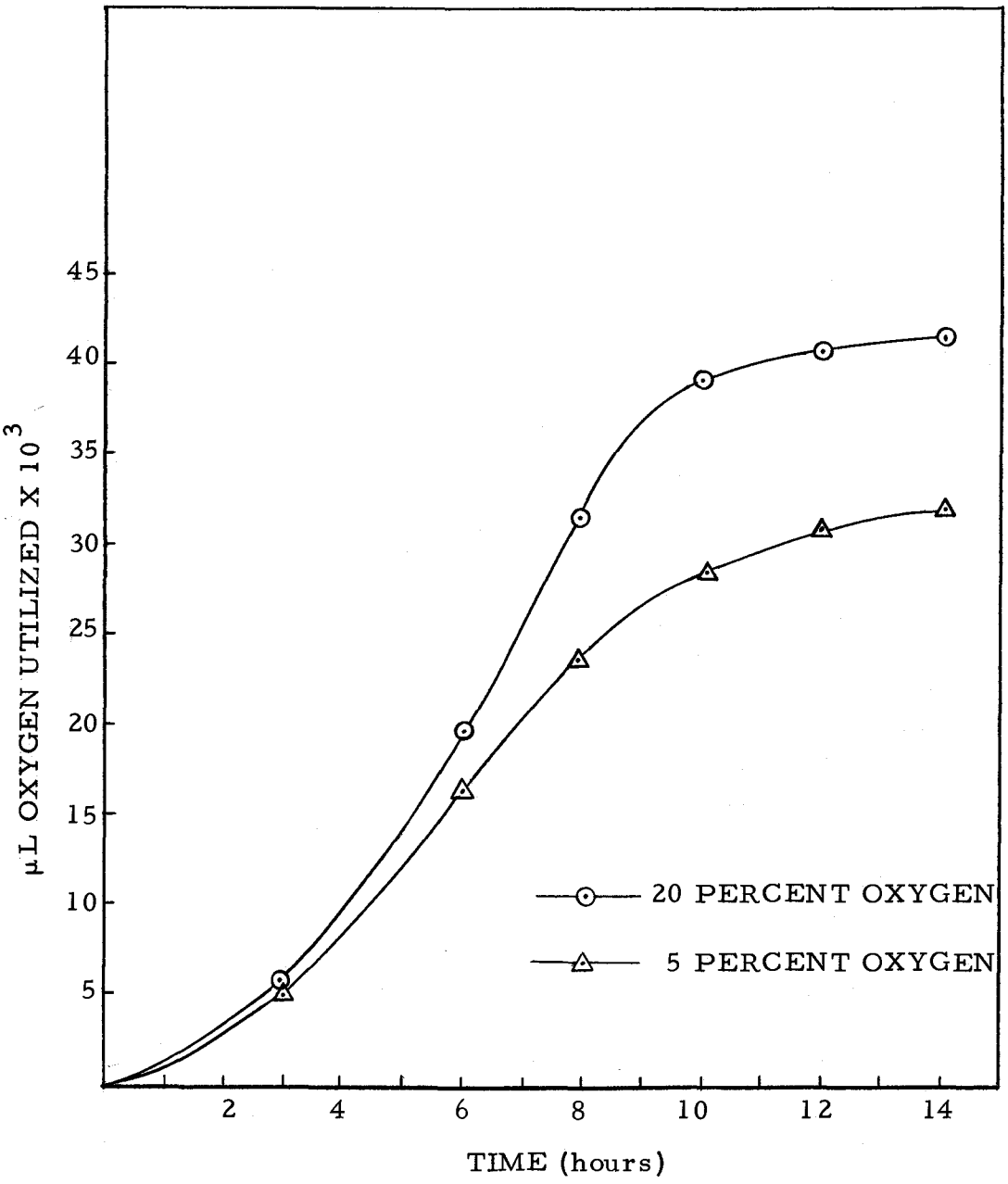


Figure 18. Cumulative oxygen consumption during nitrite production by S. natans strain ATCC 15291.

nitrate to nitrite occurred at the 5% oxygen level as that produced at the 20% level. The cells in the 5 percent oxygen atmosphere utilized only about 75 percent as much oxygen as the cells in the 20 percent atmosphere but the final cell weights were approximately equivalent. In each case glucose analyses revealed that all of the glucose was used.

