METABOLIC BASIS FOR THE PREFERENTIAL UTILIZATION OF DISACCHARIDE BY THE CELLULOSE-DECOMPOSING BACTERIUM, CELLVIERIO GILVUS (NOV. SP.)

by

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ABBREVIATIONS IN THE TEXT

G-1-P, Glucose-1-phosphate

G-6-P, Glucose-6-phosphate

F-6-P, Fructose-6-phosphate

F-1,6-P_2, Fructose-1,6-diphosphate

ATP, Adenosine triphosphate

DPN, Diphosphopyridine nucleotide

TPN, Triphosphopyridine nucleotide

Co-A, Coenzyme A

FMP, Riboflavin-5'-phosphate

"Tris", Trihydroxymethyl-amino-methane

P_i, Inorganic ortho-phosphate

P_o, Organic phosphate

MB, Methylene blue hydrochloride

These abbreviations are employed in the tables and ATP, DPN, TPN and Tris are used in the text. The full names of all other compounds are used in the text.

I. INTRODUCTION

The present scant knowledge about the biochemical transformations of cellulose, which has delayed advances in preserving cellulosic products and in improving ruminant nutrition, has prompted sporadic investigations in the past ten years. Basic programs concerning cellulose metabolism are now underway in several laboratories in this country. The Departments of Biochemistry and Nutrition and of Biology at Virginia Polytechnic Institute have instituted a program of investigating several phases of cellulose metabolism in relation to ruminant nutrition and soil fertility.

This thesis bears on some of the problems involved in the intracellular metabolism of the degradation products of cellulose. It is hoped that this study may contribute to the understanding of cellobiose and glucose metabolism in cellulose-decomposing bacteria, in the partial elucidation of the direct metabolism of dissacharides and more particularly to the anomalous preference of dissacharides to monosaccharides found to occur in many bacteria.

Biological Significance of Cellulose Degradation.

Although easily overlooked or ignored, cellulose assumes significance in nature and in our economy. Being

the major constituent of plant cell walls cellulose is exceedingly abundant in nature. The greatest mass of fixed carbon in nature is in the form of cellulose. Unfortunately, almost nothing is known about cellulose transformations in plants (Axelrod and Beevers, 1956; Reese, 1956). Altermatt (1956) has found that labeled glucose can be directly synthesized into cellulose in wheat plants.

Undoubtedly, one of the most vital roles of cellulose transformation in nature is its part in the carbon cycle. Detailed descriptions of the carbon cycle are discussed by Siu (1951) and Meyer and Anderson (1939).

The total amount of carbon available to the world is limited and is essentially interconverted between carbon dioxide and organic compounds. Should a carbon cycle not operate so as to replenish the atmosphere with carbon dioxide after the photosynthetic conversion to carbohydrates and protein, indeed, life on earth would be time-limited.

A crude estimate of carbon dioxide in the earth's atmosphere is about 2×10^{15} Kg, and plants consume about 6×10^{13} Kg annually. (Rabinowitch, 1945). Since about one-third to one-half of the weight of plant residues is cellulose, it would be possible eventually to reduce the available carbohydrate and protein foods in nature if some

of this cellulose were not returned to carbon dioxide. Of course, about one-twelfth of the carbon dioxide taken up by plants can be replaced by combustion of coal and petroleum. However, the greatest source of carbon dioxide in the air is from decomposition of plant and animal residues by microorganisms; thus, because a large portion of plant residues is cellulose, the microbial decomposition of this substance assumes importance. Microbial decomposition of cellulose is a vital process and reasonably enough we need to understand the biological transformations involved.

The importance of microbial action in soil fertility is well established not only concerning the physical structure of soil but in preserving important plant nutrients, such as phosphorus, nitrogen and potassium. Unfortunately, beyond the demonstrable dependence of these soil processes on microbial activity, very little is known. In addition, cellulose-decomposing microorganisms have had an indirect role in the formation of coal, peat, humus, and oil deposits (Waksman, 1939, 1940).

Simply the removal of plant residues from both field and forest prevents the piling up of these residues which eventually would limit not only plant growth itself but the utilization of these areas by man.

The anaerobic cellulolytic bacteria are in some degree involved in the carbon cycle to the extent that they can

degrade cellulose to organic acids assimilable by their symbiotic hosts. Of course, this symbiotic system is not only valuable to man but essential for the health and existence of the ruminant.

Economic Importance of Cellulolytic Microflora.

Heavy financial losses resulting from the microbial destruction of cellulose-containing products and materials, such as timber, fabrics and foodstuffs, have been the impetus for basic research studies with these destructive agents. Losses ranging in the multi-million-dollar class have been experienced in the United States alone. The importance of preservation of cellulosic materials is well documented (Siu, 1951). Economically, then, the destructive action of cellulolytic microorganisms accounts for much of the concern in conducting basic researches with these micro-organisms.

The popularity and necessity of the beef and dairy industries is great compared to many other protein food sources, but the efficiency in conversion of feed to food is comparatively low. Presumably, a more efficient digestibility of hay by cattle could prevent great wastes of feedstuffs, and result in great financial savings and increased food production. Therefore, the nutrition of cattle is a field of important current research. The need is seen to stretch the feed for cattle, improve the

efficiency and lower the cost of production. This requires intensive studies of the rumen microflora and the symbiotic systems intimately involved in ruminant nutrition and physiology. Cellulolytic bacteria are intimately involved in the nutrition of ruminants in which they ferment cellulose essentially to fatty acids providing a large part of the energy source for the animal.

Since a large fraction of sewage and garbage is composed of cellulosic material, the problem of cellulose destruction in waste disposal becomes critical, and microorganisms are especially efficient in this process. Nevertheless, the operation and maintenance of waste disposal plants costs enough to warrant attempts to utilize the products formed. Further, attempts may be made in the direction of obtaining useful fermentation products from such wastes; indeed, some of these efforts are already underway. The fermentation of sewage and the recovery of vitamin B-12 is an outstanding example. According to Quisenberry (1954) hunger in the world is on the increase, especially in the Far and Near East, in Africa, and in many more limited other parts of the world. It might be possible to convert waste materials into proteins so cheaply as to alleviate much of the world's hunger. A particular example of this has been the work of Gottaas et al. (1954). This group has reported that

valuable raw materials can be converted economically to nutritious protein material. This was accomplished on a pilot plant scale in which a bacterial-algal symbiotic system was used in converting organic sewage waste into algal cells which could be utilized as food.

Reese (1956) states that the enzyme "cellulase" has not been adapted to useful and industrial purposes. Some limited commercial uses are in retting treatments, preparing products from edible mushrooms and other minor uses. There are technical troubles which have prevented the exploitation of cellulosic fermentations for useful products, and these are the low concentration of fermentation products, and the impregnation of many native celluloses with lignin which reduces the availability of substrate and limits the fermentation.

Metabolism of Cellulose.

The microorganisms hold the high position in nature's society of carbon turnover at the step involving replacement of carbon dioxide in the atmosphere. They are preeminent among the agents of cellulose decomposition. The most important of these are the fungi, actinomycetes, myxobacteriales or "slime bacteria", and the anaerobic and aerobic bacteria. The natural milieu of the majority of these organisms is the soil but considerable numbers are present in the digestive tract of herbivores, especially in ruminants.

The organisms credited with the majority of cellulolytic activity in nature and responsible for most of the destructive importance to man are the fungi. Nevertheless, the relative significance of aerobic cellulolytic bacteria is not yet known, and these bacteria may be more important than thus far disclosed. Several reviews are available documenting their distribution and importance (Siu, 1951; Waksman, 1940; Tracy, 1953).

While the function of cellulases in plants would be of special concern in the synthesis of the cell wall and in cell wall alterations, it would be interesting to know if these plant enzymes are involved in the decomposition of the plant residues also. The presence of cellulases in animals is rare and in many of the instances where they are found the source of their synthesis is in doubt.

Balows and Jennison (1949) isolated thermophilic cellulase-producing bacteria from porcupine intestines and Tracy (1953) obtained cellulase-producing pathogenic bacteria from the human digestive tract. Seillière (1906) showed that the snail, Helix, produces a cellulase but the intestinal microorganisms may be responsible. Apparently, earthworms also form the enzyme according to Tracy (1953).

Some of the ciliate protozoa, oligotrichs in the genera Metadinium and Diplodinium, found in rumen contents

digest chiefly cellulose. The Metadinium swallows cellulose particles and then slowly digests them storing amylopectin (Oxford, 1955). It is still dubious if the cellulase is of animal or bacterial origin.

An important animal known to produce large amounts of cellulase is the marine wood-boring mollusc, Teredo (Lane, 1955), which causes millions of dollars worth of damage annually to naval property alone. These borers eat the wood, produce cellulase, and convert the cellulose to large quantities of glycogen. Here then is a system which can convert the beta-linked polysaccharide to an alpha-linked one.

Lasker and Giese (1956) have demonstrated that cellulose can constitute a food source for the silverfish, Ctenolepisma lenesta by prolonging life when fed to the aseptic animal. Microbial symbionts were not involved.

Cellulose itself is difficult to work with chemically and enzymatically since even its molecular weight is subject to large variation. The cellulose molecule is a definite chemical structure composed of anhydroglucose units with beta-1, 4-linkages and can be hydrolyzed quantitatively to glucose.

The physical structure of the cellulose molecule is based on the cellobiose molecule, a reducing disaccharide composed of two glucose molecules linked by a beta-1, 4-glycosidic bonding. Difficulty appears in determination of

the precise number of anhydroglucose units in the native cellulose molecule which is apparently variable. Thus the size of the molecule which can be determined only after chemical isolation may not reflect the size of the original substance. Even precise physical methods do not account for cross-linking of cellulose chains.

The crystalline or amorphous nature of cellulose affects the ease of attack by cellulases. Much of the detailed mechanism of cellulase action must await even more explicit knowledge of the chemistry and physics of the cellulose molecule. Moreover, cellulosic materials in nature are impregnated with many impurities such as lignin, waxes, pectins, proteins, and hemicelluloses, and this further complicates study of cellulase action in nature. The most formidable characteristic of cellulose to the enzymologist is its insolubility in water. Solubility of substrates is generally taken for granted and when they are not soluble as in the case of fats, some proteins, and starch grains, they usually have been treated to obtain some degree of solvation before study.

Various derivatives of cellulose such as carboxymethyl cellulose are now used in enzyme work, and although it is granted that these are not natural substrates they have allowed many valuable studies. Tracy (1953) has reviewed and evaluated the use of such derivatives.

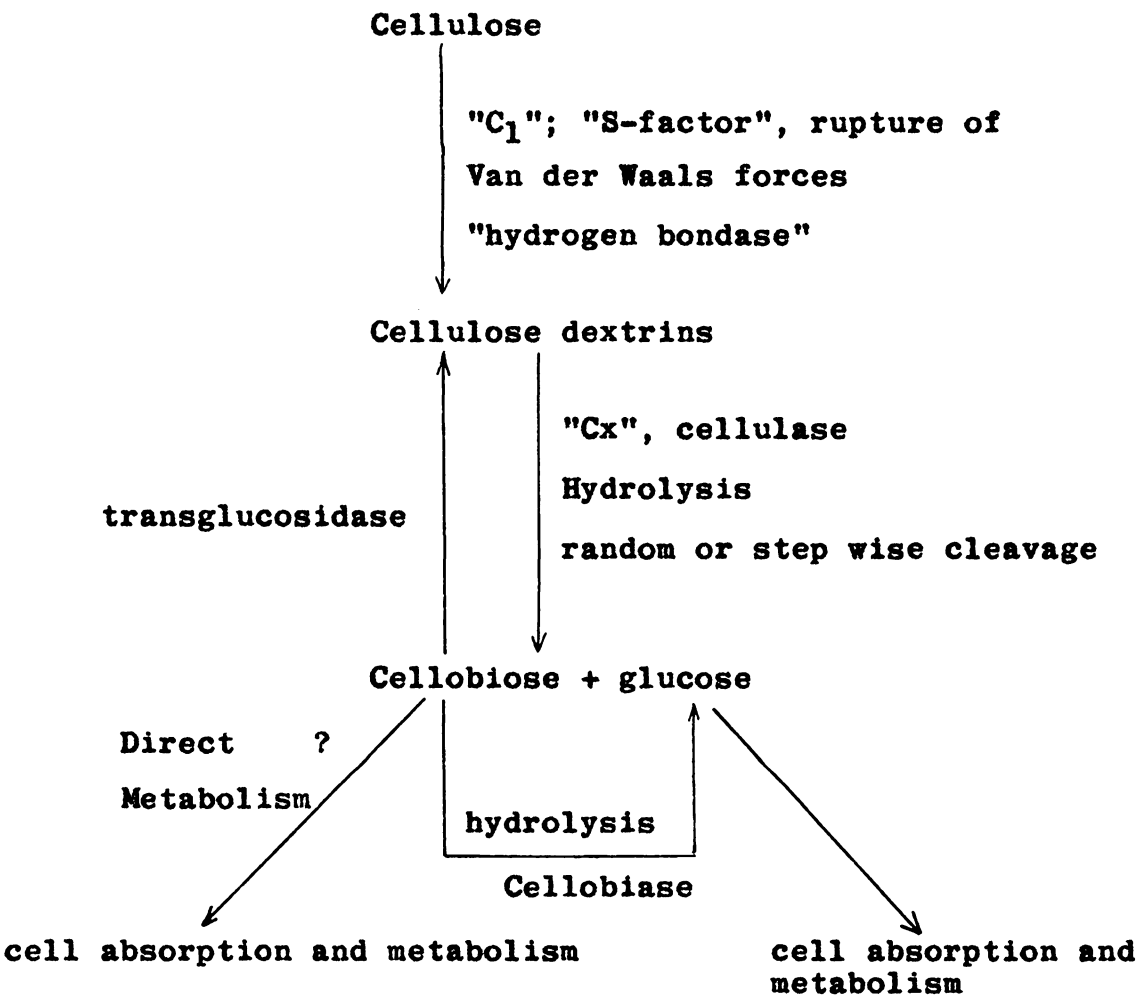
The initial action of cellulolytic enzymes may not be an hydrolysis but a solvation, dis-orientation of the subunits, or a surface action. Amorphous cellulose is more subject to attack than crystalline cellulose. Increase of surface area by grinding in a ball mill improves susceptibility to enzymatic attack.

Reese and Gilligan (1954) have discussed an enzyme, called the "S-factor", in cellulolytic filtrates which acts on the primary wall of cotton fibers and may be different from the hydrolyzing enzymes or part of a systematic complex of enzymes active in solvation, chain splitting, and hydrolysis of these chains to sugars.

The nature of cellulase enzymes is another phase of study, and evidence is accumulating that not simply one single protein species but several separate enzymes of a complex are involved even in pure cultures (Reese and Gilligan, 1954; Jermyn, 1952; and Hash, 1957). Another important question concerns the intermediate dextrins and final degradation products formed by cellulase activity. According to Reese (1956) the higher dextrins are not found since the initial reactions are slow relative to later ones. However, some of the short chain oligosaccharides have been detected (Kooiman, 1953; Hash and King, 1954). The terminal products of cellulase action will be covered more thoroughly in the literature

review since it is the metabolism of these compounds which is under study in this investigation.

To summarize the sequence of enzymatic events leading from native cellulose to glucose and cellobiose a schematic diagram is presented in view of present day knowledge.



II. REVIEW OF THE LITERATURE

The Final Extracellular Products of Cellulase Action.

The nature of the enzymatic degradation products of cellulase activity is currently an unsettled question, and reports from the literature indicate that either glucose or cellobiose or sometimes both of these sugars may be produced.

As early as 1906 Seillière isolated a glucosazone from products of cellulose digestion by Helix digestive juice, and Holden and Tracy (1950) found glucose to be the product of the action of Helix enzymes. Hungate (1944) obtained cellobiose as the only product of cellulose breakdown by autolyzing cultures of Clostridium cellobioparus from the bovine rumen.

Whitaker (1953) claimed that a purified cellulase fraction from Myrothecium verrucaria was able to produce glucose in addition to cellobiose as an end product. That the C_x enzyme fraction, which hydrolyzes a straight chain of anhydroglucose units with beta-1,4-glucosidic linkages, can form glucose directly was shown by Levinson et al. (1950). They (1951) later found that both glucose and cellobiose were produced by the enzymes of several fungal filtrates.

It is interesting to note that Reese et al. (1952) demonstrated that cellobiose inhibits the cellulase enzyme from numerous fungal filtrates but glucose does not. They interpreted this as evidence that the true product may be cellobiose. Whistler and Smart (1953) found that cellobiose appeared initially and glucose later in a mixture of cellulose and a purified cellulase from Aspergillus niger. Kitts and Underkofler (1954) failed to find sugars other than glucose as a result of the action of cell-free preparations of rumen organisms, but were careful to say that the crude preparations probably contained active cellobiase which would rapidly decompose the cellobiose. Apparently, both Reese (1956) and Whitaker (1954) believe that glucose can be formed from a single cellulase fraction. The C_x cellulase fraction from a filtrate of Myrothecium verrucaria produced predominately cellobiose as an end product while a filtrate from Trichoderma viride produced glucose although the enzyme preparation was free of cellobiase (Reese, 1956). The latter situation is considered to be the most common. Hash (1957) reported that cellulase fractions separated on a cellulose column and with no cellobiase or transglucosidase activity were able to hydrolyze cellulose dextrins forming glucose, cellobiose and higher sugars. At present the terminal degradation products of cellulase action appear to be

either glucose, cellobiose or both and the exact product formed by the activity of a specific enzyme must await definitive purification of the enzymes in the cellulase complex.

The literature cited above is essential because it is important to know something about the actual terminal products of cellulase action in a given organism especially in undertaking a study of the further metabolism of these compounds as was done in this investigation. For instance, if glucose were found to be the only product one would scarcely expect to find very active enzymes for cellobiose metabolism. On the other hand, one can anticipate the presence of disaccharide-utilizing enzymes if the disaccharide is a natural substrate for intracellular metabolism. Such information may be helpful in interpreting the over-all metabolism of cellulose by a given organism.

Disaccharide Metabolism and Non-Utilization of Monosaccharides.

The two general mechanisms for the dissimilation of disaccharides are considered as "indirect" and "direct". The indirect mechanism refers to the hydrolysis of the disaccharide into two hexoses which are subsequently metabolized by the cell. Those processes by which disaccharides are not hydrolyzed but are metabolized by phosphorolysis, transglucosidation, by oxidation to bionic

acids and possibly by other reactions are termed direct processes.

The first exponent of indirect disaccharide metabolism was Emil Fischer (1894, 1898). He realized the relationship between the occurrence of hydrolytic enzymes and fermentation of disaccharides; and also described the hydrolytic conversion of maltose to two molecules of glucose which were further metabolized to ethanol and carbon dioxide by yeast. Fischer's authority and experiments established the biochemical opinion that the initial step of disaccharide metabolism was such an hydrolysis with the formation of monosaccharides which were later assumed to be further metabolized according to the well known Embden-Meyerhof-Parnas scheme. This opinion was maintained in the biochemical world in spite of many opposing reports.

A voluminous literature has since appeared concerning the enzymes which catalyze the hydrolysis of disaccharides, such as sucrase, lactase, maltase, melibiase and others. According to DeLey and Bernaerts (1953) many reports incompatible with the theory of indirect or hydrolytic metabolism followed but were either struck down by former authority or were ignored. The difficulty with these reports was that they offered no proposals for alternative reactions although they indicated that hydrolysis was not the sole mechanism present in microorganisms.

The primary antagonist to the Fischer theory was Willstätter (1922) who claimed that in many yeast strains the amount of hydrolyzing enzyme did not account for the rate of fermentation. Maltase-poor yeast fermented the same amount of maltose as did maltase-rich yeast; furthermore, there was no adaptive formation of maltase in the maltase-poor yeast. Unfortunately, no new reaction was described with this system. This worker also demonstrated that the differences between strains were not due to the extraction methods or to the amounts of enzymes extracted and corrected for maltase inhibition by glucose.

Several reports relative to the direct and indirect theories of disaccharide metabolism appeared between the early work of Fischer and Willstätter and the definitive work of Hehre (1943), Hestrin (1943) Monod (1948) and Doudoroff (1949), but without exception there was no clear-cut evidence to prove that disaccharides could be metabolized directly. All of the arguments presented for and against the direct utilization hypothesis were conditional and indirect, and the interpretations so confusing that it is felt by this writer that discussions of these works would not aid this review in any way. A bibliography of these references relative to direct disaccharide catabolism up to 1953 may be found in the review of DeLey and Bernaerts (1953).

Evidence that lactose was utilized directly was presented by Hoff-Jørgenson, Williams and Snell (1947), and Doudoroff et al. (1949) who showed that a strain of Lactobacillus bulgaricus grows on lactose but not on galactose or glucose. This was probably a non-hydrolytic mechanism.

Proteus vulgaris was found to grow well on sucrose but not on fructose by Morel and Monod (1945). The growth was about twice as great on sucrose as on an equimolar mixture of fructose and glucose. The problem, left unanswered, then was to determine why fructose was not fermented as the free pyranose form but was fermented in the disaccharide form. Possibly, only the furanose form or other form is attacked by the enzyme and the pyranose is not. Isbell and Pigman (1938) and later Gottschalk (1947 and 1947a) found that fructofuranose spontaneously isomerized to fructopyranose, so the latter point is probably not the explanation.

It was shown recently by Blanchard and Albon (1950), De Whalley et al. (1951) and Barton-Wright (1951) that sucrase activity on sucrose not only forms monoses but also forms a trisaccharide composed of two molecules of fructose and one of glucose.

Trehalose metabolism has also received attention. Myrbäck and Vasseur (1943) found that dry yeast fermented trehalose faster than glucose but this may have been due

to a loss of hexokinase activity. O'Conner (1940) noticed that Fusarium lini Bolley grows better on trehalose than on glucose and these same results were reported by Doudoroff, Kaplan and Hassid (1943) for Pseudomonas saccharophila.

Leibowitz and Kupermintz (1940) found that glucose fermented by Escherichia coli caused an early formation of polysaccharide. Likewise Doudoroff (1949) observed that an Escherichia coli mutant formed a starch-like polysaccharide from maltose but did not respire glucose, and Hehre (1949) found that Neisseria perflava formed a glycogen-like polysaccharide from sucrose.

There were some oppositions and criticisms of the experiments that seemed to indicate a direct disaccharide process. Gottschalk (1949) critically discussed these experiments in a stimulating review and claimed to have dispelled most of the evidence. However, most of Gottschalk's arguments like those of his opposition gave no clear cut proof that disaccharides could not be metabolized directly since chemical intermediates were never isolated.

The above discussion is important essentially because it provides the background of considered attention to the possibility of non-hydrolytic reactions participating in the first stages of disaccharide utilization.

The recent work discussed below is adequate proof that disaccharides are metabolized directly and this principle has been accepted in modern biochemical thinking. Hehre (1946 and in previous papers) demonstrated that sucrose was converted to dextran and acid and gas by Leuconoctoc mesenteriodes and called the responsible enzyme dextransucrase, which is a transglucosidase. Hestrin, Avineri-Shapiro and Ashner (1943) obtained a conversion of sucrose to levan and called the enzyme levansucrase. They (1945) have proposed a mechanism for this reaction.

Monod and Torriani (1948) described an enzyme of Escherichia coli able to form polysaccharide from maltose and purified the enzyme. The polysaccharide was similar to starch giving a blue color with iodine. In Escherichia coli, at nearly the same time, (Doudoroff, 1949) a mechanism was elucidated for the direct utilization of maltose in which the enzyme, amylomaltase, catalyzed the transformation of maltose to glucose and an intracellular polysaccharide. The glucose could then be phosphorylated by the hexokinase reaction and subsequently metabolized. The polysaccharide was a readily-utilizable energy source by the formation of glucose-1-phosphate in the typical phosphorylase reaction. The mechanism of transglucosidation has been explained by Kalkar (1954). Fitting and Doudoroff (1952) have shown that maltose is phosphorylated by Neisseria meningitidis to

glucose and beta-D-glucose-1-phosphate. Delaporte and Duval (1950) observed that Phytomonas betagelatae formed a fructosan and a reducing sugar from sucrose and other sugars with a fructofuranose moiety. Doudoroff et al. (1949) found that dry cell preparations of a mutant of Escherichia coli fermenting maltose rapidly but not respiring glucose would form glucose and a polysaccharide from maltose. The reaction was reversible and glucose-6-phosphate was formed from the polysaccharide. Enzymes that convert glucose-1-phosphate to glucose-6-phosphate to fructose-6-phosphate were present. Polysaccharide increased in the presence of fluoride which inhibited the phosphoglucomutase reaction.

Hassid and Doudoroff (1950) and Hassid et al. (1951) studied the sucrose phosphorylase system wherein sucrose is phosphorylated in the presence of inorganic phosphate to fructose and glucose-1-phosphate. This enzyme is present in other bacteria (Doudoroff et al., 1943; and Doudoroff et al., 1949). Hassid et al.(1944) further obtained the synthesis of sucrose with this system but the equilibrium constant (K = 0.05) was too small to expect that this same system was important in sucrose synthesis in plants.

The sucrose phosphorylase was later shown to be a transglucosidase by Wolochow et al. (1949) and the mechanism was described. Sucrose synthesis has been

accomplished in wheat seedlings by Caputto and Leloir (1953) and with pea preparations by Bean and Hassid (1955) and shown to be mediated by uridine diphosphoglucose.

Another reaction of disaccharides is the oxidation to bionic acids. In 1947 Stodola and Wood observed the microbiological production of bionic acids from disaccharides. Several strains of Pseudomonas, particularly graveolens, formed lactobionate and maltobionate. DeLey found (1953) that Pseudomonas aromatica converts maltose to maltobionic acid. Many microorganisms apparently are able to hydrolyze these bionates to gluconate and hexose but these enzymes may be maltases. Further, it is of some doubt if these enzymes have a definite physiological function in the living cell. The catabolism of the bionic acids is being studied by DeLey (unpublished). Hestrin (1949) and Doudoroff (1951) have reviewed the problem of direct utilization of disaccharides by bacteria. Doudoroff discussed the anomoly of utilization of disaccharides and the inability to utilize constituent monosaccharides. Although new mechanisms are revealed for disaccharide metabolism the inability to use monosaccharides is yet unexplained.

