MONTANA STATE UNIVERSITY

Studies on a purified cellobiose phosphorylase

by James K Alexander

A THESIS Submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Bacteriology
Montana State University
© Copyright by James K Alexander (1959)

Abstract:
Cellobiose phosphorylase, obtained from cell-free extracts of Clostridium themocellum strain 65l, has been purified sufficiently to eliminate interfering reactions. The purified enzyme catalyzes the phosphorolysis of cellobiose with the formation of equimolar quantities of glucose-l-phosphate and glucose. The reaction is reversible for equimolar quantities of cellobiose and inorganic phosphate are formed from glucose and glucose—l-phosphate. At equilibrium the molar ratio of cellobiose and inorganic phosphate to glucose-l-phosphate and glucose is about 2 to 1. The equilibrium constant, K = (glucose-l-phosphate)(glucose)/(cellobiose)(inorganic phosphate), at 37 C and pH 7 is about 0.23.

The optimum pH for the activity of cellobiose phosphorylase appears to be about 7.0. The temperature coefficient between 20 C and 40 C is about 2.4. The effect of various inhibitors has been determined and the results suggest that a sulfhydryl group is essential for the activity of the enzyme.

The synthesis of glucosyl-D-xyloses glucosyl-L-xylose, glucosyl-D-deoxyglucose, and glucosyl-D-glucosamine apparently is catalyzed by cellobiose phosphorylase. The enzyme also catalyzes the arsenolysis of cellobiose.

Beta-glucose—l-phosphate is unable to replace alpha-glucose-l-phosphate in the synthesis of cellobiose. A Walden inversion apparently occurs in the reaction. Cellobiose phosphorylase apparently does not catalyze the exchange between glucose-l-phosphate and arsenate or between cellobiose and D-xylose. These results suggest that a ' cellobiose-enzyme-phosphate complex is formed in the reaction.

STUDIES ON A PURIFIED CELLOBIOSE PHOSPHORYLASE

by

JAMES K. ALEXANDER

A THESIS

Submitted to the Graduate Faculty

in

partial fulfillment of the requirements

for the degree of

Doctor of Philosophy in Bacteriology

at

Montana State College

Approved:

Louis De Smet
Head, Major Department

Richard H. Willard
Chairman, Examining Committee

Leon J. Johnson
Dean, Graduate Division

Bozeman, Montana
June, 1959

[redacted]K

1987

D378
Al27s
Cop. 2

TABLE OF CONTENTS

ACKNOWLEDGEMENTS 6

ABSTRACT 7

INTRODUCTION 8

REVIEW OF THE LITERATURE 10
Enzymes involved in the decomposition of cellulose 12
Phosphorylases 14

MATERIALS AND METHODS 19
Enzyme source 19
Crude enzyme preparations 20
Purified enzyme preparations 21
Protein determinations 22
Determination of the products and reactants of the cellobiose phosphorylase reaction 22
a) Glucose determinations 23
b) Cellobiose determinations 24
c) Inorganic phosphate determinations 26
d) Glucose-1-phosphate determinations 27
Phosphoglucomutase activity 27
Chromatography 27
Colorimetric determinations of reducing sugars 27
Kinase activity 28
Barbital-acetate buffer 28

RESULTS 29
A. Studies on the cellobiose phosphorylase reaction 29
Elimination of interfering reactions 29
Analyses of the products and reactants of cellobiose phosphorolysis 35
Analyses of the products and reactants of cellobiose synthesis 35
Equilibrium constant of the cellobiose phosphorylase reaction 39
Free energy calculations 42
B. Properties of cellobiose phosphorylase 44
The effect of hydrogen ion concentration on the activity of cellobiose phosphorylase 44
The effect of temperature on the activity of cellobiose phosphorylase 45
The effect of p-chloromercuribenzoate on the activity of cellobiose phosphorylase 47
The effect of $AgNO_3$ on the activity of cellobiose phosphorylase 48
The effect of phlorizin on the activity of cellobiose phosphorylase 50

130563

The effect of glucose on cellobiose phosphorolysis 51
The effect of NaF, $(NH_4)_2SO_4$, NaCl, and $MgSO_4$ on the activity of cellobiose phosphorylase 54
Specificity of cellobiose phosphorylase 56
1) The synthesis of disaccharides 56
2) The phosphorolysis of disaccharides 61
3) Beta-glucose-1-phosphate 62
4) Arsenolysis 63
C. The mechanism of the cellobiose phosphorylase reaction 67
D. Pathways of cellobiose metabolism in C. thermocellum 71

DISCUSSION 74
The specificity of cellobiose phosphorylase 81
The mechanism of the cellobiose phosphorylase reaction 87

SUMMARY 94

REFERENCES 96

FIGURES

Figure 1.	Disappearance of inorganic phosphate during the phosphorolysis of cellobiose.	36
Figure 2.	The liberation of inorganic phosphate during the synthesis of cellobiose.	38
Figure 3.	The activity of cellobiose phosphorylase at different pH values.	46
Figure 4.	Chromatographs of glucosyl-D-xylose and glucosyl-L-xylose.	60
Figure 5.	The arsenolysis of cellobiose with cellobiose phosphorylase.	66
Figure 6.	Chromatographs of reaction mixtures incubated with cellobiose and D-xylose.	70
Figure 7.	Evidence that cellobiokinase is produced by Clostridium thermocellum strain 651.	73
Figure 8.	Pathways of cellobiose utilization by Clostridium thermocellum.	74
Figure 9.	Diagramatic representation of the specificity of cellobiose phosphorylase.	85
Figure 10.	The relationship of the sucrose, maltose, and cellobiose phosphorylase reactions.	89
Figure 11.	The formation of an inverted glucose-enzyme complex from alpha-glucose-1-phosphate and sucrose phosphorylase.	90
Figure 12.	The formation of sucrose and free sucrose phosphorylase from a glucose-enzyme complex and fructose.	90
Figure 13.	An illustration of the single displacement mechanism proposed for the maltose phosphorylase reaction.	90
Figure 14.	A proposed single displacement mechanism for the cellobiose phosphorylase reaction.	92

TABLES

Table I	The activity of enzyme preparations from Clostridium thermocellum strain 651 on glucose-1-phosphate.	30
Table II	Determination of cellobiase activity in enzyme preparations from Clostridium thermocellum strain 651.	33
Table III	Analyses of the products and reactants of cellobiose phosphorolysis.	37
Table IV	Analyses of the products and reactants of cellobiose synthesis.	37
Table V	Analyses of the products and reactants of a reaction with purified cellobiose phosphorylase and approximately equilibrium amounts of cellobiose, inorganic phosphate, glucose and glucose-1-phosphate.	40
Table VI	Summary of equilibrium data for the cellobiose phosphorylase reaction.	41
Table VII	The activity of cellobiose phosphorylase at different temperatures.	47
Table VIII	The effect of p-chloromercuribenzoate on the activity of cellobiose phosphorylase.	49
Table IX	The effect of silver nitrate on the activity of cellobiose phosphorylase.	50

Table	X	The effect of phlorizin on the activity of cellobiose phosphorylase.	51
Table	XI	The effect of glucose on cellobiose phosphorolysis.	53
Table	XII	The effect of NaF, NaCl, $(NH_4)_2SO_4$, and $MgSO_4$ on the activity of cellobiose phosphorylase.	55
Table	XIII	Liberation of inorganic phosphate when cellobiose phosphorylase was incubated with glucose-1-phosphate and various other compounds.	58
Table	XIV	The change in inorganic phosphate when cellobiose phosphorylase was incubated with inorganic phosphate and different disaccharides.	62
Table	XV	Liberation of inorganic phosphate when cellobiose phosphorylase was incubated with glucose and beta-glucose-1-phosphate or alpha-glucose-1-phosphate.	64
Table	XVI	The disappearance of inorganic phosphate in a reaction mixture used as a control in the arsenolysis experiment.	65
Table	XVII	The formation of free glucose when cellobiose phosphorylase was incubated with glucose-1-phosphate and arsenate.	68
Table	XVIII	A summary of the information concerning the specificity of cellobiose phosphorylase.	83

ACKNOWLEDGEMENTS

The author wishes to thank Dr. Richard H. McBee for his guidance and advice throughout the course of this study. The author is also indebted to Dr. Nels M. Nelson and Dr. John E. Gander for their advice. The helpful suggestions of Dr. Louis DS. Smith and Dr. William G. Walter in writing this thesis are greatly appreciated.

This investigation was supported in part by a research grant from the National Institutes of Health.

ABSTRACT

Cellobiose phosphorylase, obtained from cell-free extracts of Clostridium thermocellum strain 651, has been purified sufficiently to eliminate interfering reactions. The purified enzyme catalyzes the phosphorolysis of cellobiose with the formation of equimolar quantities of glucose-1-phosphate and glucose. The reaction is reversible for equimolar quantities of cellobiose and inorganic phosphate are formed from glucose and glucose-1-phosphate. At equilibrium the molar ratio of cellobiose and inorganic phosphate to glucose-1-phosphate and glucose is about 2 to 1. The equilibrium constant, $K = \frac{(\text{glucose-1-phosphate})(\text{glucose})}{(\text{cellobiose})(\text{inorganic phosphate})}$, at 37 C and pH 7 is about 0.23.

The optimum pH for the activity of cellobiose phosphorylase appears to be about 7.0. The temperature coefficient between 20 C and 40 C is about 2.4. The effect of various inhibitors has been determined and the results suggest that a sulfhydryl group is essential for the activity of the enzyme.

The synthesis of glucosyl-D-xylose, glucosyl-L-xylose, glucosyl-D-deoxyglucose, and glucosyl-D-glucosamine apparently is catalyzed by cellobiose phosphorylase. The enzyme also catalyzes the arsenolysis of cellobiose.

Beta-glucose-1-phosphate is unable to replace alpha-glucose-1-phosphate in the synthesis of cellobiose. A Walden inversion apparently occurs in the reaction. Cellobiose phosphorylase apparently does not catalyze the exchange between glucose-1-phosphate and arsenate or between cellobiose and D-xylose. These results suggest that a cellobiose-enzyme-phosphate complex is formed in the reaction.

INTRODUCTION

Cellulose is one of the most abundant organic compounds in nature. Besides being one of the end products of photosynthesis, cellulose is a major constituent of plants.

Annually many plants die. Yet ordinarily dead plant material does not accumulate in soil or in water. Microorganisms are responsible for the decomposition of practically all dead plant material. The carbonaceous material of plants, of which cellulose composes up to 50 per cent of the dry weight, is decomposed and carbon dioxide is ultimately formed. The carbon dioxide which is liberated from this decomposition becomes available, once again, for photosynthesis. Thus, in this way cellulose participates in the carbon cycle.

Cellulose is of vital importance in the nutrition of ruminant animals. Microorganisms in the rumen ferment the cellulose of plant material and the end products of fermentation serve as energy sources for these animals.

Although the decomposition of cellulose by microorganisms is essential in nature the mechanism by which it is decomposed is not well understood. It has been generally thought that cellulose is hydrolyzed by one or more extracellular enzymes to cellobiose or glucose. Until recently the enzymatic hydrolysis of cellobiose was the only biological mechanism by which cellobiose was known to be decomposed.

The hydrolysis of cellobiose, however, did not seem to provide an adequate explanation for the decomposition of cellobiose by all

cellulolytic bacteria. Clostridium thermocellum can use cellobiose as an energy source but not glucose (McBee 1948a, 1948b). If glucose were formed from cellobiose simply by hydrolysis, it might be expected to serve as an energy source for the organism. This anamolous situation stimulated investigations in which pathways other than hydrolysis were found for cellobiose utilization.

An enzyme, cellobiose phosphorylase, which catalyzed the phosphorolysis of cellobiose was found in cell-free extracts of C. thermocellum (Sih, 1955; Sih and McBee, 1955a and 1955b). Later an enzyme, cellobiokinase, which in the presence of adenosine triphosphate and cellobiose catalyzed the formation of a cellobiose-phosphate ester, was found in extracts from the same organism (Nelson and McBee, 1957).

The purpose of the present study was to confirm and to extend the previous work on cellobiose phosphorylase. Quantitative studies with a purified enzyme were made in order to determine precisely the products and reactants of the cellobiose phosphorylase reaction. Furthermore, some of the properties of cellobiose phosphorylase were determined.

REVIEW OF THE LITERATURE

The fermentation of cellulose *in vitro* at room temperature was demonstrated by Hoppe-Seylor (1883) as long as 75 years ago. The fermentation of cellulose at temperatures above 50 C was attributed to thermophilic bacteria by Macfadyen and Blaxall (1899), who inoculated cotton suspensions with soil.

Others have studied the fermentation of cellulose with crude or partially purified bacterial cultures (Pringsheim, 1912 and 1913; Khouvine, 1923; Viljoen, Fred and Peterson, 1926; Cowles and Rettger, 1931; and Pochon, 1942). In these studies organic acids, ethanol, carbon dioxide, hydrogen, and methane were reported as fermentation products.

Viljoen *et al.*, (1926) suggested *Clostridium thermocellum* for the name of the organism with which they worked, although they probably did not have a pure culture (McBee, 1948a).

Considerable difficulty was experienced by different workers in isolating pure cultures of anaerobic cellulolytic bacteria (McBee, 1948a). Furthermore, the criteria for establishing the purity of a culture were inadequate. It was not until the work of Hungate (1944 and 1950) that these difficulties were overcome.

Hungate (1944) isolated *Clostridium cellobioparus* (*Clostridium cellobioparum*, Breed et al., 1957) in pure culture by using a new technique which consisted of eliminating oxygen from the medium with an oxygen-free gas and growing the organisms in shake tubes. The criteria which indicated that a pure culture had been obtained were essentially the same as those described by him in a later publication (Hungate, 1950).

"The organism must grow in solid cellulose medium and give isolated spots in which the cellulose is digested and in which the cellulose-digesting colony (or diffuse growth if no colony is formed) may be identified and picked. Picked colonies must give growth in solid cellulose medium when inoculated into dilution series and the numbers of colonies obtained must be approximately proportional to the number of colonies expected. From cellulose, the organism must be inoculated into a solid sugar or other clear medium in which all types of colonies which develop can be seen. A single colony in a high dilution must be subcultured in a second clear dilution series and this must be continued until two successive series give colonies of only one sort, the uniformity being determined not only macroscopically but also by microscopic examination. Any colony from this second sugar series must upon reinoculation into a solid cellulose medium give rise to cellulose-digesting colonies present in approximately the expected numbers in the various dilutions and containing cells microscopically similar to those in the sugar series. Cultures which successfully pass these tests under critical scrutiny are considered pure."

Two pure cultures of anaerobic, thermophilic, cellulolytic bacteria were isolated by McBee (1948a and 1948b) using the technique developed by Hungate. McBee (1948b and 1950) obtained four impure cultures from other workers and from each he was able to isolate a pure culture. A comparative study of the two fresh isolates and the isolates obtained

from impure cultures revealed that all were closely related. Thus these six cultures were considered as strains of C. thermocellum.

This organism is an obligately anaerobic rod which grows slowly below 50 C and which does not grow on cellulose media above 68 C (McBee, 1950). C. thermocellum is quite limited in its ability to use various substrates as energy sources. It is able to ferment cellulose, cellobiose, xylose, and hemicellulose, but it does not ferment any of the other common sugars. The fermentation products are H_2, CO_2, ethanol, formic acid, acetic acid, and lactic acid.

Another thermophilic, cellulolytic bacterium, Clostridium thermocellulaseum, has been described by Eneboe (1951). This organism differs from C. thermocellum in that it is able to ferment glucose.

Enzymes involved in the decomposition of cellulose. In studying the fermentation of cellulose Pringsheim (1912) showed that cellobiose and glucose accumulated when growth was inhibited by the addition of iodoform to the medium. The antiseptic apparently stopped growth but did not stop the activity of the extracellular enzymes. He reasoned that both cellobiose and glucose were intermediates in the fermentation of cellulose. Consequently, Pringsheim postulated that cellulose was hydrolyzed to cellobiose by cellulase and that cellobiose was hydrolyzed to glucose by cellobiase.

A mechanism for the hydrolysis of cellulose has been postulated on the basis of work with bacteria and fungi (Reese et al., 1950). In this mechanism, undegraded cellulose is attacked by an enzyme,

C_1, with the production of shorter linear polyanhydro-glucose chains. Another enzyme, Cx, hydrolyses the end products of the C_1 reaction producing small molecules capable of diffusion into the cell. Levinson et al., (1950) concluded that cellobiose was produced in the Cx reaction.

On the other hand, Whitaker (1953), working with fungi, has proposed that only one enzyme is responsible for cellulose hydrolysis. Moreover, he suggested (Whitaker, 1957) a random cleavage of glucose and cellobiose units from cellulose molecules. However, it appears that some clostridia produce cellobiose, but not glucose, during the hydrolysis of cellulose. Cellobiose accumulated under certain conditions in the medium of C. cellobioparus cultures but no glucose was found (Hungate, 1944). Since cellobiose accumulated under conditions unfavorable for cellular metabolism, glucose also would be expected to accumulate if there were random cleavage of cellobiose and glucose units from cellulose molecules. There is reason to suspect that cellobiose, but not glucose is formed during the hydrolysis of cellulose by C. thermocellum. This organism is able to use cellobiose but not glucose as an energy source (McBee, 1948a, 1948b and 1950), yet glucose does not accumulate in the medium under ordinary conditions. The accumulation of glucose would be expected if this sugar were an end product of cellulose hydrolysis but could not be utilized by the organism.

The anomalous situation whereby a disaccharide can be utilized but not its monosaccharide constituents stimulated further investigation by Sih (1955) and Sih and McBee (1955a and 1955b). They postulated that

cellobiose was cleaved by mechanisms other than hydrolysis. In cell-free extracts from C. thermocellum they found an enzyme, cellobiose phosphorylase, which catalyzed the phosphorolysis of cellobiose. Cellobiose phosphorylase was found in similar extracts from C. cellobioparus (Macmillan, 1956).

Cellobiose apparently is decomposed by another mechanism involving cellobiokinase (Nelson and McBee, 1957). Cell-free extracts obtained from C. thermocellum catalyzed the formation of a cellobiose-phosphate ester in the presence of adenosine triphosphate. The formation of this cellobiose-phosphate ester was attributed to cellobiokinase activity.

Phosphorylases. A phosphorylase is an enzyme which catalyzes the cleavage of a substrate with the uptake of inorganic phosphate. Phosphorolysis differs from hydrolysis in that phosphate, instead of water, combines with one of the cleavage products (Parnas, 1937).

In the phosphorolysis of glycogen by muscle phosphorylase, glucose-1-phosphate was formed (Cori et al., 1937). The reaction was reversible for glycogen was synthesized from glucose-1-phosphate (Cori, Schmidt and Cori, 1939). A small amount of glycogen or some related material was necessary as a primer for the synthesis of a polysaccharide with muscle phosphorylase (Cori and Cori, 1939).

Arsenate is able to substitute for phosphate in the breakdown of glycogen resulting in the arsenolysis of glycogen. Glucose-1-arsenate presumably was formed which decomposed spontaneously to glucose and arsenate (Cohn and Cori, 1948). Direct evidence that glucose-1-arsenate is formed during the arsenolysis of glycogen has been presented by Slocum and Varner

(1958) with the use of isotopic oxygen. Muscle phosphorylase, however, did not catalyze the exchange between glucose-1-phosphate and phosphate or arsenate (Cohn and Cori, 1948).

A phosphorylase was found in peas which catalyzed both the phosphorolysis and the synthesis of starch (Hanes, 1940b). As in polysaccharide synthesis with muscle phosphorylase a primer was needed for polysaccharide synthesis with potato phosphorylase (Green and Stumpf, 1942). Potato phosphorylase did not catalyze the synthesis of a polysaccharide from glucose-6-phosphate, glucose, fructose, fructose-1-phosphate, or fructose-1, 6-phosphate (Hanes, 1940b and Green and Stumpf, 1942).

Potato phosphorylase did not catalyze the exchange between glucose-1-phosphate and inorganic phosphate or inorganic arsenate (Cohn and Cori, 1948 and Katz and Hassid, 1948). The enzyme did, however, catalyze the arsenolysis of starch (Katz and Hassid, 1948).