Table 21. Oxygen consumption versus nitrite production in an electrolytic respirometer by S. natans strain ATCC 15291.*

O_2 Level of Flask during run	µl O_2 Uptake	µgm NO_2^--N	% Conversion of NO_3^- to NO_2^--N	mgm C as CO_2
5%	32,000	7000	50	27.04
20%	41,500	4000	28.6	33.21

*14,000 µg-NO_3-N/100 ml, 108 mg glucose, incubated at 25 C.

The data from the three experiments just discussed seem to indicate that S. natans is able to use the reduction of nitrate to nitrite in some manner as a substitute for normal respiration (in which atomic oxygen is the final hydrogen acceptor). The $DPNH_2 \rightarrow$ DPN reaction plus other terminal carriers have been shown to be coupled to nitrate reduction and would indeed explain the results which were obtained.

CONCLUSIONS

An attempt has been made to study the pathways of carbohydrate and nitrogen metabolism in pure cultures of S. natans and to correlate laboratory data with field data in order to better understand this organism.

The development of isolation and identification techniques were a prerequisite to growth and metabolic studies of the Berry Creek strain of S. natans. Due to the rather wide spectrum of conditions which support the growth of diverse strains of S. natans, the metabolism of two ATCC strains were studied.

Preliminary growth studies with strain BC-IV revealed that this strain had a pH range, growth characteristics and a temperature range which were comparable to published values for other strains of S. natans. One point of interest was that strain BC-IV established considerable growth at 3 C, while other investigators reported their strains were unable to grow below 5 C.

Radiorespirometric studies indicated that the Entner-Doudoroff and pentose phosphate pathways were functioning as the primary catabolic pathways in each of the three strains tested. Indirect evidence for participation of the TCA cycle and glyoxalate cycle as secondary pathways was also obtained. It was strongly indicated that S. natans in nature uses the same catabolic pathways found with

the laboratory strains. The effect of poly-β-hydroxybutyric acid production on the overall carbohydrate metabolism was investigated. Radiotracer studies of PHB helped to further confirm the operation of the above mentioned pathways. Recoveries of C-1 and C-6 from glucose in PHB gave further support to previous evidence that little or no EMP pathway was functioning. Additional ^{14}C studies on endogenous metabolism revealed that PHB was an excellent source of carbon and energy for S. natans.

Studies with TCA cycle intermediates as sole carbon sources indicated that each intermediate supported growth of strain ATCC 15291 while the remaining strains grew on all of the intermediates except citrate. The failure to grow with citrate was probably due to a permeability barrier.

Further experiments to demonstrate the operation of the TCA cycle and glyoxalate cycles were growth and radiorespirometric studies with acetate as the sole carbon source. $^{14}CO_2$ patterns and cell recovery data for acetate-1-^{14}C and acetate-2-^{14}C supported other evidence for the two pathways.

Final evidence for the TCA cycle participation was obtained by radiorespirometry with specifically labelled glutamic acid. Initial experiments with DL-glutamic acid were unsuccessful due to the inhibition of cellular activity by the D-isomer of glutamic acid. When L-glutamic acid was used as the carrier for specifically

labelled DL-glutamic acid, however, radiorespirometric patterns from the labelled-L-isomer coincided with the results expected from the operation of the TCA cycle.

The fixation of CO_2 by S. natans was shown by uptake of $^{14}CO_2$. Since only moderate amounts were fixed, it is doubtful that CO_2 fixation is required for growth of S. natans. More fixation occurred with glucose than pyruvate, possibly because of a permeability barrier to pyruvate.