Some hypotheses proposed were (1) that the utilization of free fructose might depend on simultaneous utilization of the glucose portion of sucrose so that a

phosphorylation including glucose-1-phosphate might be required for phosphorylation of fructose, (2) that even though sucrose phosphorylase may be involved in normal metabolism of sucrose, the free sucrose may not be a natural substrate and if some derivative of sucrose is phosphorylated then a derivative of fructose rather than free fructose may occur as a product, and (3) that the cell membrane may be impermeable to free fructose and only the combined molecule may pass, possibly by "active transport" at the cell surface. He, moreover, points to the inconsistencies between demonstrations of enzymes in vitro but inactivity in vivo and problems of permeability.

Very recently, Doudoroff, Palleroni et al. (1956) obtained evidence that the ability of Ps. saccharophila to use glucose was independent of the capacity to use sucrose but depends on the genetic makeup of the cells, physiological variants in a population, the inductive effect of substrate, the growth phase, and endogenous reserves. Fructose was phosphorylated in an adenosine triphosphate-dependent reaction different from the glucokinase reaction and it is probably further metabolized according to the Entner-Doudoroff scheme.

Disaccharide Preference in Cellulolytic Microorganisms.

Reports of a similar nature relative to cellobiose have

been overlooked by reviewers. A number of cellulose-decomposing bacteria that do not grow on glucose or grow better on cellobiose than on glucose have been reported but not further studied.

The first report seen in which cellulose-decomposing bacteria did not grow on glucose or other sugars but grew only on cellulose was the fine paper of Hutchinson and Clayton (1918) who made a thorough study of Spirochaeta cytophaga. These authors noted that sugars appeared to inhibit growth. In 1931 Cowles and Rettger found that Clostridium cellulosolvens, a widespread cellulose-fermenting anaerobe, would not utilize glucose. Stanier (1942) found that the chytridiaceous fungus, Rhizophylyctis rosea, attacked cellulose and cellobiose at much greater rates than glucose.

It was thought for some time that the Cytophaga group would grow only on complex polysaccharides until Stanier (1942) found that toxic substances were formed from heating glucose in the presence of salts, especially phosphates. He then was able to obtain growth of the chytrid and Cytophaga on glucose by filter sterilization.

Fuller and Norman (1943) in a survey of aerobic cellulose-decomposing soil bacteria found that Pseudomonas erythera would not grow on glucose. The writer tried to obtain this culture from Dr. Fuller and from the American

Type Culture Collection but found that it had been lost. Enebo (1949) reported that some cellulose-decomposing bacteria were more active on cellulose and cellobiose than on glucose. McBee (1948) demonstrated definitely that the anaerobic cellulose-decomposing bacterium, Clostridium thermocellum, would not grow on glucose but grew on cellobiose. Sih and McBee (1955) and McBee (1955) have extended this work and demonstrated a direct utilization of cellobiose by a cellobiose phosphorylase, the first to be reported. The phosphorylase was demonstrated by P^{32} exchange from inorganic phosphate into glucose-1-phosphate. However, the inability to utilize glucose remains somewhat of a mystery and is even more complicated by the fact that a kinase enzyme is present in extracts that will convert glucose to glucose-6-phosphate, whereas glucose accumulates extracellularly when intact cells attack cellulose or cellobiose.

McBee has postulated that cellobiose may first be phosphorylated and then undergo a phosphoroclastic split to form two molecules of glucose phosphate or that the intracellular kinase may act simultaneously with the cellobiose phosphorylase to yield two glucose phosphates. This does not, however, seem to be compatible with the fact that glucose can accumulate extracellularly upon cellulose hydrolysis unless the cellulase produces a random cleavage, a process which is not compatible with

the utilization mechanism of the cell. A reasonable explanation may be that a preferential permeability is involved.

Recently, other non-glucose-utilizing cellulose-degrading organisms have been reported. Sijpesteijn (1951) obtained such organisms from the bovine rumen. He isolated a strain of Rumminococcus flavefaciens from sheep rumen that did not utilize glucose. The products of fermentation were succinic, acetic and formic acids, and ethanol was absent (1949).

Hungate (1950) believed the colorless cocci to be the most important cellulolytic bacteria in the rumen of cattle for they were found in numbers ranging from 10 to 100 million per milliliter. These organisms utilized cellulose and cellobiose but not glucose. Hall (1952) obtained cellulose-digesting cocci from rabbit caecum that utilized cellobiose but not glucose or other mono- and disaccharides; and, the fermentation products were acetic, succinic and formic acids, ethanol, carbon dioxide, and hydrogen.

In summary then, it is known that living cells are able to decompose disaccharides in several ways, (1) by hydrolysis, (2) by conversion of half of the molecule to polysaccharide and half to free hexose (transglucosidation), (3) by phosphorolytic cleavage in which one product-molecule is esterified to phosphate and the other set free for further metabolism, and (4) by the direct oxidation to

bionic acids. Only bacteria and certain other primitive cell types have been reported to perform direct catabolism of disaccharides; nothing is known about such enzymes in plant and animal tissues. Mechanisms other than those summarized above are no doubt possible.

Pathways of Carbohydrate Metabolism.

Carbohydrates furnish the cell with both a metabolic fuel for energy and carbon skeletons for synthesis of non-carbohydrate compounds. Thus, it is important to understand the chemistry of these compounds and their enzymatic transformations so that we not only gain insight into the basis of the living system but in order that we may control these processes. Little can be done with such knowledge unless the processes can be controlled and the ramifications of this become obvious to the problems of health, food, preservation, nutrition and in the chemical and fermentation industries.

The status of our knowledge about carbohydrate metabolism, although a great deal more lucid than in 1940, is apparently far from being catalogued even in a single species or genus. In many transformations the reaction mechanisms are obscure. A great deal of attention has been paid during the past 15 years to the elucidation of so-called "metabolic pathways" which are the series of sequential reactions catalyzed for the most part by

enzymes in the cell. The function of these sequential reactions is or becomes multiferous as the knowledge increases or improves. It is by these reactions that energy may be liberated to the other cellular elements involved in endergonic processes such as growth, synthesis, excretion, locomotion, temperature regulation, light emission, osmotic pressure control, and detoxication.

In this section, then, will be considered briefly the known metabolic pathways of hexoses and disaccharides. Most consideration in research has been given to glucose, the widely distributed and almost universally utilized hexose, because of our concern about this important compound in animal and human metabolism. Glucose is important as a starting point for the alternative pathways and a basic unit for both polysaccharide synthesis and again as an intermediate product-metabolite. Thus glucose plays the main role in this discussion.

Glycolysis by the Embden-Meyerhof-Parnas Pathway (Meyerhof, 1938) was established in yeast and muscle and accounts for the conversion of glycogen by a particular series of reactions to pyruvic acid (formerly to lactic acid in muscle).

For some time biochemists considered glycolysis to be the basic pattern of carbohydrate fermentation in microorganisms. Taken as evidence for the Embden-Meyerhof

pathway for conversion of glucose to pyruvate were the following: (Gunsalus et al. 1955) (1) production and utilization of intermediates; (2) presence of enzymes that catalyze reactions of this pathway by specific demonstration; (3) sensitivity of these enzymes to known inhibitors of the muscle and yeast enzymes, i.e., iodoacetate, M/1,000 and NaF, M/50; (4) fermentation of glucose-3,4-C^{14} to carboxyl labeled lactate or to labeled CO_2 in the ethanol fermentation; (5) accumulation of phosphoglycerate in growing cells by addition of M/50 sodium fluoride; and (6) conversion of phosphate esters to glycolytic intermediates or to fermentation products.

The evidence that glycolyzing enzymes are present does not prove the operation of the scheme but is only indicative since alternate routes are now known for the conversion of glucose and hexose phosphates to the triose phosphates and pyruvate. The enzymic reactions that are now known to be unique to the Embden-Meyerhof scheme are phosphohexokinase, which phosphorylates fructose-6-phosphate to fructose-1,6-diphosphate, fructose-1,6-diphosphate-specific aldolase and triosephosphate isomerase, these enzymes converting fructose-1,6-diphosphate to an equilibrium mixture of dihydroxyacetone phosphate and glyceraldehyde-3-phosphate. Other enzymes in this pathway are common to other known pathways.

Recently, alternate pathways for fermentative and oxidative degradation of carbohydrates have been established. One of these is the so-called hexose monophosphate shunt or the Warburg-Dickens scheme. The universal distribution and relative importance of this pathway in microorganisms, animals and plants is not yet clear but its presence in Escherichia coli, Pseudomonas fluorescens, Pseudomonas saccharophila, Leuconostoc mesenteroides, yeast, animal and plant tissues has been indicated (Gunsalus, 1956). It may begin with the oxidation of glucose-6-phosphate to aldonic or ketoaldonic acid, followed by a specific aldolase cleavage with further oxidation of the two 3-carbon fragments. The transformations form a cyclic process. The pathway involved or whether a certain intermediate accumulates depends on the number and amount of enzymes since these determine the concentrations of the particular donors and acceptor intermediates.

Glucose-6-phosphate dehydrogenase in a TPN-coupled reaction dehydrogenates glucose-6-phosphate to 6-phosphogluconate mediated by 6-phosphogluconolactone and a hydrolytic reaction. Six-phosphogluconate dehydrogenase catalyzes a TPN-coupled reaction to 3-keto,6-phosphogluconate which is decarboxylated to carbon dioxide and ribulose-5-phosphate which is in equilibrium catalyzed by pentosephosphate isomerase with ribose-5-phosphate. These

two pentose phosphates catalyzed by transketolase form glyceraldehyde-3-phosphate and sedoheptulose-7-phosphate. The two products undergo a transaldolase reaction to yield erythrose-4-phosphate and fructose-6-phosphate. Glucose-6-phosphate is then regenerated from fructose-6-phosphate by isomerization. Fructose-6-phosphate may be regenerated also in two other ways; (1) condensation of glyceraldehyde and dihydroxyacetone phosphate to yield fructose-1,6-diphosphate which in the presence of a phosphatase is converted to fructose-6-phosphate and (2) condensation of tetrose phosphate and glycoaldehyde to form fructose-6-phosphate.

Criteria and experiments inconsistent with glycolysis but that explain the hexose monophosphate shunt are given by Neilands and Stumpf (1955). Another pathway, the Entner-Doudoroff or 6-phosphogluconate shunt, has been demonstrated (Entner and Doudoroff, 1952; MacGee and Doudoroff, 1954) in Pseudomonas saccharophila in which 6-phosphogluconate is first dehydrated to 2-keto,3-deoxy, 6-phosphogluconate by a dehydrase. This compound is cleaved by a specific aldolase to pyruvic acid and glyceraldehyde-3-phosphate. This pathway has been established in species of Pseudomonas and in Escherichia coli (Kovachevich and Wood, 1955).

Thus, at the present time we have three established pathways that may be inter-related in the cell. There is

little doubt that others occur and that variations on the known patterns will appear (Gunsalus et al. 1956).

Entner and Stanier (1951) showed that Pseudomonas fluorescens oxidized glucose to gluconic acid with a constitutive enzyme. Exclusion of a phosphorylation mechanism for glucose oxidation was based on the fact that this oxidation was insensitive to M/50 sodium fluoride, was not stimulated by ATP (Stokes and Campbell, 1951) and that phosphate esters could not be extracted from freshly harvested glucose-grown cells (Campbell and Norris, 1950). The presence of both a hexose monophosphate pathway and a direct oxidation to gluconic and 2-keto-gluconic acid has furthered our understanding of carbohydrate metabolism in Pseudomonas. Oxidation to gluconate would not release energy at the substrate level but a cytochrome system mediates hydrogen transport and may thus form high energy phosphate by oxidative phosphorylation. Evidence for participation of an electron transport system was found with extracts and particulate preparations of Pseudomonas fluorescens (Wood, 1953).

No evidence for a pyridine nucleotide-linked reaction as found in mammalian liver (Strecker and Korkes, 1951) or for a flavin-linked glucose oxidase in molds (Coulthard et al.) was obtained, and cytochrome oxidase was not implicated. Two pathways for glucose oxidation are thus revealed (Gunsalus et al., 1956) and no Embden-Meyerhof

pathway was present in Pseudomonas aeruginosa (Campbell, 1950) and Pseudomonas fluorescens (Wood and Schwerdt, 1955). In one case, glucose is oxidized to gluconate which is phosphorylated to 6-phosphogluconate and this is converted to pyruvate and triose phosphate by the Entner-Doudoroff enzymes. In the other scheme, glucose is oxidized to 2-keto-gluconate before phosphorylation by a 2-keto-gluconokinase to 2-keto-6-phosphogluconate and then is converted by an unknown reaction sequence to pyruvate and a 3-carbon fragment.

The knowledge of the known pathways for carbohydrate metabolism facilitates the exploration of glucose and cellobiose metabolism in a new microorganism such as the Cellvibrio gilvus used here.

III. OBJECTIVES OF THE INVESTIGATION

The objective of this research was the elucidation of the metabolic basis of the preferential utilization of cellobiose over glucose by an aerobic cellulolytic bacterium. More specific objectives were the following:

(1) To demonstrate the occurrence of the phenomenon of preferential utilization of the disaccharide, cellobiose, over its constituent monosaccharide, glucose, in a cellulose-decomposing bacterium.

(2) To define the physiological areas with which the differences were associated in order to make more specific approaches possible. By this is meant the confining of the problem to a specific physiological event in the cell such as a disturbance in active sugar absorption, a permeability barrier, an enzyme deficiency, or an intracellular enzyme reaction.

(3) To attempt explanation of the anomalous disaccharide preference by detailed examination of the carbohydrate metabolism with respect to the physiological events indicated in (2) above.

IV. SPECIAL MATERIALS AND METHODS

In view of the fact that many of the methods employed in the investigation were closely related to the experiments and to the interpretations thereof, it was fitting to include a description of them in the experimental section along with the details of the more important chemicals and materials.

Materials and Sources.

The reagent grade anhydrous D-glucose was obtained from the J. T. Baker Chemical Company and the cellobiose was obtained from Eastman Organic Chemicals. The disodium salt of adenosine triphosphate was secured from the Pabst Laboratories. Riboflavin-5'-phosphate was a product of the California Foundation for Biochemical Research. Coenzyme-A was a lyophilized preparation from the Pabst Laboratories.

The barium salts of glucose-6-phosphate and fructose-6-phosphate in addition to the dipotassium salt of glucose-1-phosphate and the magnesium salt of fructose-1,6-diphosphate were obtained from Nutritional Biochemical Corporation.

The casein hydrolyzate (enzymatic) was a product of General Biochemicals Incorporated and contained 12.9% total nitrogen and 3.7% amino nitrogen. The yeast extract

used in the growth media was Bacto-yeast Extract manufactured by Difco Laboratories.

The crystalline bovine albumin used as a standard for protein analysis was obtained from General Biochemicals Corporation.

The alumina employed for grinding cells was Levigated Alumina No. a-542 from Fisher Scientific Company. The cation exchange resin was Amberlite-IR-120 (H) from Rohm and Haas Company. An IR-4B resin was used for the anion exchange column.

Dow Antifoam-A (silicone defoamer) employed in the liquid culture media undergoing aeration was obtained from the Dow Corning Corporation.

General Methods.

The organism, Cellvibrio gilvus, was maintained on a stock medium of Fuller and Norman (see experimental section) in which the cellulose dextrins and yeast autolyzate were replaced with 0.5% cellobiose or 0.1% ground Whatman No. 1 filter paper and 0.3% yeast extract. Both stock and running cultures were maintained and stored at 4° C and transfers were made every two weeks or a month.

To grow large quantities of cells 3 or 6 liter Pyrex serum bottles were fitted with sparger stones and connected by sterilized tubes containing non-absorbent cotton to an aspirator and the open air. The addition of 0.025 g of

enzymatic casein hydrolyzate to the above medium caused a rapid growth that produced a very heavy suspension of cells which were enzymatically very active after 18 hours of growth with very strong aeration, when inoculated with a concentrated suspension from a 24-hour cellobiose agar slant. About 1 ppm of Dow Antifoam-A was added to the medium before autoclaving for 20 minutes at 15 pounds pressure.

When preparing inocula several 24-hour cultures were made prior to the preparation of the inoculum since this was found to increase the rate of growth. Each time a culture was grown it was tested for contamination by examination of a gram-stain or a nigrosin stain.

Oxygen uptake measurements were made as described by Umbreit et al. (1951) and the specific conditions of the experiments are given in the experimental section. The flasks and manometers were calibrated by a mercury method from triplicate determinations.

Growth was measured by the increase in turbidity as measured with the Klett-Summerson photoelectric colorimeter (blue filter) or the Bausch and Lomb photoelectric colorimeter at 415 mμ wavelength.

All pH measurements were made using a glass electrode with a Leeds and Northrup, Model 7664 pH meter. A pH meter was also used for the titration experiments.

Reducing sugars were determined by the titrimetric method of Somogyi (1945) and by the microcolorimetric method of Nelson (1944) and Somogyi (1952).

The total and inorganic phosphorus were determined by the microcolorimetric method of Fiske and Subbarow (1925). Total nitrogen was determined by the microcolorimetric kjeldahl method of Johnson (1941). Protein was estimated by the method of Lowry et al. (1951). Carbon analyses were made by the wet combustion method of Van Slyke (1951, 1954) using a modified apparatus.

The methods used for the fermentation balance and fractionation of the products were basically as described by Pelczar et al. (1955). Viscosimetric measurement was accomplished with an Ostwald viscometer. The other methods are described in the experimental section except for those that were altered or devised.

Determination of Intracellular Inorganic and Total Phosphate and Total Nitrogen in Bacterial Cells.

The methods used were basically the fractionation procedure of LePage and Umbreit (1943) and Umbreit et al. (1951), the microcolorimetric phosphorus method of Fiske and Subbarow (1925), and the microkjeldahl method of Johnson (1941). The turbidity of a cell suspension free of clumps in isotonic saline or buffer was adjusted so that a 1:10 dilution had a 25% transmission or higher at 430 millimicrons wavelength. Dilutions of 1:2 or 1:4 of

this stock solution were suitable for analysis; i.e., within the range of the standard curve for phosphorus and nitrogen analyses. Fifteen milliliters of this suspension and 5 ml of the substrate solution were then mixed.

Phosphorus analyses were begun by placing 5 ml of this cell suspension into Pyrex test tubes, adding 5 ml of acetone, shaking for 1 hour, and removing the acetone by oven drying at 80° C.

The residue was then mixed thoroughly with 10 ml of cold 10% trichloroacetic acid and stored for 12 hours at 0° C. The contents were then transferred quantitatively to cellulose nitrate centrifuge tubes and centrifuged at 0° C for 30 to 60 minutes at 12,000 rpm (Servall SS-1 head).

The supernatant was decanted and saved. Cold extraction of the residue with 2 ml of 5% trichloroacetic acid using 5 hour extraction periods was then repeated until no phosphate could be detected in the extract (usually four times). The supernatants were then pooled and made up to 10 ml volume. Inorganic and total phosphorus were then determined on 2 to 5 ml portions of the pooled extract.

Modifications of Paper Chromatographic Methods for the Separation of Hexose Phosphates.

The method of Bandurski and Axelrod (1951) was tried initially using methanol, formic acid, water (80-15-5),

and methanol, ammonium hydroxide, water (60-10-30) in two-dimensional systems with little success. The spots were large and diffuse and the separations were not dependable. According to Mortimer (1952) a large percentage of water increases the diffusion of the spots and this is made worse by the use of the free phosphate esters that have been treated with cation exchange resin in the hydrogen form such as Dowex-50 in 0.2 N HCl. Mortimer has changed the solvent systems and has obtained more success by substituting formamide for the water. The method described by Wade and Morgan (1955) is apparently a fine way to separate and detect nearly all of the known phosphate esters found in biological materials. However, this method involves two-dimensional systems using first ionophoresis and then paper chromatography and is extensive in technique, equipment and time.

It was desired to obtain a one dimensional solvent system that would definitely separate ortho-phosphate, glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-diphosphate and possibly adenosine triphosphate. The systems used by Mortimer were tried using both Whatman No. 1 and No. 52 paper (the latter is an acid washed paper). Movement on Whatman No. 52 paper was much more restricted than on the No. 1 paper. This was probably because of the compactness of cellulose fibers in this heavier paper. Spots moved well on

Whatman No. 1 paper in the solvent, methyl cellosolve, methyl ethyl ketone, ammonia (7-2-3) and in ethyl acetate, formamide, pyridine (1-2-1). Separation of the above sugar phosphates in these solvents was particularly good in the methyl cellosolve, methyl ethyl ketone, ammonia system. However, even with this system movement of the spots was retarded and it was desired to obtain more movement of the spots with the expectation that greater movement would allow complete separation of the above phosphate esters which were already moving apart.

This solvent system was found to be somewhat improved by the addition of a small quantity of water and the solvent system methyl ethyl ketone, methyl cellosolve, ammonia and water (7-2-3-1) was used. The distances traveled by the esters and their R_p values are shown in Table 1. This system also separates fructose-1,6-diphosphate and fructose-6-phosphate very well from other esters shown in Table 2. Compare with the data in Table 1. The distances were measured from the origin to the leading edge of the spots since one can obtain more accurate and reproducible measurements in this way than by trying to find the center of density of the spots. The spray reagent was that of Hanes and Isherwood (1949) and after drying for five to seven minutes at 85° C the papers were placed in a moist chamber with a trace of hydrogen sulfide for 20 minutes. It is imperative, of course, that the Whatman No. 1 paper

TABLE 1

PAPER CHROMATOGRAPHIC SEPARATION OF

HEXOSE PHOSPHATES IN IMPROVED SOLVENT SYSTEM*

Phosphate Esters	Distance Traveled cm	R_p Value = $\frac{\text{cm moved by compd. x 100}}{\text{cm moved by } P_i}$
P_i	15.6	100
G-1-P	27.7	173.5
G-6-P	24.5	154.0
F-1,6-P_2	7.2	4.6
All of above	Same as for each above	Same as for each above
Mixture of enzyme, phosphate and cellobiose	15.2 and 24.4	97.5 and 154.0
ATP		93.4

*Solvent: methyl cellosolve, methyl ethyl ketone, NH_4OH, H_2O (7-2-3-1)

TABLE 2

PAPER CHROMATOGRAPHIC IDENTIFICATION OF

FRUCTOSE-6-PHOSPHATE

Phosphate Esters	Distance Traveled cm	R_p Value = $\frac{\text{cm moved by compd. x 100}}{\text{cm moved by } P_i}$
P_i	10.6	100
F-6-P	25.6 and 25.3	242
Unknown preparation from cells, barium fraction.		
Barium removed by ion exchange	25.1 and 10.7	237 and 101

*Solvent: methyl cellosolve, methyl ethyl ketone, ammonia, and water (7-2-3-1).

be washed with ethylene-diamine-tetraacetic acid or with HCl solution, with ammonia solution and then with distilled water several times.

Approximate amounts of the esters detectable by the Hanes and Isherwood method were as small as 10-15 micrograms of glucose-1-phosphate and of glucose-6-phosphate. This solvent system is not too good for identification of glucose-1-phosphate and glucose-6-phosphate but one can first spray the papers with aniline hydrogen phthalate in butanol (Block, 1952) and detect the esters with free aldehyde groups and subsequently spray with the phosphate reagent to detect all spots.

The preparations of the free phosphate esters by either ion exchange to remove barium or by making the ammonium salts were satisfactory. More compact spots were usually obtained with the ammonium salts but the acid treatment to remove barium was, of course, touchy and the esters were not stable if stored at pH 5.0. Equivalent addition of ammonium sulfate to precipitate barium was adequate except in the case of glucose-6-phosphate where acid was required to remove the barium. The treatment with Dowex-50 in the hydrogen form in 0.2 N HCl solution until dissolution and then adjustment of the pH to 5.0 (the green endpoint of brom cresol green) worked well and samples could be stored several

weeks (4-6) even though the spots obtained were a bit diffuse.

The size of the spots applied apparently affects the amount of diffusion on the paper and not more than 1 lambda should be applied at one time. The method for preparing the free phosphates according to Wade and Morgan (1955) appeared to be a good one.

Where one has sufficient quantity of sample it would probably be preferable to precipitate the esters as the barium salts to remove all other compounds according to the procedures of Umbreit et al. (1948) or to partially fractionate the esters as was done by Krahl (1956).

For definite separation of glucose-1-phosphate from glucose-6-phosphate other systems are available and the methanol, ammonia, water (6-3-1) system is satisfactory for confirmation. However, ortho-phosphate smears in this solvent and one must depend on R_{G-1-P} values.

Another solvent system which was found useful for identification of fructose-6-phosphate was methyl cellosolve, methyl ethyl ketone, formamide (7-2-3). No report has been seen on this combination but it separated the compounds very well. Given in Table 3 are the data obtained for the identification of fructose-6-phosphate from an enzymatic sugar phosphate preparation from bacterial cells.

TABLE 3

PAPER CHROMATOGRAPHIC IDENTIFICATION OF

FRUCTOSE-6-PHOSPHATE IN A NEW SOLVENT SYSTEM*

Phosphate Esters	Distance Traveled cm	R_p Value = $\frac{\text{cm moved by compd. x 100}}{\text{cm moved by } P_i}$
P_i	27.5	100
F-6-P	15.0	54.5
Unknown sugar phosphate fraction from cells, barium fraction. Barium removed by ion exchange	13.0 and 27.5	47.3 and 100

*Solvent: methyl cellosolve, methyl ethyl ketone, formamide (7-3-2)

V. EXPERIMENTAL RESEARCH AND RESULTS

Isolation of Cellulolytic Bacteria.

Procedure. The samples used for isolation were selected from a wide variety of sources usually in an aerobic environment. The sources, locations, and their sample numbers are described in Table 4. The soil samples were deflocculated with 0.5% sodium metaphosphate (Gamble, 1951) in order to remove or desorb as many cells as possible from soil particles. The samples were then serially diluted and used to inoculate the media after appropriate dilutions were obtained. Colonies forming clear zones were transferred to cellobiose agar slants and tested for purity by streaking on cellobiose agar plates. The initial test for purity was based on formation of uniform colonies on the agar plates.