Sucrose phosphorylase was found to be produced by Pseudomonas saccharophilia (Douderoff et al., 1943). This enzyme catalyzes both the phosphorolysis and the synthesis of sucrose according to the equation, sucrose + inorganic phosphate = alpha-glucose-1-phosphate + fructose. The equilibrium constant, K = (sucrose)(inorganic phosphate)/(glucose-1-phosphate)(fructose) at 30 C is approximately 0.05 at pH 6.6 and 0.09 at pH 5.8 (Douderoff, 1943).

Sucrose phosphorylase is able to catalyze the synthesis of 4 different disaccharides besides sucrose. The following reactions are catalyzed by sucrose phosphorylase (Hassid and Douderoff, 1950): (1)alpha-D-glucose-

1-phosphate ⇌ L-sorbose ⇌ alpha-D-glucopyranosyl-alpha-L-sorbofuranoside ⇌ phosphate.

(2) alpha-D-glucose-1-phosphate ⇌ D-xyloketose ⇌ alpha-D-glucopyranosyl-beta-D-xyloketofuranoside ⇌ phosphate

(3) alpha-D-glucose-1-phosphate ⇌ L-araboketose ⇌ alpha-D-glucopyranosyl-alpha-L-araboketofuranoside ⇌ phosphate

(4) alpha-D-glucose-1-phosphate ⇌ L-arabinose ⇌ 3-(alpha-D-glucopyranosyl)-L-arabinopyranose ⇌ phosphate

In the presence of sucrose and arsenate, sucrose phosphorylase catalyzed the formation of glucose and fructose (Douderoff et al., 1949). Glucose-1-arsenate apparently was formed but decomposed spontaneously to glucose and arsenate.

Sucrose phosphorylase has been shown to act as a transglucosidase as well as a phosphorylase. The enzyme has the ability to act as a glucose acceptor and donor for one of its substrates in the absence of other glucose donors or acceptors. The first indication of the transglucosidase activity of the enzyme was the observation that labelled inorganic phosphate readily equilibrated with glucose-1-phosphate in the absence of any other substrate (Douderoff et al., 1947). The reaction for the exchange between labelled inorganic phosphate and glucose-1-phosphate was postulated as glucose-1-phosphate* ⇌ enzyme ⇌ glucose-enzyme ⇌ phosphate*. Likewise, the exchange between glucose-1-phosphate and arsenate was catalyzed by the enzyme (Douderoff et al., 1949). The enzyme catalyzed the exchange between sucrose and labelled fructose, D-glucose-1-fructoside* (sucrose) ⇌ enzyme ⇌ glucose-enzyme ⇌ fructose*

(Wolochow et al., 1949). The transglucosidase activity is further indicated by the ability of the enzyme to catalyze the exchange between D-glucose-xyloketoside and fructose and the exchange between sucrose and sorbose (Doudoroff et al., 1947) as shown in the following equations:

(1) D-glucose-1-fructoside + sorbose = D-glucose-sorboside + fructose

(2) D-Glucose-1-xyloketoside + fructose = D-glucose-1-fructoside + xyloketose.

The postulated glucose-enzyme complex explains all the reactions catalyzed by sucrose phosphorylase. The phosphorolysis of sucrose apparently occurs according to the following scheme:

(1) D-glucose-1-fructoside + enzyme = glucose-enzyme + fructose

(2) glucose-enzyme + phosphate = glucose-1-phosphate + enzyme.

Maltose phosphorylase has been found in cell-free extracts of Nesseria meningitidis (Fitting and Scherp, 1952). Both the synthesis and the phosphorolysis of maltose is catalyzed by maltose phosphorylase (Fitting and Doudoroff 1952a and 1952b) in accordance with the equation:

Maltose + inorganic phosphate = beta-glucose-1-phosphate + glucose

The equilibrium constant, K = (Maltose) (phosphate)/(glucose-1-phosphate) (glucose), is about 4.4 at 37 C (Fitting and Doudoroff, 1952b).

In the reaction, maltose, with an alpha linkage, is phosphorolyzed and a phosphate ester with a beta linkage is formed. Alpha-glucose-1-phosphate can not substitute for beta-glucose-1-phosphate in the synthesis of maltose. Thus, a Walden inversion occurs in the reaction.

Glucosyl-D-xylose as well as maltose can be synthesized with maltose

phosphorylase (Fitting and Douderoff, 1952b). No reaction occurred, however, when maltose phosphorylase was incubated with beta-glucose-1-phosphate and D-fructose, D-galactose, L-glucose, D-mannose, D-ribose, D-arabinose, alpha-methyl glucoside, alpha-D-glucose-1-phosphate, maltose, trehalose, cellobiose, and D-gluconic acid.

In contrast to sucrose phosphorylase, maltose phosphorylase did not catalyze the exchange between glucose-1-phosphate and inorganic phosphate, or between maltose and glucose. Maltose phosphorylase catalyzed the arsenolysis of maltose but it did not catalyze the arsenolytic decomposition of glucose-1-phosphate. These data indicate that maltose phosphorylase did not form a glucose-enzyme complex as has been postulated for sucrose phosphorylase (Douderoff et al., 1947). It appeared that both a glucose donor and acceptor had to be present in order for the transfer of glucose to take place. Consequently, a maltose-enzyme-phosphate complex was postulated to be formed in the reaction.

Cellobiose phosphorylase was demonstrated to be present in cell-free extracts from C. thermocellum strain 157 (Sih, 1955; and Sih and McBee 1955a and 1955b). The reaction for the phosphorolysis of cellobiose presumably is cellobiose + inorganic phosphate = alpha-glucose-1-phosphate + glucose. The synthesis of cellobiose from alpha-glucose-1-phosphate and glucose was demonstrated by Sih, Nelson, and McBee (1957). The cellobiose phosphorylase reaction apparently resembles the maltose phosphorylase reaction in that a Walden inversion occurs.

MATERIALS AND METHODS

Enzyme source. An active cellobiose phosphorylase was obtained in cell-free extracts of C. thermocellum strain 651. The isolation, cultural characteristics, and physiology of this thermophilic, cellulolytic organism have been described by McBee (1948a, 1948b, 1950, and 1954).

The medium used to cultivate this organism contained the following percentages of material: NaCl, 0.3; $(NH_4)_2SO_4$, 0.1; K_2HPO_4, 0.05; KH_2PO_4, 0.05; $MgSO_4 \cdot 7H_2O$, 0.01; $CaCl_2 \cdot 2H_2O$, .001; sodium thioglycollate, 0.02; yeast extract, 0.1; cellulose, 0.25; and $NaHCO_3$, 0.5. A small amount of resazurin was added to the medium as an oxidation-reduction indicator because in order for growth to occur, it was necessary that the medium be sufficiently reduced to turn resazurin colorless. Suspensions of cellulose were prepared by the method described by Hungate (1950). The medium was made up to the desired volume with tap water and sterilized at 121 C for 20 to 60 minutes depending upon the amount of medium. Sodium bicarbonate, in 5 per cent solution which had been sterilized by filtration, was added to tubes containing 10 ml of medium. For larger amounts of media, dry $NaHCO_3$ was sterilized in a hot air oven at 160-180 C for 2 hours and added to the medium immediately before inoculation.

For obtaining cells to be used in making the cell free extracts, 20 L cultures were grown. A series of cultures of increasing size were grown in order to obtain inoculum for 20 L cultures. The first of these, (10 ml), was inoculated with one ml of an old 100 ml culture stored at

room temperature, and after growth, was used to inoculate 90 ml of medium. This 100 ml culture was used as inoculum for 1400 ml of medium which in turn was used to inoculate 18.5 L of medium.

All cultures were flushed with carbon dioxide after inoculation in order to lower the pH and to eliminate oxygen from the medium. The technique of obtaining anaerobic conditions by flushing the medium with an oxygen-free gas has been described by Hungate (1950). The cultures were incubated at 55 C. Much faster growth resulted in the 20 L cultures when the medium was stirred constantly. Hence, these cultures were stirred with a magnetic mixer.

The first sign of growth in the cultures was the evolution of gas from the medium. This persisted throughout growth and for a period after all the visible cellulose had disappeared. All of the cellulose was usually digested in 1 to 3 days. Incubation was discontinued after all the cellulose had disappeared but before the gas had ceased to evolve. The 20 L cultures were harvested with a Sharples centrifuge at a speed of about 40,000 to 50,000 rpm yielding 15 to 25 g of cells (wet weight).

Crude enzyme preparations. Cell-free extracts were prepared by grinding the cell paste with glass beads (average diameter, 200 microns) in a Waring blendor with a semi-micro container (Lamanna and Mallette, 1954). An ice bath, surrounding the container, served to keep the material cool during grinding. Fifteen to 30 g of cell paste, 7 to 15 ml of distilled water, and 20 to 40 g of glass beads were added to the blendor. The cells were ground for 30 to 60 minutes and then 20 to 40 ml

of distilled water were added. Mixing was resumed until an even suspension was obtained. The cell debris, unbroken cells and glass beads were centrifuged down at approximately 6,000 X G for 1 hour. The supernatant fluid was used as a crude enzyme source.

Purified enzyme preparations. In order to study the cellobiose phosphorylase reaction in the absence of interfering reactions it was necessary to purify the crude enzyme preparations.

Phosphoglucomutase was the only interfering enzyme which was found in the crude enzyme preparations. Phosphoglucomutase was not inactivated by cold storage as Sih reported (1955). It was important to eliminate this enzyme because it converts glucose-1-phosphate, formed by phosphorolysis, to glucose-6-phosphate. Two other possible interfering enzymes, cellobiase and glucose-1-phosphatase, were not found in the crude enzyme preparations.

Ammonium sulfate fractionation appeared to be a suitable method for purification of the enzyme because it had been used in the purification of other bacterial phosphorylases (Fitting and Douderoff, 1952b and Weimberg and Douderoff, 1954). The following procedure, developed by trial and error, rendered the enzyme preparations free from interfering enzymes.

1) The crude enzyme preparation (containing 15 to 25 mg protein/ml) was made up to 0.27 saturation with a cold, saturated solution of ammonium sulfate.
2) The material was allowed to stand in the cold for 1 hour.
3) The material was centrifuged for 1 hour in the cold at about 6000

X G. The precipitate was discarded.

4) The supernatant fluid was made up to 0.64 saturation with ammonium sulfate.

5) The material was allowed to stand in the cold for 1 hour.

6) The material was centrifuged in the cold for 1 hour at about 6000 X G, the supernatant fluid was discarded, and the precipitate was dissolved in distilled water.

This entire procedure was repeated twice on the material containing the dissolved precipitate.

The activity per mg of protein of one of these purified enzyme preparations was about twice that of the crude enzyme material.

The purified enzyme preparations were found to be free from phosphoglucomutase, cellobiase, and glucose-1-phosphatase activity. Therefore, it was possible to study the cellobiose phosphorylase reaction with these preparations in the absence of known interfering reactions.

Protein determinations. The amount of protein in the enzyme preparations was estimated by the Biuret reaction according to the method developed by Levin and Braven (1951). A standard curve was made with known amounts of egg albumin. It was assumed that the color development with egg albumin was equal to the color development with an equal amount of bacterial protein. Color intensities were measured with a Klett-Summerson photoelectric colorimeter using a green filter (540 millimicrons).

Determination of the products and reactants of the cellobiose phosphorylase reaction. In certain experiments it was necessary to

determine the amount of change in the products and reactants of the cellobiose phosphorylase reaction. The products of cellobiose phosphorolysis are glucose and glucose-1-phosphate (Sih et al., 1957). In selecting methods to determine the amount of these compounds it did not seem feasible to attempt to determine glucose in the presence of cellobiose by colorimetric methods because cellobiose would interfere. Conversely, colorimetric determinations of cellobiose would be complicated by glucose interference. Therefore, glucose oxidase was used to determine the amount of glucose. The amount of cellobiose was estimated by determining the increase in free glucose resulting from the hydrolysis of cellobiose.

a) Glucose determinations. Amounts of glucose were determined manometrically with glucose oxidase (Keilin and Hartree, 1948) in the Warburg respirometer (Umbreit et al., 1957). In the glucose oxidase reaction glucose is oxidized to gluconate by the following reaction:

1) $C_6H_{12}O_6 + 1/2\ O_2 = C_6H_{12}O_7$

In the presence of catalase and ethanol twice as much oxygen is taken up per mole of glucose.

2) $C_6H_{12}O_6 + O_2 + RCH_2OH = C_6H_{12}O_7 + H_2O + RCHO$

The glucose oxidase was prepared by suspending 3 g of "DeeO" (a crude glucose oxidase preparation obtained from the Takamine Co.) in 100 ml of distilled water. The suspension was allowed to stand at room temperature for 1 hour except for brief periods of shaking. Then the particulate material was centrifuged down and the supernatant fluid was used as a source of glucose oxidase.

The main compartment of the Warburg vessels contained an aliquot of the unknown sample, or a known amount of glucose or cellobiose, 0.1 ml of a solution of catalase (technical grade obtained from the Nutritional Biochemicals Corporation), and 0.1 ml of 20 per cent (W/V) ethanol. In the sidearm of the vessels there was 0.5 ml of the glucose oxidase preparation. The total volume was 2.5 ml. These experiments were conducted at 37 C in an atmosphere of air.

The addition of catalase to the system increased the amount of oxygen uptake about 30 per cent, resulting in about 90 per cent of the oxygen uptake predicted in reaction 2.

The amount of glucose was estimated by comparing the amount of oxygen uptake with the amount of oxygen uptake from a glucose standard after a correction factor had been introduced. This correction factor was necessary because weak beta-glucosidase activity in the glucose oxidase preparations hydrolyzed some of the cellobiose in the sample, resulting in a falsely high value for glucose. The correction value was calculated from the oxygen uptake in a control vessel containing approximately the same amount of cellobiose that was contained in the experimental sample. The amount of cellobiose in the experimental sample was estimated from the change in the concentration of inorganic phosphate. The amount of glucose formed in a control vessel was subtracted from the amount of glucose found in an experimental sample and the difference was assumed to be equal to the amount of free glucose in the experimental sample.

b) Cellobiose determinations. Cellobiose was determined manometrically

by measuring the amount of glucose resulting from the hydrolysis of cellobiose. Cellobiose was hydrolyzed enzymatically by the beta-glucosidase present in the glucose oxidase preparation. The glucose resulting from the hydrolysis of cellobiose was determined manometrically as described above.

The beta-glucosidase in the crude glucose oxidase preparation was relatively inactive. However, a beta-glucosidase inhibitor, which was soluble in 50 per cent acetone, could be separated from the beta-glucosidase and glucose oxidase. After the crude glucose oxidase preparation had stood in 50 per cent acetone for one hour in the cold, the precipitate was centrifuged out. Several washings of the precipitate in cold acetone removed the remaining water. The precipitate was dissolved in water and contained both beta-glucosidase and glucose oxidase. The acetone-precipitated-preparation hydrolyzed cellobiose much faster than did the crude glucose oxidase preparation. The glucose oxidase in the acetone-precipitated-preparation oxidized the glucose formed from cellobiose hydrolysis.

The original amount of free glucose in an unknown sample as well as the glucose formed from the hydrolysis of cellobiose contributed to the oxygen uptake in these determinations. Therefore, the amount of oxygen uptake from the original free glucose, determined previously as described above, was subtracted from the total oxygen uptake in the vessel in order to determine the amount of glucose formed in the hydrolysis of cellobiose. The amount of oxygen uptake in a vessel containing a known amount of cellobiose was used as a standard in calculating the amount of cellobiose in

the unknown sample.

The vessel contents, temperature, and atmosphere for the determination of cellobiose were the same as for the determination of glucose.

c) Inorganic phosphate determinations. In the phosphorolysis of cellobiose, inorganic phosphate is esterified, resulting in the formation of glucose-1-phosphate. There is a decrease in the amount of inorganic phosphate equal to the amount of glucose-1-phosphate formed. Conversely, in the synthesis of cellobiose with this enzyme inorganic phosphate is liberated in an amount equal to the amount of glucose-1-phosphate utilized. Consequently, it was possible to estimate the amount of cellobiose phosphorylase activity by the change in the amount of inorganic phosphate.

Inorganic phosphate determinations were made by the method of Dryer et al., (1957) or by the method of Fiske and Subbarow (1925) as described by Umbreit et al., (1957). The color intensities were measured with a Klett-Summerson photoelectric colorimeter with a red filter (660 millimicrons). A phosphate standard was included in all determinations.

Samples were prepared for phosphate determinations by boiling the reaction mixtures for 3 minutes. Boiling served to facilitate protein precipitation and to stop the reaction by heat inactivation of the enzyme. An aliquot of the reaction mixture was diluted with equal parts of 10 per cent trichloroacetic acid and was then further diluted with distilled water. The protein, coagulated by heat and by the acid, was centrifuged down and the amount of inorganic phosphate in the clear supernatant fluid was determined. The difference in the amount of inorganic phosphate at the

beginning of the reaction and at the end of any given time interval was assumed to be due to cellobiose phosphorylase activity.

Glucose-1-phosphate determinations. The amount of acid-labile phosphate was measured in order to determine the amount of glucose-1-phosphate. Equal amounts of reaction mixture and 2 N HCl were boiled for 7 minutes, diluted, and centrifuged. A second sample received the same treatment except that it was boiled for 3 minutes before the acid was added instead of for 7 minutes after the acid was added. Both samples were analyzed and the difference in the amount of inorganic phosphate was assumed to be equivalent to the amount of glucose-1-phosphate.

Phosphoglucomutase activity. Phosphoglucomutase activity was estimated by measuring the disappearance of acid-labile phosphate when the enzyme preparation was incubated with glucose-1-phosphate. The conversion of glucose-1-phosphate to glucose-6-phosphate was assumed if there was a decrease in acid-labile phosphate.

Chromatography. Descending chromatography of sugars was done using a butanol-pyridine-water solvent in a ratio of 3:2:1.5 (Chargaff et al., 1948). Schleicher and Schuell filter paper, grade number 589 green, was used in all chromatography.

Aniline-acid-phthalate was used as a color developer (Barr, 1954). This reagent contained 6.64 g phthalic acid to which 40 ml of distilled water were added and then heated until the phthalic acid was dissolved. To this, 340 ml of isopropanol and 3 ml of aniline were added. The final volume was made up to 400 ml with distilled water.

Colorimetric determinations of reducing sugars. In experiments in

which the amount of glucose was measured in the absence of cellobiose it was possible to use colorimetric methods. In these determinations the method developed by Saifer et al., (1941) was used and the amount of glucose was estimated from a standard curve.

In experiments conducted to determine cellobiase activity the method of Levinson et al., (1950) was used. The reducing sugars were measured by the method described by Noetling and Bernfeld (1948).

Kinase activity. Kinase activity was determined manometrically by the method of Colowick and Kalckar (1943). In this method the acid formed from the kinase reaction causes the formation of carbon dioxide from a bicarbonate solution. The amount of carbon dioxide liberated is a measure of the kinase activity.

The main compartment of the Warburg vessels contained 0.3 ml of 0.22 M $NaHCO_3$, 0.2 ml of 0.1 M $MgSO_4$, 0.2 ml of 0.5 M NaF, 0.2 ml of 1.6 per cent sodium thioglycollate, and 0.9 ml of the crude enzyme preparation. The sidearm of the Warburg vessels contained 0.3 ml of 0.1 M cellobiose, 0.1 M glucose, or water as well as 0.1 ml of 0.22 M $NaHCO_3$, and 0.2 ml of 0.1 M adenosine triphosphate. The total volume was 2.4 ml. The reaction mixtures were incubated at 37 C in an atmosphere of 95 per cent nitrogen and 5 per cent carbon dioxide.

Barbital-acetate buffer. A stock solution of M/7 barbital-acetate buffer was made by dissolving 1.94 g sodium acetate and 2.94 g sodium barbital in 100 ml of carbon-dioxide-free distilled water. The stock solution was diluted to a M/35 concentration and this solution was adjusted to the desired pH. The M/35 solution was added to the reaction mixtures.

RESULTS

A. Studies on the cellobiose phosphorylase reaction

Although the cellobiose phosphorylase reaction has been reported previously by Sih (1955), Sih and McBee (1955a and 1955b), and Sih et al., (1957), no quantitative data on the reaction are available. Sih (1955) showed that cell free extracts from C. thermocellum strain 157 catalyzed the esterification of inorganic phosphate in the presence of cellobiose. Glucose and glucose-1-phosphate were formed from the phosphorolysis of cellobiose. Cellobiose synthesis from glucose-1-phosphate and glucose, catalyzed by cellobiose phosphorylase, was reported by Sih et al., (1957). These studies indicate that the following reaction is catalyzed by cellobiose phosphorylase: Cellobiose + inorganic phosphate = glucose + glucose-1-phosphate.