A diagram of the pathways which have been indicated to be present in S. natans is shown in Figure 18.

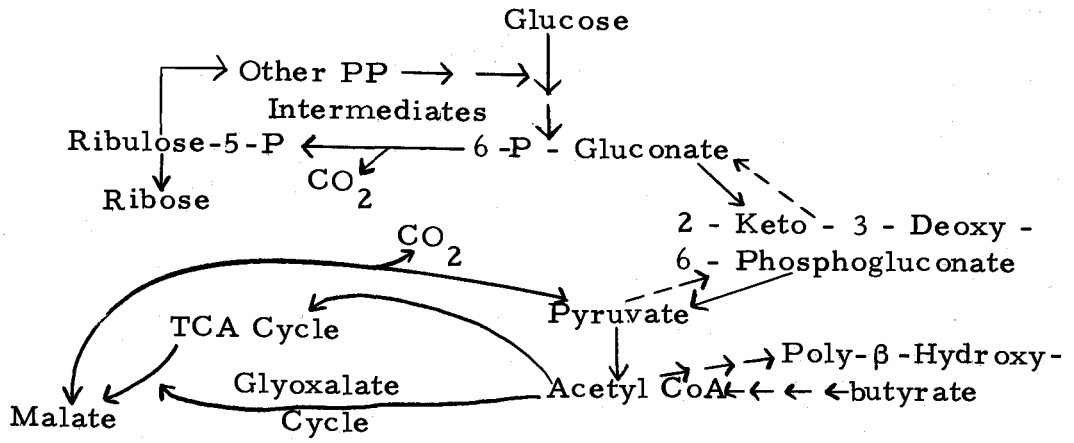


Figure 18. Suggested major metabolic routes for S. natans.

The role of NO_3^- as a contributor to cellular respiration at low oxygen levels has been suggested. No nitrogen gas is produced in

this set of reactions and NO_2^- appears to be the sole end product as DPN H_2 is oxidized to DPN.

SUMMARY

The carbohydrate and nitrogen metabolism of S. natans has been studied in considerable detail. Some of the more significant results are:

1. The results of growth studies agree in general with those obtained by other workers with the exception that reasonably good growth was obtained at 3 C.

2. Radiorespirometric studies indicated that glucose is catabolyzed via the ED and PP pathways in conjunction with the TCA and/or glyoxylate cycles.

3. The results of the incorporation of specifically labelled glucose into PHB indicated that little or no EMP pathway was functioning in S. natans.

4. Radiorespirometric studies with natural slime material which was composed almost entirely of Sphaerotilus cells suggest that S. natans has the same pathways functioning in nature as those found for pure strains.

5. The carbohydrate metabolism of S. natans appeared to be significantly affected by the participation of the pathways for PHB synthesis and degradation.

6. PHB is used in preference to other cellular carbon sources during endogenous respiration and may contribute to the survival of

S. natans in nature.

7. D-(-)-β-hydroxybutyrate dehydrogenase, a requisite enzyme in the pathway for PHB catabolism was assayed and found to be present in substantial quantity.

8. Intermediates of the TCA cycle (except for citrate) may serve as the sole sources of carbon for S. natans.

9. Growth studies and radiorespirometric studies with acetate as the sole source of carbon indicated that the TCA cycle and glyoxalate cycle are operative in S. natans.

10. The presence of an active TCA cycle was further substantiated by radiorespirometry of specifically labelled glutamic acid.

11. The D-isomer of glutamic acid is not utilized by S. natans and actually inhibits growth probably by tying up enzymes.

12. CO_2 is fixed by S. natans in moderate amounts in the presence of suitable condensing substrates.

13. The conversion of NO_3^- to NO_2^- with the accompanying $DPNH_2 \longrightarrow$ DPN reaction has been suggested as a mechanism for the growth of S. natans at low oxygen levels.

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