Media for Isolation of Cellulolytic Bacteria. The media used were selected to support growth of those cellulose-decomposing organisms (1) that required only cellulose and inorganic salts, (2) those that might require amino acids and vitamins, (3) those that required crude filter paper or cellulose in a finely ground state, and (4) those that required or

TABLE 4

SOURCES OF SOIL SAMPLES USED TO ISOLATE AND SCREEN CELLULOLYTIC MICROORGANISMS FOR NON-UTILIZATION OF GLUCOSE

Sample No.	Source and Location
1	Rotting stump soil; Virginia Polytechnic Institute (V. P. I.) Campus
2	Oak leaf mulch soil, moist, aerobic; V. P. I. Campus
3	Sawdust mulch soil, moist, aerobic; V. P. I. Campus
4	Sawdust-compost, moist, aerobic; V. P. I. Campus
5	Road bog; Natural Bridge Forest Floor, Virginia
6	Mixed deciduous mulch; Route 11, Bank of Purgatory Creek, Buchanan, Virginia
7	Plowed lespedeza soil; Shenandoah Valley, Virginia
8	Plowed alfalfa soil; Shenandoah Valley, Virginia
9	Plowed alfalfa soil; Shenandoah Valley, Virginia
10	Forest floor; Amherst County, Virginia
11	Bovine rumen ingesta
12	Bovine feces
13	Meadow soil; Bedford County, Virginia
14	Compost, one year old; V. P. I. Campus
15	Fresh bovine feces
16	Old bovine feces

could utilize the cellulose dextrins but not natural cellulose.

The liquid medium of Dubos (1928) contained $NaNO_3$, 0.05 g; K_2HPO_4, 1.0 g; $MgSO_4$, 0.5 g; KCl, 0.5 g; $Fe_2(SO_4)_3.9H_2O$, a trace; 1 liter of distilled water at pH 7.5 and filter paper strips. The Fuller and Norman medium (1942) contained $NaNO_3$, 10 g; K_2HPO_4, 1.0 g; KCl, 0.5 g; $MgSO_4.7\ H_2O$, 0.5 g; yeast extract, 1%; agar, 15 g; 1 liter of distilled H_2O at pH 7.0. Cellulose dextrins, prepared according to Fuller and Norman, were added at the 0.1% level.

In some instances 100 mg of nitrogen as enzymatic casein hydrolyzate was added to each liter of medium as a supplementary amino acid source. Occasionally it was found profitable to substitute ground filter paper for cellulose dextrins since rapid fungal growth on the dextrin medium obscured the bacterial colonies.

The filter paper or absorbent cotton was partly decomposed in the cold for 12 hours in 1 N HCl, washed, filtered, and ground in a ball mill for 72 hours. About 0.1% was added to the medium.

Because the majority of soil cellulose-decomposing bacteria are mesophilic most of the incubations were carried out at 25 or 30° C. However, several

thermophilic cellulose-decomposing bacteria were isolated by incubations at 55° C in moist chambers approaching 100% humidity (Murray, 1944).

Isolated Microorganisms.

Group I. These aerobic mesophilic, cellulose-decomposers from cultivated field soil and manure were isolated on the Fuller and Norman medium supplemented with casein hydrolyzate. Among ten cultures two very small cocci, labeled FNC-4 and FNC-8, grew on cellulose and on cellobiose but not on glucose. Some organisms appeared to grow better on cellobiose than on glucose. Growth of all these cultures was very poor. In general these organisms were not very active in decomposing ground filter paper cellulose.

Group II. Nine cultures labeled A through I were isolated from moist, aerobic oak leaf mulch soil on Dubos' medium containing filter paper. Cultures were selected for variety in colonial morphology on isolation plates. All cultures grew on glucose and cellobiose. Cultures J through S were isolated from mixed forest floor soil (#6) on Dubos' medium. These organisms varied in morphology from minute cocci

and short rods to narrow, large and oval rods. All cultures grew on both glucose and cellobiose. Among 52 other cultures isolated from Soil Sample Nos. 1, 2, 3, 4, 5, 6, including a large variety of morphotypes, all but four cultures grew well on glucose; cultures S, FNC-4, FNC-8 and Y grew slightly.

Group III. Cultures labeled M-1 to M-53 were isolated on the modified cellulose medium of Fuller and Norman from Soil Sample Nos. 10, 15, 2, 5, forest floor soil, fresh bovine feces, aerobic moist oak leaf mulch and forest bog soil. Of these organisms M-29, M-31, M-45, M-46, and M-47 through M-52 grew very, very slightly on glucose broth and no growth could be seen on glucose agar plates. It was later found that after long incubation a very slight growth did appear in some cultures.

Group IV. Twenty thermophilic cellulolytic bacteria were isolated on cellulose agar at 55° C from fresh bovine feces and from moist aerobic oak leaf mulch since these sources had proven to be the most fruitful of 16 sources for cellulose-decomposers inactive in the utilization of glucose. Growth occurred on

glucose with all but four of these organisms, designated T-6, T-9, T-11, and T-12.

Dilutions of 10^{-7} to 10^{-9} were made from bovine rumen ingesta and plated on Fuller and Norman's medium containing casein hydrolyzate. While some colonies appeared on the plates with casein hydrolyzate no clear zones indicating cellulose hydrolysis were noted. Plates were incubated aerobically at 37° and 55° C, for several weeks.

It was noted in the process of testing certain cultures for cellulolytic activity that the presence of casein hydrolyzate frequently enhanced growth but retarded the hydrolysis of cellulose.

Cellulolytic Bacteria Unable to Grow on Glucose.

Ability of Isolated Organisms to Grow on Glucose, Cellobiose, and Cellulose Without Vitamins and Amino Acids. Those organisms which produced clear zones on cellulose or cellulose dextrin agar plates were tested for ability to grow in the absence of added vitamins or amino acids in the form of yeast extract both in the presence and in the absence of cellulose on Dubos' medium. Of the 23 cultures tested without yeast extract, only one organism grew on cellulose and

cellobiose, six grew on cellulose in presence of glucose, three grew on cellobiose alone and 11 grew on glucose alone. These data indicate that there are definite requirements of the cellulolytic bacteria for either vitamins or amino acids or both since all tested organisms decomposed cellulose in the presence of yeast extract.

Ability of 25 Selected Cultures to Produce Clear Zones and to Grow on Glucose or Cellobiose. Twenty-five cultures were retested for ability to produce a diffusible cellulase and to hydrolyze ground cellulose in the Fuller and Norman medium (containing yeast extract). They were also tested for growth on agar plates with glucose and cellobiose. All but one of the cultures produced well-defined clear zones around the colonies and this same culture refused to grow on cellobiose. All remaining 25 cultures, M-29 through M-53, grew very well on cellobiose, but 13 of these failed to grow or growth was not visually detectable on glucose agar.

Purification of a Cellulose-Decomposing Bacterium Preferring Cellobiose to Glucose.

The selected cultures were first streaked on cellulose agar after dilution and shaking in sterile water blanks. Cultures M-29, M-43, M-45, M-46, M-47 and M-52 were then

transferred from well-isolated colonies into both cellulose and cellobiose broth of the Fuller and Norman medium containing yeast extract. After incubation for 24 hours at 30° C smears were made, stained with crystal violet and examined. Each culture appeared to contain a given morphology but morphology differed from culture to culture.

Organism M-29 was obtained from well-isolated colonies on cellulose agar plates and diluted and streaked on cellobiose and cellulose agar plates. Colonies were checked for identity and in all cases no differences were observed. These cells were short rod-shaped gram-negative bacteria. Cells examined after growth on cellobiose broth were much enlarged and elongated and this occurred in every case with this supposedly pure culture. It was thought that the organism was either contaminated or that cultural conditions were causing pleomorphism and enlargement of the cells. It was not possible to obtain further purity in terms of consistent morphology.

Organism M-52 was comparatively active in cellulose decomposition but no growth resulted in glucose broth in 24 hours. It appeared to have homogeneous morphology; so it was purified in the following manner. Purity was based on (1) identical colonies appearing on pour and streak plates, (2) gram-stain character, (3) homogeneous morphology and (4) the criterion used by McBee (1948) to insure purity of cellulose-decomposing bacteria. This

principle is based on the sustained ability to decompose cellulose after several culture generations of growth on cellobiose and glucose. Contaminants would grow on the simple sugars, overgrow the cellulolytic organism and yet not grow when transferred back to cellulose.

After streaking numerous times on cellulose and cellobiose agar an inoculum from cellulose slants of organism M-52 was diluted in sterile water blanks and shaken with glass beads on a mechanical shaker for 30 minutes, to facilitate the separation of imbedded contaminants. One loop from each dilution tube was transferred to cooled cellulose agar roll-tubes, mixed, and poured into sterile petri plates. After incubation at 30° C small colonies appeared and developed the clear zones indicative of cellulose hydrolysis. Small, well-isolated colonies were transferred to cellobiose and glucose agar. All colonies appeared identical. The surface colonies were circular, white to opaque and convex, but deep colonies were more transparent and diffuse. Both the deep and surface colonies had the same cellular morphology and gram-stain character. Colonies were transferred to cellobiose agar, and after growth appeared smears were made and stained. Colonial and cell morphology were uniform and all cells were gram-negative. This procedure was repeated ten times more, and even after

growth on glucose and re-transfer to cellulose agar the same results occurred.

Further evidence for purity was obtained by transferring to cellulose roll-tubes and testing for clear zones. These colonies when transferred back and forth on cellulose, cellobiose and glucose agar retained both their ability to hydrolyze cellulose and a consistent colonial morphology, gram-character, and cell morphology on cellulose.

Characterization of Organism M52-3.

Methods. Stock cultures were maintained on the medium of Fuller and Norman in which 0.1% of ground Whatman No. 1 filter paper and 0.5% Difco yeast extract were substituted for cellulose-dextrins and autolyzed yeast preparation. Cultures were also maintained on this medium using cellobiose instead of cellulose. The cultures were stored at 4° C and could be kept for two or more months.

Morphology was determined using cells grown on cellulose and on cellobiose media for 24 hours at 30° C. The stains employed to determine morphology were crystal violet, Kopeloff's and Hucker's (Committee, S. A. B.) gram-stain and nigrosin.

Flagellation was determined on both 18 and 24-hour cultures using Leifson's and Bailey's flagella stains (Committee, S. A. B.).

Colonial morphology descriptions were made on cellulose agar after two or three days and on cellobiose agar in 24 to 48 hours.

Cells were examined for spores using the malachite green-safranin procedure of Schaeffer and Fulton (Committee, S. A. B.).

The optimum temperature and pH values were obtained by measuring growth in cellobiose broth using the Bausch and Lomb or the Klett-Summerson photoelectric colorimeters.

The oxygen relation was determined in deep agar stab tubes and in broth containing cysteine under CO_2 gas.

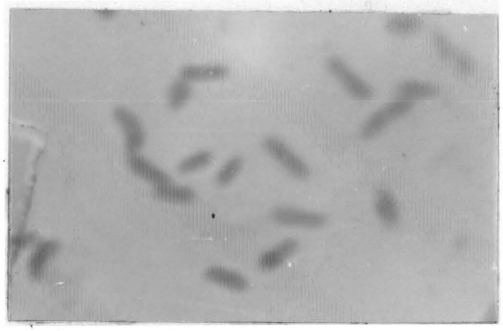
The fermentation products were determined by carbon distribution analyses of the fractionated compounds (Van Slyke, 1951, 1954).

Description. Since the morphology of this organism is not consistent when grown on media other than cellulose, several photomicrographs were made under various environmental conditions. These are shown in Plate 1 in which different stains were employed. The magnification was about 1200X and the photographs were enlarged about three times (final magnification ca. 3600X). Photograph A is of a gram-stain of the organism after 24 hours growth in cellulose broth.

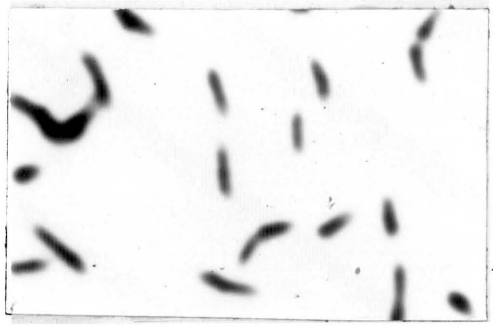
PLATE 1

PHOTOMICROGRAPHS OF CELLVIBRIO GILVUS

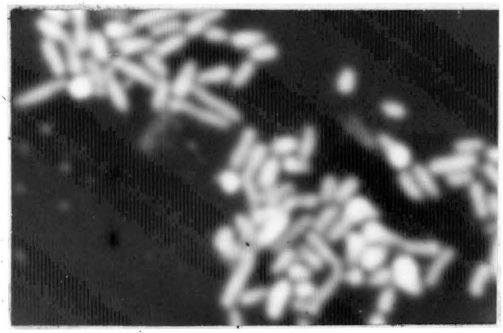
(All Photographs Magnified
Approximately 3600X)



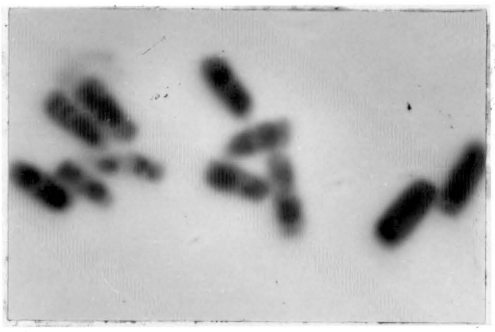
A. Gram Stain after 24 Hours Growth on Cellobiose



B. Crystal Violet Stain after 24 Hours Growth on Cellobiose



C. Nigrosin Stain after 16 Hours Growth on Cellobiose

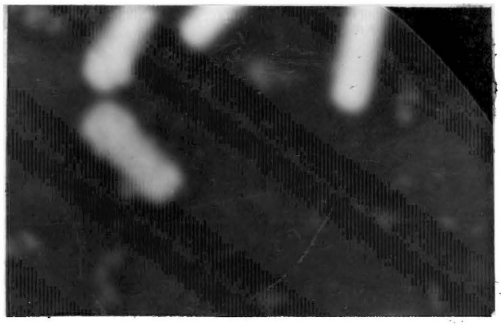


D. Crystal Violet Stain after 3 Hours Growth on Cellobiose

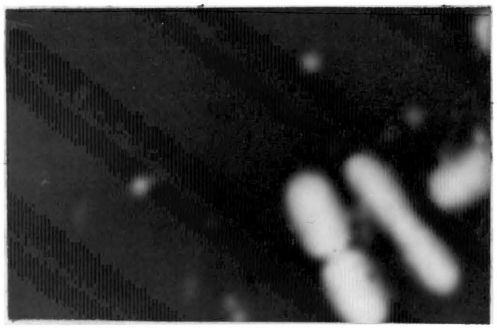
PLATE 2

PHOTOMICROGRAPHS OF CELLVIBRIO GILVUS

(All Photographs Magnified
Approximately 3600X)



E. Nigrosin Stain after 3 Hours Growth on Cellobiose



F. Nigrosin Stain after 4 Hours Growth on Cellobiose

G. Cell Wall Stain after 4 Hours Growth on Cellobiose

Some morphological variation can be seen. Short to long rods occur and many show slight curvature. In the freshly isolated culture the cells were estimated to range in size from 0.3 - 0.5 by 0.8 to 1.5 microns when grown on cellulose agar and stained with crystal violet.

More extreme morphological variation is evident from Photograph B which is of a crystal violet stain of a 24-hour culture grown on cellobiose broth. Large inclusion bodies are evident in some cells.

A negative (nigrosin) stain of a 16-hour cellobiose culture is shown in Photograph C. Much of the cell curvature is lacking in these young cultures but shows more conspicuously in 24-hour cultures.

Large cell bodies or chromatic bodies are seen (Photograph D) in the crystal violet stain of a three-hour old culture grown on cellobiose broth. Usually two such bodies were seen per cell. The large size of these cells is especially interesting. Such giant cells always appear in young growing cultures from three to 12 hours old. Most bacteria swell during physiological youth during formation of nucleoprotein. However, these cells appeared to grow to tremendous size and apparently disintegrated. One can see large ghost cells with many small rods and

refractile bodies in the area which apparently grow into "normal" cells. Photographs of nigrosin stains taken at 3, 4, and 16 hours are shown in Plate 2, Photographs E and F, and Plate 1, Photograph C. Such variation occurs in the pleuropneumonia group and in the anaerobic non-spore-forming gram-negative genus Bacteroides (Burrows, 1949). Whether or not this organism does possess L variation and passes through a filterable form in addition to transverse fission would provide an interesting study in basic bacteriology. Photograph G is of a cell wall stain (Webb, 1954) showing that the giant cells four hours old are themselves cells and not spore cases containing the cells.

The size of cells grown on cellobiose broth for 24 hours at 30° C was estimated to average 0.75 by 2.25 microns with sizes ranging from 0.75 to 1.5 by 1.5 and 3.75 microns as measured on smears stained with nigrosin. Under some conditions of growth the cells become exceedingly large and elongated and some curvature can still be seen.

Generally, the cells were straight or slightly curved rods with rounded ends occurring as single cells, in pairs and in chains. In addition to terminal chromatic granules, there were some rather

involuted forms with large bodies. No capsules were detected.

The cells were definitely gram-negative in all instances with monotrichous flagella, usually attached subterminally or terminally. Spores were not present even in old cultures. Motility was very pronounced with rapid turning, twisting and translatory motion as determined on both 18-hour cultures grown in cellobiose broth and on broth cultures stored for as long as two months.

Growth was aerobic to facultative. Growth occurred under anaerobic conditions, but aeration increased the cell yield greatly.

Surface colonies on cellulose agar containing yeast extract were slightly raised, convex to flat and opaque to white and pale yellow. Distinct clear zones of cellulose hydrolysis appeared early. Deep colonies were opaque to translucent and spreading or diffuse.

Cellobiose-yeast extract-mineral salts medium provided excellent growth in broth. Yeast extract or a mixture of amino acids and vitamins was required for growth. A faint ochre to yellow pigment developed slowly on cellulose agar. No growth occurred on nutrient broth or agar. Growth also occurred on

carboxymethyl-cellulose, and a cellulase active on carboxymethyl-cellulose is present after growth on cellobiose as determined viscosmetrically. The pH optimum was about 7.0 with a range of 5.0 to 9.5. The optimum temperature was 30° C with a range of from 25° C to 35° C. The organism did not produce catalase, form indol, reduce nitrate, form H_2S or produce acetylmethyl carbinol. Gelatin was not used and no growth occurred on it. Citrate served well as the sole source of carbon. Ammonium phosphate did not serve as a sole source of nitrogen, and ammonia production in the presence of amino acids was very slight.

Fermentation of carbohydrates showed acid and no gas was formed from cellobiose, glucose, sucrose or maltose. Only scant growth occurred on glucose. Growth occurred on mannitol but no acid and gas were formed. Growth occurred on starch, fructose, galactose, mannose, lactose. No growth occurred on ribose, glucuronic acid or methyl-glucoside.

The products of fermentation from cellobiose included CO_2, volatile acids, volatile neutral compounds, non-volatile neutral compounds, non-volatile acids and gluconic acid.

The source was fresh bovine feces, and the probable habitat is dung or soil.

The only satisfactory classification is in the genus Cellvibrio, but no species name could be assigned (Breed, 1947). The organism has in common with the Bacteroides the possibility that it may have L variation. Since the older species descriptions in the genus Cellvibrio are not very comprehensive the species name might be either ochraceus variety cellobiopreferans or a new species named cellobiopreferans or gilvus. Since the species are differentiated largely on the color produced on cellulose agar the species name gilvus is proposed.

Decomposition of Cellulose by Cellvibrio gilvus.

One milliliter of a 24-hour culture was inoculated into each of three 250 ml flasks containing 100 ml of Fuller and Norman's basal medium with 0.5% Difco yeast extract at pH 7.0 and about 0.1% filter paper cellulose ground for 72 hours in a ball mill. These flasks and three uninoculated controls were incubated at 30° C for ten days. The cells were then hydrolyzed by making the final mixture 0.5 N with NaOH and autoclaving at 121° C for 30 minutes. The cellulose was recovered in tared porous glass filters, washed, dried and weighed. The results given in Table 5 show that this organism possesses rather strong capacity to decompose cellulose even in stagnant culture.

TABLE 5

CELLULOSE DECOMPOSITION BY CELLVIBRIO GILVOUS IN TEN-DAY STAGNANT CULTURES

	Sterile Controls				Inoculated Flasks			
	1	2	3	Avg.	1	2	3	Avg.
Final Cellulose (mg)	116.3	111.2	116.8	114.8	38.4	47.2	37.9	41.2
Cellulose Digested (mg)	0.0	0.0	0.0	0.0	76.4	67.6	76.9	73.6
Cellulose Digested (%)	0.0	0.0	0.0	0.0	66.5	59.4	67.1	64.3

Optimum Growth Temperature of Cellvibrio.

In order to determine growth quantitatively by the turbidimetric method, the salt content of Fuller and Norman's medium was reduced to avoid a precipitate which ordinarily forms. No apparent reduction in growth was seen and the broth medium was clear. This medium contained $NaNO_3$, 0.5 g; K_2HPO_4, 0.5 g; KCl, 0.3 g; $MgSO_4$, 0.3 g; and Difco yeast extract 3.0 g. Tubes containing 9 ml of medium were sterilized by autoclaving at 15 pounds for 15 minutes, and 1 ml of a 3% cellobiose solution that had been sterilized by filtration through a Morton sintered glass filter was added aseptically to each tube. These tubes were inoculated with 0.1 ml of a 24-hour culture grown on cellobiose broth. Growth was then measured on triplicate tubes as the increase in optical density in a Bausch and Lomb photoelectric colorimeter at 415 millimicrons zeroed against a sterile broth control.

Growth curves obtained during incubation at the different temperatures are illustrated in Figure 1. It can be seen that the optimum growth temperature was about 30° C. The organism has a rather wide growth-temperature range from about 20° to 40° C.

Extracellular Fermentation Products of Cellulose.

Identification of the sugars produced by Cellvibrio growing on cellulose was accomplished by paper

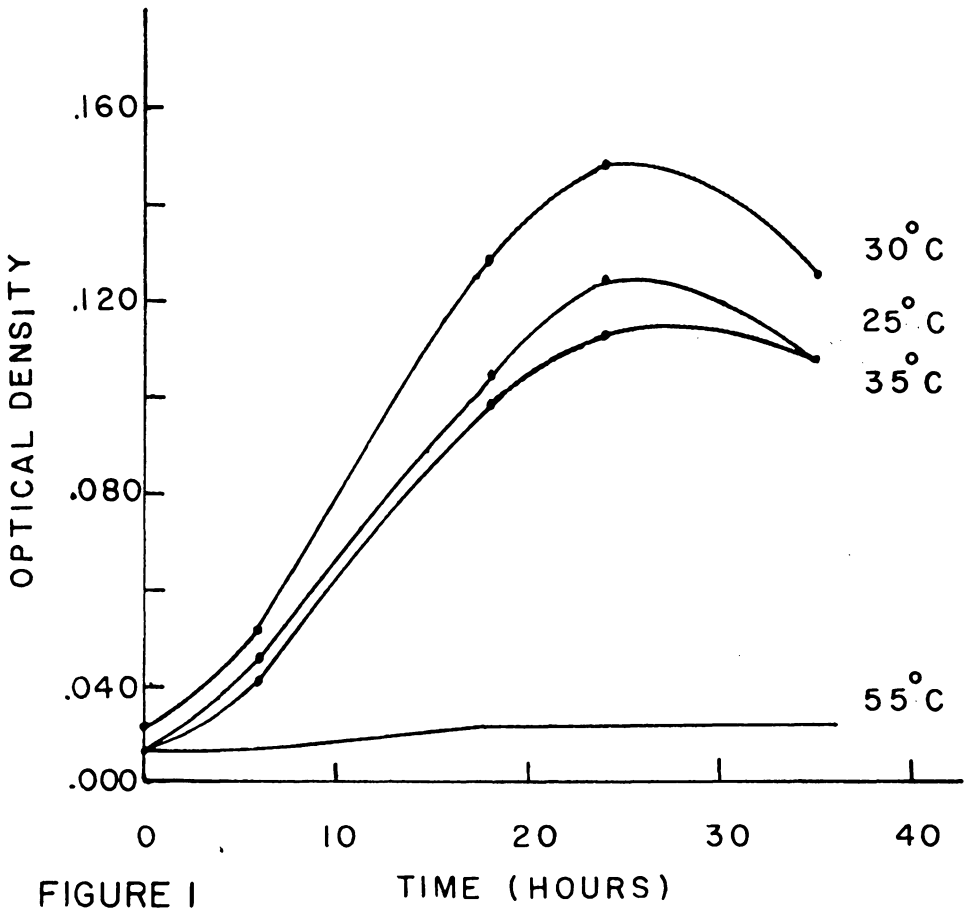


FIGURE I

TEMPERATURE-GROWTH RELATIONSHIP OF CELLVIBRIO GILVUS IN CELLOBIOSE BROTH

chromatography. Whatman No. 1 chromatography paper was spotted with 10 lambda of 0.01 M solutions of known cellobiose and of glucose as standards. Twenty and 40 lambda spots of four and ten day old cellulose broth cultures were applied after removal of cellulose and cells by filtration. The solvent system was isopropanol, water, acetic acid (6-3-1 volume ratio) and the spots were developed with 1% benzidine in acetic acid and heated at 110° C for 20 minutes. The R_f values for the compounds are presented in Table 6.

A definite spot corresponding to the R_f of cellobiose appeared in both cases but no glucose spot was detected even when 1.0 ml of the medium was spotted. It appears that either cellobiose is the primary product of the extracellular enzymes or that glucose is quantitatively taken up by the cells. Other spots did appear, but their identity is obscure other than that they were not glucose.

Effect of Vitamins, Amino Acids and Trace Minerals on the Growth of Cellvibrio gilvus.

A basal medium consisting of $NaNO_3$, 0.5 g; KCl, 0.25 g; $MgSO_4$, 0.25 g; and K_2HPO_4, 0.5 g per liter of distilled water was prepared at pH 7.5. Twenty milliliter portions were placed in each of ten flasks and 2.0 mg of glucose or cellobiose was added per milliliter of solution. To these flasks were then added several treatments. The trace mineral supplement was about 1.0 mg/l of $CaCO_3$ as the

TABLE 6

PAPER CHROMATOGRAPHIC MOVEMENT OF SUCARS ACCUMULATING IN CELLULOSE CULTURES

Sample	R_f Values x 100		Unknown Spots
	4-day Culture	10-day Culture	
Glucose	76.5	75	
Cellobiose	59.8	60.5	
Cellulose culture (20 λ)	59.8	60.0	73 - (green spot)
Cellulose culture (40 λ)	59	-	

Solvent: Isopropanol, acetic acid, H_2O, (6-3-1)

trace mineral carrier in addition to a trace of $Fe_2(SO_4)_3.(NH_4)_2SO_4$ as a source of iron. Yeast extract was added at the level of 0.1%. Amino acids were added as enzymatic casein hydrolyzate at the 0.5% level. The vitamin supplement contained 1 mg/l of thiamine hydrochloride, riboflavin, para-aminobenzoic acid, niacin, biotin, pantothenic acid, pyridoxal, and folic acid. Tubes containing 5.0 ml of broth were inoculated with 0.2 ml of a 24-hour culture and incubated at 30° C. Growth was measured by the increase in turbidity in the Klett-Summerson photoelectric colorimeter against a sterile broth blank.