One of the objectives of the present investigation was to study the cellobiose phosphorylase reaction quantitatively. For this study it was essential to eliminate interfering reactions so that any changes in the system could be attributed to cellobiose phosphorylase.

Elimination of interfering reactions. The method of purifying the crude enzyme preparations has been described in the section on materials and methods. The only interfering enzyme which was encountered in the crude enzyme preparations was phosphoglucomutase. When the crude enzyme preparation was incubated with glucose-1-phosphate a decrease in acid-labile phosphate resulted (table I). Presumably phosphoglucomutase catalyzed the formation of glucose-6-phosphate from glucose-1-phosphate resulting in a decrease of acid-labile phosphate.

TABLE I

The activity of enzyme preparations from Clostridium thermocellum strain 651 on glucose-1-phosphate

Time in hr	Micromoles of inorganic phosphate/ml			
	Crude(Boiled)	Crude	Purified(Boiled)	Purified
0 After Hydrolysis*	5.9	5.8	4.8	4.8
0 Before Hydrolysis	2.8	2.6	0.7	1.3
0 Acid-labile	3.1	3.2	4.1	3.5
1 After Hydrolysis	6.4	3.4	4.7	4.5
1 Before Hydrolysis	2.5	2.9	0.8	1.1
1 Acid-labile	3.9	0.5	3.9	3.4
Change in acid-labile phosphate due to Phosphoglucomutase	+ 0.8	-2.7	-0.2	-0.1
Change in inorganic phosphate before hydrolysis, due to glucose-1-phosphatase	+ 0.3	+ 0.3	+ 0.1	-0.2

*Hydrolysis in 1 N HCl for 7 minutes in boiling water bath

Each ml of reaction mixture contained 3.8 micromoles of glucose-1-phosphate, 5 micromoles of $MgSO_4$, and 10 micromoles of cysteine. The tubes with the crude enzyme preparations contained 6.5 mg protein/ml; those with the purified enzyme, 1.5 mg protein/ml. The reaction mixtures were buffered with M/100 barbital-acetate buffer, pH 7.5, and incubated at 37 C. $MgSO_4$ was added because it has been shown that magnesium stimulated phosphoglucomutase activity (Najjar, 1948). Cysteine was added as a chelating agent because phosphoglucomutase can be activated by metal-binding agents (Sutherland, 1949).

There was no decrease in acid-labile phosphate with the purified enzyme preparation (table I). Therefore, it appeared that phosphoglucomutase activity was eliminated from the purified enzyme preparation. It is possible that this loss of phosphoglucomutase activity was due to the removal of the coenzyme, glucose-1,6-diphosphate (Caputto et al., 1948), and not due to the removal of phosphoglucomutase itself. This possibility was not investigated because it was important only to eliminate phosphoglucomutase activity.

No glucose-1-phosphatase activity was found in either the purified or the crude enzyme preparations. There was no significant increase in inorganic phosphate before acid hydrolysis as would be expected if glucose-1-phosphate had been hydrolyzed enzymatically (table I).

Considerable difficulty was encountered in determining whether the enzyme preparations contained cellobiase, since it appeared that cellobiase was active only in the presence of the glucose oxidase preparation. The apparent cellobiase activity, indicated by the formation of glucose, was stimulated by the addition of both boiled and unboiled glucose oxidase. At first it appeared that a coenzyme in the glucose oxidase preparation was needed for cellobiase activity. Later, however, it was found that the crude glucose oxidase preparations contained considerable amounts of inorganic phosphate. The addition of inorganic phosphate to the system which contained cellobiose and cellobiose phosphorylase apparently allowed glucose to be formed by phosphorolysis instead of by hydrolysis.

Further complications were encountered in attempts to remove the inorganic phosphate from the glucose oxidase by precipitating the enzyme

with 50 per cent acetone. Unexpectedly, it was found that the glucose oxidase, which originally had little beta-glucosidase activity, had a relatively active beta-glucosidase after acetone precipitation. A beta-glucosidase inhibitor, which was soluble in 50 per cent acetone, was removed from the glucose oxidase preparation by this treatment. The acetone soluble fraction inhibited beta-glucosidase activity when it was added to the precipitated glucose oxidase.

It seemed advisable to determine the cellobiase activity with another method. In the following experiment cellobiose phosphorylase preparations were incubated with cellobiose. Portions of the mixture were examined colorimetrically for an increased number or reducing groups. An increase in the reducing intensity would be expected from cellobiase activity because two moles of glucose would be formed per mole of cellobiose. Cellobiase activity was also estimated by determining the disappearance of cellobiose. Reaction mixtures, to which inorganic phosphate had been added, served as controls and the disappearance of cellobiose was compared to the disappearance of inorganic phosphate.

Both purified and crude enzyme preparations were incubated with cellobiose for 2 hours at 37 C. No significant increase in reducing groups was found in these reaction vessels (table II, part 1) as would be expected from cellobiase activity. Likewise, there was no significant increase in reducing groups in the control vessels containing cellobiose, inorganic phosphate and either the purified or the crude enzyme. An increase in the number of reducing groups would not be expected from phosphorolysis because the products of this reaction would be glucose,

TABLE II

Determination of cellobiase activity in enzyme preparation from Clostridium thermocellum strain 651.

Time in hr.	Reaction Mixture			
	Purified Enzyme Cellobiose	Purified Enzyme Cellobiose Phosphate	Crude Enzyme Cellobiose	Crude Enzyme Cellobiose Phosphate
	Part 1. Change in Number of Reducing Groups (Klett units)			
0	169	167	167	171
2	168	167	169	170
	Part 2. Disappearance of Cellobiose (micromoles/ml)			
0	20.0	19.7	20.2	20.0
2	20.4	18.1	19.9	16.7
	Amount of Change			
	+0.4	-1.6	-0.3	-3.3
	Part 3. Disappearance of Inorganic Phosphate (micromoles/ml)			
0	0.4	8.1	0.8	8.5
2	0.1	5.5	0.4	5.7
	Amount of Change			
	-0.3	-2.6	-0.4	-2.8

Each ml of reaction mixture contained 20 micromoles of cellobiose. The reaction mixtures with the purified cellobiose phosphorylase contained 1.5 mg protein/ml; those with the crude enzyme, 4.2 mg protein/ml. The control vessels contained 8 micromoles of inorganic phosphate/ml. The reaction mixtures were incubated at 37 C. The initial pH was approximately 7.0.

a reducing sugar, and glucose-1-phosphate, a non-reducing ester.

Cellobiase activity was also estimated by the decrease in cellobiose. In order to determine the decrease in cellobiose it was necessary first to remove any glucose which might have been formed. Glucose was removed by incubating aliquots of the reaction mixture with equal volumes of a crude glucose oxidase preparation for 15 minutes at 37 C. The amount of cellobiose then was determined colorimetrically. No significant decrease in cellobiose was detected in the reaction mixtures containing cellobiose with either the purified or the crude enzyme (table II, part 2). There was, however, a significant decrease in the amount of cellobiose in the control vessels containing inorganic phosphate. In the control vessels with the purified enzyme 1.6 micromoles of cellobiose per ml were decomposed, and with the crude enzyme 3.3 micromoles of cellobiose per ml were decomposed. Cellobiose presumably was decomposed in the control vessels by phosphorolysis.

The amount of cellobiose which disappeared in the reaction mixtures (table II, part 2) was approximately equal to the amount of inorganic phosphate which disappeared (table II, part 3). This indicates that cellobiose was decomposed by phosphorolysis and not hydrolysis.

It is concluded that there was no cellobiase activity from either the purified or the crude enzyme preparations. No significant increase in the number of reducing groups resulted when the enzyme preparations were incubated with cellobiose (table II, part 1) which suggests that there was no cellobiase activity. Moreover, there was no significant decrease in cellobiose when the enzyme preparations were incubated with

cellobiose (table II, part 2), which further indicates that there was no cellobiase activity.

These experiments showed that the purified preparations were free from known interfering substances. With this accomplished, it was possible to make a quantitative study of the changes in products and reactants of the cellobiose phosphorylase reaction.

Analyses of the products and reactants of cellobiose phosphorolysis. An experiment was designed to determine the amount of change in the products and reactants from the phosphorolysis of cellobiose. A reaction mixture of purified enzyme, cellobiose and inorganic phosphate was incubated for 80 minutes at 37 C. The disappearance of inorganic phosphate was determined at 20 minute intervals (figure 1). The reaction apparently reached equilibrium within 60 minutes.

Analyses of the products and reactants of the reaction indicate that equimolar quantities of glucose and glucose-1-phosphate were formed from cellobiose and inorganic phosphate (table III). Furthermore, the molar quantities of cellobiose and inorganic phosphate that were utilized were approximately equal to the molar quantities of glucose and glucose-1-phosphate formed in the reaction. It is concluded that only glucose and glucose-1-phosphate are the products of cellobiose phosphorolysis.

Analyses of the products and reactants of cellobiose synthesis. From the preceding experiment it seemed likely that equimolar quantities of cellobiose and inorganic phosphate would be formed from glucose and glucose-1-phosphate. Furthermore, the amount of cellobiose and inorganic phosphate formed in this reaction would be expected to be equal to the

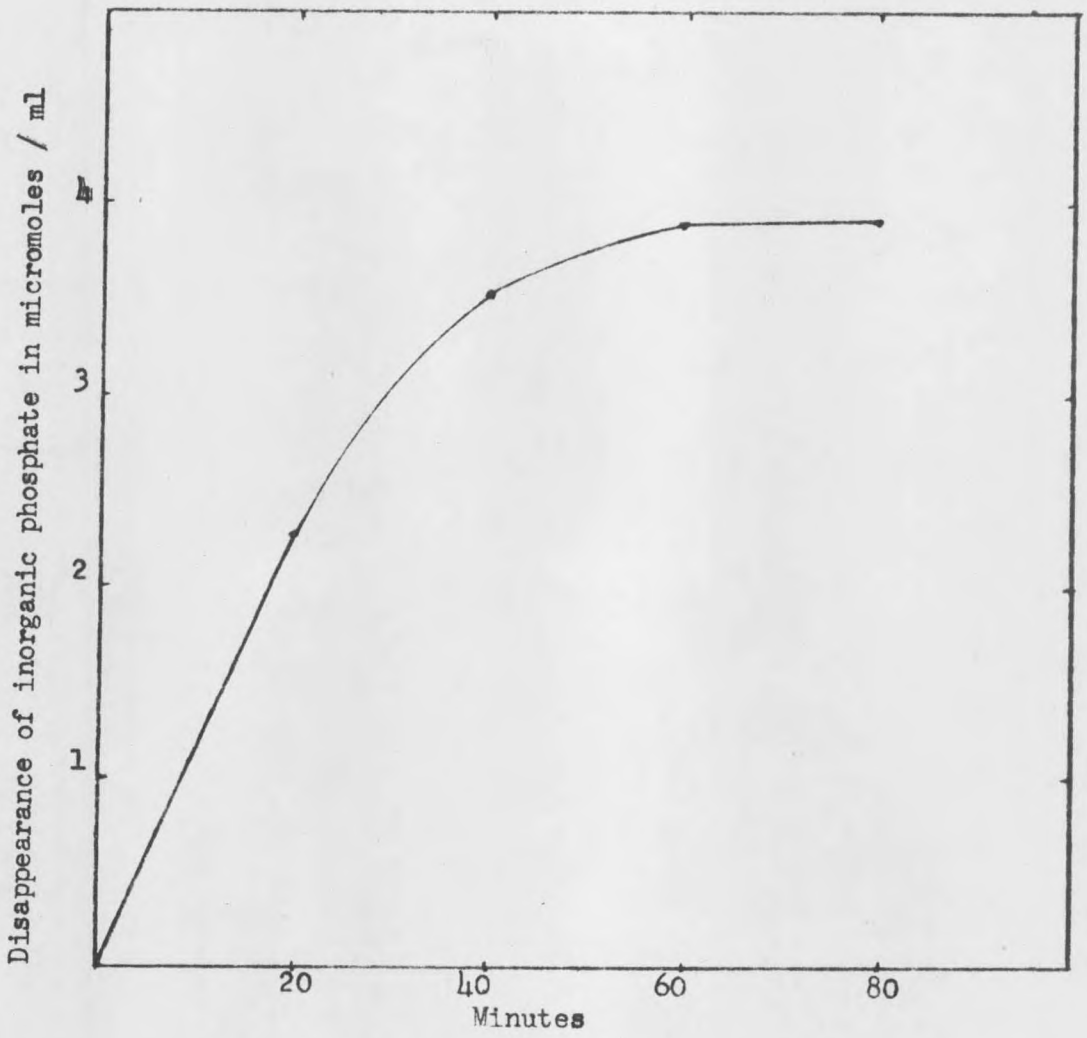


Figure 1. Disappearance of inorganic phosphate during the phosphorolysis of cellobiose. To each ml of reaction mixture, 15 micromoles of inorganic phosphate, 10 micromoles of cellobiose, and purified cellobiose phosphorylase were added. The reaction mixture was buffered with M/140 barbital-acetate buffer, pH 7.0, and incubated at 37 C.

TABLE III

Analyses of the products and reactants of cellobiose phosphorolysis.

	Micromoles/ml		
	Initial	Final	Amount of Change
Cellobiose	15	10.4	-4.6
Inorganic phosphate	10	6.1	-3.9
Glucose-1-phosphate	0	3.9	+ 3.9
Glucose	0	4.2	+ 4.2

The reaction mixture was the same as in figure 1.

amount of glucose and glucose-1-phosphate utilized. This was investigated by measuring the liberation of inorganic phosphate in a reaction mixture containing enzyme, glucose and glucose-1-phosphate as shown in figure 2. Equilibrium apparently was reached in about 60 minutes.

Analyses of the products and reactants of this reaction are summarized in table IV. The molar quantities of cellobiose and inorganic phosphate formed in the reaction are equal to the molar quantities of glucose-1-phosphate and glucose utilized.

TABLE IV

Analyses of the products and reactants of cellobiose synthesis.

	Micromoles/ml		
	Initial	Final	Amount of Change
Cellobiose	0	5.9	+ 5.9
Inorganic phosphate	1.1	6.9	+ 5.8
Glucose-1-phosphate	7.6	1.8	- 5.8
Glucose	10.0	4.5	- 5.5

The reaction mixture was the same as in figure 2.

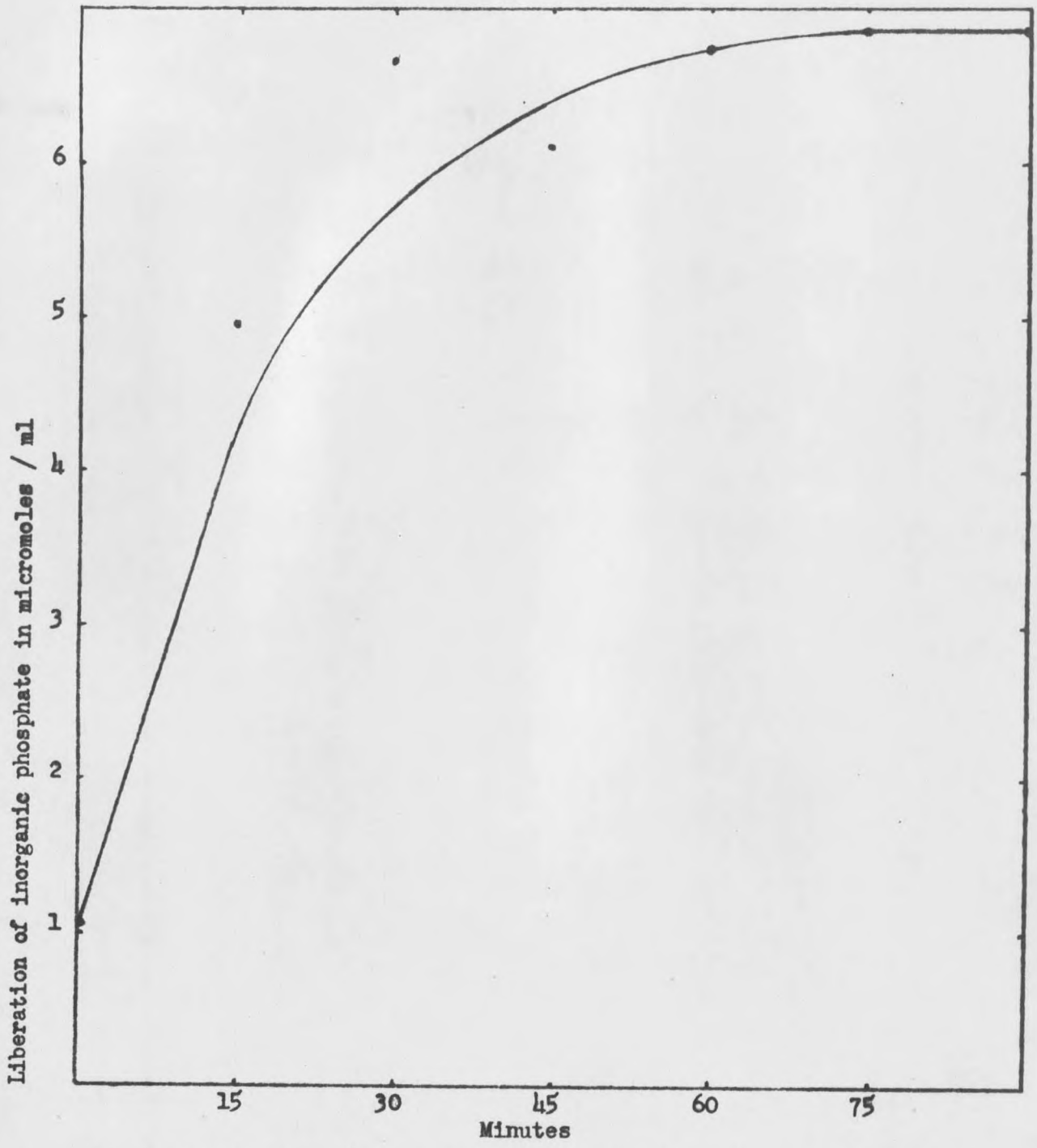


Figure 2. The liberation of inorganic phosphate in a reaction mixture in which each ml contained purified cellobiose phosphorylase (2.5 mg protein), 7.64 micromoles of glucose-1-phosphate, 10 micromoles of glucose, and 1.06 micromoles of inorganic phosphate. The reaction mixture was buffered with M/70 barbital-acetate buffer, pH 7.0, and incubated at 37 C.

Therefore, the phosphorolysis of cellobiose can be reversed resulting in the synthesis of cellobiose from glucose and glucose-1-phosphate.

Equilibrium constant of the cellobiose phosphorylase reaction. In the experiments shown in figures 1 and 2, it appeared that the reaction had reached equilibrium because there was no further change in the amount of inorganic phosphate. However, other factors could have stopped the reaction such as inhibition by the products or inactivation of the enzyme. If the true equilibrium had been reached, the same equilibrium constant should have been obtained whether the reactants were cellobiose and inorganic phosphate, or glucose and glucose-1-phosphate.

The equilibrium constant (K) was calculated from the molar quantities of products and reactants at the end of the reaction according to the following equation:

$$K = \frac{(\text{glucose})(\text{glucose-1-phosphate})}{(\text{cellobiose})(\text{inorganic phosphate})}$$

From the data in table III, K was found to be 0.26, and from the data in table IV, K was found to be 0.20. These two values for the equilibrium constant are in fair agreement and are within the limits of experimental error. Therefore, it is concluded that equilibrium was reached in both reaction mixtures.

In another experiment the enzyme was incubated with approximately equilibrium amounts of cellobiose, inorganic phosphate, glucose, and glucose-1-phosphate. The purpose of this experiment was to determine if any interfering reactions could be detected. The results in table V show the initial and final amounts of the products and reactants. The

equilibrium constant calculated from the data in table V is 0.25. Apparently there were no significant interfering reactions.

TABLE V

Analyses of the products and reactants of a reaction with purified cellobiose phosphorylase, and approximately equilibrium amounts of cellobiose, inorganic phosphate, glucose, and glucose-1-phosphate.

	Micromoles/ml		
	Initial	Final	Amount of Change
Cellobiose	7.5	7.5	0
Inorganic Phosphate	7.8	7.5	-0.3
Glucose	7.5	7.5	0
Glucose-1-phosphate	1.9	1.9	0

The reaction mixture was buffered with M/280 barbital-acetate buffer, pH 7.0, and incubated for 30 minutes at 37 C.