The data are given in Table 7. The addition of trace minerals made no real difference in the growth of this organism; however, a very slight improvement in growth was detected. The essentiality of amino acids or peptides is amply demonstrated. Yeast extract was required for growth but it may be replaced by the addition of the vitamins and amino acids. Much better growth was obtained by addition of these supplements than with yeast extract. That amino acids were serving primarily as a nitrogen source rather than as an energy source was suspected since only a small amount of ammonia (11.5 ug/ml) was formed in 30 hours.

TABLE 7

EFFECT OF VITAMINS AND AMINO ACIDS ON THE GROWTH OF CELLVIBRIO GILVUS IN BROTH MEDIUM AT pH 7.5 AND 30° C AS MEASURED IN KLETT-SUMMERSON UNITS OF TURBIDITY AFTER 30 HOURS

Ingredients*				Turbidity	
				Substrates	
Trace Minerals	Yeast Extract	Vitamins	Amino Acids	Glucose	Cellobiose
0	+	0	0	90	74
+	0	0	0	27	24
+	+	0	0	94	78
+	0	+	0	50	47
+	0	+	+	119	122

* Ingredients described on Page 77.

Carbon Distribution in the Fermentation Products.

The inoculum for the fermentation was prepared by using a 2% inoculum from a 24 hour culture on cellobiose agar after washing into sterile 0.85% NaCl solution. The composition of the fermentation culture medium is presented in Table 8. Cellobiose was sterilized by filtration in the Morton filter and added to the autoclaved basal medium.

It was found that a small quantity of yeast extract in the magnitude of 100 ug was not sufficient to produce maximum growth, but upon addition of .01% enzymatic casein hydrolyzate and the vitamin mixture described in Table 8, a very heavy growth was obtained. In addition, sodium bicarbonate was added as a source of CO_2 in the event the organism might have a CO_2 requirement.

The fermentation train used was basically as described by Pelczar, et al. (1955). The CO_2 produced was absorbed in ascarite tubes and determined gravimetrically. The fermentation flask was incubated at 32° C for approximately 72 hours, whereupon all dissolved carbon dioxide was released by titrating with sulfuric acid to pH 4 and continuing aeration for five minutes. The final volume of the fermentation mixture was adjusted to 525 ml and 200 ml were subjected to the fractionation procedure described in Figure 2.

Carbon analyses were then carried out by a wet digestion method (Van Slyke et al., 1951, 1954) in which

TABLE 8

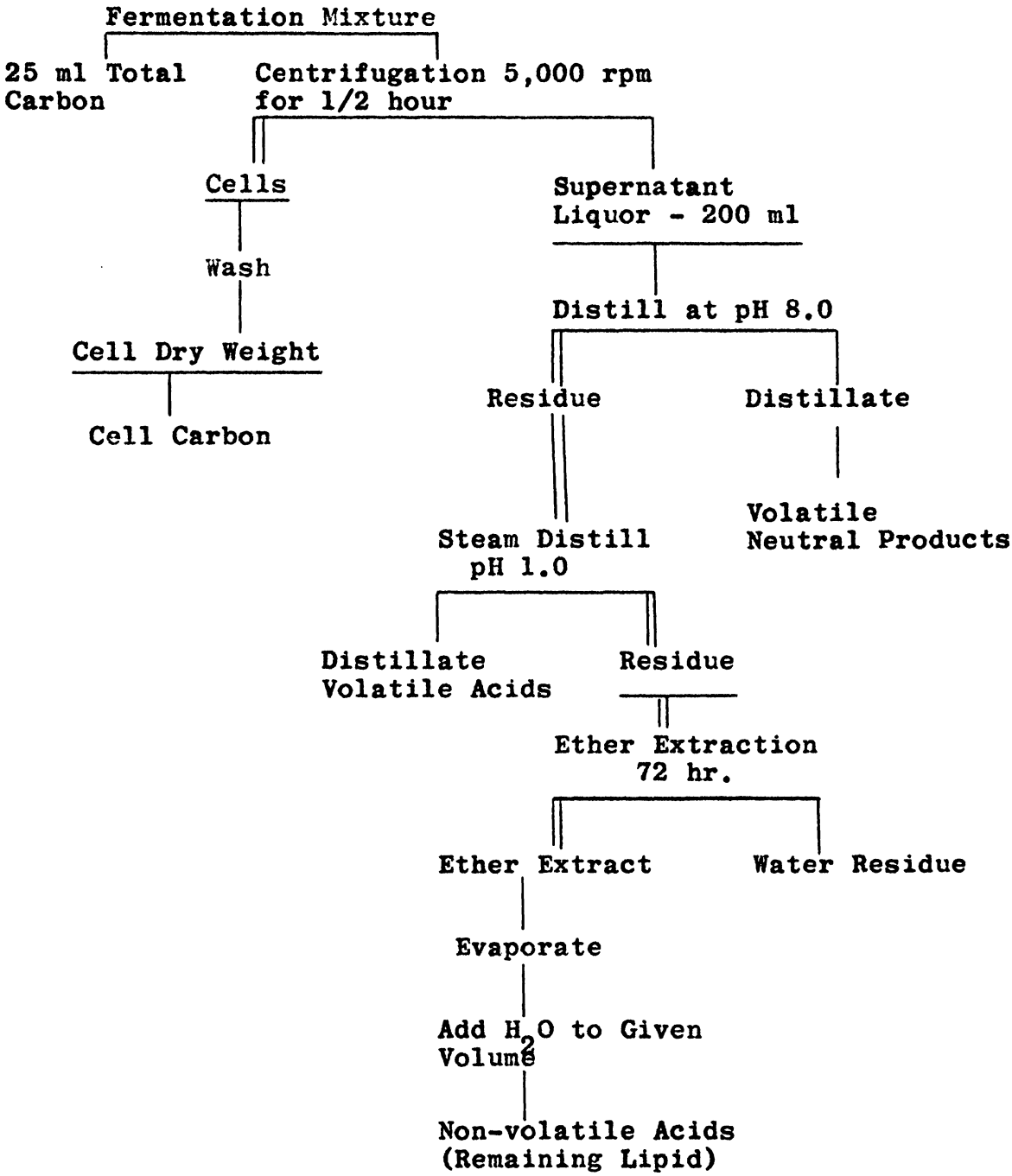
COMPOSITION OF MEDIUM USED TO DETERMINE CARBON BALANCE

$NaNO_3$	0.5 g
$MgSO_4$	0.25 g
KCl	0.25 g
Yeast extract (DifCo)	0.05 g
K_2HPO_4-KH_2PO_4 Soln.	500 ml (.067M at pH 7.0)
Casein hydrolyzate(enzymatic GBI)	0.05 g
Cellobiose	5.0 g
$NaHCO_3$	0.03 g
Vitamin mixture	1 ml/100 ml media
Thiamin	10 mg
Riboflavin	10 mg
Pyridoxal	2 mg
Pantothenate	10 mg
Niacin	10 mg
PABA	2 mg
Biotin	0.1 mg
Folic Acid	0.1 mg
H_2O (dist.)	100 ml

pH = 7.0

FIGURE 2

FRACTIONATION SCHEME FOR FERMENTATION PRODUCTS



the oxidizing reagent was an anhydrous mixture of chromic, iodic, phosphoric and sulfuric acids. The carbon dioxide was collected in standard $Ba(OH)_2$ and the excess $Ba(OH)_2$ was titrated with standard acid. The carbon in milligrams was calculated from the equation

$$\frac{12}{2} \times \left(\begin{array}{l}\text{ml of std. acid} \\ \text{to titrate blank}\end{array} - \begin{array}{l}\text{ml of std. acid} \\ \text{to titrate sample}\end{array}\right) \times \begin{array}{l}\text{Normality} \\ \text{of acid}\end{array} = \text{mg Carbon}$$

The accuracy of the carbon analysis method used was demonstrated when several glucose analyses containing 1 mg per ml yielded 0.997 to 1.01 mg carbon. Carbon analyses were carried out on the fractions described in Figure 2 and these data are presented in Table 9. The total cell dry weight per 500 ml of medium was 334.9 mg obtained from triplicate analyses of 50 ml portions. Cell carbon analyses showed that the carbon content on a dry weight basis was 44%. The percentages of carbon recovered in the combined fractions, fermentation mixture and cell free extract plus cells are given in Table 9. The presence of an aldehyde or ketone in the neutral volatile fraction was indicated by formation of an osazone with 24-dinitrophenylhydrazine.

The data suggest that the organism is not highly efficient in obtaining energy from cellobiose since a large percentage of end products were not completely oxidized.

TABLE 9

CARBON DISTRIBUTION IN FERMENTATION PRODUCTS FROM CELLOBIOSE

Fractions From Fermentation	Carbon mg/500 ml	Percent Carbon
CO_2	141.02	12.5
Cells	146.46	13.0
Volatile neutral products	216.45	19.2
Volatile acids	119.17	10.6
Non-volatile acids	279.72	24.9
Water residue	214.04	19.8
Total	1,116.86	
Total carbon in control flask	1,257.2	
Total carbon after fermentation (does not include CO_2)	960.81	
Cell-free liquor	761.25	

The occurrence of significant amounts of carbon in the several fractions suggested that end products included volatile and non-volatile acids, aldehydes or ketones, alcohol and CO_2.

It was difficult to limit the character of the carbon substrate since both vitamins and casein hydrolyzate were necessary for obtaining heavy growth. However, the non-cellobiose carbon amounted to about 40 mg (theoretical) or about 2% of total carbon in the fermentation medium. The 44% carbon per cell dry weight falls in the usual range of cell dry weight carbon content.

Comparison of the Growth of Cellvibrio gilvus on Glucose and Cellobiose.

Buffer solutions of 0.067 M K_2HPO_4 and KH_2PO_4 were admixed in proportions to give solutions with pH values of 5.0, 5.5, 6.0, 6.5, 7.0 and 7.5. For each 250 ml of buffered solution $NaNO_3$, 0.125 g; KCl, 0.05 g; $MgSO_4$, 0.05 g; and yeast extract, 0.25 g were added. The sugars were sterilized by filtration and added aseptically as aqueous solutions to the tubed media to give a final concentration of 0.05 M on a hexose basis. The matched tubes were then read for changes in Klett-Summerson readings with the blue filter against the broth blanks. The tubes contained 5 ml of medium and were inoculated with 0.1 ml of a 24-hour culture. Tubes containing cellobiose were inoculated with cellobiose-grown cells and

those containing glucose were inoculated with a glucose-grown inoculum. These inocula were aseptically standardized at the same optical density. Controls containing no added sugar were run to determine the difference caused by the yeast extract or substances carried over in the inoculum.

The data are illustrated graphically in Figure 3, in which glucose and cellobiose treatments at pH 7.0 are shown. Growth on cellobiose ranged from 30% to 46% more than on glucose.

The concentrations of the substrates provided the cells with the same amount of carbohydrate on a hexose basis. The same relative differences in growth have been obtained several times. Whether the inoculum was from cellobiose or glucose-grown cells made no apparent difference in the growth response except that cellobiose-grown inocula carried over enough substrate when transferred to glucose media to give a transitory growth response like that seen in the control curve. It can also be seen that eventually the growth rate in glucose broth approaches that obtained on cellobiose.

Effect of pH on Growth in Glucose and Cellobiose Broth.

The methods and procedures in this experiment were identical to those described in the foregoing experiment. The procedure for preparing the tubes of broth medium at

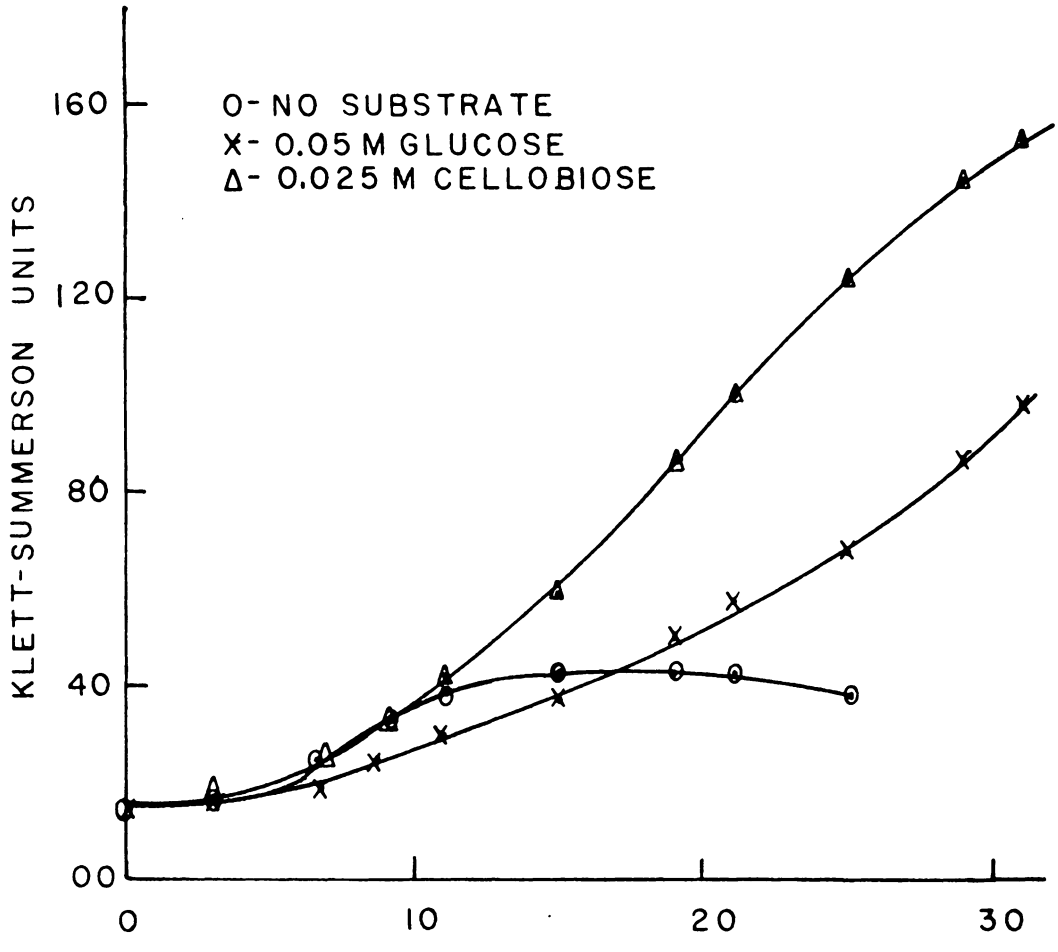


FIGURE 3

GROWTH OF CELLVIBRIO GILVUS IN BROTH MEDIA CONTAINING EQUIVALENT AMOUNTS OF GLUCOSE AND CELLOBIOSE

the different pH's was carried out in the following manner.

Buffer solutions were prepared by mixing 0.067 M K_2HPO_4 and KH_2PO_4 solutions and the pH adjusted using a pH meter. The salts and yeast extract were added in the same concentrations as before and the solutions were sterilized by autoclaving for 15 minutes at 12 pounds of pressure/square inch. The sugar solutions were sterilized separately by filtration and added aseptically to the sterile broth medium to give a final concentration of 0.05 M glucose or 0.025 M cellobiose.

Five milliliters of this broth was then transferred aseptically into matched colorimeter tubes and each tube was inoculated with 0.1 ml of an 18-hour old culture of Cellvibrio grown on glucose or cellobiose depending on the medium inoculated. Both inocula were standardized turbidimetrically. The tubes were then incubated at 30° C in triplicate with triplicate control uninoculated tubes.

The results shown in Figures 4 and 5 demonstrate the pH ranges and optima for growth. The optimum pH for growth on glucose was 6.5 with an optimum range of 6.0 to 7.0. Cellobiose broth supported the best growth at pH 7.0. This range also determined previously was from 5.5 to 9.5. The different pH optima for these two sugars may indicate the pH optima for separate enzymes in the metabolism of glucose and cellobiose.

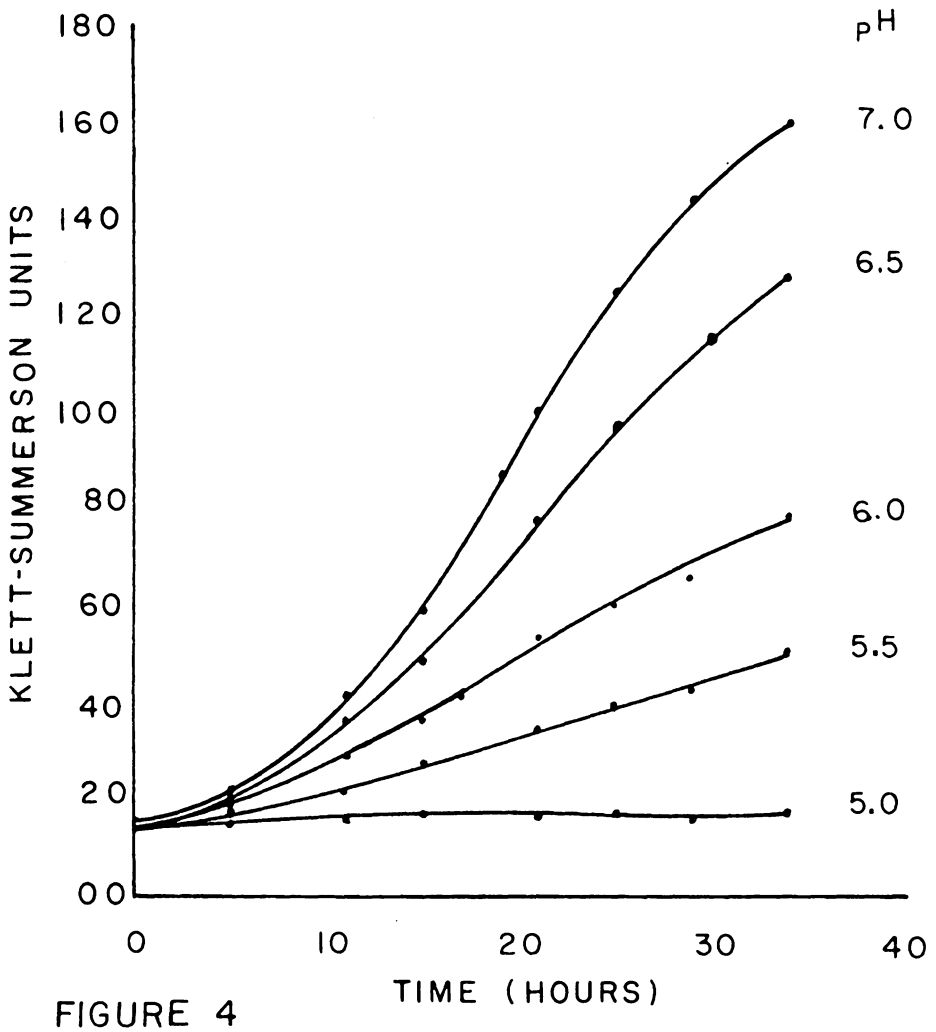


FIGURE 4

EFFECT OF pH ON GROWTH IN CELLOBIOSE BROTH AT 30° C

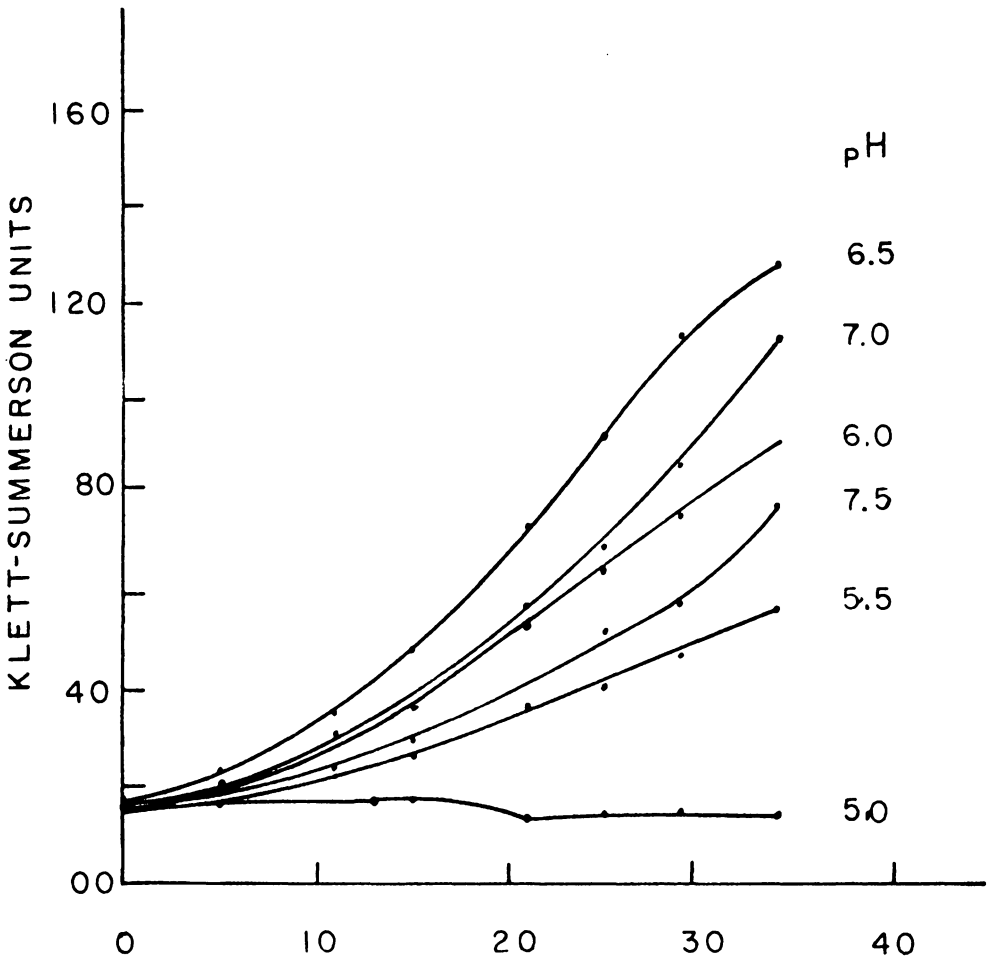


FIGURE 5

EFFECT OF pH ON GROWTH IN GLUCOSE BROTH AT 30° C

Evidence for the Ability of Cellvibrio to Grow on Glucose.

The organism was transferred from cellulose agar into glucose broth tubes and incubated at 30° C. Growth appeared after 24 hours. These cultures served as inocula and a loop of the suspension was transferred again into glucose broth and after growth occurred it was repeated and a transfer also made into cellobiose broth. Growth resulted in both media and it was thus found that the organism could use the glucose substrate, however slowly, and was not subsisting on the other substrates transferred in the inoculum. The organism also retained the ability to grow rapidly on cellobiose after several generations on glucose indicating a stable constitutive enzyme system for metabolizing cellobiose.

Oxidation of Glucose and Cellobiose by Intact Cells.

The lag in growth of Cellvibrio on glucose had implied the possibility of an adaptive hexokinase or a surmountable permeability barrier. In order to test these possibilities respiration experiments were conducted to determine if glucose was oxidized by an adaptive system. Cells were grown on cellobiose-yeast extract-mineral salts-broth for 24 hours with aeration, harvested by centrifugation and washed two times in 0.067 M potassium phosphate buffer at pH 7.0. The suspension was filtered through glass wool to dislodge

clumps of cells. The turbidimetrically-standardized suspension was used immediately after preparation. The Warburg vessels contained 1.0 ml of cell suspension, 1 ml of 0.028 M glucose or 0.014 M cellobiose solution or 0.5 ml of each, 0.8 ml of buffer in the main compartment and 0.2 ml of 10% KOH in the center wells. All flasks were pre-equilibrated for 15 minutes. Substrate was placed in side arms and the temperature was 39° C.

The results in Figure 6 show immediate and rapid oxidation of glucose by intact resting cells grown on cellobiose. No evidence of adaptive oxidation of glucose is seen. The oxidation of glucose was approximately 10% less than that obtained with cellobiose. These data minimize the possibility of either impermeability or adaptation to glucose.

Furthermore, a comparison of the oxidation rates of a mixture of equivalent amounts of cellobiose and glucose to the oxidation of equivalent amounts of either sugar alone was made. The approximate ratios of oxygen assimilation for glucose, cellobiose and a mixture of both sugars were 100:110:140. Thus it would appear that two metabolic pathways were operating simultaneously or that a catalytic effect of one sugar on the other was functioning. In a different experiment it was found that minute quantities of cellobiose gave no significant stimulation of glucose

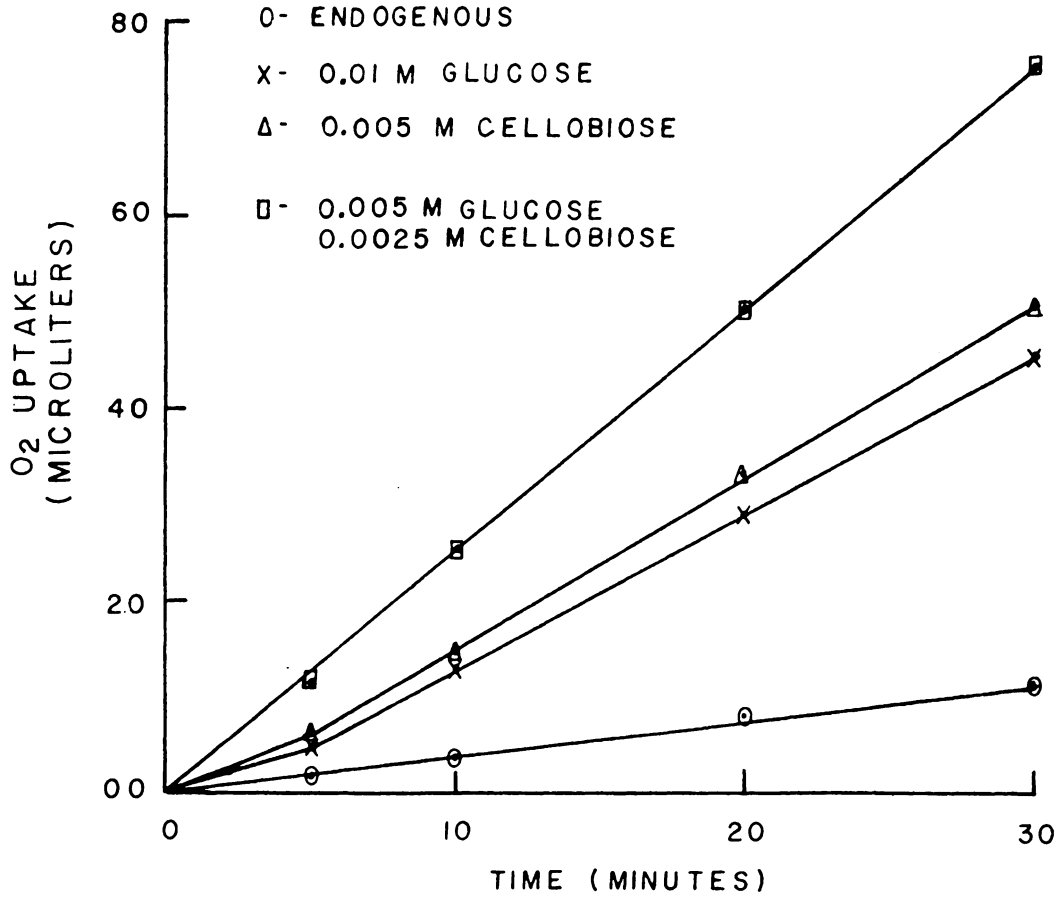


FIGURE 6

OXIDATION OF EQUIVALENT CONCENTRATIONS OF GLUCOSE AND CELLOBIOSE BY INTACT CELLS OF CELLVIBRIO GILVUS AT pH 7.0 AND 39°C GROWN ON CELLOBIOSE

oxidation, and so the independent pathways hypothesis seem more plausible.

Esterification of Inorganic Phosphate by Intact Cells in the Presence of Glucose and Cellobiose.

Previous experiments had shown that cellobiose affords more rapid growth than could be obtained on glucose. The slow growth on glucose suggested the possibility that either an adaptive hexokinase or other adaptive glucose-utilizing enzyme systems might be involved; however, the immediate and rapid oxidation of glucose by intact resting cells grown on cellobiose eliminated the possibility of adaptive oxidation of glucose. On the other hand, the cells did oxidize cellobiose at a rate about 10% greater than that of glucose whereas growth differences were very pronounced. It thus appeared possible that the energy yield from the oxidation of glucose might be restricted.

In order to assess this possibility, cells grown on cellobiose were starved to reduce the organic intracellular phosphates and were incubated aerobically in phthalate buffer at pH 6.5 and 30° C in the presence of glucose, cellobiose and a mixture of the two sugars. The intracellular phosphate distribution was then determined.

Cells were grown in mass on cellobiose-yeast extract-mineral salts agar slants for 24 hours and then washed from the agar with 0.005 M potassium phthalate buffer or 0.85% NaCl solution. Agar slants were used to keep the

phosphate, present in the medium, as low as possible in the cell mass. The cells were washed and centrifuged until the supernatant was free of inorganic, extracellular phosphate. This usually required three or four washings. These cells were then filtered through glass wool to remove any agar or cell clumps that might introduce error in the analyses of samples.

The reaction mixtures contained 15 ml of a turbidimetrically standardized cell suspension in 0.005 M potassium phthalate buffer at pH 6.5 and 5 ml of a 0.04 M glucose or a 0.02 M cellobiose solution giving a final concentration of 0.01 M glucose or 0.005 M cellobiose. The mixtures were incubated on a rotary shaker at 30° C in 125 ml Erlenmeyer flasks with indented bottoms to provide good aeration by breaking the liquid surface. Aliquots were taken at zero, 15 and 30 minute intervals. The method used in determination of organic phosphate is described in the Methods section.

The data obtained for the esterification of inorganic phosphate by intact resting cells are given in Table 10. Here it can be seen that respiration of both glucose and cellobiose results in phosphate esterification and that the accumulation of organic phosphates is greater in the case of glucose or a mixture of the two sugars than in the case of cellobiose alone. This elevation of intracellular organic phosphates in the presence of glucose

TABLE 10

ESTERIFICATION OF INORGANIC PHOSPHATE BY RESTING CELLS IN THE PRESENCE OF EQUIVALENT CONCENTRATIONS OF GLUCOSE, CELLOBIOSE AND BOTH SUGARS

Time (Min.)	Endogenous	Cellobiose 0.005 M	Glucose 0.01 M	Cellobiose, 0.0025 M Glucose, 0.005 M
		Total Phosphorus (µg/5 ml Cell Suspension)		
0	56.85	54.85	45.6	45.1
15	56.80	55.1	47.6	46.6
30	51.25	54.8	47.6	46.0
		Inorganic Phosphorus		
0	17.5	17.3	17.5	17.0
15	17.4	14.65	10.25	9.0
30	17.25	15.50	6.88	6.5
		P_o Change (Micrograms per 5 ml Cell Suspension*)		
15	- 0.90	≠ 2.65	≠ 7.25	≠ 8.00
30	≠ 0.25	≠ 1.80	≠ 10.62	≠ 10.50

* 1 ml suspension = 110 µg N; cellobiose-grown cells.

may mean that the further metabolism of certain phosphate esters is impeded by slow further metabolism of phosphate esters different from those formed from cellobiose.

It is evident, at least, that cellobiose-grown cells not only oxidize glucose rapidly but also esterify inorganic phosphate in the course of both glucose and cellobiose oxidation. Thus the hexokinase deficiency hypothesis does not appear to be the basis of the disaccharide preference.

One explanation for the low increase in organic phosphates from cellobiose is that most of the organic phosphates of the pathway for cellobiose oxidation may have been esterified already because the cells were grown on cellobiose. The relatively larger increase of esters with glucose may result from a different pathway from that involved in cellobiose metabolism. Another reason may have been that the enzyme reactions for esterification of cellobiose were so rapid that formation of the esters was almost instantaneous but the esterification of glucose actually lagged enough to be measured.

Sugar Utilization and Acid Production of Cellvibrio gilvus.

The question next desired to be answered was whether the entire cellobiose molecule was used more rapidly than glucose or whether glucose would accumulate after initial hydrolysis or phosphorolysis of the disaccharide. Also

during the course of pH-growth studies it was observed that some discrepancy occurred in the amount of acid produced from glucose and cellobiose, so quantitative studies were made to determine if this was a real difference.

In order to compare the utilization of glucose and cellobiose by intact cells and to compare the relative acid production from each sugar, the following experiment was carried out. The basal medium used for growth contained $NaNO_3$, 0.125 g; KCl, 0.05 g; $MgSO_4$, 0.05 g; enzymatic casein hydrolyzate, 0.005%; yeast extract, 0.125 g (0.05%); 0.04 M substrate in 250 ml of 0.067 M potassium phosphate buffer at pH 6.5 and at pH 7.0. The sugar solutions were sterilized by filtration in a Morton sintered glass filter to prevent partial hydrolysis of the disaccharide and decomposition of glucose. Flasks containing 25 ml of sterile medium were prepared at both pH 6.5 and 7.0 containing either glucose or cellobiose. The inoculum was 0.1 ml of a 24-hour agar culture suspended in 0.85% NaCl solution. The flasks were then incubated in stagnant culture for 36 hours at 30° C. Control flasks at zero time were also sampled.

After 36 hours, 5 ml samples were removed and reducing sugar was determined by the Somogyi titrimetric copper reduction method (1945). To analyze the cellobiose it was found necessary first to remove any glucose that

might be formed from the cellobiose before determining residual cellobiose. This was done by treatment with Baker's yeast to ferment the glucose as described later. The remaining 20 ml of culture was then titrated with standard 0.01 N NaOH solution to pH 8.5.

The data obtained by comparison of the utilization of glucose and cellobiose and the acid production per 100 mg and per millimole are presented in Table 11. The ability of this organism to utilize cellobiose in preference to glucose is well illustrated since about 130% more cellobiose was utilized at pH 6.5, and at pH 7.0 about 590% more was used. These data were supporting evidence that a direct disaccharide reaction was involved and further established the disaccharide preference phenomenon. The data also support the pH growth data that the optimum for glucose is 6.5 and for cellobiose, 7.0.

Acid production differed in about the same magnitude at both pH values. From 50 to 58% more acid/unit wt. of sugar was formed from glucose. Even on a millimole basis 30% more acid was formed from glucose. The difference in acid production pointed out essentially that glucose could yield more acid than cellobiose and that a wasteful process might be involved. Moreover, alternate pathways for the metabolism of the sugars or differences in the reaction kinetics might be operating.

TABLE 11

COMPARISON OF GLUCOSE AND CELLOBIOSE UTILIZATION AND ACID PRODUCTION BY CELLVIBRIO GILVUS AT pH 6.5 AND 7.0 AFTER 36 HOURS INCUBATION AT 30° C

	pH 6.5		pH 7.0	
	Glucose	Cellobiose	Glucose	Cellobiose
Mg sugar utilized/ 20 ml media	52.0	118.0	15.4	107.4
ME acid/100 mg sugar	0.378	0.216	0.298	0.176
ME acid/mM sugar	0.680	0.522	0.556	0.426

Data represent averages of triplicate analyses.

Effect of Removal and Addition of Inorganic Phosphate on the Respiration of Cellvibrio.

Previous experiments indicated that a direct reaction was responsible for the rapid utilization of cellobiose. Indeed it was probable that a phosphorylase was involved. To find if cellobiose utilization was the result of hydrolysis or phosphorolysis the effect of inorganic phosphate upon the respiration of the substrate was determined. In addition it was desirable to know if the extraction of inorganic phosphorus would reduce the intracellular concentration enough to affect phosphorylation.

Cells were grown on a cellobiose broth with strong aeration at 30° C and harvested by centrifugation. These cells were then washed four times in 0.85% saline or 0.067 M potassium phthalate buffer at pH 6.5 to remove the inorganic phosphate. The supernatants were examined for inorganic phosphate.

The Warburg vessels contained 1.8 ml of the standard cell suspension in the same phthalate buffer and 1 ml of glucose or cellobiose solution at 0.01 M concentration in buffer solution. Center wells contained 0.2 ml of 10% KOH solution. One milliliter of a 0.01 M KH_2PO_4 solution was added as the inorganic phosphate source in some of the flasks and distilled water to the others to make a total flask volume of 3.0 ml. The flasks were

shaken at 34° C and pre-equilibrated to temperature for 15 minutes. The results are shown in Table 12. In this experiment the cells were not starved but used immediately after washing.

It can be seen from the data that addition of inorganic phosphate to cells washed free of phosphate increased the respiration of cellobiose by about 53% and that addition of phosphate to flasks containing glucose made no apparent difference in respiration. Finally, the respiration of a mixture of glucose and cellobiose in the presence of phosphate was partially additive and greater than that obtained from either substrate alone. This was shown before and taken as evidence for divergent pathways.

Utilization of Glucose and Cellobiose by Cell-Free Extracts.

This experiment was carried out to determine if the disaccharide preference demonstrated for growth, respiration, and utilization of sugar with intact cells was characteristic of the intracellular enzymes. Should this be the case the possibility of differences in permeability to the sugars and in the active transport enzymes at the cell barrier might be rejected and the cause could then be ascribed to the enzyme systems within the cell.

In preliminary cell-free experiments it was evident that large differences existed in sugar utilization rates.

TABLE 12

CARBOHYDRATE OXIDATION BY INTACT CELLS WASHED FREE OF INORGANIC PHOSPHATE AFTER 55 MINUTES AT 30° C

Substrate	O_2 Uptake (Microliters)
None	47
Cellobiose	59
Glucose	84
Cellobiose + P_i	86
Glucose + P_i	85
Glucose + Cellobiose + P_i	100

Furthermore, 1 ml of a reaction mixture containing cellobiose, enzyme and phosphate was evaporated, extracted with pyridine and chromatographed in isopropanol, water, acetic acid (6,3,1). After spraying the paper with a solution of benzidine, 0.5 g; acetic acid, 200 ml; ethanol, 80 ml; and heating at 110° C for 20 minutes, a definite glucose spot was obtained indicating that some of the cellobiose was converted to glucose. On the basis of this glucose formation, it was then necessary to remove this reducing sugar by yeast fermentation from samples to which cellobiose was added in order to obtain accurate values for cellobiose utilization when measured by the Nelson Somogyi microcolorimetric method (1944).

Differential sugar analysis (Pan, et al. 1953) for cellobiose was accomplished using a suspension of 10 g of Fleishman's bakers yeast/100 ml water washed five times by centrifugation. A bottom layer of starch was first removed by taking off the yeast top layer. It was found that 0.5 to 1.0 ml of this suspension when incubated with a sample containing 100 µg glucose for 30 minutes at 30° C would remove from 92 to 95% of the glucose. On the other hand, cellobiose reducing values before and after yeast treatment did not change. The yeast and protein were removed prior to analysis for reducing sugars by addition of $ZnSO_4$ and $Ba(OH)_2$ solutions which, in addition, removed the hexose phosphates (Somogyi, 1945). Treatment

with $Ba(OH)_2$ and $ZnSO_4$ was shown to be necessary since protein, ATP and hexose phosphates increased the reducing values greatly.

The Nelson colorimetric method was also affected by the presence of Tris buffer but the effect was shown to be subtractable by using a Tris-containing blank.

Preparation of Cell-Free Enzymes. Cells were grown on a yeast extract, mineral salts medium with 0.05% casein hydrolyzate with glucose or cellobiose as substrate. Vigorous aerobic conditions were provided by use of a sparger stone connected to an aspirator. Cultures were grown at 30° C from 16 to 24 hours. Cells were washed two times in M/30 Tris buffer at pH 7.0 in the cold after harvesting at 4000 rpm in the Servall refrigerated centrifuge for 30 minutes. The wet cells were then either stored by freezing at -16° C or used as needed. These cells were ground for five to ten minutes at 0° C with two volumes of alumina and extracted with five volumes of M/30 Tris buffer at pH 7.0. Alumina and cell debris were removed by centrifuging at 12,000 rpm (Servall SS-1 head) at 0° C for 20 to 30 minutes. The clear supernatant was stored at -16° C.

One milliliter of the soluble enzyme preparation for cellobiose-grown cells containing 0.94 mg protein/ml (Lowry et al. 1951) or from glucose-grown

cells containing 0.71 protein/ml was allowed to react with 0.4 ml of sugar solution (glucose or cellobiose (3.0 mg/ml)) with additions of 0.6 ml ATP (3.3 micromoles) or 0.2 ml potassium phosphate (3.3 micromoles P) adjusted to pH 7.0 and 2 ml volume. The reactions were carried out at 30° C for 60 minutes and stopped by boiling for one minute at 100° C. Protein was removed and 1.0 ml samples were analyzed for reducing sugar by the Nelson colorimetric method (1944).

The results are given in Table 13. It appears that ATP is required for the utilization of glucose by these soluble enzymes. Phosphate apparently caused an increase in the reducing value in the presence of glucose and retarded its conversion. The ability of glucose-grown cells to utilize cellobiose better than glucose indicates again that the cellobiose enzyme system is constitutive. Cellobiose conversion is apparently not dependent on ATP. Further evidence is seen for the presence of a cellobiose phosphorylase because of the phosphate stimulation of cellobiose conversion.

Utilization of Sugars by Cell-Free Extracts of Cellobiose-Grown Cells.

Table 14 shows the results obtained from determination of the amounts of sugars utilized by cell-free extracts of

TABLE 13

EFFECT OF ATP AND P_i ON UTILIZATION OF GLUCOSE AND CELLOBIOSE BY CELL-FREE EXTRACTS OF GLUCOSE-GROWN CELLS

Addenda				Change in Reducing Sugar Micromoles/
Glucose	Cellobiose	Phosphate	ATP	Hour/2 ml
0	0	0	0	0.0
+	0	0	0	+ 0.25
+	0	+	0	+ .65
+	0	+	+	- .45
0	+	0	0	- .64
0	+	+	0	- 1.22
0	+	+	+	- .43

TABLE 14

UTILIZATION OF GLUCOSE, CELLOBIOSE AND INORGANIC PHOSPHATE BY CELL-FREE EXTRACTS OF CELLOBIOSE-GROWN CELLS (MICROMOLES/HOUR) AT pH 7.0

Addenda				Amount of Sugar Used (μM/2 ml)	Loss in P_i (μg/2 ml)
Glucose	Cellobiose	Phosphate	ATP		
0	0	0	0	0.0	0.0
+	0	0	0	1.0	0.0
+	0	+	0	0.33	-
+	0	0	+	2.84	10.0
+	0	+	+	2.84	-
0	+	0	0	4.20	80.0
0	+	+	0	7.25	160.0
0	+	0	+	3.10	-
0	+	+	+	3.86	-

cells grown on cellobiose. Other addenda were inorganic phosphate and ATP. Phosphate depressed the disappearance of reducing sugar when added to the glucose reaction, but ATP increased the glucose utilization about three fold. This is good evidence for the presence of a hexokinase in the phosphorylation of glucose. ATP can apparently overcome the inhibition by phosphate.

Stimulation of the utilization of cellobiose by inorganic phosphate was demonstrated also. The almost two-fold increase when inorganic phosphate is added is strongly indicative of a cellobiose phosphorylase. ATP tends to suppress the utilization of cellobiose both in the absence and presence of phosphate.

The important demonstration of this experiment, nevertheless, was that the disaccharide preference was characteristic of the intracellular enzyme systems. Other factors such as differential permeability, adaptive hexokinase formation, hexokinase deficiency and active transport across the cell barrier were thus eliminated as possible explanations of the growth and respiration phenomenon.

Here, then, was a basic stepping stone suggesting a fundamentally enzymatic explanation of the disaccharide preference for growth, utilization and respiration.

Esterification of Inorganic Phosphate by Cell-Free Enzymes in the Presence of Cellobiose.

In conjunction with the foregoing sugar utilization experiment, inorganic phosphate analyses were made on the reaction mixtures at zero time and after 60 minutes. The Fiske-Subbarow micro-method for phosphorus (1925) was used. The micrograms of phosphorus esterified as phosphate are given in Table 14.

These data show that the utilization of cellobiose by soluble enzymes is accompanied by esterification of phosphate. Little if any phosphate was esterified with glucose except in the presence of ATP.

Accumulation of Fructose-6-Phosphate in Cells Grown on Glucose or Cellobiose.

Quantitative studies showed that inorganic phosphate was esterified with cellobiose but not with glucose. The next step was to determine if qualitative differences might exist in the hexose phosphates accumulating in cells grown on the two sugars.

Cells were grown on a medium containing $NaNO_3$, 1 g; KCl, 0.25 g ; $MgSO_4$, 0.25 g; K_2HPO_4, 1.0 g; yeast extract, 5.0 g; enzymatic casein hydrolyzate, 1.0 g; and substrate (glucose or cellobiose) 6.0 g per liter for 48 hours at 30° C. The cells were harvested and washed two times in 0.01 M Tris buffer at pH 7.0. Approximately 2.0 g of wet cells were obtained from 3 liters of each

culture. These cells were extracted with 10% and then with 5% trichloroacetic acid in the cold and the barium salts of the extracted phosphate esters were prepared after barium and alcohol fractionation according to the method of Umbreit et al. (1951). Total phosphorus was determined on the trichloroacetic acid extracts. Cellobiose-grown cells contained approximately 6.5 mg total P/2 g wet cells whereas glucose-grown cells contained only 2.1 mg total P/2 g wet cells.

The barium salts were converted to free acids by the ion exchange method of Wade and Morgan (1954) or by acidifying with H_2SO_4, removing the barium sulfate precipitate and adjusting the pH to 5.0 with NH_4OH. Samples containing 5 and 10 lambda of a solution with 50 mg of the barium salts/5 ml were then spotted on acid- and NH_4OH-washed papers and chromatographed in two solvents, methyl-ethyl-ketone, methyl-cellosolve, ammonia, water (7-2-3-1 volume ratio) and methyl-cellosolve, methyl-ethyl-ketone, formamide (7-3-2).

The R_{Pi} values obtained are given in Table 15. Only one phosphate compound was detected except for a trace of inorganic phosphate. In both solvents tested the compound appeared chromatographically to be fructose-6-phosphate. The methyl-cellosolve, methyl-ethyl-ketone, formamide solvent was previously shown to separate

TABLE 15

PAPER CHROMATOGRAPHY OF HEXOSE PHOSPHATES EXTRACTED FROM INTACT CELLS GROWN ON GLUCOSE AND CELLOBIOSE.

Compound or Sample	R_p Values*	
	methyl-cellosolve, methyl-ethyl-ketone, NH_4OH, H_2O, (7-2-3-1)	methyl-cellosolve, methyl-ethyl-ketone, formamide (7-3-2)
P_i	100	100
F-6-P	241	54
G-1-P	173.5	
G-6-P	154.0	
F-1,6-P_2	4.6	
Phosphate Esters of Glucose-Grown Cells	239	48
Phosphate Esters of Cellobiose-Grown Cells	238	48

* $R_p = \frac{\text{distance compound movement x100}}{\text{distance of phosphate movement}}$

fructose-6-phosphate very well from inorganic phosphate, glucose-6-phosphate, glucose-1-phosphate and fructose-1, 6-diphosphate.

Paper Chromatographic Survey for the Products Formed by Cell-Free Extracts from Glucose and Cellobiose.

A rapid paper chromatographic method for sugars, sugar acids, phosphates and related compounds was employed in an attempt to obtain some information about the intermediates formed by soluble enzymes as employed in the experiment above. Gordon et al. (1956) used the solvent system: isopropanol, pyridine, water, acetic acid (8-8-4-1 vol. ratio) in an ascending system for two hours at 30° C and obtained good separations of many compounds. The system was checked with glucose, cellobiose, glucuronic acid, glucose-1-phosphate and glucose-6-phosphate. Good separations were obtained but the esters moved very slowly.

The reaction mixtures described in the experiment of the utilization of glucose and cellobiose by cell-free extracts were used. Two milliliters of the soluble enzyme preparation containing 0.94 mg protein/ml prepared as given before was allowed to react with 0.2 ml of sugar solution (3.0 mg/ml) with additions of 3.3 micromoles of ATP or 3.3 micromoles of phosphate phosphorus. All solutions were buffered at pH 7.0 with M/30 Tris buffer. The reactions were allowed to proceed

at 30° C for 60 minutes and were stopped by boiling for one minute at 100° C.

The R_f values of the products in these reaction mixtures are shown in Table 16. Glucose is apparently a product formed from cellobiose hydrolysis or phosphorolysis. Spots with low R_f value appeared at approximately the same positions as the hexose phosphates. The R_f value of known glucose-6-phosphate was 0.57. It can be seen from the table that a spot appeared at an R_f value of .036 from the glucose-ATP reaction mixture. The R_f of known glucose-1-phosphate was .15 and spots appeared in this region from the cellobiose reaction mixtures. Thus it was reasonable to attempt to identify the products of these reactions further using more accurate methods of paper chromatography.

Formation of Glucose from Cellobiose by Cell-Free Enzyme Preparations.

The reaction mixtures described in the experiment to determine utilization of glucose and cellobiose were chromatographed on Whatman No. 1 chromatography paper in a solvent system containing butanol, pyridine and water (7-4-1 volume ratio) for 18 hours, sprayed with aniline hydrogen phthalate in butanol and developed by heating for five minutes at 105° C (Block et al., 1952). The R_f values were glucose, 0.71 and cellobiose, 0.38. In all the reaction mixtures containing cellobiose, well-defined

spots with the same R_f as glucose appeared when 20 or 40 lambda volumes were spotted. Only glucose appeared from reaction mixtures containing glucose.

Formation of Glucose from Cellobiose by Cell-Free Enzyme Preparations.

Reaction mixtures from the preceding experiment were spotted on washed Whatman No. 1 papers and chromatographed in a solvent containing methyl cellosolve, methyl ethyl ketone, ammonium hydroxide and water (7-2-3-2 volume ratio) and the phosphate esters developed by the Hanes-Isherwood reagent (1949).

The data obtained for the formation of phosphate esters are given in Tables 17 and 18. A spot corresponding to glucose-6-phosphate appeared from the cellobiose-phosphate

TABLE 16

PAPER CHROMATOGRAPHIC MOVEMENT OF THE PRODUCTS OF SUGAR METABOLISM BY CELL-FREE EXTRACTS OF *CELLVIBRIO GILVUS*

Addenda				R_f Values		
Glucose	Cellobiose	P_i	ATP	Glucose	Cellobiose	Unidentified Hexose Phosphates
0	0	0	0	-	-	-
+	0	0	0	.57	-	.13; .31
+	0	+	0	.61	-	-
+	0	0	+	.60	-	.16; .036
+	0	+	+	.60	-	-
0	+	0	0	.60	.48	.19
0	+	+	0	.61	.45	.10
0	+	0	+	.56	.48	.13
0	+	+	+	.60	.50	-

Solvent: Isopropanol, pyridine, acetic acid, water, (8-8-4-1, volume ratio)

TABLE 17

PAPER CHROMATOGRAPHIC SEPARATION OF HEXOSE PHOSPHATES PRODUCED BY CELL-FREE ENZYMES

Solvent System: Methyl-cellosolve, methyl-ethyl-ketone, ammonium hydroxide, water, (7-2-3-1 volume ratio)

Compound	Distance Traveled cm	R_p Value
Phosphate	15.6	100
Glucose-1-phosphate	27.7	173.5
Glucose-6-phosphate	24.5	154
Fructose-1,6-diphosphate	7.2	4.6
Mixture of above	Same as above	
Reaction mixture Cellobiose Phosphate Cell-free enzymes	15.2; 24.4	97.5; 154

TABLE 18

PAPER CHROMATOGRAPHIC SEPARATION OF PHOSPHATE ESTERS FORMED FROM GLUCOSE AND CELLOBIOSE BY CELL-FREE ENZYME PREPARATIONS

Reaction Mixtures						
Addenda					Time (Minutes)	
Enzyme	Cellobiose	Glucose	Phosphate	ATP	0	60
+	+	0	0	0	None	99; 158
+	+	0	+	0	None	96; 145
+	0	+	0	0	None	97
+	0	+	0	+	None	131

R_p of known compounds on the same chromatograms were P_i, 100; G-1-P, 173; G-6-P, 157.