In several other experiments the equilibrium constant was estimated by determining only the change in inorganic phosphate. In these experiments it was assumed that the reaction had reached equilibrium if the last readings showed no further change in inorganic phosphate. The data of these and the previous experiments are summarized in table VI.

In the experiments in which only the change in inorganic phosphate was measured it was assumed that equimolar quantities of inorganic phosphate, cellobiose, glucose, and glucose-1-phosphate reacted. This assumption is supported by the data in tables III and IV, which indicate that equimolar changes in the products and reactants of the reaction occurred.

TABLE VI

Summary of equilibrium data for the cellobiose phosphorylase reaction.

Experiment number	Equilibrium Constant	Source of Data	Direction of Reaction*
1	0.26	Table III	Forward
2	0.20	Table IV	Reverse
3	0.25	Table V	Near Equilibrium
4	0.22	Pi determination**	Forward
5	0.25	Pi determination	Reverse
6	0.22	Pi determination	Reverse
7	0.20	Pi determination	Forward
Average	0.23		

* In the forward reaction the reactants were cellobiose and inorganic phosphate. In the reverse reaction the reactants were glucose and glucose-1-phosphate.

** Pi = Inorganic Phosphate.

Only a slight change is made in the calculated equilibrium constant by including the last 4 experiments in table VI. The average value of the equilibrium constant for all 7 experiments is 0.23; the average value of the equilibrium constant from the first 3 experiments is 0.24. Therefore, it appears that the true equilibrium constant for the cellobiose phosphorylase reaction is approximately 0.23.

It has been shown that the molar quantities of glucose and glucose-1-phosphate formed in the phosphorolysis of cellobiose are equal to the molar quantities of cellobiose and inorganic phosphate utilized. The reaction is reversible, for equimolar quantities of cellobiose and inorganic phosphate are formed from glucose and glucose-1-phosphate.

These data confirm the previous work with cellobiose phosphorylase (Sih, 1955, Sih and McBee, 1955a, 1955b, and Sih et al., 1957) and indicate that their results were due to the activity of one enzyme. It is concluded that the equation for the cellobiose phosphorylase reaction is:

Cellobiose + inorganic phosphate = glucose + alpha-glucose-1-phosphate.

Free energy calculations. The information from which the free energy change of the cellobiose phosphorylase reaction and the free energy content of cellobiose can be calculated could not be found in the literature. However, the free energy change could be calculated once the equilibrium constant of the cellobiose phosphorylase reaction had been determined. With the value of 0.23 for the equilibrium constant the standard free energy change for the cellobiose phosphorylase reaction was estimated by the following equation:

$$\Delta F^{o} = -RT \ln K = 880 \text{ cal}$$

Where ΔF^{o} = change in standard free energy

R = gas constant (1.987)

T = absolute temperature (298.1)

K = 0.23

The standard free energy change for the phosphorolysis of cellobiose was estimated to be about 880 cal. The temperature at which the experiments were conducted was about 310 A. Using this value in the equation the free energy change is about 910 cal. In the following calculations a value of 900 cal will be used for the standard free energy change of the reaction.

The standard free energy of hydrolysis of cellobiose can be estimated by adding the two equations:

Cellobiose + HPO_4^{-2} = Glucose + Glucose-1-phosphate ΔF^o = 900 cal

Glucose-1-phosphate + H_2O = Glucose + HPO_4^{-2} ΔF^o = -4,800 cal*

Cellobiose + H_2O = 2 Glucose ΔF^o = -3,900 cal

* The value for the free energy of hydrolysis of glucose-1-phosphate was taken from Burton and Krebs (1953).

The standard free energy of formation of cellobiose was estimated to be -378.2 k cal. In this calculation the standard free energy of formation of glucose (-219.38 k cal) and water (-56.69 k cal) were taken from Burton and Krebs (1953). These values are for standard conditions. An additional error was introduced into the calculations by assuming that the equilibrium constant was the same at 298 A as at 310 A.

The equilibrium constant for the cellobiose phosphorylase reaction appears to be the same as the equilibrium constant of the maltose phosphorylase reaction. The equilibrium constant, K = (maltose)(phosphate) /(glucose)(beta-glucose-1-phosphate), was found to be 4.4 (Fitting and Doudoroff, 1952b), or 1/K = 0.23 which is the same as the equilibrium constant for the phosphorolysis of cellobiose. From this equilibrium constant the free energy of hydrolysis of maltose has been estimated to be approximately -4,000 calories (Kalckar, 1954). This estimation is quite close to the -4,200 cal for the free energy of maltose estimated by Burton and Krebs (1953), and the -3,900 calories estimated for the free energy of hydrolysis of cellobiose in the present work.

In estimating the free energy of hydrolysis of maltose, Kalckar assumed that alpha-glucose-1-phosphate and beta-glucose-1-phosphate had about the same free energy of hydrolysis. If this assumption is correct then cellobiose and maltose would be expected to have about the same free energy of hydrolysis. These results suggest that the configuration at the C-1 atom makes little difference in the energy level of cellobiose, maltose, alpha-glucose-1-phosphate, or beta-glucose-1-phosphate.

B. Properties of cellobiose phosphorylase.

The following experiments were designed to define some of the properties of cellobiose phosphorylase. In these experiments the effect of hydrogen ion concentration, temperature, inhibitors, and the specificity of the enzyme were studied.

The effect of hydrogen ion concentration on the activity of cellobiose phosphorylase. The effect of hydrogen ion concentration on the activity of cellobiose phosphorylase was determined by measuring the amount of activity at different pH values. Reaction mixtures, varying only in pH, containing purified cellobiose phosphorylase, cellobiose, and inorganic phosphate were incubated at 37 C. The uptake of inorganic phosphate was measured in order to indicate the amount of enzyme activity.

In these experiments the reaction mixtures were buffered with M/70 barbital-acetate buffer. Solutions containing buffer, phosphate, and cellobiose were adjusted to various pH values and then the enzyme was added. The pH of the complete reaction mixture could not be predicted accurately because the addition of the enzyme caused the pH to change.

The pH values reported were determined at the end of the reaction with a Beckman pH meter (model GS). No significant change in the pH occurred during the course of the reaction.

The optimum pH for activity of the enzyme is about 7, according to the results in figure 3. The enzyme is active over quite a large pH range; activity has been detected at pH 4.6 and at pH 8.1, however, no activity was observed at pH 4.1 or at pH 8.7. The activity was not measured between pH 4.1 and 4.6 or between pH 8.1 and 8.7.

Difficulty was encountered in determining the optimum pH of the enzyme because there was very little difference in activity at pH values close to 7. Later it was learned that glucose inhibits the phosphorolysis of cellobiose. It is possible that inhibition by the glucose formed in the reaction may have obscured the effect of the hydrogen ion concentration. If this is true the effect of the hydrogen ion concentration could probably be determined more readily by measuring the rate of synthesis instead of the rate of phosphorolysis of cellobiose.

The effect of temperature on the activity of cellobiose phosphorylase. An experiment was designed to determine the relative activity of cellobiose phosphorylase at temperatures between 20 C and 40 C. Difficulty was encountered in determining the relative activity in this temperature range within a single incubation period. A short incubation period (about 10 minutes) was needed to approach initial rate conditions at 40 C; however, the amount of activity occurring at 20 C in this time interval was so slight that it could not be measured accurately. Therefore, it was necessary to vary the time interval as well as the temperature, in order to

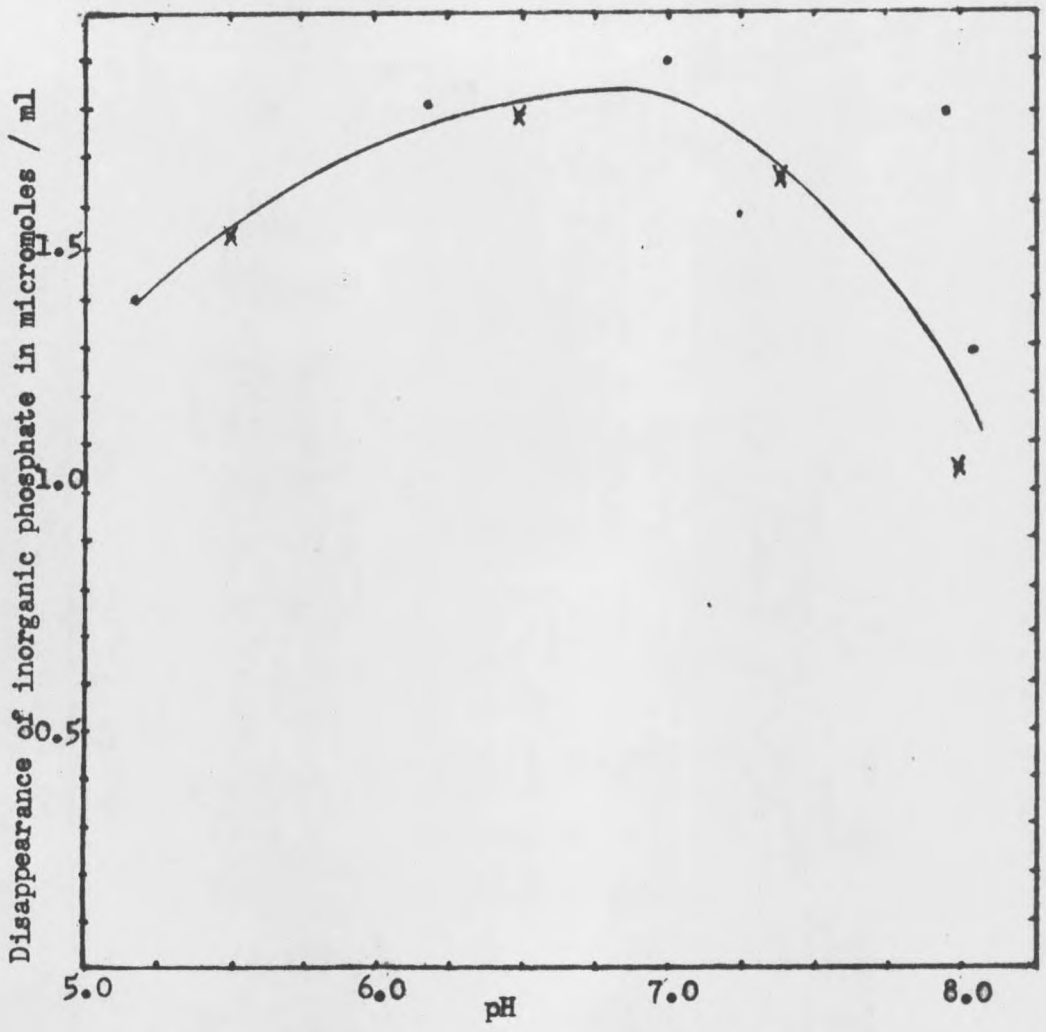


Figure 3. The activity of cellobiose phosphorylase at different pH values. The encircled points represent one experiment, the points represented by x's were taken from another experiment. In both experiments each ml of reaction mixture contained 10 micromoles of cellobiose, 14 micromoles of inorganic phosphate, and purified cellobiose phosphorylase. The reaction mixtures were buffered with M/70 barbital-acetate buffer.

determine the relative activity at different temperatures.

The relative activity of cellobiose phosphorylase at different temperatures is shown in table VII. According to the results the Q_{10} between 20 C and 30 C is 2.2, whereas between 30 C and 40 C the Q_{10} is 2.5. This discrepancy is probably due to experimental error. On the basis of these data and the results of other experiments it is concluded that the Q_{10} value of cellobiose phosphorylase is about 2.4. This value of 2.4 lies well within the range of 1.3 to 3.5 for the Q_{10} value of most enzymes (Sizer, 1943).

TABLE VII

The activity of cellobiose phosphorylase at different temperatures.

	Temperatures and Incubation Period		
	20 C 40 min	30 C 20 min	40 C 10 min
Disappearance of inorganic phosphate in micromoles/ml	0.66	0.72	0.91
Disappearance of inorganic phosphate in micromoles/ml/min	0.016	0.036	0.091

Each ml of reaction mixture contained purified cellobiose phosphorylase, (2 mg protein) 15 micromoles of cellobiose, 7.5 micromoles of inorganic phosphate, and 1 micromole of ethylenediaminetetraacetic acid.

The effect of p-chloromercuribenzoate on the activity of cellobiose phosphorylase. Many enzymes are known to contain sulfhydryl groups (Tauber, 1949). Sulfhydryl groups which are essential for the activity of enzymes may be located near the site of substrate attachment or may actively participate in the reaction (Neilands and Stumpf, 1955).

An experiment was conducted to obtain evidence of cellobiose phosphorylase having an essential sulfhydryl group. For this purpose, p-chloromercuribenzoate was selected as an enzyme inhibitor because organic mercurial compounds are the most specific reagents for sulfhydryl groups (Boyer, 1954). Furthermore, p-chloromercuribenzoate combines reversibly with cysteine or glutathione (Barron and Singer, 1945). Cysteine and glutathione apparently compete with the sulfhydryl groups of enzymes for p-chloromercuribenzoate.

Cellobiose phosphorylase activity was completely inhibited by 2 X 10^{-5} M or 4 X 10^{-5} M p-chloromercuribenzoate, whereas 1 X 10^{-5} M p-chloromercuribenzoate caused about 50 per cent inhibition (table VIII).

The inhibition by p-chloromercuribenzoate was reversed by cysteine. The enzyme retained practically all of its activity when 1 X 10^{-4} M cysteine was added along with 1 X 10^{-5} M p-chloromercuribenzoate. About 25 per cent of the activity was retained when 1 X 10^{-4} M cysteine was added along with 2 X 10^{-5} M p-chloromercuribenzoate. Presumably cysteine exerted its protective effect by competing with sulfhydryl groups of the enzyme for p-chloromercuribenzoate. These data suggest that a sulfhydryl group either participates in the cellobiose phosphorylase reaction or is situated near the point of substrate attachment.

The effect of $AgNO_3$ on the activity of cellobiose phosphorylase. That an essential sulfhydryl group is present in cellobiose phosphorylase is further supported by an experiment using $AgNO_3$ as an inhibitor. It was found that $AgNO_3$ completely inhibited the activity and that this inhibition could be reversed by cysteine (table IX). 1 X 10^{-4} M $AgNO_3$

TABLE VIII

The effect of p-chloromercuribenzoate on the activity of cellobiose phosphorylase.

Molarity of p-chloromercuri-benzoate	Molarity of Cysteine	Miromoles of inorganic phosphate liberated/ml	Per cent inhibition
0	0	1.2	0
1×10^{-5}	0	0.7	42
2×10^{-5}	0	0	100
4×10^{-5}	0	0	100
1×10^{-5}	1×10^{-4}	1.0	7
2×10^{-5}	1×10^{-4}	0.3	75

Each ml of reaction mixture contained purified cellobiose phosphorylase, (2 mg protein), 15 micromoles of cellobiose, and 10 micromoles of inorganic phosphate. The reaction mixtures were buffered with M/100 barbital-acetate buffer, pH 7.0, and incubated for 1 hour at 37 C.

completely inhibited cellobiose phosphorylase activity. With 1×10^{-4} M $AgNO_3$ and 1×10^{-4} M cysteine only slight activity was obtained. However, a hundred-fold excess of cysteine completely reversed the inhibition of $AgNO_3$, for with 1×10^{-4} M $AgNO_3$ and 1×10^{-2} M cysteine no inhibition was obtained. The inhibition by $AgNO_3$ presumably resulted from the silver ions combining with sulfhydryl groups of the enzyme.

The results of another experiment conducted under essentially the same conditions as those summarized in tables VII and VIII indicated that 1×10^{-4} M merthiolate (sodium ethyl mercuri thiosalicylate) completely inhibited cellobiose phosphorylase activity. This experiment provides further evidence that cellobiose phosphorylase contains an essential

Table IX

The effect of silver nitrate on the activity of cellobiose phosphorylase.

Molarity of $AgNO_3$	Molarity of cysteine	Disappearance of inorganic phosphate (micromoles/ml)	Per cent inhibition
0	0	2.9	-
1×10^{-4}	0	0	100
1×10^{-4}	1×10^{-6}	0.1	97
1×10^{-4}	1×10^{-4}	0.3	91
1×10^{-4}	1×10^{-2}	3.0	0
0	1×10^{-6}	2.7	7
0	1×10^{-4}	2.8	3
0	1×10^{-2}	2.7	7

Each ml of reaction mixture contained 15 micromoles of cellobiose, 10 micromoles of inorganic phosphate and purified cellobiose phosphorylase (2 mg protein). The reaction mixtures were buffered with M/100 barbital-acetate buffer pH 7.0, and incubated at 37 C for 1 hour.

sulfhydryl group.

The effect of phlorizin on the activity of cellobiose phosphorylase. It has been found that phlorizin inhibits muscle phosphorylase (Cori et al., 1939 and 1943) and sucrose phosphorylase (Douderoff, 1943). Thus, it was of interest to determine the effect of phlorizin on cellobiose phosphorylase. About 50 per cent inhibition of cellobiose phosphorylase activity was obtained with 5×10^{-3} M phlorizin but there was hardly any inhibition with 1.25×10^{-3} M phlorizin (table X). In this range of concentrations the inhibitory effect was proportional to the concentration of phlorizin.

Approximately the same concentrations of phlorizin were required to inhibit cellobiose phosphorylase as were required to inhibit sucrose phosphorylase (Douderoff, 1943).

TABLE X

The effect of phlorizin on the activity of cellobiose phosphorylase.

Phlorizin M X 10^{-3}	Uptake of inorganic phosphate in micromoles per ml.	Per cent inhibition
0	1.5	-
1.25	1.4	8
2.5	1.2	21
5.0	0.6	56

Each ml of reaction mixture contained 15 micromoles of cellobiose, 10 micromoles of inorganic phosphate and purified cellobiose phosphorylase (2 mg protein). The reaction mixtures were buffered with M/100 barbital-acetate buffer pH 7.0 and incubated for one hour at 37 C.

The effect of glucose on cellobiose phosphorolysis. Sih (1955) reported that cellobiose phosphorolysis was inhibited by glucose. He observed less phosphate esterification in reaction mixtures with glucose included than in reaction mixtures without added glucose. However, Sih did not rule out the possibility that the decreased esterification of phosphate in the reaction mixtures containing added glucose resulted from equilibrium being reached.

The following experiment was designed to determine the effect of glucose on cellobiose phosphorolysis. In order to rule out the attainment of equilibrium as an explanation for the effect of glucose, the reaction was stopped before equilibrium was reached.

To a series of reaction mixtures, containing the same amount of enzyme and inorganic phosphate, varying amounts of cellobiose and glucose were added. For controls, similar reaction mixtures containing no added glucose were included in the experiment. The total volume of each reaction mixture was one ml. The attainment of equilibrium was prevented by stopping the reaction after a 20 minute incubation period by inactivating the enzyme in a boiling water bath.

It was found that glucose inhibited cellobiose phosphorolysis. In a reaction mixture containing 7.5 micromoles each of cellobiose and glucose the activity was only 17 per cent of that in a similar reaction mixture containing no added glucose (table XI). When the amount of glucose was doubled , (15 micromoles), no enzyme activity was detected. In the reaction mixtures containing 15 micromoles of cellobiose about 73 per cent inhibition was caused by the addition of 7.5 micromoles of glucose, whereas the addition of 15 micromoles of glucose completely inhibited the activity. In the reaction mixture containing 30 micromoles of cellobiose about 75 per cent inhibition resulted from the addition of 7.5 micromoles of glucose and 82 per cent inhibition from 15 micromoles of glucose.

There is some indication that increased amounts of cellobiose were able partially to overcome the inhibition of glucose, which suggests that glucose is a competitive inhibitor. Increased amounts of cellobiose were able to overcome in part the inhibition of glucose except in the reaction mixture with 30 micromoles of cellobiose and 7.5 micromoles of glucose. In this reaction mixture the activity was about the same as

in a similar reaction mixture containing only 15 micromoles of glucose.

TABLE XI

The effect of glucose on cellobiose phosphorolysis.

Micromoles/ml Added to reaction mixture		Uptake of inorganic phosphate	Per cent inhibition
Cellobiose	Glucose		
7.5	0	1.47	-
7.5	7.5	0.25	83
7.5	15.0	0	100
15.0	0	1.73	-
15.0	7.5	0.47	73
15.0	15.0	0	100
30.0	0	2.22	-
30.0	7.5	0.55	75
30.0	15.0	0.40	82

Each ml of reaction mixture contained purified cellobiose phosphorylase (3 mg protein), 7.5 micromoles of inorganic phosphate, and 0.75 micromoles of ethylenediaminetetraacetic acid. The reaction mixtures were incubated at 37 C for 20 minutes.

It is concluded that glucose inhibits cellobiose phosphorolysis. The decreased uptake of inorganic phosphate in reaction mixtures containing added glucose can be attributed to glucose inhibition because equilibrium was not reached in any of the tubes. The reaction mixture in which the least amount of change would be required for equilibrium to be reached was the one containing 15 micromoles of glucose and 7.5 micromoles of cellobiose. Approximately one micromole of inorganic

phosphate would be expected to be taken up in this reaction mixture at equilibrium. Since the amount of uptake of inorganic phosphate in any of the reaction mixtures with added glucose did not even approach 1 micromole, the possibility that equilibrium was reached is excluded.

The inhibition of cellobiose phosphorylase by glucose is an example of an enzyme being inhibited by a product of the reaction which it catalyzes. Similar results have been obtained with other phosphorylases, in which glucose inhibits the enzyme and also is a product of phosphorolysis (Green and Stumpf, 1942; Cori et al., 1943; and Douderoff, 1943).

The effect of NaF, $(NH_4)_2SO_4$, NaCl, and $MgSO_4$ on the activity of cellobiose phosphorylase. It is well known that magnesium stimulates the activity of certain enzymes (Lardy, 1951). Magnesium sulfate was added as an enzyme activator by Sih (1955) in his work with cellobiose phosphorylase, however, no evidence was presented to show that the enzyme was activated by magnesium sulfate. In the following experiment the effect of magnesium sulfate on the activity of cellobiose phosphorylase was investigated. With concentrations of 0.01 M or 0.02 M magnesium sulfate there was about 15 per cent inhibition of the activity as shown in table XII. These data indicate that increased amounts of magnesium sulfate have little or no effect on the activity.

If magnesium stimulated cellobiose phosphorylase activity, it might be expected that the enzyme would be sensitive to fluoride, for Warburg and Christian (1942) have shown that a magnesium fluoro-phosphate complex is formed which may render magnesium unavailable for an enzyme. It was found that relatively high concentrations of fluoride inhibited cellobiose

phosphorylase activity. Only slight inhibition was found with 0.1 M NaF, whereas 0.2 M NaF almost completely inhibited the activity (table XII). The effect of NaF on the activity probably can be attributed to fluoride because equal amounts of NaCl caused little or no inhibition.

TABLE XII

The effect of NaF, NaCl, $(NH_4)_2SO_4$ and $MgSO_4$ on the activity of cellobiose phosphorylase.

Compound added	Molarity	Uptake of inorganic phosphate in micromoles/ml	Per cent inhibition
None		2.46	-
NaF	0.1	1.94	21
NaF	0.2	0.12	95
NaCl	0.1	2.40	3
NaCl	0.2	2.22	10
$(NH_4)_2SO_4$	0.1	2.22	10
$(NH_4)_2SO_4$	0.2	2.19	11
$MgSO_4$	0.01	2.06	16
$MgSO_4$	0.02	2.10	15

Each ml of reaction mixture contained 15 micromoles of cellobiose, 10 micromoles of inorganic phosphate and purified cellobiose phosphorylase (2 mg protein). The reaction mixtures were buffered with M/100 barbital-acetate buffer, pH 7.0, and incubated for one hour at 37 C.

Although increased amounts of magnesium sulfate did not stimulate cellobiose phosphorylase activity, the possibility that magnesium activates

the enzyme is not excluded. If only traces of magnesium are needed it is possible that sufficient amounts for activation were present in the reaction mixtures because the enzyme preparations were not dialyzed. Furthermore, it is possible that fluoride exerts its inhibitory effect by binding traces of magnesium in the reaction mixture.

The effect of ammonium sulfate on the activity of cellobiose phosphorylase was investigated because Cori et al., (1943) found that muscle phosphorylase was inhibited by ammonium sulfate. It was especially important to consider this inhibition because the purified cellobiose phosphorylase preparations contained a small amount of ammonium sulfate which was carried over from the purification procedure. Cellobiose phosphorylase was not inhibited significantly by 0.1 M or 0.2 M ammonium sulfate as shown in table XII. Thus it seems likely that the small amount of ammonium sulfate in the purified enzyme preparations did not effect the activity.

Specificity of cellobiose phosphorylase. In studying the specificity of cellobiose phosphorylase, experiments were conducted to determine whether the enzyme could catalyze the synthesis of disaccharides from glucose-1-phosphate and various other compounds. The ability of the enzyme to catalyze the phosphorolysis of several disaccharides was determined. Experiments were conducted to determine the specificity of cellobiose phosphorylase for beta-glucose-1-phosphate and arsenate.

1) The synthesis of disaccharides. Bacterial phosphorylases have been shown to catalyze the synthesis of a number of different disaccharides. Sucrose phosphorylase catalyzed the synthesis of five different disaccharides

ides (Hassid and Doudoroff, 1950). Evidence that maltose phosphorylase catalyzed the synthesis of glucosyl-D-xylose, as well as maltose, was presented by Fitting and Doudoroff (1952b). Thus it appeared that a study of the ability of cellobiose phosphorylase to catalyze the synthesis of disaccharides would be of value. Cellobiose phosphorylase apparently is able to catalyze the synthesis of several different disaccharides. When the enzyme was incubated with glucose-1-phosphate and glucose, D-xylose, L-xylose, 2-deoxyglucose, or glucosamine, inorganic phosphate was liberated (table XIII). This liberation of inorganic phosphate suggests that the following reactions are catalyzed by cellobiose phosphorylase:

D-glucose + glucose-1-phosphate = glucosyl-D-glucose + inorganic phosphate

D-xylose + glucose-1-phosphate = glucosyl-D-xylose + inorganic phosphate

L-xylose + glucose-1-phosphate = glucosyl-L-xylose + inorganic phosphate

2-deoxyglucose + glucose-1-phosphate = glucosyl-D-deoxyglucose + inorganic phosphate

glucosamine + glucose-1-phosphate = glucosyl-D-glucosamine + inorganic phosphate.

Chromatography appeared to be a convenient method to obtain confirmative evidence that disaccharides were formed from D-xylose and L-xylose. It appeared that small amounts of the disaccharides could be separated from the monosaccharides in the reaction mixtures by this method. Since the pentose-containing disaccharides would be expected to be closely related to cellobiose, it seemed likely that their mobility would be approximately the same as cellobiose.

TABLE XIII

Liberation of inorganic phosphate when cellobiose phosphorylase was incubated with glucose-1-phosphate and various other compounds.

Expt. #	Compound added	Micromoles of inorganic phosphate liberated/ml 1 hr.	2 hr.
1	D-glucose	4.40	5.51
1	D-xylose	3.61	5.31
1	L-xylose	4.14	5.87
1	2-deoxyglucose	2.35	3.48
1	gluconate	0.44	0.22
1	glucuronate	0.22	0
1	glucose-6-phosphate	0.19	0.19
2	D-glucose	4.35	5.63
2	D-xylose	4.08	5.80
2	L-xylose	3.93	5.58
2	D-ribose	0	0.13
2	D-sorbitol	0.03	0.10
3	D-glucose	4.37	5.34
3	delta-gluconolactone	0	0.52
3	glucosamine	1.78	3.04
3	N-acetylglucosamine	0	0.32
4	D-glucose	5.33	6.37
4	2-deoxyglucose	1.40	2.69
4	glucosamine	1.08	1.64
5*	D-glucose	1.05	1.79
5	L-arabinose	0	0
5	D-xylose	0.34	0.71
5	L-fructose	0	0
5	D-galactose	0	0
5	D-mannose	0.10	0
5	N-acetylglucosamine	0	1.10
5	2-deoxyglucose	0.31	0.71

* The enzyme preparation used in experiment 5 was far less active than those used in other experiments.

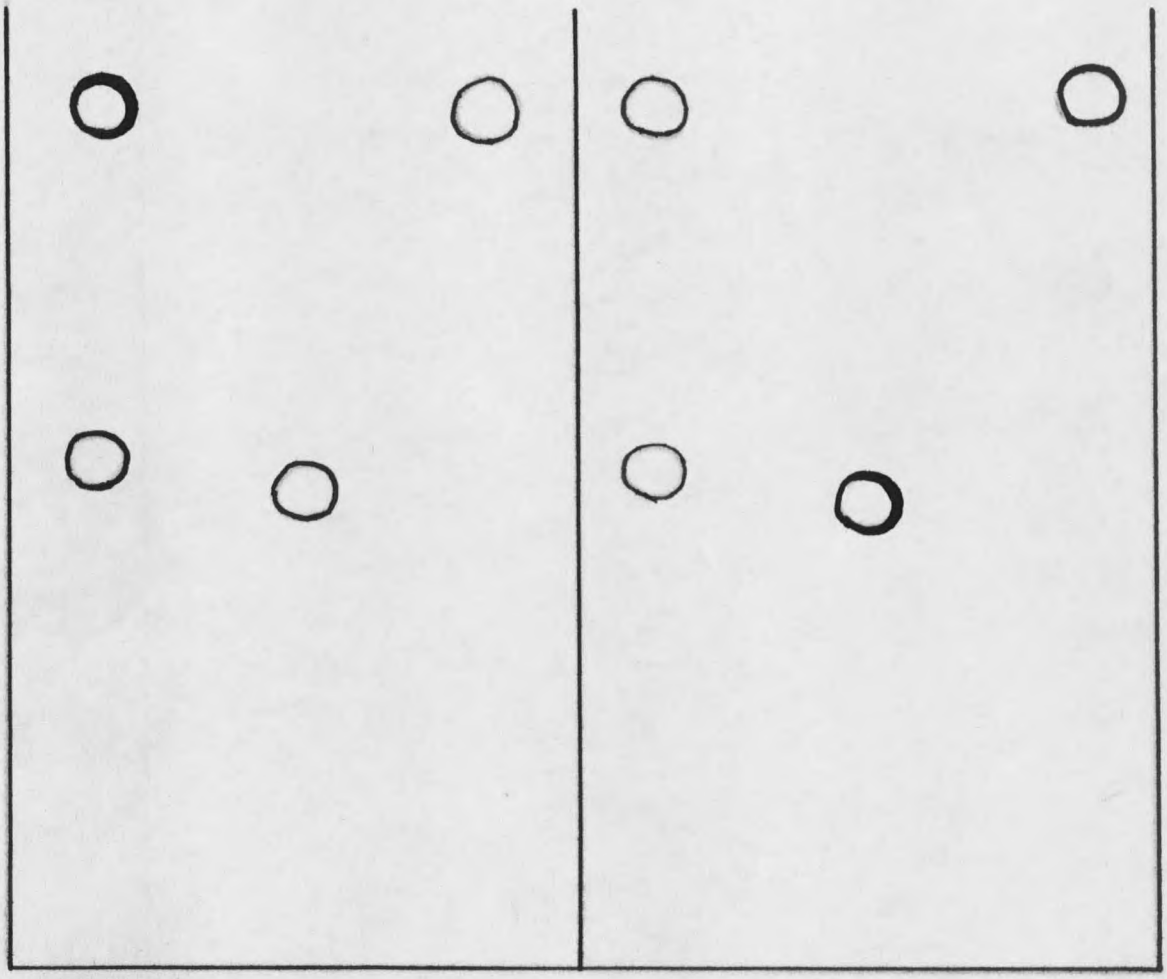
In experiments 1, 2, 3, and 5, each ml of reaction mixture contained 7.64 micromoles of glucose-1-phosphate and 15 micromoles of the compound being tested for activity. In experiment 4, each ml of reaction mixture contained 11.5 micromoles of glucose-1-phosphate and 10 micromoles of the compound being tested. One micromole of ethylenediaminetetraacetic acid was added to each ml of reaction mixture.

The aniline-acid-phthalate color reagent, developed by Barr (1954), appeared to be suitable for detecting pentose-containing disaccharides. With this solvent a reddish-pink color develops spontaneously with pentoses but not with hexoses. Since the pentose constituent of the disaccharides was expected to occupy the reducing portion of the molecule it seemed likely that the pentose-containing disaccharides would develop a color similar to that of free pentoses. By heating the chromatographs, hexoses develop a brown color which is distinguishable from the reddish-brown color shown by pentoses after heating.

Chromatographs of reaction mixtures, made after incubating cellobiose phosphorylase, glucose-1-phosphate, and D-xylose or L-xylose, indicated that disaccharides with pentose constituents had been formed. A representation of these chromatographs is shown in figure 4. Reddish-pink spots were observed from compounds with R_f values slightly greater than that of cellobiose and about one-half the R_f value of D-xylose or L-xylose. These reddish-pink spots presumably were due to disaccharides with pentose constituents. Upon heating at 110 C for 10 minutes, the reddish-pink color changed to a reddish-brown color similar to the color of pentoses but distinguishable from the brown color of cellobiose.

It appears that there is considerable variation in the rate of syntheses of the different disaccharides. The rate of phosphate liberation, and presumably disaccharide synthesis, was slower with 2-deoxy-glucose and glucosamine than with glucose. The relative amounts of phosphate liberated in the first hour were 5.33 micromoles with glucose,

Chromatograph 1 Chromatograph 2



L-xylose Cellobiose L-xylose D-xylose Cellobiose D-xylose
G-1-P* G-1-P*

*G-1-P = glucose-1-phosphate

Figure 4. Chromatographs of reaction mixtures incubated with cellobiose phosphorylase, glucose-1-phosphate and D-xylose or L-xylose. The lower spot on the left in chromatograph 1 presumably is due to glucosyl-L-xylose. The lower spot on the left in chromatograph 2 presumably is due to glucosyl-D-xylose. The top spots in both chromatographs represent D-xylose or L-xylose. The liberation of inorganic phosphate in these reaction mixtures is shown in table XIII, experiment 1.

1.40 micromoles with 2-deoxyglucose, and 1.08 micromoles with glucosamine.

If 2-deoxyglucose and glucosamine attach to the enzyme at the same position as glucose does, it might be expected that the reaction would be slower with these compounds than with glucose. It seems possible that a hydrogen group substituted for a hydroxyl group of the C-2 atom, as in deoxyglucose, could alter the rate of the reaction. Furthermore, a more drastic substitution at this position, such as the amino group of glucosamine, might be expected to alter the rate of the reaction more than would the substitution by a hydrogen group.

The rate of phosphate liberation with glucose appears to be slightly greater than with D-xylose or with L-xylose. It is doubtful, however, whether these slight differences in rates are significant because these data do not represent initial rates.

No significant amount of inorganic phosphate was released when the enzyme was incubated with glucose-1-phosphate and gluconate, glucuronate, glucose-6-phosphate, D-ribose, D-sorbitol, delta-gluconolactone, N-acetyl-glucosamine, L-arabinose, L-fructose, D-galactose, or D-mannose (table XIII). These compounds apparently are unable to accept the glucosyl moiety of glucose-1-phosphate.

2) The phosphorolysis of disaccharides. Cellobiose phosphorylase was unable to catalyze the phosphorolysis of gentiobiose, salicin, lactose, or maltose for no significant amount of inorganic phosphate disappeared when the enzyme was incubated with inorganic phosphate and these sugars (table XIV). However, when the enzyme was incubated with inorganic phosphate and

cellobiose, a relatively large amount of inorganic phosphate disappeared. These results confirm the work of Sih (1955) with salicin, lactose and maltose.

It seems likely that the enzyme would catalyze the phosphorolysis of the four new compounds which appear to have been synthesized. These compounds, however, were not available in sufficient quantity for investigation.

TABLE XIV

The change in inorganic phosphate when cellobiose phosphorylase was incubated with inorganic phosphate and different disaccharides.

Disaccharide	Change in inorganic phosphate (micromoles/ml) 1 hour	2 hours
cellobiose	- 2.52	- 2.64
gentiobiose	- 0.25	- 0.12
salicin	≠ 0.31	≠ 0.14
lactose	0	≠ 0.09
maltose	≠ 0.25	≠ 0.06

Each ml of reaction mixture contained purified cellobiose phosphorylase, 15 micromoles of disaccharide, 7.5 micromoles of inorganic phosphate, and 1 micromole of ethylene diaminetetraacetic acid. The reaction mixtures were incubated at 37 C.

3) Beta-glucose-1-phosphate. In the phosphorolysis of maltose, which is an alpha-glucoside, beta-glucose-1-phosphate is formed and the enzyme is specific for this ester in the synthesis of maltose (Fitting and Doudoroff, 1952b). This indicates that a Walden inversion occurs in the

reaction. Sih et al., (1957) demonstrated that a Walden inversion occurs in the cellobiose phosphorylase reaction by synthesizing a beta-glucoside from alpha-glucose-1-phosphate and glucose. It was found in the present investigation that beta-glucose-1-phosphate was unable to replace alpha-glucose-1-phosphate as a glucosyl donor in the synthesis of cellobiose. There was no inorganic phosphate released when cellobiose phosphorylase was incubated with beta-glucose-1-phosphate and glucose (table XV). However, inorganic phosphate was liberated in a similar reaction mixture containing alpha-glucose-1-phosphate instead of beta-glucose-1-phosphate. These data confirm the evidence presented by Sih et al., (1957), that a Walden inversion occurs in the cellobiose phosphorylase reaction.

4) Arsenolysis. Arsenate is able to substitute for phosphate in some phosphorolytic reactions (Douderoff, 1947; Cohn and Cori, 1948; Katz and Hassid, 1948; and Fitting and Douderoff, 1952b) resulting in the arsenolysis of the substrate. Therefore, the ability of cellobiose phosphorylase to catalyze the arsenolysis of cellobiose was investigated.

The arsenolysis of cellobiose presumably would result in the formation of glucose-1-arsenate and glucose. Glucose-1-arsenate is unstable (Slocum and Varner, 1958) and spontaneously dissociates into glucose and inorganic arsenate. Therefore, the reaction would be the same as for the hydrolysis of cellobiose and 2 moles of glucose would be formed per mole of cellobiose.

For each ml of reaction mixture, containing 5 micromoles of arsenate, nearly 24 micromoles of glucose were formed (figure 5). At equilibrium

TABLE XV

Liberation of inorganic phosphate when cellobiose phosphorylase was incubated with glucose and beta-glucose-1-phosphate or alpha-glucose-1-phosphate.

Reaction mixture	Compound added	Micromoles of inorganic phosphate liberated per ml	
		30 min.	60 min.
1	alpha-glucose-1-phosphate	2.00	3.19
2	alpha-glucose-1-phosphate	2.32	3.27
3	beta-glucose-1-phosphate	0.15	0.07
4	(Boiled) beta-glucose-1-phosphate	0.03	-0.09

Each ml of reaction mixture contained purified cellobiose phosphorylase (2 mg protein), 20 micromoles of glucose and 6 micromoles of either beta-glucose-1-phosphate or alpha-glucose-1-phosphate. Reaction mixture #2 contained 10 micromoles of $BaCl_2$ per ml and served as a control because the beta-glucose-1-phosphate solution contained a small amount of barium. Reaction mixture #4 was boiled at 0 time. The reaction mixtures were buffered in M/140 barbital-acetate buffer, pH 7.0, and incubated at 37 C.

less than 5 micromoles of glucose would have been formed if glucose-1-arsenate were stable. It seems likely that glucose-1-arsenate was formed, but decomposed spontaneously.

In a similar reaction mixture, containing phosphate instead of arsenate, 4.9 micromoles of glucose per ml were formed (figure 5). Only 3.7 micromoles of glucose per ml would be expected to be formed at equilibrium. According to the phosphate analysis of the same reaction mixture (table XVI) 2.3 micromoles of inorganic phosphate per ml were esterified. This

relatively large amount of glucose formed may have been due in part to the hydrolysis of cellobiose by the beta-glucosidase in the glucose oxidase preparation. Corrections were made for the beta-glucosidase activity; however, these corrections may not have been adequate.

Another reaction mixture containing cellobiose phosphorylase and cellobiose, but no arsenate or phosphate, was incubated under similar conditions. The glucose analysis indicated that 1.3 micromoles of glucose per ml were formed in this reaction mixture (figure 5). The formation of glucose in this reaction mixture may have been due to the beta-glucosidase present in the glucose oxidase preparation.