Solvent: methyl-cellosolve, methyl-ethyl-ketone, ammonia, water, (7-2-3-1)

reaction mixture, but fructose-6-phosphate appears at almost the same position, so either of these is possible. A spot with a lower R_p value was obtained from glucose and ATP and was probably glucose-6-phosphate. ATP retards the movement of other esters on paper chromatograms.

In the previous experiment glucose apparently was not converted to hexose phosphate and so the experiment was repeated using a fresh enzyme preparation to avoid the possible loss of hexokinase activity. Cells were grown in the manner described before on a glucose-cellobiose medium except that the buffer used to extract the enzymes was 0.1 M Tris-HCl at pH 7.0.

The reagents used in these reactions were sugar, 100 micromoles/2 ml; phosphate, 50 μM/2 ml; ATP, 25.0 μM/2 ml plus magnesium ion, 9×10^{-6} M; TPN, 5 μM/2 ml and enzyme, 1.0 ml containing 0.70 mg protein/ml.

Tubes were prepared with the reagents and a portion of the enzyme was boiled before adding to the zero time control tubes. Tubes containing active enzymes were incubated for 60 minutes at 30° C. In tubes containing ATP the pH was maintained as needed by titration with 0.2 N NaOH. The reactions were stopped by placing the tubes in a boiling water bath for two minutes. To examine these reaction mixtures 50 lambda aliquots were spotted on papers and chromatographed for sugars and phosphate esters.

The results obtained by examining for sugars by paper chromatography in a butanol, pyridine, water solvent (6-4-3 volume ratio) for 18 hours and developing the spots with aniline hydrogen phthalate are presented in Table 19. These data showed that glucose was produced as a product from cellobiose but not when ATP or TPN was added. Glucose disappearance required the presence of ATP when using the soluble enzyme preparations.

The data obtained by paper chromatography of the phosphate esters in the above reaction mixtures are given in Table 20. Papers were spotted with 100 lambda of the reaction mixtures and placed in a descending solvent system of methyl-cellosolve, methyl-ethyl-ketone, ammonium hydroxide and water (7-2-3-1 volume ratio). The R_p values were obtained for known esters and for the spots which developed.

The data reveal the formation of glucose-6-phosphate and fructose-1,6-diphosphate from glucose and ATP. With addition of TPN a spot for fructose-6-phosphate appeared. Fructose-6-phosphate was the only hexose phosphate detected in mixtures containing cellobiose and phosphate. With the addition of TPN no fructose-6-phosphate but glucose-1-phosphate and glucose-6-phosphate appeared. With TPN and ATP only the glucose-1-phosphate appeared.

Although certain of the data cannot be explained, it is apparent that glucose can be converted to glucose-6-phosphate and further to fructose-1,6-diphosphate in the

TABLE 19

REDUCING SUGAR FORMATION FROM CELLOBIOSE AND GLUCOSE IN THE PRESENCE OF CELL-FREE ENZYME PREPARATIONS DETERMINED BY PAPER CHROMATOGRAPHY

R_g (glucose) = 100;
R_g (cellobiose) = 70.6;
Others unidentified

	Addenda					R_g Values
Enzyme	Glucose	Cellobiose	Phosphate	ATP	TPN	
+	0	0	0	0	0	0
+	+	0	0	0	0	98.5
+	+	0	0	+	0	29; 23
+	+	0	0	+	+	29; 23
+	0	+	0	0	0	96;70.6; 28; 23
+	0	+	+	0	0	70; 36; 26
+	0	+	+	0	+	70; 32
+	+	+	+	+	0	71; 34; 9.5

Solvent: butanol, pyridine, water (6-4-3, volume ratio).

TABLE 20

PAPER CHROMATOGRAPHY OF PHOSPHATE ESTERS PRODUCED FROM GLUCOSE AND CELLOBIOSE BY CELL-FREE ENZYME PREPARATIONS

	Addenda					R_p values Known Compounds and Products				
Enzyme	Glucose	Cellobiose	P_i	ATP	TPN	G-1-P	G-6-P	F-6-P	F-1,6-P_2	Unknown Products
						187	152	267	39	
+	0	0	0	0	0					
+	+	0	0	+	0	188	132		36	
+	+	0	0	+	+	175	141	294	32	175
+	0	+	+	0	0			294		
+	0	+	+	0	+	189	152			303
+	+	+	+	+	+	189				303

Solvent: methyl-cellosolve, methyl-ethyl-ketone, NH_4OH, H_2O (7-2-3-1, volume ratio).

presence of ATP. Cellobiose can be converted to fructose-6-phosphate only in the presence of inorganic phosphate.

The Effect of ATP, TPN and Glucose on the Formation of Phosphate Esters from Cellobiose in the Presence of Cell-free Enzyme Preparations.

The fact that fructose-6-phosphate accumulates in cells growing on cellobiose and that no fructose-1,6-diphosphate was formed by cell-free preparations from cellobiose but was detected in reaction mixtures containing glucose and ATP led to an experiment to determine whether fructose-1,6-diphosphate would be formed from cellobiose, phosphate, and ATP. It was also desired to find if high glucose concentration would inhibit the cellobiose phosphorylase or result in accumulation of a compound that might be formed prior to glucose-1-phosphate.

The reagents used in these reaction mixtures were cellobiose, 600 micromoles; glucose, 250 micromoles; P (KH_2PO_4) 150 micromoles; ATP, 25 micromoles; TPN, 10 micromoles; and 4 ml of the enzyme preparation containing 0.71 mg protein/ml prepared as described before with a total reaction volume of 5 ml. The reaction was allowed to proceed for 80 minutes at 37° C at pH 7.0 in 0.01 M Tris buffer. The reactions were stopped by placing the tubes in a boiling water bath for two minutes.

The evidence obtained by paper chromatography of 50 lambda samples in methyl cellosolve, methyl ethyl ketone, NH_4OH, and water (7-2-3-1, volume ratio) and development of the spots is shown in Table 21.

Glucose-1-phosphate was formed in all reaction mixtures except that to which glucose was added. This was confirmed using another solvent (methanol, NH_4OH, H_2O, 6-3-1) as seen in Table 22. Apparently the addition of glucose inhibited the cellobiose phosphorylase reaction. It did not have the effect of resulting in the accumulation of other esters. No spot corresponding tc glucose-6-phosphate was detected, nor was fructose-1,6-diphosphate detected even when ATP was added.

Isolation of Glucose-1-Phosphate, a Product of the Action of Cellobiose Phosphorylase on Cellobiose.

The reaction mixture used to produce glucose-1-phosphate contained the following reagents: cellobiose, 5 g; potassium phosphate buffer at pH 7 containing phosphorus at half the molar concentration of cellobiose; fluoride, 6×10^{-3}M; magnesium ion, 9×10^{-6}M and 20 ml of enzyme preparation containing 1 mg/ml of protein.

The cell-free enzyme source was prepared by growing cells on cellobiose mineral salts broth for 18 hours at 30° C. After washing the harvested cells two times in

TABLE 21

FORMATION OF GLUCOSE-1-PHOSPHATE FROM CELLOBIOSE BY CELL-FREE ENZYME PREPARATIONS AS DETECTED BY PAPER CHROMATOGRAPHY

	Addenda					R_p Values
Enzyme	Cellobiose	P_i	ATP	TPN	Glucose	G-1-P - 190
+	+	+	0	0	0	199
+	+	+	+	0	0	200
+	+	+	+	+	0	195
+	+	+	0	+	0	189
+	+	+	0	0	+	-

Solvent: methyl cellosolve, methyl ethyl ketone, NH_4OH, H_2O, (7-2-3-1, volume ratio).

TABLE 22

FORMATION OF GLUCOSE-1-PHOSPHATE FROM CELLOBIOSE BY CELL-FREE ENZYME PREPARATIONS AS DETECTED BY PAPER CHROMATOGRAPHY

Addenda				R_{G-1-P} Values			
Enzyme	Cellobiose	P_i	TPN	G-1-P (100)	G-6-P (96)	F-6-P (105)	F-1,6-P_2 (71.5)
+	+	+		102		110	
+	+	+	+	101		110	

Solvent: methanol, NH_4OH, H_2O (6-3-1, volume ratio).

0.01 M Tris buffer at pH 7.0, the cells were ground with two times their volume of alumina and extracted with five volumes of cold water or 0.01 M Tris buffer. Protein content of this preparation was 1.16 mg/ml. The reaction with substrate was allowed to proceed for 12 hours at 30° C after which inorganic phosphate was removed as the magnesium ammonium phosphate complex which precipitated from solution at pH 10.0. Analysis of the phosphate hydrolyzed in 10 minutes by 1 N HCl showed that approximately 760 mg of glucose-1-phosphate had been produced.

The mixture was then passed through cation and anion exchange resins according to the method of McCrady and Hassid (1944) with a few modifications. The elution of glucose-1-phosphate from the anion exchange resin was followed by analysis of ten-minute hydrolyzable phosphate as measured by the method of Fiske and Subbarow (1925). The elution curve is given in Figure 7.

The calculated amount of glucose-1-phosphate from the eluted fractions, including Fractions 7 through 11, was approximately 520 mg. The ester was finally crystallized from a mixture of methanol and acetone. Four volumes of methanol were added to 90 ml of the eluted and condensed solution of glucose-1-phosphate and acetone added until persistent turbidity occurred. This mixture was held at -16° C for

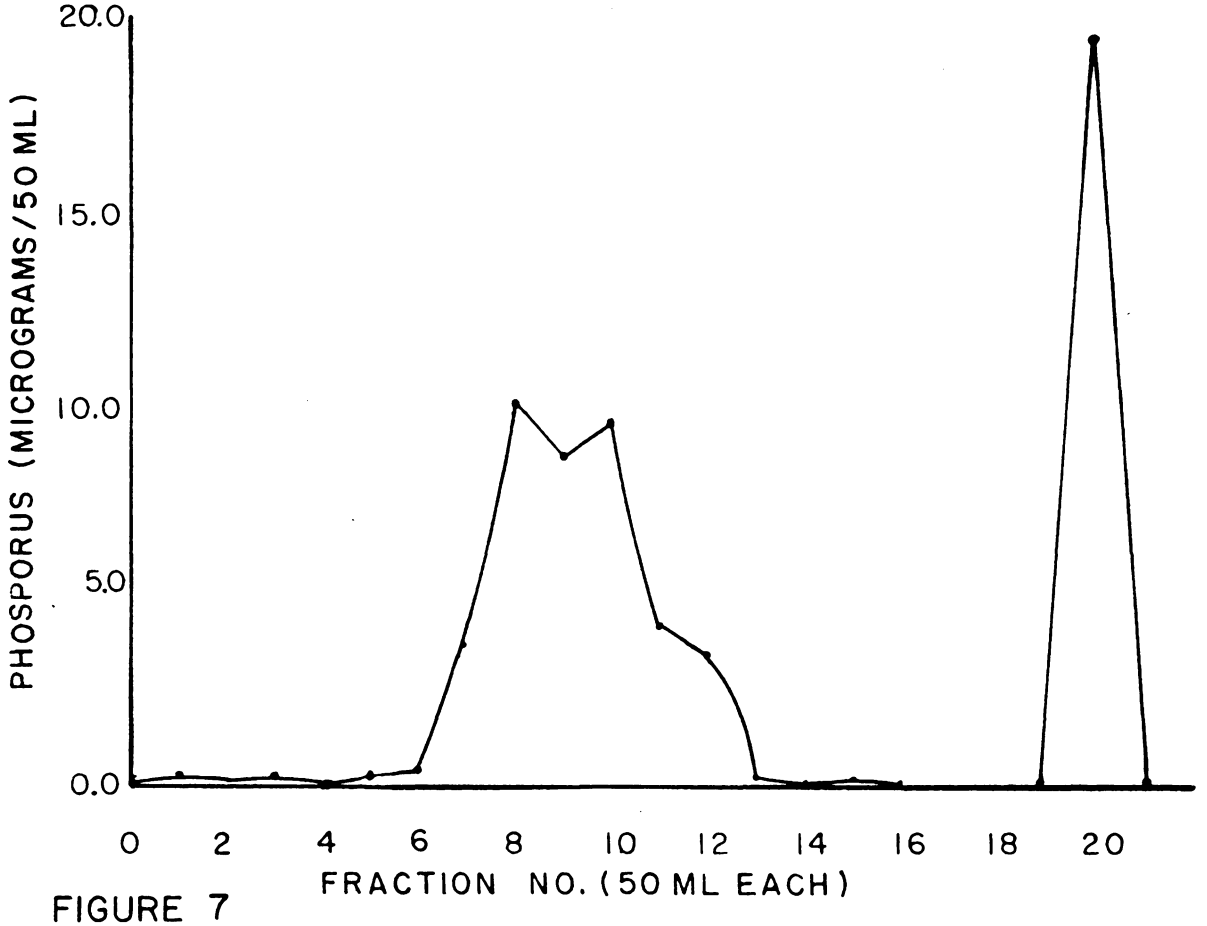


FIGURE 7

ELUTION OF GLUCOSE-I-PHOSPHATE AND UNKNOWN PHOSPHATE FROM ANION EXCHANGE COLUMN

48 hours. After this time nearly all of the ester had crystallized as the dipotassium salt which was recovered by filtration and washed with acetone.

Characterization of the Glucose-1-Phosphate Formed from Cellobiose by the Action of Cellobiose Phosphorylase.

Prior to crystallization, Fractions 7 through 11 were pooled and chromatographed using 10 and 20 lambda samples. In the methyl cellosolve, methyl ethyl ketone, NH_4OH, water (7-2-3-1 volume ratios) solvent, only one spot appeared from each fraction and the R_p values were the same as those obtained with known glucose-1-phosphate.

The crystalline potassium salt was further identified by paper chromatography. A solution containing 0.5 mg/ 0.1 ml of the isolated ester in water was prepared and 10 and 20 lambda samples were placed on the papers and allowed to separate. The results are presented in Table 23. The known sample of a dipotassium alpha-D-glucose-1-phosphate (General Biochemicals) was used as a standard known. According to R_f and R_p values the ester appeared to be glucose-1-phosphate.

After ten-minute hydrolysis of the isolated ester the sugar moiety proved to be glucose by paper chromatography. The molar ratio of glucose to phosphorus was 1.03, the same for both the known and isolated compound.

TABLE 23

IDENTITY OF THE PHOSPHATE ESTER ISOLATED BY PAPER CHROMATOGRAPHY AFTER METABOLISM OF CELLOBIOSE AND P_i BY CELL FREE ENZYMES

	P_i	α-D-G-1-P	G-6-P	Isolated Ester	Fraction #20
R_f	.19	.33	.28	.32	.39
R_p	100	180	152	170	210

Solvent: Methyl cellosolve, methyl ethyl ketone, NH_4OH, H_2O, (7-2-3-1, volume ratio)

That this isolated ester was the alpha-D-glucose-1-phosphate was determined by optical rotation measurement. The known compound from General Biochemicals had a specific optical rotation of $(\alpha)_D^{23^\circ} = +69^\circ$ (C = 1% in H_2O) and the isolated ester $(\alpha)_D^{23^\circ} = +67^\circ$ (C = 1% in H_2O) for an average of seven readings. This establishes the configuration of the phosphate group on the number 1 carbon as alpha. The beta-D-glucose-1-phosphate has a specific rotation of $(\alpha)_D^{20^\circ} = +12^\circ$ (C = 1% in H_2O) (Reithel, 1945).

During the course of isolation of the glucose-1-phosphate another phosphate compound was eluted from the anion exchange column entirely in Fraction 20. The compound was resistant to hydrolysis in N HCl since the phosphorus was liberated only after four days at room temperature. It contained 7.1% carbon, gave a negative anthrone test and was apparently formed only in preparations containing collobiose but not with glucose. Its R_f and R_p values are also given in Table 23.

Enzymic Synthesis of Cellobiose.

The soluble cell-free enzyme preparation was prepared from cellobiose-grown cells after 18 hours growth and used immediately after grinding with alumina and extracting into 0.1 M Tris buffer. Five milliliters of this preparation containing 1.0 mg of protein per ml was incubated with 0.5 g

glucose and 0.5 g alpha-D-glucose-1-phosphate, 6 x 10^{-3} M sodium fluoride, 9 x 10^{-6} M magnesium chloride in a total volume of 10 ml. The reaction was carried out at pH 7.0 at 30° C, and 1 ml samples were removed at 0, 1, 3 and 16 hours. To these was added 0.2 ml of 10% trichloroacetic acid to precipitate protein. After centrifuging, the supernatants were neutralized to pH 5.0 with ammonia. One-hundred lambda samples were spotted on Whatman No. 1 paper and chromatographed in a solvent containing isopropanol, water and acetic acid (6-3-1 volume ratio) and sprayed with diphenylamine-aniline-phosphate reagent (Buchan and Savage, 1952).

Spots corresponding to cellobiose were present in all samples after one hour incubation. The R_f of the known standard was 0.52 compared to 0.51 for the synthesized product. Two milliliters of the reaction mixture after 16 hours incubation was streaked at the origin of Whatman No. 1 paper, and the sugars were separated for 20 hours in the isopropanol, water, acetic acid solvent. The cellobiose was located by cutting off a thin strip of the chromatogram and developing the reducing sugar spots with aniline hydrogen phthalate spray reagent (Block, 1952). The area containing the synthetic compound was cut out and eluted with water. The eluate was condensed in vacuo and again chromatographed yielding an R_f which was nearly the same as that of the known cellobiose. Maltose was colored blue with diphenylamine-

aniline-phosphate spray reagent whereas cellobiose gave a grey-blue. The synthesized compound (170 micrograms) produced a grey-blue spot. The ratio of reducing sugar before and after hydrolysis of the compound (200 micrograms) in 1 N HCl for 45 minutes at 121° C was 2.2 indicating a reducing disaccharide.

A cellulase fraction from Myrothecium verrucaria known to possess cellobiase activity split the compound to glucose, but it was also active on maltose.

The Fate of Glucose-1-Phosphate.

A 2 ml reaction mixture containing glucose-1-phosphate, 0.05%; enzyme preparation containing 0.87 mg of protein/ml, 1 ml; sodium fluoride, 6×10^{-3} M; and $MgCl_2$, 9×10^{-6} M, was allowed to react for 60 minutes at 30° C at pH 7.0. Trichloroacetic acid extracts were neutralized after removal of protein, spotted on Whatman No. 1 papers and chromatographed in the methyl cellosolve, methyl ethyl ketone, ammonia, water (7-2-3-1 volume ratio) solvent as before.

The R_p values are tabulated in Table 24. Glucose-6-phosphate did not appear in the reaction mixtures. Three new spots appeared both in the presence and absence of fluoride. The spot with an R_p of 146 was eluted after streaking the origin of a paper with 1 ml of the mixture. The eluted compound was then chromatographed in methyl

TABLE 24

PHOSPHATE ESTER REACTION PRODUCTS FROM GLUCOSE-1-PHOSPHATE IN THE PRESENCE OF CELL-FREE ENZYME PREPARATIONS

Addenda				R_p Values			
Enzyme	Glucose-1-phosphate	Fluoride	ATP	P_i (100)	G-1-P (208)	G-6-P (170)	F-6-P (140)
+	+	0	0			140; 250	
+	+	+	0			146; 250	
+	+	0	+			-	-

Solvent: methyl cellosolve, methyl ethyl ketone, NH_4OH, H_2O (7-2-3-1, volume ratio).

cellosolve, methyl ethyl ketone, formamide (7-3-2 volume ratio) simultaneously with known compounds.

The R_p values obtained were P_i, 100; glucose-1-phosphate, 92; glucose-6-phosphate, 100; fructose-6-phosphate, 117; eluted compound, 117. Thus fructose-6-phosphate appeared to be formed from glucose-1-phosphate in the presence of fluoride. No glucose-6-phosphate could be detected.

It would appear that glucose-1-phosphate may be converted to fructose-6-phosphate without the mediation of glucose-6-phosphate since glucose-6-phosphate did not appear even in the presence of fluoride. The enzyme preparations used in this attempt to follow the fate of glucose-1-phosphate are too complex to allow detection of the exact conversions taking place. Further solution of these problems will require the fractionation and study of the specific enzymes involved in these conversions.

Pyruvic Acid Produced from Fructose-1,6-Diphosphate by Cell-free Enzyme Preparations.

A reaction mixture containing 0.5 ml of a soluble alumina-ground enzyme preparation with fructose-1,6-diphosphate, 20 micromoles; DPN, 5 micromoles; TPN, 5 micromoles; and methylene blue, 0.5 micromoles in 0.4 ml of 0.1 M potassium phosphate buffer at pH 7.0, incubated for 45 minutes at 30° C was examined for the formation of

pyruvic acid by the paper chromatographic method of Cavallini and Frontali (1954).

Samples were deproteinized with sodium tungstate, 10%; H_2O; and 2/3 N $(NH_4)_2SO_4$ (60-20-20 volume ratio). The ratio of deproteinizing reagent to sample was 1-5 (v/v). The dinitrophenyl hydrazone was prepared by adding 0.1 ml of 0.2% 2,4-dinitrophenyl hydrazine in 2 N HCl to 2 ml of sample and held at room temperature for 20 minutes. The 2,4-dinitrophenylhydrazone derivatives were then extracted with ethyl ether until all color was removed. The ether was evaporated in vacuo and traces of water absorbed over $CaCl_2$ and KOH in a desiccator.

The hydrazones were partitioned between 1 ml of 1 N NH_4OH and 1 ml of chloroform and sedimented by centrifugation. The supernatant ammonia solution contained the hydrazones of keto acids. Two-tenths of a milliliter of the ammonia solution wasspotted on chromatography paper strips and the derivatives allowed to separate in a descending solvent of n-butanol, ethanol and water (40-10-50 volume ratio) for six hours. The strips were dried and spots were detected by the color of the hydrazones.

Three separate spots appeared from the reaction mixture at distances (leading edge measurements) of 7.0, 10.8 and 15.8 cm. The known pyruvic acid hydrazone

moved 10.6 cm. The 15.8 cm spot was probably residual 2,4-dinitrophenylhydrazine since it did not form a red color in 1 N NaOH solution.

The spots were eluted with alcohol containing 10% HCl evaporated to dryness and taken up in 1 N NaOH solution. The absorption spectrum was then determined for the known pyruvic acid and the eluted compound from the reaction mixture. Both compounds showed peaks at around 400 millimicron wavelength using a "Spectronic 20" Bausch and Lomb photoelectric colorimeter. However, the instrument did not permit determination of the other peak occurring for pyruvate at 760 millimicrons.

Since the extraction procedures are known to extract nearly all of the pyruvic acid hydrazone to the near exclusion of the non-acidic keto derivatives (Friedmann and Haugen, 1943) and because of the movement using paper chromatography, the presence of pyruvic acid is very probable.

Oxidation of Glucose and Cellobiose by a Ground Cell Preparation Saturated with Fructose-1,6-Diphosphate.

The rationale of this experiment is as follows. Enough evidence has been obtained about the metabolism of sugars and hexose phosphates to suggest that the conventional Embden-Meyerhof pathway of glucose metabolism is intact in this organism and that fructose-1,6-diphosphate is converted to pyruvic acid. The use of inhibitors to block this

system in an attempt to demonstrate alternate pathways was possible, but these same inhibitors might also block an alternate pathway for the oxidation of glucose or cellobiose.

It was thought that if ground cell-free enzymes or preparations, in which disintegrated cells were reconstituted with the soluble enzymes, could be obtained that would oxidize fructose-1,6-diphosphate with optimum cofactor concentrations, this oxidation system could be saturated with fructose-1,6-diphosphate such that the addition of more of this ester would not yield more oxygen uptake. Having assumed that such a system could be obtained it was further postulated that if alternate pathways for oxidation of glucose and cellobiose did exist in these cells, addition of these substrates would result in a stimulation of oxygen uptake above that obtained with a saturating amount of fructose-1,6-diphosphate, provided terminal electron transport systems were not saturated. The same enzyme preparation would have to be used to determine first the cofactor requirements, second to estimate the saturation concentration of fructose-1,6-diphosphate, and of course to measure the effects of sugar additions in the final experiment. This experiment was then attempted as described below.

Oxidation of Fructose-1,6-Diphosphate by Soluble Enzyme Preparations.

Initial experiments in which soluble enzyme preparations made by alumina grinding or lyophylization and grinding in dry form under vacuum by the method of Gunsalus and Umbreit (1945) were unsuccessful in that oxidation of fructose-1,6-diphosphate did not occur even in the presence of methylene blue, ATP, DPN, and TPN. However, it was found that a slight stimulation of oxidation did occur in the presence of these cofactors with very fresh concentrated preparations. Apparently the electron transport system was either absent or inactive in these preparations. Thus other methods of preparation were attempted.

Oxidation by Soluble Enzymes Reconstituted with the Cell Debris.

Fresh washed cells were ground with 2 volumes of alumina and also with 2 volumes of glow beads in the usual manner with mortar and pestal but for a period of 20 minutes in the cold to obtain a high percentage of disrupted cells. After extraction of the soluble enzymes the precipitated debris was removed from the alumina or glass beads and added to the soluble portion. A suspension was formed and this preparation was tested for its oxidizing capacity on fructose-1,6-diphosphate and fructose-6-phosphate in the presence of cofactors.

Warburg vessels contained 10 micromoles of fructose-6-phosphate or fructose-1,6-diphosphate, 10 micromoles phosphorus as potassium phosphate; adenosine triphosphate, 5 micromoles; diphosphopyridine nucleotide, 5 micromoles; triphosphopyridine nucleotide, 5 micromoles and 0.5 ml of 1/5000 methylene blue all at pH 7.0. Flasks were shaken at 30° C and oxygen uptake measured.

The glow bead-ground preparation yielded an endogenous rate almost as high as that obtained with substrate. The alumina ground preparation, however, did produce a small but significant oxidation of the esters above that obtained by the endogenous control. The oxidation rates are shown in the graph of Figure 8. These data indicated that cell debris was necessary for the oxidation of these esters and that significant levels of O_2 uptake could be obtained in this manner.

Enzyme-Debris Preparation.