In spite of the small discrepancies the results of the arsenolysis experiment clearly indicate that cellobiose phosphorylase was able to catalyze the arsenolysis of cellobiose.

TABLE XVI

The disappearance of inorganic phosphate in a reaction mixture used as a control in the arsenolysis experiment.

Hours	Disappearance of inorganic phosphate in micromoles per ml
1	0.03
2	1.44
4	2.12
5	2.37
11	2.31

The reaction mixture was the same as the reaction mixture in figure 5.

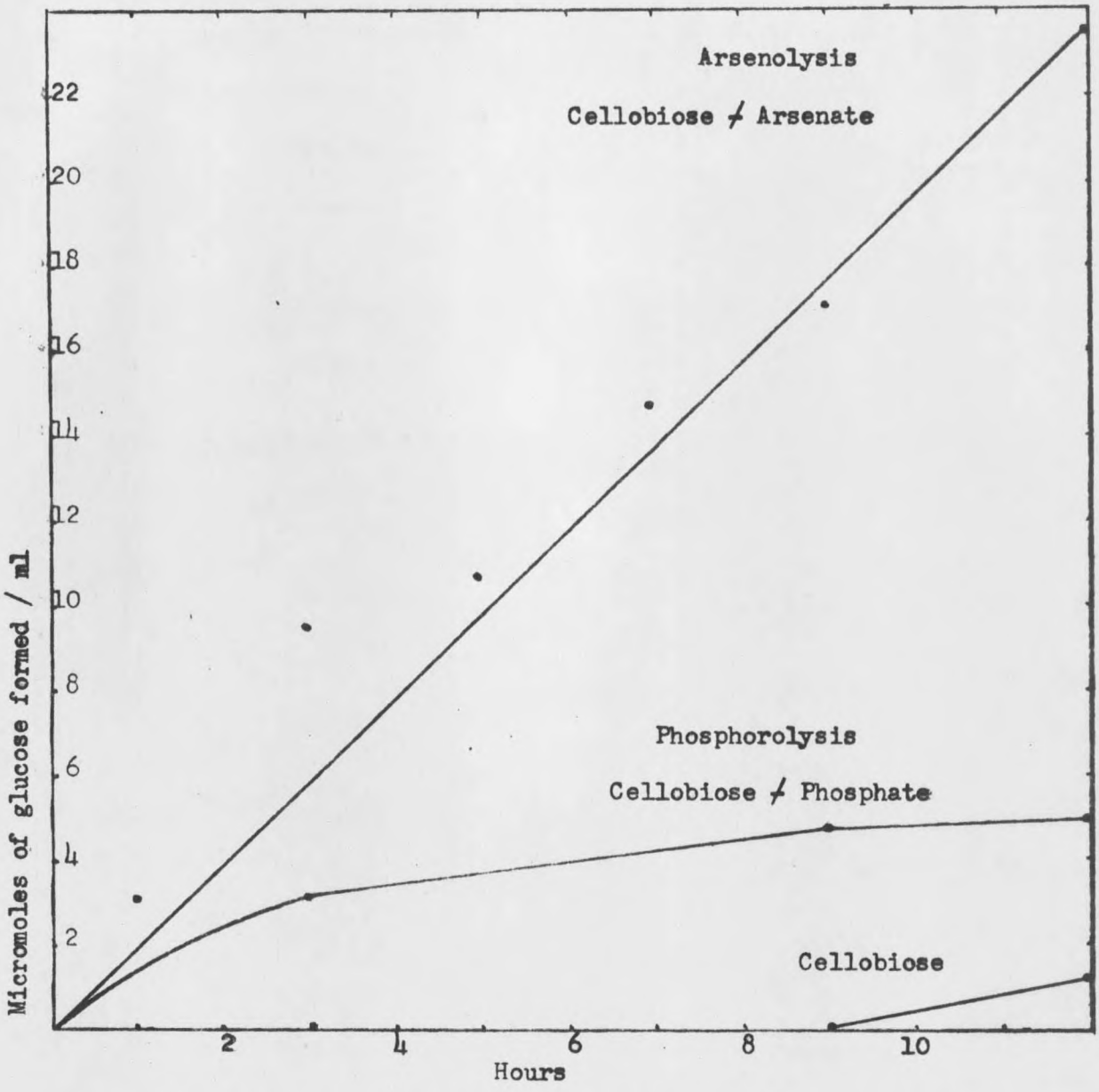


Figure 5. The arsenolysis of cellobiose with cellobiose phosphorylase. Each ml of reaction mixture contained 50 micromoles of cellobiose and purified cellobiose phosphorylase (2.5 mg protein). The arsenolysis reaction mixture contained 5 micromoles of inorganic arsenate / ml. The phosphorolysis reaction mixture contained 5 micromoles of inorganic phosphate / ml. A third reaction mixture contained no arsenate or phosphate. The reaction mixtures were buffered in M/100 barbital-acetate buffer pH 7.0 and incubated at 37 C.

(C) The mechanism of the cellobiose phosphorylase reaction.

The mechanism of phosphorylase reactions has been postulated on the basis of the exchange reactions catalyzed by the enzymes. Maltose phosphorylase is unable to catalyze the exchange between glucose-1-phosphate and inorganic phosphate or inorganic arsenate (Fitting and Douderoff, 1952b). Furthermore, this enzyme does not catalyze the exchange between maltose and glucose. On the basis of these results Fitting and Douderoff postulated that a maltose-enzyme-complex was formed in the reaction. That a Walden inversion occurs in both the cellobiose and maltose phosphorylase reactions suggested that the mechanism of these two reactions may be the same.

An experiment was conducted to determine whether cellobiose phosphorylase catalyzed the exchange between glucose-1-phosphate and arsenate. If this exchange occurred glucose-1-arsenate would be formed which presumably would undergo spontaneous decomposition resulting in the formation of glucose and inorganic arsenate. It was found that no significant amount of free glucose was formed when the enzyme was incubated with glucose-1-phosphate and arsenate (table XVII).

It has been shown that arsenate is able to substitute for phosphate in the decomposition of cellobiose (figure 5). Therefore, it might be inferred that the exchange reaction between glucose-1-phosphate and inorganic phosphate does not occur because the exchange between glucose-1-phosphate and inorganic arsenate does not occur.

The following experiment was conducted in order to determine the ability of cellobiose phosphorylase to catalyze the exchange between

TABLE XVII

The formation of free glucose when cellobiose phosphorylase was incubated with glucose-1-phosphate and arsenate.

Hours	Formation of glucose in micromoles per ml	
	Glucose-1-phosphate and arsenate	Glucose-1-phosphate
1	0.22	
2	0.29	
3		0.26
5	0.25	
7	0.31	
12	1.01	0.52

Each ml of reaction mixture contained purified cellobiose phosphorylase (2.5 mg protein/ml), and 38 micromoles of glucose-1-phosphate. One reaction mixture contained 5 micromoles of inorganic arsenate per ml, the other contained none. The reaction mixtures were buffered in M/100 barbital-acetate buffer and incubated at 37 C. Glucose was determined by the method of Saifer et al., (1941).

cellobiose and D-xylose. If this exchange took place, glucosyl-D-xylose would be expected to be formed in the absence of inorganic phosphate by reaction (a).

(a) cellobiose + D-xylose = glucosyl-D-xylose + glucose

In this experiment, if glucosyl-D-xylose had been formed in the exchange reaction between cellobiose and D-xylose, presumably it could be detected on a chromatograph. The aniline-acid-phthalate color developer

forms a reddish-pink color with D-xylose and glucosyl-D-xylose but no color is formed with cellobiose or glucose before the chromatograph is heated. It was shown that no spot corresponding to glucosyl-D-xylose was formed in a reaction mixture which contained cellobiose phosphorylase, cellobiose and D-xylose (figure 6).

As a control, another reaction mixture was included in the experiment which differed from the above in that the reaction mixture contained a small amount of inorganic phosphate. It was reasoned that in the presence of a small amount of inorganic phosphate the following reactions would occur:

(b) cellobiose + inorganic phosphate = glucose + glucose-1-phosphate

(c) glucose-1-phosphate + D-xylose = glucosyl-D-xylose + inorganic phosphate

The inorganic phosphate liberated in reaction (c) presumably would be able to react again in reaction (b). Reactions (b) and (c) apparently did occur for glucosyl-D-xylose was detected on the chromatograph of this reaction mixture as shown in figure 6.

The results indicate that a small amount of inorganic phosphate must be present in order for glucosyl-D-xylose to be formed under these conditions. Therefore, it is concluded that the exchange between cellobiose and D-xylose is not catalyzed by cellobiose phosphorylase. Likewise, the exchange between cellobiose and glucose would not be expected to be catalyzed by cellobiose phosphorylase.

The inability of cellobiose phosphorylase to catalyze the exchange reactions between glucose-1-phosphate and arsenate, and cellobiose and D-xylose is analogous to the results obtained with maltose phosphorylase

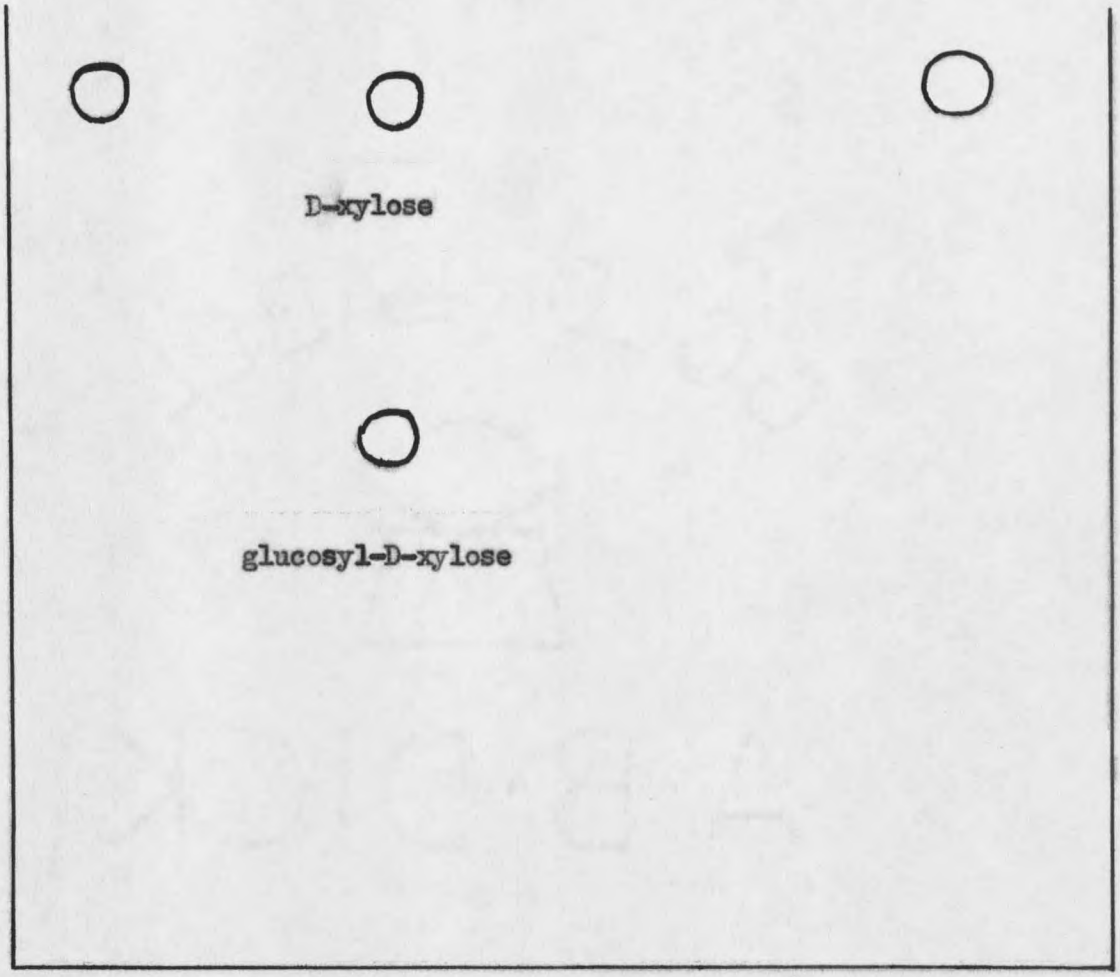


Figure 6. Chromatograph of reaction mixtures incubated with cellobiose and D-xylose. Reaction mixture 1 contained no inorganic phosphate. Reaction mixture 2 contained 0.4 micromoles of inorganic phosphate/ml. Each ml of reaction mixture contained 20 micromoles of cellobiose, 20 micromoles of D-xylose, and purified cellobiose phosphorylase (3 mg protein). Only the spots which developed before heating are shown. Each spot had a reddish-pink color characteristic of pentoses. Each reaction mixture was incubated for 1 hour at 37 C.

(Fitting and Douderoff, 1952b). This similarity in results suggests that the cellobiose and maltose phosphorylase reactions have similar mechanisms.

D) Pathways of cellobiose metabolism in C. thermocellum. In addition to cellobiose phosphorylase, C. thermocellum has been reported to utilize cellobiose by a kinase reaction (Nelson and McBee, 1957). In this reaction, cell free extracts from C. thermocellum, in the presence of cellobiose and adenosine triphosphate, catalyzed the formation of a cellobiose-phosphate ester.

Nelson and McBee used strain 157 of C. thermocellum in their study (personal communication). A different strain (651) of C. thermocellum was used in the present study, therefore, an experiment was conducted to determine whether strain 651 produced cellobiokinase.

In this experiment a cell free extract from C. thermocellum, strain 651, was incubated with cellobiose, adenosine triphosphate, and $NaHCO_3$. The acid liberated during the phosphorylation of cellobiose, resulted in the liberation of carbon dioxide from $NaHCO_3$ as shown in figure 7.

In the control vessels, two containing glucose and two containing no added substrate, less than half as much carbon dioxide was liberated as in the vessels containing cellobiose. It seems likely that most of the carbon dioxide liberated in the control vessels was due to adenosine triphosphatase activity. The additional activity in the vessels containing cellobiose presumably was due to cellobiokinase activity. It appears that strain 651 as well as strain 157 of C. thermocellum produces cello-

kinase.

Cellobiase does not appear to be produced by this organism (table II), which indicates that cellobiose is not utilized by hydrolysis. Therefore, it is suggested that cellobiose is utilized by C. thermocellum by two different pathways, one involving cellobiose phosphorylase and the other involving cellobiokinase.

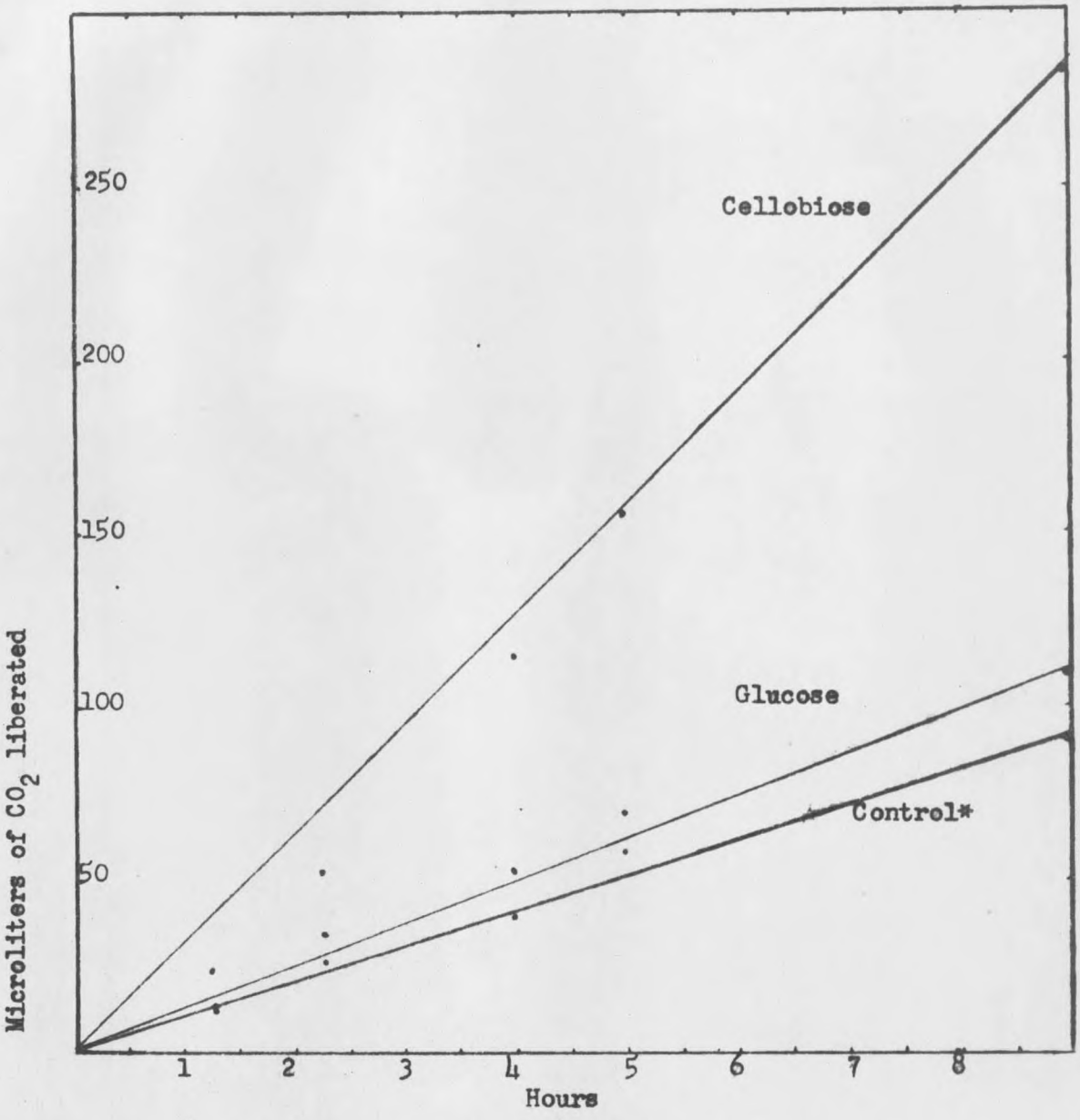


Figure 7. Evidence that cellobiokinase is produced by Clostridium thermocellum, strain 651. The readings represent the average value from two vessels. *The control vessels contained no cellobiose or glucose. The liberation of carbon dioxide in these vessels was probably due to adenosine triphosphatase activity.

DISCUSSION

Cellobiose is one of the few carbohydrates utilized by C. thermocellum. It seems likely that cellobiose is the end product of cellulose hydrolysis and that cellulose is the most abundant substrate for this organism in nature. Besides cellulose and cellobiose, hemicellulose and xylose are the only other substrates known to be fermented by C. thermocellum. The pathways of cellobiose utilization by this organism are shown in figure 8.

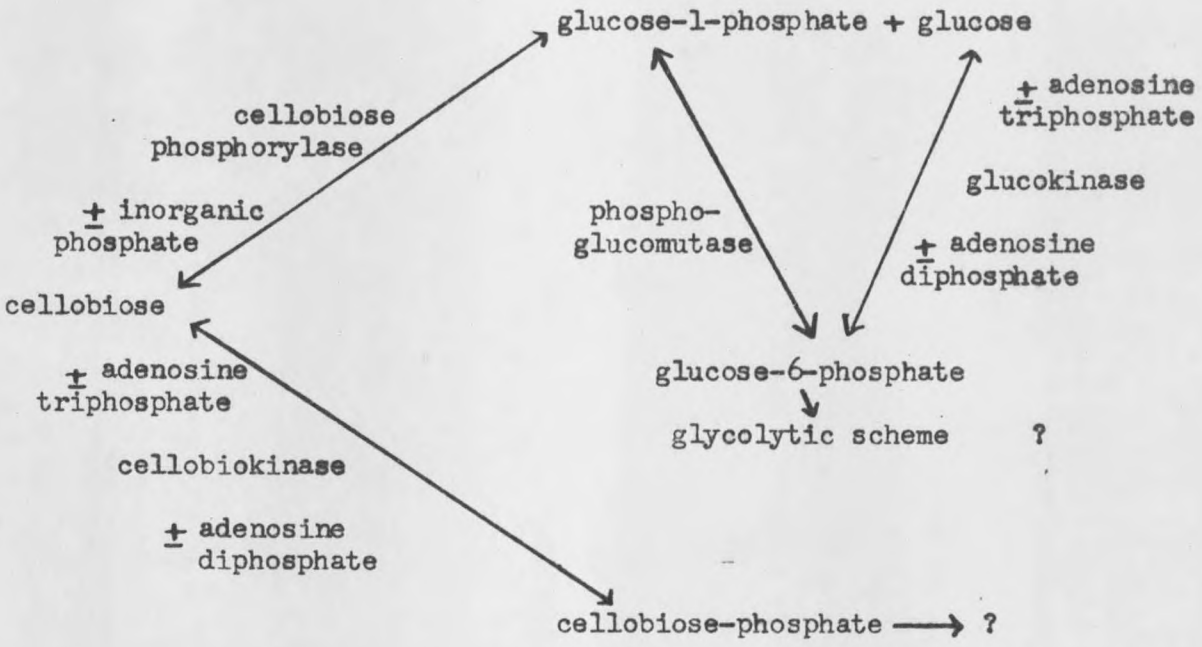


Figure 8. Pathways of cellobiose utilization by Clostridium thermocellum. The products of phosphorolysis presumably are converted to glucose-6-phosphate by glucokinase and phosphoglucomutase. An active phosphoglucomutase has been found in cell-free extracts (table I). Although the results of this study do not prove that glucokinase is produced (figure 7) this enzyme has been found in cell-free extracts of C. thermocellum

(Nelson and McBee, 1957). Glucose-6-phosphate, the product of both the phosphoglucomutase reaction and the glucokinase reaction, presumably can be metabolized through a conventional pathway such as the Embden-Meyerhof scheme.

The fate of the cellobiose-phosphate ester formed in the cellobiokinase reaction is unknown. It has been suggested that cellobiose-phosphate acts as a substrate for the phosphorylase (Sih, 1955 and Sih and McBee, 1955b). However, the inability of cellobiose phosphorylase to catalyze the condensation of glucose-1-phosphate and glucose-6-phosphate or the condensation of two molecules of glucose-1-phosphate renders this possibility unlikely. If cellobiose-1-phosphate were able to act as a substrate for cellobiose phosphorylase then this compound would be expected to be synthesized from glucose-1-phosphate. It has been shown, however, that when glucose-1-phosphate was incubated with cellobiose phosphorylase no inorganic phosphate was released (table I). The lack of phosphate liberation in this system indicates that cellobiose-1-phosphate was not synthesized.

Likewise, there was no significant amount of phosphate released when the enzyme was incubated with glucose-1-phosphate and glucose-6-phosphate (table XIII) as would happen if cellobiose-6-phosphate had been formed. Since neither cellobiose-1-phosphate nor cellobiose-6-phosphate appears to have been synthesized, these esters would not be expected to be phosphorolyzed.

The specificity of phosphorylases for the glucosyl moiety of various substrates appears to be absolute. Thus, it would seem unlikely

that cellobiose phosphorylase could act upon a cellobiose-phosphate ester such as cellobiose-6-phosphate in which the phosphate radicle is attached to the glucosyl moiety.

No evidence that cellobiose is decomposed by mechanisms other than those involving cellobiose phosphorylase or cellobiokinase was found. It appears that cellobiase is not produced by C. thermocellum (table II).

Of the two enzymes which act upon cellobiose, the phosphorylase appears to be more active than the kinase in vitro. A comparison of the rates of activity of the two enzymes (figure 1 and figure 7) indicates that cellobiose phosphorylase reacts about 5 to 10 times as fast as cellobiokinase. These rates, however, do not necessarily reflect the relative rates of activity in vivo, because the rate of the kinase reaction may have been limited by experimental conditions. That the kinase activity was not maximal is suggested by the fact that little or no glucokinase activity was found (figure 7). Yet it might be expected that the rate of glucokinase activity is almost equal to the rate of phosphorylase activity in vivo for ordinarily glucose does not accumulate in the culture medium. It is possible, however, that glucose accumulates inside the cell and inhibits phosphorylase activity. This possibility leads to the suggestion that the utilization of glucose is a limiting factor in the growth of the organism.

Although the equilibrium constant is unfavorable for the phosphorolysis of cellobiose the equilibria of the reactions in which the products of phosphorolysis would be expected to enter favor the decomposition of the products. At equilibrium the molar ratio of cellobiose and inorganic

phosphate to glucose and glucose-1-phosphate is about 2 to 1. Glucose-1-phosphate presumably is converted to glucose-6-phosphate by phosphoglucomutase which has been shown to be present in cell-free extracts of the organism (table I). At equilibrium the molar ratio of glucose-1-phosphate to glucose-6-phosphate is about 1 to 19 (Colowick and Sutherland, 1942). Glucose presumably is converted to glucose-6-phosphate by glucokinase. The molar ratio of glucose and adenosine triphosphate to glucose-6-phosphate and adenosine diphosphate at equilibrium is about 1 to 40 (based upon K_t = 1550, Robbins and Boyer, 1957). Thus the thermodynamics of the reactions by which cellobiose is phosphorolyzed and the phosphorolytic products are converted to glucose-6-phosphate favor the formation of glucose-6-phosphate.

Cellobiose phosphorylase apparently acts as an energy saving device for C. thermocellum. For each mole of cellobiose which is decomposed by phosphorolysis, one mole of glucose-1-phosphate is formed which presumably retains the energy of the glucosidic linkage of cellobiose. Glucose-1-phosphate apparently is converted to glucose-6-phosphate which can enter the glycolytic scheme without the expenditure of energy by the organism. In contrast, the hydrolysis of cellobiose would form two glucose units, both of which would require adenosine triphosphate for the formation of glucose-6-phosphate. Therefore, for each mole of cellobiose phosphorolyzed, one mole of adenosine triphosphate would be conserved.

If one considers the conversion of cellobiose to lactate by a combination of hydrolysis and glycolysis, 4 moles of adenosine triphosphate per mole of cellobiose would be expended by the organism with a net gain

of 4 moles of adenosine triphosphate. On the other hand, the conversion of cellobiose to lactate by a combination of phosphorolysis and glycolysis would require the expenditure of only 3 moles of adenosine triphosphate per mole of cellobiose with a net gain of 5 moles of adenosine triphosphate. Thus, up to 20 per cent more energy could be obtained by the phosphorolysis of cellobiose than by the hydrolysis of cellobiose.

Although the direct utilization of cellobiose by C. thermocellum led to the demonstration of cellobiose phosphorylase, the ability of the organism to utilize cellobiose but not glucose remains unexplained. The direct utilization of sucrose by certain pseudomonads, which appears to be analogous to the direct utilization of cellobiose by C. thermocellum, has been discussed by Doudoroff (1951). It is interesting to note that the ability of certain bacteria to utilize sucrose more rapidly than either glucose or fructose led to the discovery of sucrose phosphorylase by Doudoroff. Several explanations for the direct utilization of sucrose were proposed. The possibility that the furanose form of fructose was the product of sucrose phosphorolysis and was utilized more readily than the pyranose form was discredited. It appeared that a rapid equilibrium was attained between the pyranose and furanose form of fructose.

Another possibility, that the utilization of free fructose depended upon the utilization of the glucose portion of sucrose, was quite convincingly eliminated. Intact cells adapted to sucrose utilized the glucose portion of glucosido-sorboside but excreted sorbose into the medium. When fructose and glucosido-sorboside were supplied together,

fructose was not utilized, which indicated that the utilization of fructose was not dependant on the glucose portion of a disaccharide.

That a derivative of sucrose, such as a sucrose-phosphate ester, was phosphorolyzed instead of sucrose was also considered. In such a reaction a fructose-phosphate ester rather than free fructose would be expected to be the product of phosphorolysis. Evidence was obtained that sucrose was phosphorylated in the presence of adenosine triphosphate and dried cell suspensions. Douderoff suggested that the sucrose-phosphate ester which appeared to have been formed serves as a substrate for sucrose phosphorylase. The possibility that sucrose-phosphate is phosphorolyzed, however, is not compatible with the inability of sucrose phosphorylase to catalyze the condensation of fructose-1,6-diphosphate, fructose-1-phosphate, or fructose-6-phosphate, with glucose-1-phosphate.

Another alternative which was proposed was that sucrose is permeable to the cells but fructose is not. It was further suggested that sucrose phosphorylase catalyzes the transport of sucrose across the permeability barrier. That fructose is impermeable to the cells is suggested by the inability of intact cells to catalyze the exchange between either labelled fructose and unlabelled sucrose or unlabelled fructose and sucrose labelled in the fructose portion. The question of the permease activity of sucrose phosphorylase, however, remains unanswered.

It seems appropriate to consider the direct utilization of cellobiose by C. thermocellum in relation to the discussion presented by

Douderoff. It is possible that glucofuranose is liberated during the phosphorolysis of cellobiose and that glucose is utilized by the organism in this form. Glucofuranose, however, is unstable and presumably it readily forms glucopyranose. Furthermore, no mechanism is known whereby glucofuranose is utilized but glucopyranose is not utilized. It seems unlikely that glucokinase would catalyze the phosphorylation of glucofuranose without acting upon glucopyranose.

The transphosphorylation involving glucose-1-phosphate and glucose appears to have no bearing on the direct utilization of cellobiose. Transphosphorylation would not result in the phosphorylation of both of the glucose units of cellobiose.

The possibility that a cellobiose-phosphate ester acts as a substrate for cellobiose phosphorylase seems unlikely because of the inability of the enzyme to catalyze the condensation of glucose-phosphate esters (see page 75).

Finally, that cellobiose, but not glucose, is permeable to the cells, appears to have some merit. The impermeability of glucose to the cells is a possible explanation for the inability of C. thermocellum to ferment glucose. Cellobiose apparently is permeable to the cells; however, it is not certain whether cellobiose or a derivative of cellobiose is transported across the permeability barrier. Douderoff (1951) has suggested that the phosphorylation of sucrose in the presence of adenosine triphosphate provides a mechanism for transporting sucrose across the cell membrane. Likewise, the transport of cellobiose across the permeability barrier may be dependent on cellobiokinase activity.

On the other hand, it seems equally likely that cellobiose phosphorylase catalyzes the transport of cellobiose across the permeability barrier. If cellobiose phosphorylase were able to act as a permease one might expect that the organism could grow in a medium containing the end-products of cellobiose phosphorolysis as well as in a medium containing cellobiose and phosphate. Cellobiose phosphorylase, however, would not be expected to catalyze the transport of glucose across the permeability barrier in the absence of glucose-1-phosphate. Thus the permease activity of cellobiose phosphorylase could explain the ability of C. thermocellum to use cellobiose but not glucose. At least there seems to be a tendency for organisms which utilize disaccharides directly to decompose the disaccharides by phosphorolysis. Sucrose, maltose, and cellobiose phosphorylases have been found in organisms which utilize disaccharides more readily than the monosaccharide constituents.

The specificity of cellobiose phosphorylase. The ability of cellobiose phosphorylase to catalyze the condensation of glucose-1-phosphate with D-glucose, D-xylose, 2-deoxyglucose, or glucosamine can be explained by assuming that the enzyme is relatively non-specific for the C-2 and C-5 groups of these substrates. It seems reasonable to assume further that these compounds are attached to a glucosyl radicle through a 1-4, beta-glucosidic linkage as in cellobiose. The enzyme presumably attaches to different substrates at similar positions on the molecules. On the basis of these assumptions certain interpretations can be made in regard to the specificity of cellobiose phosphorylase.

It appears that the enzyme is fairly specific for groups attached to C-1 (table XVIII). Cellobiose phosphorylase is unable to catalyze the condensation of two molecules of glucose-1-phosphate which indicates that a phosphate can not replace an OH group at C-1. The failure of delta-gluconolactone and gluconate to react suggests that a double bonded oxygen group cannot be attached to C-1. Gluconate, however, may not have reacted because of the absence of a ring structure. Likewise, it is possible that D-sorbitol did not react because of the absence of a ring structure.

The H and OH groups of C-2 cannot be reversed because mannose did not react. However, 2-deoxyglucose did react, although the reaction was not as fast as with glucose. Thus, the OH group cannot replace the H group of C-2, but the OH group of C-2 can be replaced by H. These data are difficult to interpret in terms of an enzyme-substrate complex. It might be expected that the hydrogen bonding due to an OH group would be stronger than the bonding due to a H group. The enzyme, therefore, might be expected to attach more firmly to mannose than to 2-deoxyglucose. Mannose, however, seemed to have no inhibitory effect on the synthesis of cellobiose, which indicates that it has no affinity for the enzyme.

The enzyme appears to be relatively non-specific for the OH group of C-2 in glucose. This OH group can be replaced by H as in 2-deoxy-glucose, or by NH_2 as in glucosamine. The specificity is limited, however, because this OH group could not be replaced by the acetylamine group of N-acetylglucosamine.

The position of the H and OH groups attached to C-3 apparently can

A summary of the information concerning the specificity of cellobiose phosphorylase

Carbon atom number	Group attached to carbon atom	Evidence
1	OH	not replaceable by phosphate in glucose-1-phosphate
	O	failure of delta-gluconolactone to react
	*	failure of gluconate or sorbitol to react
2	H	D-mannose does not react
	OH	replaceable by H in deoxyglucose
	OH	replaceable by NH_2 in glucosamine
	OH	not replaceable by acetylamine in N-acetylglucosamine
3	H and OH cannot be reversed	D-ribose does not react
4	H and OH cannot be reversed	D-galactose does not react
5	CH_2OH	replaceable by H in D-xylose
6	OH	not replaceable by phosphate in glucose-6-phosphate
	OOH	glucuronate does not react

* Lack of ring structure may have prevented these reactions.

not be reversed for D-ribose did not react. D-ribose differs from D-xylose, which was readily condensed with glucose-1-phosphate, in the position of the H and OH groups of C-3.

Likewise, the position of the H and OH groups attached to C-4 cannot be reversed for D-galactose did not react. This positional effect might be expected because the oxygen from this OH group probably forms the glucosidic bond in cellobiose.

The ability of both D-glucose and D-xylose to react indicates that the CH_2OH group can be replaced by an H group. Therefore, the enzyme appears to be somewhat non-specific for the groups attached to C-5. The failure of glucuronate to react indicates that C-6 cannot be a carboxyl group. Moreover, the failure of glucose-6-phosphate to react indicates that the OH group of C-6 cannot be replaced by a phosphate radicle.

The substitutions which apparently can be made in the reducing portion of cellobiose are shown in figure 9A. The encircled groups are those which probably cannot be substituted. The arrows with cross-bars indicate that the positions of the H and OH groups cannot be reversed.

The observation that L-xylose was able to react was surprising in view of the other information on the specificity of the enzyme. L-xylose would not be expected to combine with a glucosyl group through C-4 because the H and OH groups on C-2, C-3, and C-4 would be reversed from the position in which they occur in other compounds which react (figure 9A and 9C). It seems possible, however, that L-xylose could attach to the glucosyl group through C-2. This type of linkage seems to conform to the specificity requirements of the enzyme. It appears that C-4 of L-xylose could occupy the same position as C-2

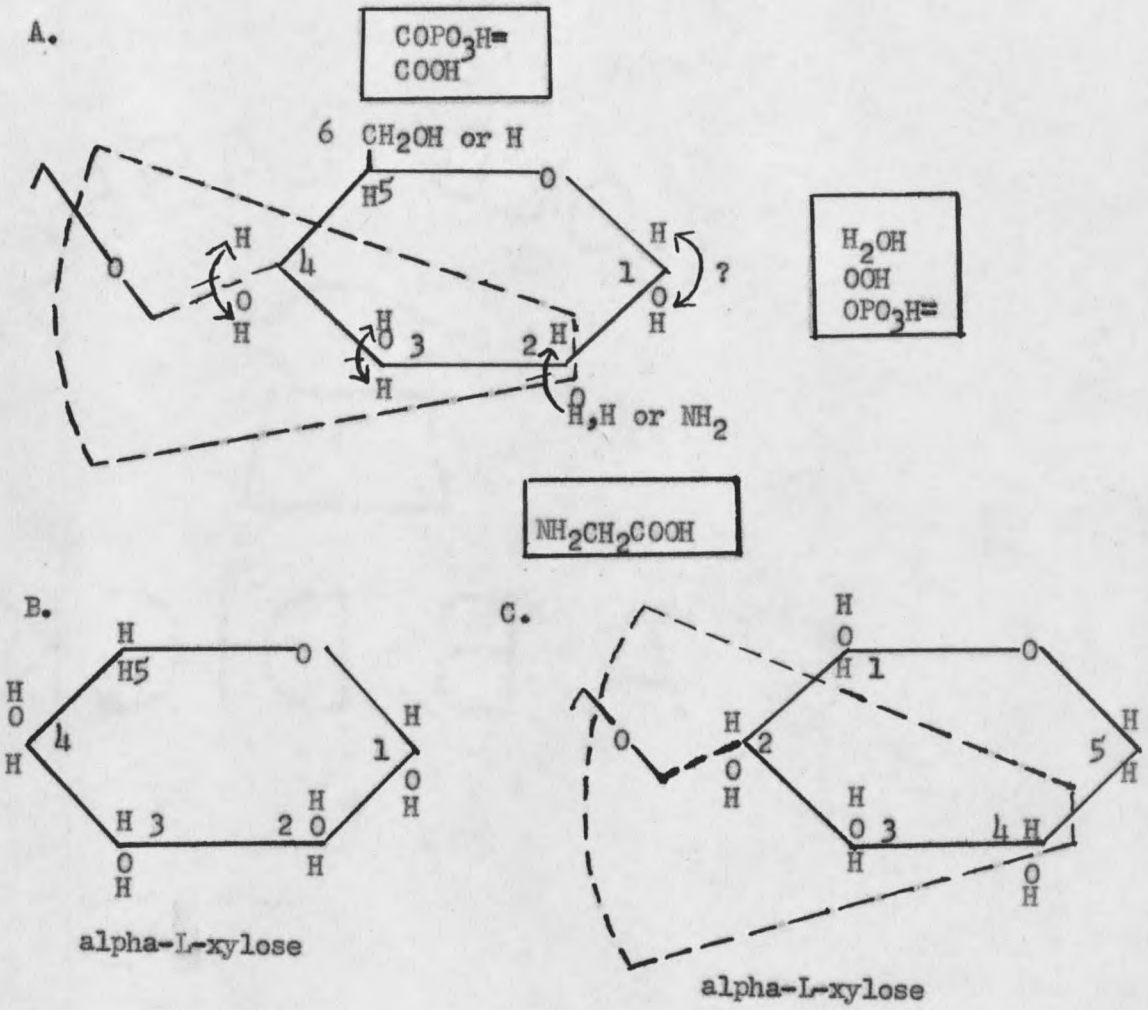
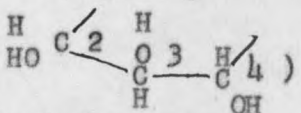
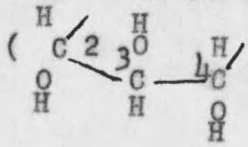
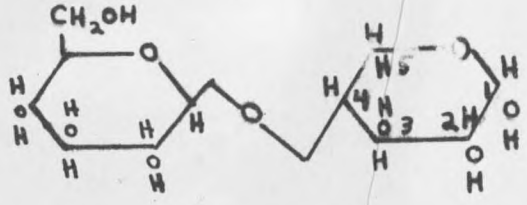


Figure 9. Diagramatic representation of the specificity of cellobiose phosphorylase.

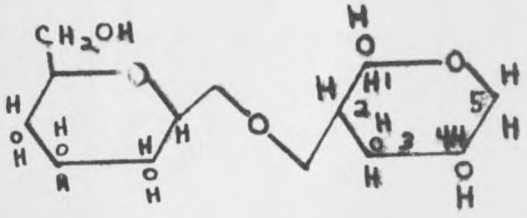
in D-glucose, D-xylose, 2-deoxyglucose, or glucosamine. Furthermore, the sequence of H and OH attached to C-2, C-3, and C-4 () would be similar to the sequence in the H and OH groups in D-glucose and D-xylose ().

The encircled groups in figure 9A and 9C illustrate the similarity in structure of L-xylose to D-glucose, D-xylose, 2-deoxyglucose, and glucosamine. The attachment of L-xylose to a glucosyl group through C-2 would result in the formation of a disaccharide with a 1, 2 glucosidic linkage. Evidence for the formation of such a compound could be obtained by determining its ability to form an osazone, because both C-1 and C-2 must be free for osazone formation.