The enzyme preparation used in all the following experiments was, of necessity, the same preparation kept at -16° C in the interim between experiments. The organism was grown on the usual cellobiose medium for 18 hours at 30° C. The cells were harvested by centrifuging at 6,000 rpm (Servall SS-1 head). A top loose gum layer was removed until none remained. The cells were washed two times in 0.01 M Tris

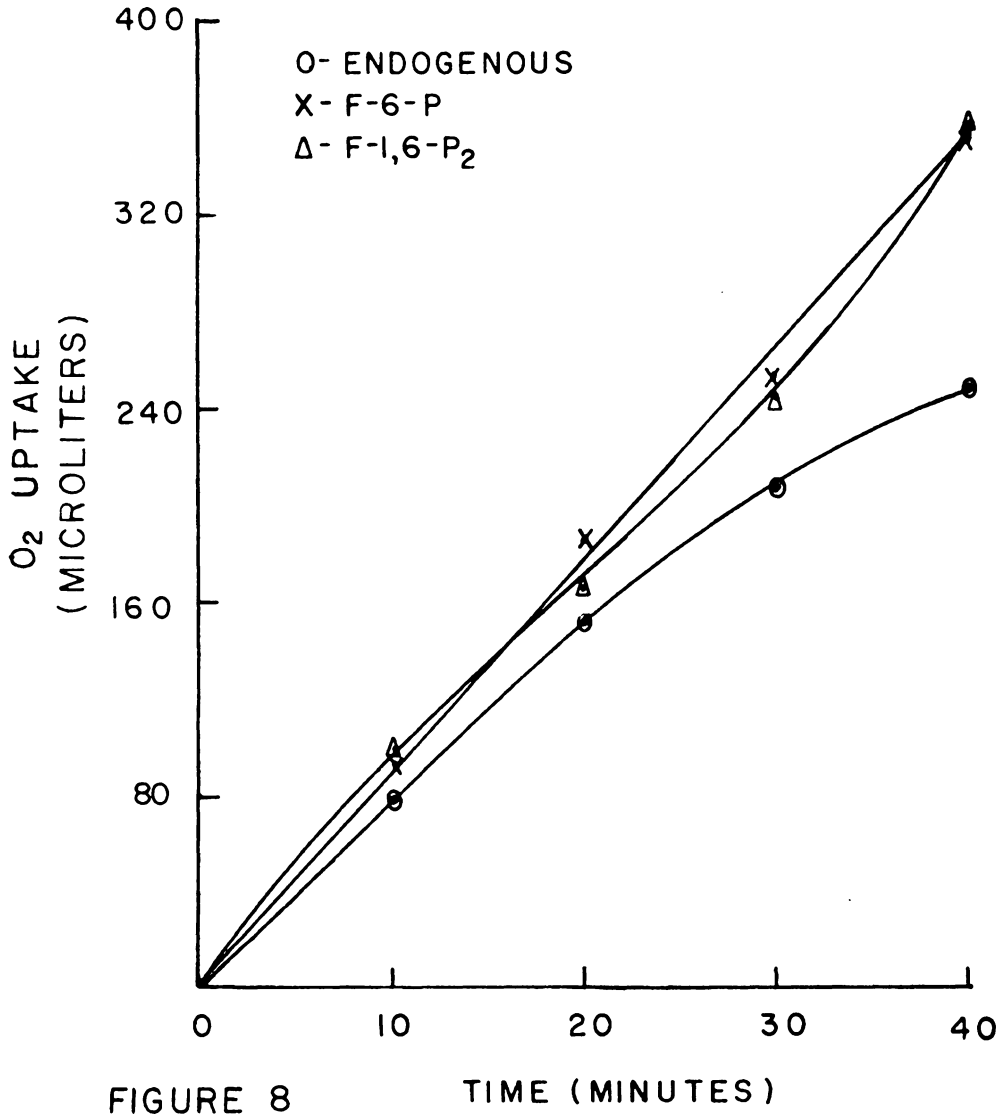


FIGURE 8

OXIDATION OF FRUCTOSE-1,6-DIPHOSPHATE AND FRUCTOSE-6-PHOSPHATE BY GROUND CELL PREPARATIONS

buffer at pH 7.0. Approximately 12 g wet weight of cells were obtained from 3 liters of liquid medium.

These cells were ground with 2 volumes of levigated alumina three times for 20 minutes each time at 0° C to -4° C, checking microscopically for the disintegration of cells. The mass was centrifuged at 6,000 rpm and the debris added back to the soluble viscous enzymes. After thorough suspension this mixture was again centrifuged at 3500 rpm for ten minutes. The supernatant containing considerable debris was poured off from the bottom layer of cells and alumina. Both portions were tested for disintegrated cells. Practically all cells in the supernatant were disrupted as seen by examination of nigrosin and methylene blue stains. One milliliter of this material was used per flask in the following experiments.

One can distinguish between the intact and disrupted cells in a nigrosin or methylene blue stain since intact cells are clear and highly refractile in the nigrosin stain whereas the disrupted cells are dull and the stain is seen inside these cell walls. With methylene blue the intact cells retain the stain, but it is washed from the disrupted ones. According to the counts of intact and disrupted cells only about 11% of the total were intact or appeared to be intact.

Cofactor Requirements for the Oxidation of Fructose-1,6-Diphosphate by Ground Cells.

Determination of the cofactor requirements for obtaining the maximum oxidation of fructose-1,6-diphosphate by the ground-cell preparation described above was carried out by adding all but one of the cofactors to the flasks. The reagents added to the Warburg vessels were fructose-1,6-diphosphate, 10 micromoles; diphosphopyridine nucleotide, 1.0 micromole; triphosphopyridine nucleotide, 1.0 micromole; adenosine triphosphate, 5 micromoles; coenzyme-A, 0.1 micromoles, and methylene blue, 0.5 micromoles in 0.1 M potassium phosphate buffer at pH 7.0. Substrates and cofactors other than methylene blue were prepared in 0.1 M Tris buffer at pH 7.0. These concentrations served as "1 X" concentrations in the experiment.

Warburg flasks contained 3 ml of reaction mixture plus 0.2 ml of 10% KOH and papers in the center well. One milliliter of the enzyme preparation was added per flask. The substrate was placed in the side arms. After temperature equilibration for 20 minutes at 30° C the substrate was dumped and oxygen uptake was measured periodically.

The results obtained in microliters of oxygen consumed after one hour are presented in Table 25. The addition of "2 X cofactor" concentrations stimulated oxidation only a small amount, 7%. The decrease in O_2 uptake caused by lack

TABLE 25

EFFECT OF COFACTORS ON OXIDATION OF FRUCTOSE-1, 6-DIPHOSPHATE BY GROUND-CELL PREPARATIONS (MICROLITERS PER HOUR)

Flask Contents*					O_2 Uptake
Enzyme	Cofactors X	Cofactors 2X	F-1,6-P	Other	
+	-	-	-	-	75
+	+	-	-	-	73
+	+	-	+	-	85
+	-	+	+	-	91
+	-	+	-	-	71
+	+	-	+	No DPN	68
+	+	-	+	No TPN	83
+	+	-	+	No CO-A	73
+	+	-	+	No MB	27
+	+	-	+	-	83

* 1 ml enzyme, 10 micromoles F-1,6-P_2, 0.1M Tris buffer pH 7.0

of DPN was about 20%. Triphosphopyridine nucleotide deletion reduced 0_2 uptake 2.4%. Coenzyme-A deletion reduced it 14%. Methylene blue deletion reduced it about 68%. Thus, methylene blue, DPN and coenzyme-A were required to obtain significant oxidation of fructose-1,6-diphosphate.

Saturation of the Oxidizing System with Fructose-1,6-Diphosphate.

The concentration of fructose-1,6-diphosphate required to saturate the enzyme systems involved in its oxidation was determined by measuring the 0_2 uptake obtained at increased levels of this substrate concentration. The same ground-cell preparation described above was used. The Warburg experiment was carried out as described in the foregoing experiment. The concentrations of fructose-1,6-diphosphate used were: 10, 20, 40, 100, and 200 micromoles per flask. The graph in Figure 9 relates the total microliters of oxygen uptake obtained in 140 minutes to the concentrations of fructose-1,6-diphosphate.

It is seen that concentrations greater than 100 micromoles per flask did not significantly increase the oxygen uptake. The change from a concentration of 150 micromoles to 200 micromoles of substrate apparently was about 2.0 microliters of oxygen.

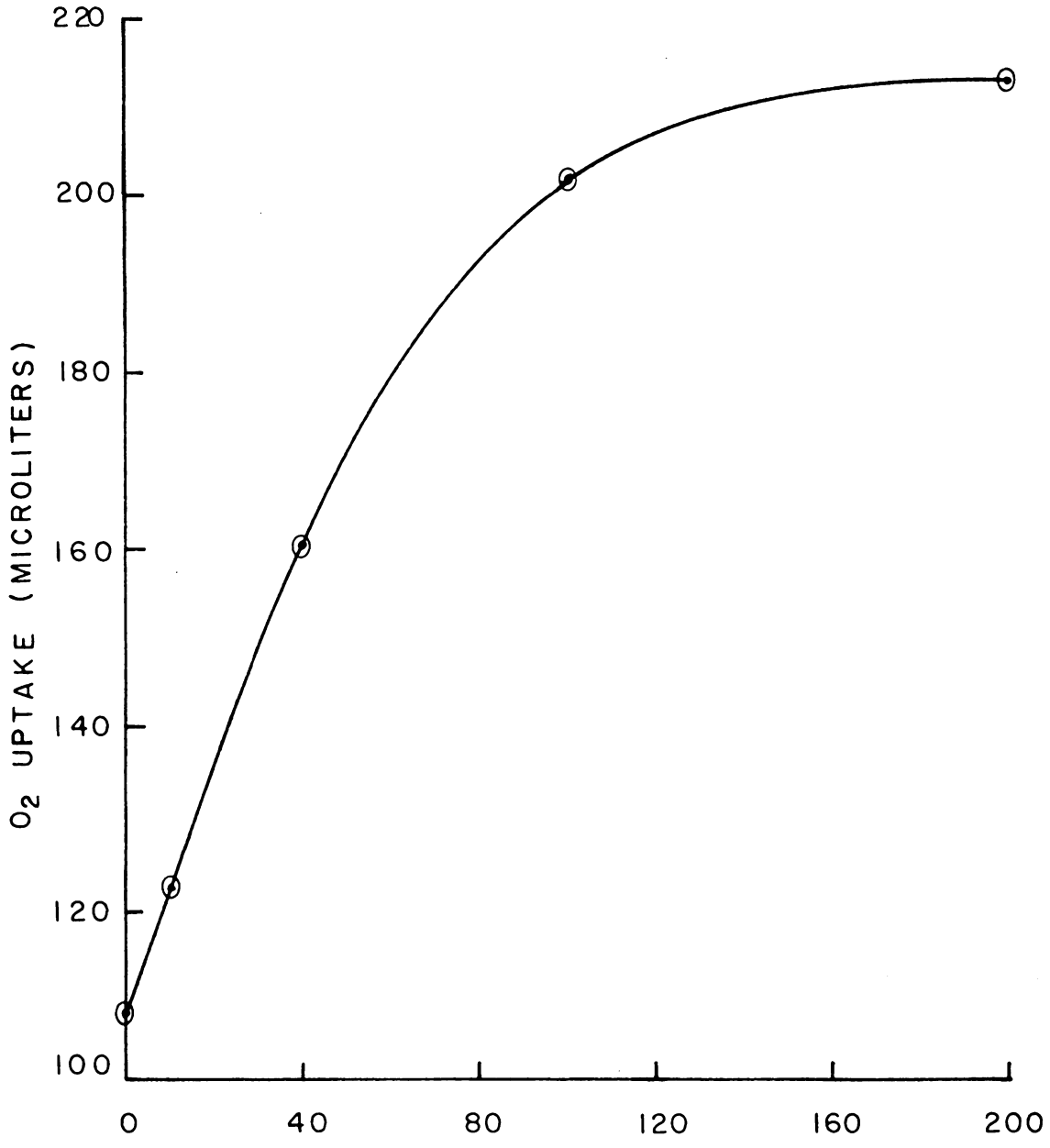


FIGURE 9

EFFECT OF SUBSTRATE CONCENTRATION ON OXIDATION OF FRUCTOSE-1,6-DIPHOSPHATE BY GROUND-CELL PREPARATIONS

Oxidation of Glucose and Cellobiose by Ground-Cell Preparations Saturated with Fructose-1,6-Diphosphate.

Since 200 micromoles of fructose-1,6-diphosphate was ample to obtain the maximum oxidation of this compound, it was used throughout this experiment with the same ground-cell preparation containing about 2.17 mg of protein per milliliter as in the two experiments above. The only difference in this preparation was that the remaining intact cells were removed by centrifuging for 15 minutes at 3500 rpm (Servall SS-1 head). The "1 X" concentration of cofactors was used in this experiment.

The treatments in this experiment were the addition of 10 micromoles of glucose or 5 micromoles of cellobiose in the presence and absence of ATP. The Warburg experiment was accomplished in the manner already described.

The results obtained after 65 minutes in terms of oxygen consumption are given in Table 26. The removal of all but 11% of the intact cells probably accounts for the low endogenous rate. The high rate of oxidation obtained with cofactors and substrate compared to the low endogenous rate further indicated that a cell-free enzyme system was operating since these cofactors and methylene blue, are non-diffusable to bacterial cell membranes. The addition of 10 micromoles of glucose with ATP stimulated the O_2 uptake 27.5%; without ATP it was even greater, 74%.

TABLE 26

OXIDATION OF GLUCOSE AND CELLOBIOSE BY GROUND-CELL PREPARATIONS SATURATED WITH FRUCTOSE-1,6-DIPHOSPHATE AND FORTIFIED WITH COFACTORS

Addenda					
F-1,6-P_2	Cofactors*	ATP	Glucose	Cellobiose	Microliters O_2 Per 65 Min.
0	0	0	0	0	18
+	0	0	0	0	57
+	+	+	0	0	77
+	+	+	+	0	108
+	+	0	+	0	134
+	+	+	0	+	110
+	+	0	0	+	132
+	0	0	+	0	117
+	0	+	+	0	102
+	0	0	0	+	115
+	0	+	0	+	49

* Cofactors = DPN, TPN, CO-A, Methylene Blue.

The results obtained by addition of 5 micromoles of cellobiose were of about the same order of magnitude as those obtained for glucose.

This marked capacity for oxidation of glucose and cellobiose by enzyme systems already saturated with fructose-1,6-diphosphate strongly indicates that free glucose and the glucose moiety produced by cellobiose phosphorylase are oxidized by an enzyme system alternate to the conventional Embden-Meyerhof scheme and the citric acid cycle.

Included in the experiment were the same treatments as above but cofactors were all deleted. The values so obtained were much greater than obtained with fructose-1,6-diphosphate alone.The decrease of O_2 uptake resulting from the deletion of cofactors from the glucose treatment was about 24 microliters. The increase caused by addition of cofactors to the fructose-1,6-diphosphate treatment was 20 microliters. Thus the cofactors may have stimulated oxidation of the ester but not of glucose. It appeared that these cofactors were not essential for the alternate direct oxidation of glucose. Adenosine triphosphate exerted a very strong depressing action on the alternate oxidation of glucose and cellobiose.

Gluconic Acid Formation from Glucose and Cellobiose.

After evidence was obtained for an alternate direct oxidation of glucose and cellobiose independent of the Embden-Meyerhof pathway, it seemed likely that one of the products of glucose oxidation might be gluconic acid. The reaction mixtures in the flasks saturated with fructose-1, 6-diphosphate supplemented with glucose and cellobiose were examined by paper chromatography for gluconic acid. This reaction mixture contained ground cell preparation; 1 ml; fructose-1,6-diphosphate, 200 micromoles; methylene blue, 0.5 micromoles; ATP, 5 micromoles; DPN, 1 micromole; TPN, 1 micromole; coenzyme-A, 0.1 micromole; and glucose, 10 micromoles or cellobiose, 5 micromoles. Another reaction mixture consisting of 2 ml of a cell-free enzyme preparation (0.87 mg protein/ml), 0.5 g glucose, 6×10^{-3} M sodium fluoride and 9×10^{-6} M $MgCl_2$ was also examined.

Protein was precipitated with trichloroacetic acid and centrifuged in the cold; and the samples were neutralized to pH 4.0 with NH_4OH solution. Fifty lambda aliquots were spotted on chromatography paper and separation of the organic acids was accomplished in a solvent system containing isopropanol, pyridine, water and acetic acid (8-8-4-1) for 6 hours (Gordon et al., 1956).

The compounds were detected by spraying with 10% potassium ferrocyanide (w/v), drying in air, and examining.

Papers were then sprayed with ferric ammonium sulfate in 70% ethanol and dried in air. Gluconic acid produced a spot after the first spray (Martin, 1955).

The known sodium salt of gluconic acid was acidified to pH 2.0 with 0.01 N HCl solution and 20 micrograms were applied at the origin. Glucose and cellobiose known compounds were also spotted to avoid misleading spots from the samples.

The R_f values obtained were as follows: glucose, 0.41; cellobiose, 0.18; gluconic acid, 0.25; reaction mixture (1), 0.26; reaction mixture (2), 0.26; and reaction mixture (3) 0.25. Known glucuronic acid moved close to the front, well-separated from the other compounds. Thus gluconic acid was identified by paper chromatography to be present in the reaction mixtures supplemented with glucose or cellobiose. It was also formed by a fluoride insensitive system. Apparently the free glucose and the glucose released from phosphorylation of cellobiose are partially oxidized directly to gluconic acid by ground-cell preparation. These data emphasize the presence of an alternate pathway for oxidation of glucose in this species of Cellvibrio.

VI. DISCUSSION OF THE RESULTS

Cellulolytic Bacteria.

The aerobic mesophilic bacteria isolated from field soil on the Fuller and Norman cellulose dextrin medium that produced clear zones of hydrolysis were generally weak or not active in cellulose hydrolysis on cellulose agar. At the lower dilutions of soil (10^{-3} and 10^{-4}) which were found necessary for development of several bacterial colonies, fungal growth would overtake the plate before development of clear zones. It was found that the Dubos medium and the Fuller and Norman medium in which ground filter paper was substituted for cellulose dextrins were more suitable for isolation of active cellulose-decomposing bacteria from soil.

Only about 10% of a total of 154 organisms isolated did not grow or grew very slowly on glucose. This is only a rough estimate of the cellulose decomposers isolated from several sources on three different media. About 50% of these non-glucose-using bacteria were isolated from fresh cow feces. These rough estimates are now only of value as a basis for further attempts to isolate such organisms and the further evaluation of the ecology and significance of these and similar bacteria.

It is significant to note that several different morphological types were isolated. These included very minute cocci and rods of various sizes that were short, narrow, large and oval. Large cocci, in singles and tetrads, bacilli, and long narrow rods also were found. The absence of the cytophaga group of bacteria was conspicuous.

The inability of several organisms to grow on cellulose in the absence of yeast extract was strong indication of vitamin and/or amino acid requirements since most of the organisms tested decomposed cellulose only in the presence of yeast extract.

Twenty-five of the cultures active in cellulose hydrolysis that grew sparcely or not at all on glucose were retested for these characteristics. All but one organism produced clear zones and 13 failed to grow on glucose agar.

The cursory descriptions of the cellulose-decomposing bacteria in the literature and especially in "Bergey's Manual of Determinative Bacteriology" (Breed, 1947) lead to difficulty in identification of these organisms. Presumably, many of these organisms have not yet been isolated or described and they have been largely ignored since the early work of Winogradski, Waksman, Stanier and Imschnetskii.

The Organism.

The organism chosen for investigation in this work was isolated from fresh bovine feces and its purity established after rigorous criteria were met. The description of this organism has been presented and it has been tentatively identified as a member of the genus Cellvibrio. The cells are not flexous, but rigid, in singles and in chains, having motility with flagella; no photosynthetic pigments are apparent and the cells are not attached by a stalk. Thus the organism was placed in the class Schizomycetes, Order, Eubacteriales and Suborder, Eubacteriineae. This order includes the true bacteria. The absence of endospores, inability to grow on inorganic media, presence of polar flagella, straight and curved rods, gram-negative character, the presumed non-pathogenicity to plants, lack of violet pigment, probability of non-symbiosis on the roots of Leguminosae, inactivity on litmus milk in 14 days, and inability to liquefy gelatin eliminated all but one family in Eubacteriineae. Thus it could not be placed in the families Rhizobiaceae or Corynebacteriaceae but is in the family Pseudomonaceae, and in the tribe, Spirilleae, since the cells are not straight or branching rods but are curved and straight.

Growth on ordinary culture media is feeble and the cells are long, slightly curved rods with rounded ends. The ability of the culture to decompose cellulose is marked. These characteristics place the organism in the genus Cellvibrio. The cells are not spindle shaped, do not produce green colonies, and more than one chromatic granule per cell is seen. These criteria eliminate the possibility of the genus Cellfalcicula.

Difficulty arises in classification according to species. It is unlike vulgaris since it grows poorly on glucose agar but is similar to fulvus in its scanty growth on glucose agar but not in formation of egg to red-colored pigment. It is unlike flavescens by the lack of ability to grow on nutrient peptone agar. The distinctive character or ochraceus in rapid ochre-yellow pigment formation is not particularly distinctive of this organism, but a pale yellow pigment does develop on cellulose agar. Because the other species in this genus are characterized and named mainly on the pigmentation and because this culture does not appear to represent a previously described species, the name Cellvibrio gilvus is proposed, the name being descriptive of its pale yellow pigment.

This organism is moderately strong in the decomposition of cellulose being able to decompose about 64% in stagnant cultures in 10 days. The other characteristics are presented in the Experimental section.

Hydrolysis Products of Cellulose. Paper chromatography revealed the presence of cellobiose but not glucose in stagnant cellulose cultures at 4 and 10 days of incubation. It is thus possible that the cellulase produced by this organism may hydrolyze cellulose, releasing disaccharide units and not glucose units from the cellulose chains. Other reports concerning the mechanism of cellulase action of other organisms favor a random cleavage mechanism (Whitaker, 1954). Other spots were detected on the paper chromatograms. Their identity is obscure other than that they were not glucose.

The Effect of Vitamins, Amino Acids and Trace Minerals on the Growth of Cellvibrio Gilvus.

Trace minerals in calcium carbonate and iron when added to media made no real difference in the resulting growth of the organism. The requirement for amino acids or peptides in enzymatic casein hydrolyzate was definitely shown. Yeast extract could be replaced by the addition of 0.5% enzymatic casein hydrolyzate and 1 ppm of the vitamins thiamine hydrochloride, riboflavin, para-aminobenzoic acid, niacin, biotin, pantothenic acid, pyridoxal and folic acid. The detailed nutritional requirements were not investigated. It is probable that the nitrogen requirements are met by amino acids in this organism since sodium nitrate medium supplemented with the vitamin mixture did not support growth.

Carbon Distribution in Fermentation Products.

The carbon present in the cells was about 44% of the dry weight. The carbon balance data suggest that this strain of Cellvibrio is not highly efficient in obtaining energy from cellobiose because a large percentage of the fermentation products (about 54%) was not completely oxidized. The fermentation products containing significant amounts of carbon included volatile and non-volatile acids and volatile neutral compounds. The formation of an osazone with 2,4-dinitrophenylhydrazine suggested that a ketone or aldehyde was present in the neutral volatile fraction. The non-volatile acid fraction contained about 45.5% of the carbon in the fermentation products. Presumptive evidence has been obtained that gluconic acid is one of these non-volatile acid products.

Metabolism of Glucose and Cellobiose by Intact Cells.

Comparison of the Growth of Cellvibrio Gilvus on Glucose and Cellobiose. The purpose in isolation and purification of the organism was to investigate the metabolic basis for disaccharide preference. This was well demonstrated in the comparison of growth on glucose and cellobiose when the concentration of the substrates supplied the cells with

equivalent carbohydrate on a hexose basis. The rate of growth on cellobiose ranged from 30 to 46% more than could be obtained from glucose.

The lag in growth on glucose suggested that (1) the organism might be deficient in an enzyme important in glucose metabolism (2) that the cells were adapting to glucose and synthesizing an adaptive hexokinase or (3) that a differentially permeable cell membrane barrier was responsible for the different growth rates.

In order to test these hypotheses respiration experiments were conducted to further elucidate the nature of the anomoly.

Oxidation of Glucose and Cellobiose by Intact Cells. Intact cells grown on cellobiose oxidized glucose immediately without lag and at a rapid rate, only about 10% less than that obtained with cellobiose. These data dispensed the possibilities given above to explain the slow growth on glucose. Neither permeability, adaptation, nor hexokinase deficiency could explain the disaccharide preference on the basis of the respiration data. Obviously, both substrates diffused into the cells at comparable rates and there was no time limitation for the synthesis of an adaptive hexokinase. Certainly, the enzymes required for the oxidation of glucose were present.

It thus seemed possible that glucose was not affording the cells with sufficient energy to attain the growth rate seen with cellobiose. It was decided that there might be differences in the energy-yielding hexose phosphates and high energy phosphates such as adenosine triphosphate that would accumulate in intact resting cells when presented with glucose and cellobiose.

Esterification of Inorganic Phosphate in the Presence of Glucose and Cellobiose. Inorganic phosphate was esterified by respiring, resting, intact cells in the presence of either glucose or cellobiose and the accumulation of organic phosphates was greater in the presence of glucose or a mixture of the two sugars than in the case of cellobiose alone. This might represent the accumulation of organic phosphates as a result of the slow further metabolism of organic phosphates different from those formed from cellobiose. Nevertheless, it is evident that cellobiose-grown cells could not only oxidize glucose rapidly but also esterified inorganic phosphate in the course of glucose oxidation. This experiment provided further evidence that hexokinase deficiency was not the basis of the disaccharide preference.

A possible explanation for the relatively low increase in organic phosphates in the presence of

cellobiose is that the normal amounts of organic phosphate of the cellobiose pathway may have been present already because the cells were grown on cellobiose. The larger increase in organic phosphates formed from glucose might imply that phosphate ester synthesis from glucose occurred by a different pathway than that involved in the metabolism of cellobiose. An alternative possibility may be that the enzyme reactions for esterification of cellobiose were so rapid that formation of the esters was almost instantaneous but esterification of glucose lagged enough to be measured.

The growth of this strain of Cellvibrio had revealed distinct differences in the magnitudes of the growth rates on glucose and cellobiose. It was desired to find if the entire cellobiose molecule was used more rapidly than glucose or if glucose would accumulate upon the initial hydrolysis or phosphorolysis of the disaccharide. Comparison of the utilization of these sugars revealed that about 130% more cellobiose than glucose was used at pH 6.5 and about 590% more at pH 7.0. These large differences strongly supported the evidence for the disaccharide preference and that a direct disaccharide reaction was indeed involved and not a simple hydrolysis.

Moreover, the amount of acid produced from each sugar was found to be another reflection of the differences in the metabolism of glucose and cellobiose. Fifty to fifty-eight percent more acid per 100 mg of sugar was formed from glucose than from cellobiose; and even on a molar basis about 30% more acid was formed from glucose. It was difficult to assess the metabolic significance of this information except to suppose that enzyme reaction rates were different for the sugars or that alternate pathways for metabolism of the two sugars were operating. The greater acid production from glucose implied that a wasteful process might be involved in glucose utilization.

Since it appeared that cellobiose was not simply hydrolyzed and further metabolized as glucose but was directly metabolized in some other manner, a likely reaction was the direct phosphorylation by a cellobiose phosphorylase.

The Effect of Removal and Addition of Inorganic Phosphate on Respiration of Cellvibrio. The effect of removal and addition of inorganic ortho-phosphate on intact cells was a depression and stimulation, respectively, of the respiration rate showing that cellobiose metabolism was dependent on phosphate and strongly indicating the

presence of a cellobiose phosphorylase. The respiration of glucose was not significantly affected by the addition or removal of phosphate. The oxygen uptake obtained from a mixture of both sugars was greater than either alone as shown in previous experiments.

Carbohydrate Metabolism of Cell-free Enzyme Preparations. Utilization of Glucose and Cellobiose by Cell-free Extracts. In an attempt to define the specific area with which the disaccharide preference, already demonstrated, was associated a study of the utilization of glucose and cellobiose by intracellular soluble enzymes revealed that the differences were in fact characteristic of the enzyme systems inside the cell. It was also found that adenosine triphosphate was required for the utilization of glucose strongly indicating the presence of a hexokinase.

The ability of preparations from glucose-grown cells to utilize cellobiose was taken as evidence that the cellobiose phosphorylase is a constitutive enzyme. Cellobiose conversion was not dependent on ATP. The two-fold increase in cellobiose utilization in the presence of added phosphate was convincing evidence for the presence of a cellobiose phosphorylase. Two interesting effects were noted in addition. Phosphate depressed

the disappearance of glucose and even increased the reducing value but this effect was reversed by ATP. Moreover, ATP definitely suppressed the utilization of cellobiose.

This experiment thus demonstrated that the disaccharide preference was characteristic of the intracellular enzymes. Thus, the other hypotheses including differential permeability, adaptive hexokinase formation, hexokinase deficiency and the presence of an "active transport" mechanism were eliminated by these experiments. In addition, the presence of a hexokinase and a cellobiose phosphorylase was suggested.

Furthermore, inorganic phosphate analyses of the reaction mixtures in the sugar utilization experiment showed that phosphate disappearance accompanied the utilization of cellobiose.

Accumulation of Fructose-6-phosphate in Growing Cells. Since the quantitative studies with cell-free preparations showed that inorganic phosphate was esterified with cellobiose but not with glucose and the phosphate addition also increased cellobiose disappearance, it was logical to determine if qualitative differences might exist among the phosphate esters accumulating in growing cells using glucose or cellobiose.

It was found that only fructose-6-phosphate could be detected in the isolated phosphate ester fractions from cells grown on both glucose and on cellobiose. It should be noted that the total phosphate level was about three times greater in the cellobiose-grown cells than in glucose-grown cells on a fresh weight basis. This could mean that cellobiose is a more efficient substrate for the formation of organic phosphates in the cell.

The significance of the accumulation of large amounts of fructose-6-phosphate in growing cells might lead, on further investigation, to knowledge of the importance of different pathways of carbohydrate metabolism. Fermenting yeast contain largely glucose-6-phosphate and the amounts of glucose-1-phosphate, glucose-6-phosphate, and fructose-6-phosphate were close to the equilibrium values (Trevalyn et al. 1954) predicted by the reactions known in yeast to be 95/5 and 70/30, respectively (Neilands and Stumpf, 1955).

Thus, the pathways of metabolism or the relative quantities of given enzymes in this Cellvibrio strain are different from those in yeast. Apparently, fructose-6-phosphate plays an important role in the carbohydrate metabolism of this cellulose-decomposing organism.

Hexose Phosphates Formed by Soluble Enzymes. Because of the equilibrium conditions and quantities of various enzymes existing in intact growing cells the intermediate compounds escape detection and to detect these compounds one must work with cell-free enzymes, under artificial conditions, in vitro.

A preliminary survey of the products formed from glucose and cellobiose by cell-free enzymes showed that glucose was a product of cellobiose phosphorolysis or hydrolysis. It was found that several hexose phosphates could be detected in the reaction mixtures. Thus more accurate methods of paper chromatography for identification of phosphate esters were employed.

The formation of glucose from cellobiose was further confirmed by using other paper chromatographic solvents and spray reagents.

In the first experiments it was found that cellobiose was converted to glucose-6-phosphate or to fructose-6-phosphate, and glucose apparently gave rise to glucose-6-phosphate only. It was further found, using very fresh enzyme preparations, that glucose was produced or accumulated from reactions of cellobiose but not when either ATP or TPN was added. Glucose disappearance required the presence of ATP.

The soluble enzymes were able to carry out all the reactions in the conversion of glucose to glucose-6-phosphate and to fructose-1,6-diphosphate in the presence of ATP. Furthermore, cellobiose was converted to fructose-6-phosphate.

Especially interesting is the lack of formation of fructose-1,6-diphosphate from cellobiose even in the presence of ATP. Glucose-1-phosphate was the only hexose phosphate that appeared in the presence of both of the above cofactors and when ATP was deleted fructose-6-phosphate and apparently glucose-6-phosphate also appeared. Further investigation of the effects of ATP and TPN on these reactions should be made in the eventual elucidation of the reaction mechanisms.

Reactions carried out at 37° C containing soluble enzymes, cellobiose and phosphate resulted in the accumulation of glucose-1-phosphate and fructose-6-phosphate. This phosphorylase reaction was shown to be inhibited by glucose. Again, no fructose-1,6-diphosphate could be detected in the reaction mixture containing ATP.

Therefore, glucose appeared to be metabolized via the conventional Embden-Meyerhof pathway to fructose-1,6-diphosphate, but cellobiose was phosphorylated in the absence of ATP and required inorganic phosphate to

form glucose-1-phosphate and glucose. The glucose-1-phosphate was then converted to fructose-6-phosphate. Certainly, then, some basic difference must occur in the hexose phosphate conversions of the two sugars involving fructose-6-phosphate and glucose-1-phosphate.

Cellobiose Phosphorylase. The recent interests in disaccharide phosphorylases and the single report of a cellobiose phosphorylase prompted an extensive investigation of this reaction in Cellvibrio gilvus. A cellobiose phosphorylase was demonstrated to be present in this organism by the isolation and characterization of alpha-D-glucose-1-phosphate from reaction mixtures containing soluble enzymes, cellobiose, inorganic phosphate and sodium fluoride. The reversibility of the reaction was also shown by the formation of cellobiose from glucose and alpha-D-glucose-1-phosphate.

The mechanism of phosphorylation of starch in yeast and plants and of glycogen in animals does not involve breaking of the phosphate bond, and the sugar products retain the alpha configuration about the number 1 carbon atom. On the other hand, Fitting and Doudoroff (1952) found the maltose phosphorylase of Neisseria meningitidis to have a different mechanism of phosphorolysis, and an inversion of the phosphate

occurred yielding from the alpha linked maltose the beta-D-glucose-1-phosphate. The Cellvibrio cellobiose phosphorylase mechanism then must also involve an inversion of the phosphate bond in a similar manner. This finding is important because it gives vision into the mechanism of conversion of the alpha and beta linked sugars and polysaccharides in biological systems. This reaction may also be useful in the synthesis of specifically labeled cellobiose for study of the reaction mechanisms of phosphorylation, transglucosidation and cellulose synthesis.

While isolating the glucose-1-phosphate from the reaction mixture another phosphate compound was isolated from an anion exchange column. It was found to be resistant to acid hydrolysis, contained about 7.1% carbon, and gave a negative anthrone test. The chemical nature of this compound should be further investigated since it was apparently formed from cellobiose but not from glucose.

Returning to the transformations of the hexose phosphates in cell-free enzyme preparations, the metabolic fate of glucose-1-phosphate was the next important step to attack. Cells and tissues containing phosphorylases are able to convert glucose-1-phosphate to glucose-6-phosphate catalized by the

enzyme, phosphoglucomutase. Sodium fluoride is known to inhibit this reaction in addition to the isomerase reaction. Thus one might expect that glucose-1-phosphate in the presence of fluoride would not be converted to other esters or that some glucose-6-phosphate would appear. On the contrary, glucose-6-phosphate did not appear but considerable amounts of fructose-6-phosphate did accumulate. It then appeared that glucose-1-phosphate was converted to fructose-6-phosphate without the mediation of glucose-6-phosphate or this mutase was insensitive to fluoride.

The enzyme preparations used in this study were too complex to obtain specific facts about the exact reactions taking place. For such studies, the specific enzymes must be fractionated from the others for complete and detailed study of the reaction mechanisms and because one lifetime is limited this interesting phenomenon is left for posterity.

Alternate Pathways for Glucose and Cellobiose Oxidation in Ground-Cell Preparations Saturated with Fructose-1,6-diphosphate.

Evidence was previously obtained about the metabolism of the sugars and hexose phosphates to suggest that the conventional Embden-Meyerhof pathway of glucose metabolism is intact in this organism. Ground-cell preparations were

shown to oxidize fructose-1,6-diphosphate and fructose-6-phosphate. Soluble cell-free preparations were not successful in oxidizing these esters even in the presence of the terminal hydrogen acceptor, methylene blue.

The cofactor requirements for the oxidation of fructose-1,6-diphosphate were found to be DPN, Co-A, and methylene blue. TPN and FMP had almost no effect. Under optimum conditions of cofactor concentrations, the concentration of fructose-1,6-diphosphate required to reach the maximum rate of oxidation was obtained such that if more were added or formed metabolically the oxygen uptake would not increase.

Using the same ground-cell preparation with saturating concentrations of fructose-1,6-diphosphate oxygen uptakes were found to be greatly increased when either glucose or cellobiose was added to the reaction vessels. The large increase in oxidizing capacity of glucose and cellobiose above that obtained where the enzyme systems were completely saturated with fructose-1,6-diphosphate strongly indicates that free glucose and either cellobiose or the glucose formed from cellobiose phosphorylase are oxidized by alternate enzyme systems other than the Embden-Meyerhof pathway and the citric acid cycle. Furthermore, it was apparent that the cofactors were not essential for the alternate direct oxidation of glucose.

In addition, it was found that ATP exerts a very strong depressing action on the alternate oxidation of both glucose and cellobiose. One explanation may be that ATP is driving the phosphorylation reactions and piling up hexose phosphates and thus reducing the available substrate for the direct oxidation enzymes.

Such a direct oxidation of glucose leads one to suspect that gluconic acid might be formed. These reaction mixtures were examined for this compound and it was found by paper chromatographic analysis to be present.

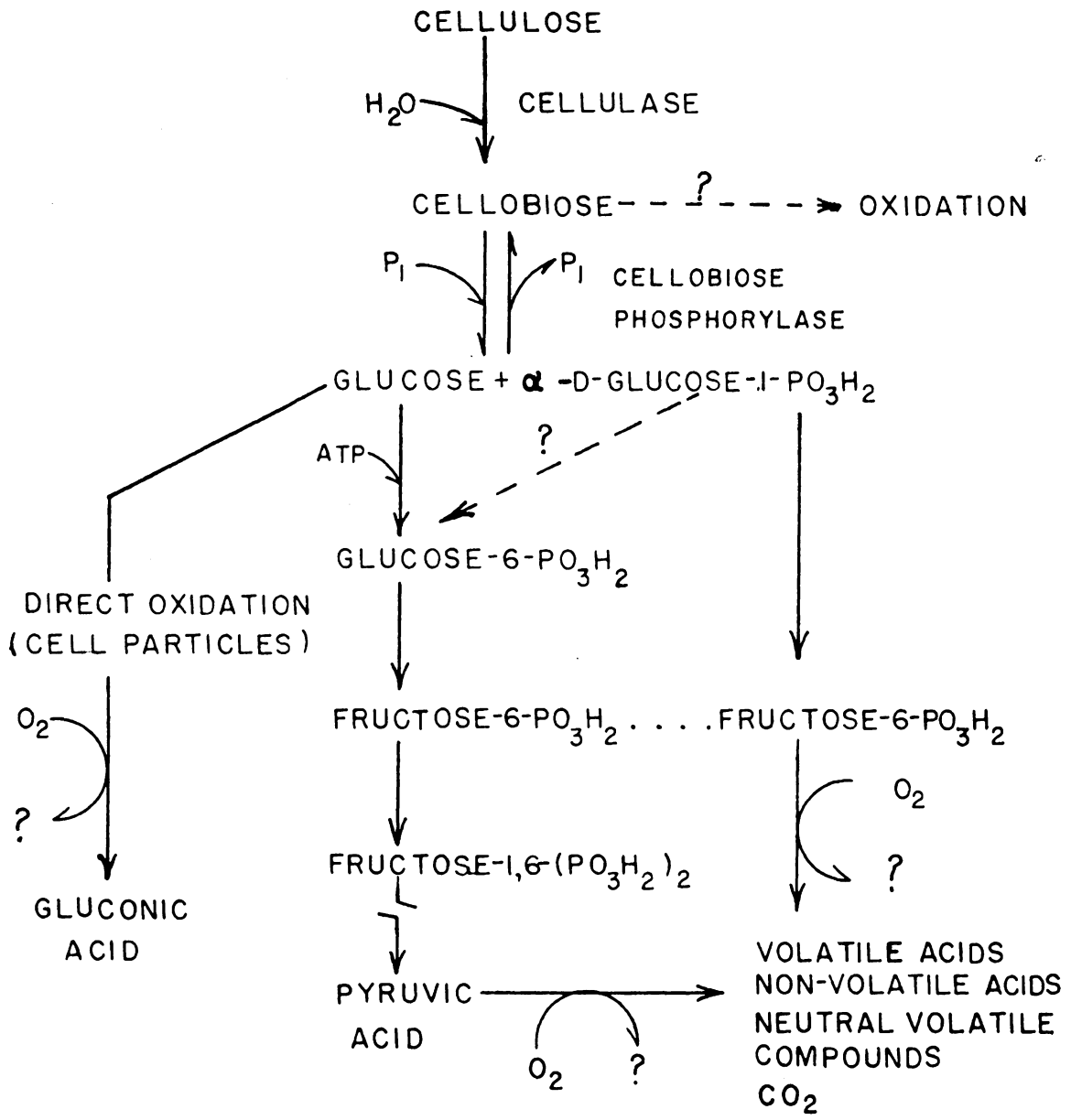
Explanation of Disaccharide Preference.

From all of the foregoing data it is possible to propose the pathways for the metabolism of glucose and cellobiose in this strain of Cellvibrio. These reactions are summarized in Plate 3. Glucose can be metabolized by two pathways. It can be phosphorylated to glucose-6-phosphate by hexokinase and ATP, transformed to fructose-6-phosphate by a phosphohexoisomerase, and further phosphorylated with ATP by a phosphofructokinase to form fructose-1,6-diphosphate. Fructose-1,6-diphosphate can be converted to pyruvic acid. Glucose can also be oxidized directly by an alternate pathway to gluconic acid.

Cellobiose is phosphorylated by a cellobiose phosphorylase in a reversible reaction to alpha-D-glucose-1-phosphate and glucose. The glucose-1-phosphate then undergoes an

PLATE 3

PROPOSED PATHWAYS OF CARBOHYDRATE METABOLISM IN CELLVIBRIO GILVUS (NOV. SP.)



unknown series of reactions to form fructose-6-phosphate which may then take part in the Embden-Meyerhof scheme or may be metabolized in another way. Since no attempt was made to demonstrate the hexosemonophosphate shunt it is not known if fructose-6-phosphate is involved in this pathway. Although cellobiose was oxidized in some manner other than by the Embden-Meyerhof pathway it was possible that the bionic acid could be formed. However, it was not detected and it seems more likely that cellobiose is first cleaved by the phosphorylase.

What then is the most logical explanation of the preferential growth, utilization and respiration obtained from the disaccharide and the slow growth and utilization of glucose? The answer seems to reside in several factors. The very active direct phosphorylation of cellobiose yielding a hexose phosphate with less energy expense than the ATP-requiring phosphorylation of glucose shows cellobiose to be a more efficient energy-yielding substrate. The relatively weak hexokinase could limit the formation of hexose phosphates which in turn would impede the energy obtained as high energy phosphate compounds such as ATP necessary for growth and reproduction of the cells. Moreover, a great portion of available glucose must be wasted in the direct oxidation to gluconic acid. This process,

wasteful to the economy of the cell could account for the disaccharide preference. Finally, should the direct oxidation of glucose involve the formation of hydrogen peroxide it could account for the poor growth on glucose. However, the organism was catalase-negative and this is not thought to be an important factor in this explanation.

VII. CONCLUSIONS

1. Numerous cultures of aerobic cellulose-decomposing bacteria were isolated from a wide variety of soil types, compost and dung. Approximately 10% of 154 organisms attacking cellulose grew slowly or not at all on glucose and about 50% of these were isolated from bovine feces. Many of these organisms exhibited organic nitrogen or vitamin requirements.
2. A cellulolytic bacterium isolated from fresh bovine feces was purified and identified as a member of the genus Cellvibrio gilvus. Its relationship to known species was discussed.
3. The ability of Cellvibrio gilvus to decompose filter paper cellulose was shown. Cellobiose was found as a product of hydrolysis of cellulose yet glucose was not detected. A source of organic nitrogen was required for growth. Excellent growth was obtained on a mineral salts medium containing cellulose, a vitamin mixture and casein hydrolyzate.
4. Carbon distribution analysis of the fermentation products of cellobiose showed that volatile and non-volatile acids, volatile neutral products and carbon dioxide were formed.

5. A preference for the disaccharide, cellobiose, and weaker activity on glucose were demonstrated by growth rate which was from 30 to 46% greater with cellobiose than with glucose.
6. An investigation was conducted to explain the disaccharide preference and slow growth with glucose.
7. Intact cells oxidized glucose and cellobiose immediately without lag. The rate of oxidation from glucose was about 10% less than from cellobiose. The approximate ratios of oxygen assimilation for equivalent amounts of glucose, cellobiose and a mixture of glucose and cellobiose were 100:110:140. Thus a greater respiration rate was obtained in the presence of both sugars than with either alone when the total amount of hexose was the same in each case.
8. Inorganic phosphate was esterified by respiring, resting, intact cells presented with glucose or cellobiose.
9. A comparison of the utilization of glucose and cellobiose by intact cells revealed that about 130% more cellobiose was used at pH 6.5 and about 590% more at pH 7.0.
10. Fifty to fifty-eight percent more acid per 100 mg of sugar was produced from glucose than from cellobiose. On a molar basis about 30% more acid was formed from glucose.

11. The removal and addition of inorganic phosphate in suspensions of intact cells caused a decrease and a stimulation, respectively, of the rate of respiration of cellobiose. The presence of inorganic phosphate had no effect on the respiration of glucose.
12. Soluble cell-free enzymes utilized 7.25 micromoles of cellobiose in the presence of phosphate but only 2.84 micromoles of glucose in the presence of ATP when the cells were grown on cellobiose. ATP was required for glucose utilization by these soluble enzyme preparations. Inorganic phosphate addition increased the cellobiose utilization about two-fold. Soluble enzyme preparations from glucose-grown cells also utilized more cellobiose than glucose. Phosphate depressed the utilization of glucose while ATP inhibited the disappearance of cellobiose. The utilization of cellobiose was accompanied by esterification of inorganic phosphate.
13. The accumulation of fructose-6-phosphate in cells grown on cellobiose and on glucose was demonstrated. Cellobiose-grown cells contained approximately 6.5 mg total phosphorus per 2 grams of wet cells whereas glucose-grown cells contained only 2.1 mg total phosphorus per 2 grams of wet cells.

14. Glucose was formed as a product from cellobiose by cell-free enzyme preparations.

15. Glucose was converted to glucose-6-phosphate, fructose-6-phosphate and fructose-1,6-diphosphate by soluble enzyme preparations in the presence of ATP. Fructose-1,6-diphosphate was converted to pyruvic acid by soluble enzymes in the presence of DPN, TPN and methylene blue.

16. A cellobiose phosphorylase was demonstrated to be present in this species of Cellvibrio. The products were alpha-D-glucose-1-phosphate and glucose. The reversibility of the reaction was shown by the synthesis of cellobiose from glucose and alpha-D-glucose-1-phosphate. A conversion of alpha-D-glucose-1-phosphate to glucose-6-phosphate could not be obtained but fructose-6-phosphate was formed.

17. A ground cell preparation virtually free of intact cells was obtained that could oxidize fructose-1, 6-diphosphate and fructose-6-phosphate in the presence of DPN, coenzyme-A and methylene blue.

18. The ground cell preparations in which the oxidizing systems were saturated with fructose-1,6-diphosphate and cofactors were able to oxidize glucose and cellobiose at greatly increased rates above that obtained with the ester.

19. Gluconic acid was detected in the final reaction mixtures from the oxidation of glucose and cellobiose.
20. An explanation for the anomolous preference of disaccharide in this species of Cellvibrio was given which was compatible with the data.
21. The pathways for the metabolism of glucose and cellobiose were proposed.

VIII. SUMMARY

Numerous aerobic cellulose-decomposing bacteria were isolated from a wide variety of sources and some of these grew slowly or not at all on glucose and exhibited organic nitrogen requirements. A cellulolytic bacterium isolated from fresh bovine feces was characterized and identified in the genus Cellvibrio. Its species name was discussed and some of the growth requirements obtained.

The carbohydrate metabolism of intact cells and of cell-free enzymes was investigated in order to explain the preferential utilization of disaccharide demonstrated in this organism. Evidence for the metabolic pathways of glucose and cellobiose was obtained and a proposed scheme of these pathways was made. A cellobiose phosphorylase was demonstrated to be present in this organism.

Finally, an explanation of the disaccharide preference was presented which was based on the knowledge of carbohydrate metabolism of this organism.

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ABSTRACT OF THE DISSERTATION

METABOLIC BASIS FOR THE PREFERENTIAL UTILIZATION OF DISACCHARIDE BY THE CELLULOSE-DECOMPOSING BACTERIUM, CELLVIBRIO GILVUS (NOV. SP.)

by

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A cellulose-decomposing bacterium isolated from bovine feces was purified, identified as a member of the genus, Cellvibrio, and the new species name gilvus was proposed.

Cellulose decomposition was demonstrated and cellobiose was the only hydrolytic sugar product. Excellent growth was obtained on mineral salts medium containing cellobiose, a vitamin mixture, and organic nitrogen (casein hydrolyzate) which was required for growth. Volatile and non-volatile acids, volatile neutral compounds, and carbon dioxide constituted the fermentation products.

A preference for cellobiose was shown by a 30 to 46% greater growth rate than resulted on glucose. An investigation was conducted to explain this disaccharide preference.

Intact cells oxidized glucose and cellobiose immediately and the rate of glucose oxidation was 10% less than obtained from cellobiose. Thus, hypotheses that adaptive hexokinase, hexokinase deficiency or impermeability to glucose could explain the preference were dispelled. Oxygen assimilation ratios of equivalent amounts of glucose, cellobiose and a mixture of these were 100:110:140. Alternate metabolic pathways were indicated.

Resting cells grown on cellobiose esterified inorganic phosphate in the presence of glucose or cellobiose showed that these sugars were metabolized to phosphate esters.

Relative rates of utilization of both sugars revealed that 130% more cellobiose was used at pH 6.5 and 590% more at pH 7.0. On a molar basis 30% more acid was formed from glucose than from cellobiose. A phosphorylase was indicated by the stimulation of cellobiose respiration by inorganic phosphate.

Disaccharide preference was associated with the intracellular enzymes because soluble enzymes utilized 7.25 uM of cellobiose but only 2.8 uM of glucose. The adenosine triphosphate requirement for glucose utilization indicated hexokinase activity. Inorganic phosphate increased cellobiose utilization two-fold and was accompanied by esterification. Soluble enzymes from glucose-grown cells produced a constitutive cellobiose enzyme. Phosphate depressed glucose utilization while adenosine triphosphate depressed cellobiose utilization.

Fructose-6-phosphate was the only ester detected in cells grown on either sugar. Cellobiose-grown cells contained 6.5 mg total P/2 g cells whereas glucose-grown cells contained only 2.1 mg.

Glucose was converted to glucose-6-phosphate, fructose-6-phosphate and fructose-1,6-diphosphate by cell-free enzymes in the presence of adenosine triphosphate. Fructose-1,6-diphosphate was metabolized to pyruvic acid. This was evidence for an Embden-Meyerhof pathway.

A cellobiose phosphorylase was demonstrated as a reversible reaction producing glucose and alpha-D-glucose-1-phosphate. Conversion of glucose-1-phosphate to glucose-6-phosphate was not obtained but fructose-6-phosphate was formed.

Ground cells oxidized fructose-1,6-diphosphate and fructose-6-phosphate in the presence of diphosphopyridine nucleotide, coenzyme-A and methylene blue. These preparations in which oxidizing systems were saturated with fructose-1,6-diphosphate and cofactors oxidized glucose and cellobiose at greatly increased rates above that obtained from the ester. Gluconic acid was detected in the final reaction mixtures.

An explanation of the disaccharide preference resides in several factors. The very active direct phosphorylation of cellobiose yielding a hexose phosphate at less expense energy-wise than the adenosine triphosphate-requiring phosphorylation of glucose showed cellobiose to be a more efficiant energy-yielding substrate. The weak hexokinase would limit the formation of hexose phosphates which in turn could impede the energy obtained as high energy phosphate compounds necessary for growth and reproduction. A portion of available glucose was probably wasted in the direct oxidation to gluconic acid. Finally, a proposed scheme for the metabolic pathways of glucose and cellobiose in Cellvibrio gilvus was presented.