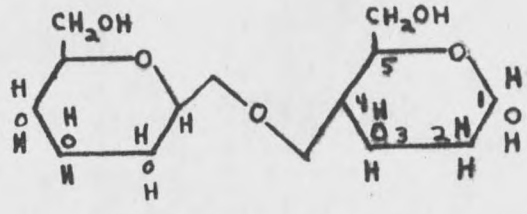
On the basis of the information on the specificity of the enzyme it is suggested that s syntheses of the following compounds are catalyzed by cellobiose phosphorylase:



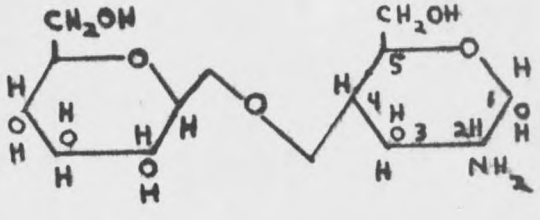
glucosyl-D-xylose



glucosyl-L-xylose



glucosyl-deoxyglucose



glucosyl-D-glucosamine

It is possible that cellobiose phosphorylase is able to catalyze synthesis of other disaccharides. For example L-idose and L-threose have structural similarities at C-2, C-3 and C-4, which suggest that these compounds would react. Unfortunately neither L-idose nor L-threose could be obtained for experimentation. Further studies on the specificity of cellobiose phosphorylase and the inhibitory effect of different substrates might provide valuable information on the points of attachment of the enzyme to a substrate.

The ability of cellobiose phosphorylase to catalyze the syntheses of various compounds may be important in the synthesis of polysaccharides. The compounds synthesized with cellobiose phosphorylase might serve as building blocks in the synthesis of more complex compounds. Although it appears that these disaccharides have not been encountered before, it is possible that they exist in nature. Cellobiose phosphorylase presumably could catalyze the phosphorolysis of these disaccharides with the formation of compounds which could be further metabolized.

The mechanism of the cellobiose phosphorylase reaction. The occurrence of an inversion in the cellobiose phosphorylase reaction suggests that the mechanism of this reaction is similar to the mechanism of the maltose phosphorylase reaction. The inability of cellobiose phosphorylase to catalyze the exchange reactions between glucose-1-phosphate and arsenate or between cellobiose and D-xylose further indicates the similarity of the mechanism of the two reactions. Maltose phosphorylase catalyzes neither the exchange between glucose-1-phosphate and phosphate or arsenate nor the exchange between maltose and glucose

(Fitting and Douderoff, 1952b). On the basis of the inability of maltose phosphorylase to catalyze these exchange reactions a maltose-enzyme-phosphate complex was postulated to be formed in the reaction.

In contrast to maltose phosphorylase, sucrose phosphorylase catalyzed the exchange reactions between glucose-1-phosphate and phosphate or arsenate (Douderoff et al., 1947) and between sucrose and fructose (Wolochow et al., 1949). On the basis of these exchange reactions a glucose-enzyme complex was postulated to be formed. Thus a fundamental difference in the mechanism of the sucrose phosphorylase reaction and the maltose or cellobiose phosphorylase reactions is apparent as illustrated in figure 10.

Further interpretations of the mechanisms of phosphorylase reactions have been made by Koshland (1951, 1954a, and 1954b). Koshland (1951, and 1954a) has proposed a double displacement mechanism for the sucrose phosphorylase reaction as shown in figures 11 and 12. As the carbon-oxygen bond of glucose-1-phosphate begins to break, the electron donor site, D, on the enzyme shares its electrons with C-1 of glucose. An inverted glucose-enzyme complex is formed and $HOPO_3^{=}$ is liberated (figure 11). In the second stage of the reaction, fructose accepts glucose from the enzyme with the formation of sucrose and free enzyme (figure 12). The double displacement mechanism offers an explanation for the retention of the configuration of the C-1 atom of glucose and takes into account the cleavage of the carbon-oxygen bond which has been reported by Cohn (1949). The glucose-enzyme intermediate explains the exchange reactions catalyzed by sucrose phosphorylase.

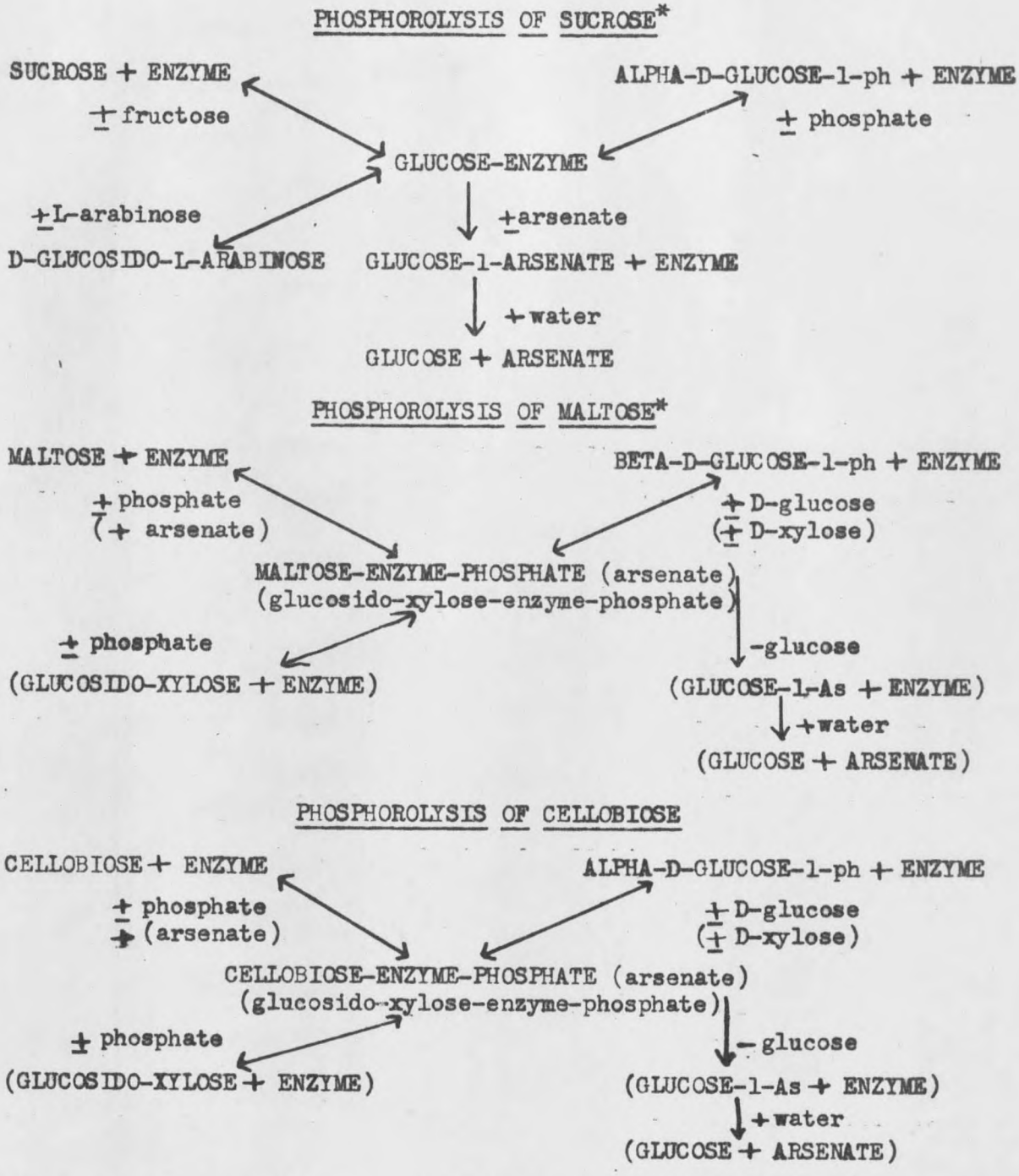


Figure 10. The relationship of the sucrose, maltose, and cellobiose phosphorylase reactions

*After Fitting and Douderoff(1952b)

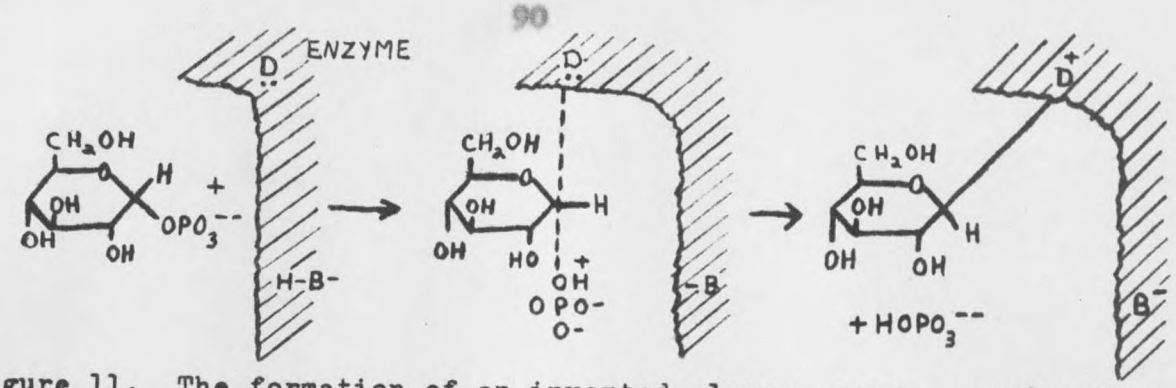


Figure 11. The formation of an inverted glucose-enzyme complex from alpha-glucose-1-phosphate and sucrose phosphorylase (After Koshland, 1954a).

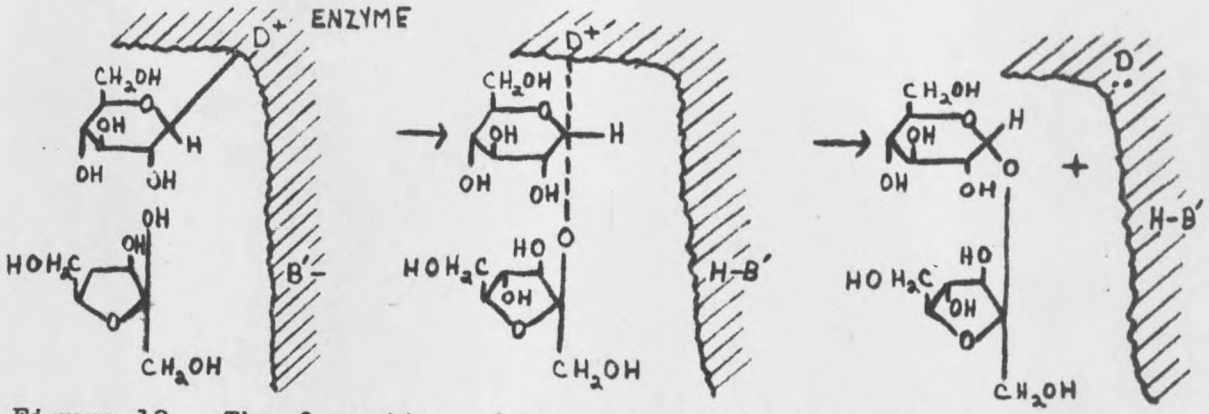


Figure 12. The formation of sucrose and free sucrose phosphorylase from a glucose-enzyme complex and fructose (After Koshland, 1954a).

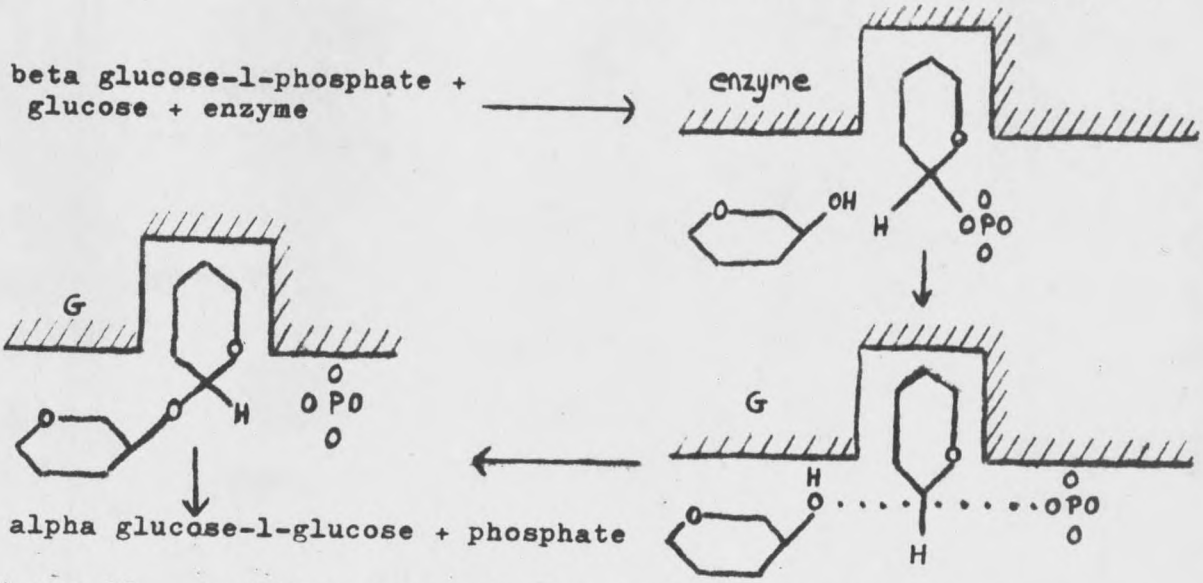


Figure 13. An illustration of the single displacement mechanism proposed for the maltose phosphorylase reaction (After Koshland,1954b).

In contrast to the mechanism of the sucrose phosphorylase reaction a single displacement mechanism has been proposed for the maltose phosphorylase reaction (Koshland, 1954b) which is shown in figure 13. In this mechanism glucose replaces phosphate directly without the formation of a glucose-enzyme intermediate. When the bond between the phosphate-oxygen and the carbon atom starts to break, the bond between the glucose-oxygen and the carbon atom begins to form. The single displacement mechanism explains the inversion of the configuration of the C-1 atom of the non-reducing glucose portion and predicts that a cleavage occurs between the carbon-oxygen bond of glucose-1-phosphate. This mechanism is consistent with the exchange and specificity data for maltose phosphorylase. In order for an exchange to occur between phosphate and glucose-1-phosphate, phosphate would have to occupy the same position as free glucose (G in figure 13). Since closely related analogs of glucose, such as mannose do not react (Fitting and Douderoff, 1952b), it seems unlikely that phosphate would be able to occupy this position. Thus the inability of maltose phosphorylase to catalyze this exchange and the specificity of the enzyme are in agreement with the single displacement mechanism.

It appears that the single displacement mechanism is applicable to the cellobiose phosphorylase reaction. The essential difference between the maltose phosphorylase and the cellobiose phosphorylase reactions appears to be the configuration of the glucosidic linkages in the substrates. Therefore, a single displacement mechanism for the cellobiose phosphorylase reaction is proposed, which is analogous to the mechanism of the maltose phosphorylase reaction (figure 14).

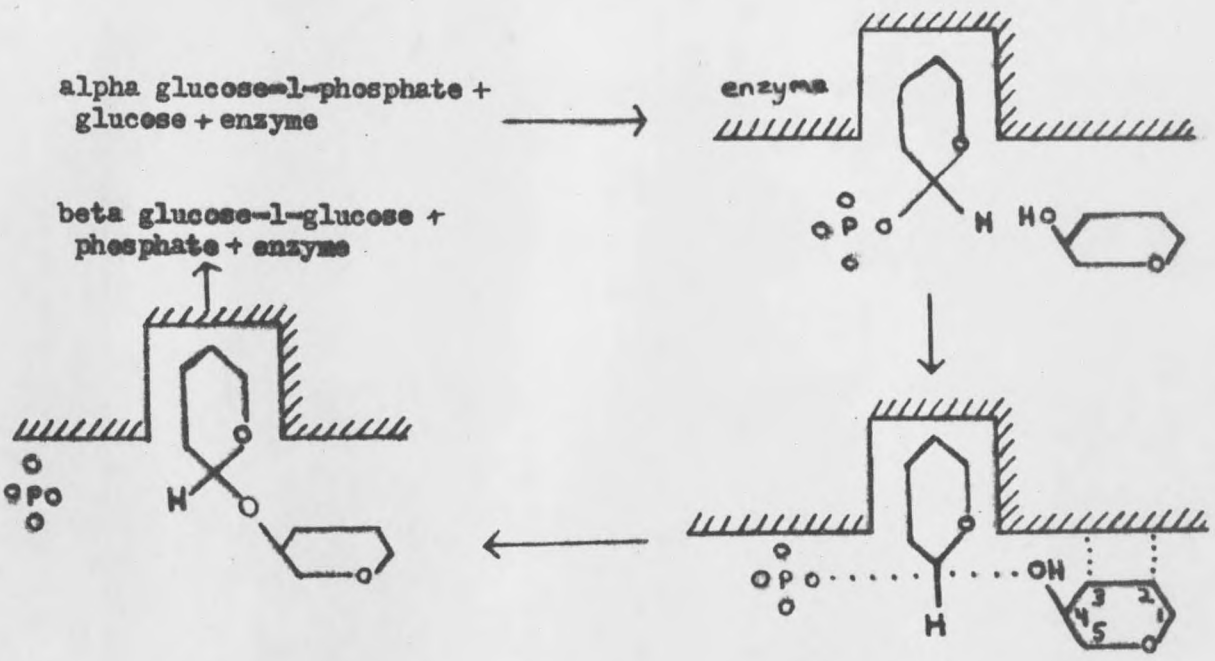


Figure 14. A proposed single displacement mechanism for the cellobiose phosphorylase reaction.

In the cellobiose phosphorylase reaction, as in the maltose phosphorylase reaction, the single displacement mechanism explains the inversion of the C-1 atom of the glucosyl moiety. If this mechanism is correct it follows that a cleavage occurs between carbon and oxygen atoms of glucose-1-phosphate. The single displacement mechanism is consistent with the information on the exchange reactions catalyzed by cellobiose phosphorylase and the specificity of the enzyme. It is interesting to note that the cellobiose-enzyme-phosphate complex shown in figure 14 illustrates the close proximity of the C-2 and C-3 atoms of the reducing portion to the enzyme. It seems reasonable to expect a close relationship of the enzyme to these positions in view of the

specificity of the enzyme (table XVIII and figure 9).

Since the cellobiose phosphorylase and the maltose phosphorylase reactions appear to have the same mechanism and differ only in the configuration of the substrates, it seems possible that the sites on the enzymes to which the substrates attach are similar. In fact, the active surface of one enzyme may be essentially a mirror image of the other. This relationship is consistent with the information on the specificity of the two enzymes. D-glucose, D-xylose, phosphate, and arsenate were able to act as glucose acceptors with both enzymes. However, D-galactose, D-mannose, D-ribose, and gluconate were unable to act as glucose acceptors with either enzyme. Another significant similarity is the free energy change which appears to be nearly the same in the two reactions. Thus, a comparitive study of cellobiose phosphorylase and maltose phosphorylase might provide valuable information on the mechanism of enzyme action.

SUMMARY

Cellobiose phosphorylase, obtained from cell-free extracts of Clostridium thermocellum strain 651, was purified sufficiently to remove interfering enzymes. The purified enzyme catalyzed the phosphorolytic decomposition of cellobiose with the formation of equimolar quantities of alpha-glucose-1-phosphate and glucose. The reaction was reversible for equimolar quantities of cellobiose and phosphate were formed from alpha-glucose-1-phosphate and glucose. The equilibrium constant, K = (glucose-1-phosphate)(glucose)/(cellobiose)(phosphate) at pH 7 and 37 C was found to be about 0.23.

The optimum pH for the activity of the enzyme is about 7.0. The temperature coefficient between 20 C and 40 C is about 2.4. The effect of various inhibitors on the activity of the enzyme was determined and evidence was obtained which suggests that a sulfhydryl group is essential for the activity of the enzyme.

It appears that D-glucose, D-xylose, L-xylose, 2-deoxyglucose, glucosamine, phosphate, and arsenate can act as glucose acceptors in the reaction. It is suggested that the synthesis of glucosyl-D-xylose, glucosyl-L-xylose, glucosyl-2-deoxyglucose, and glucosyl-D-glucosamine can be catalyzed by cellobiose phosphorylase. The arsenolysis of cellobiose was demonstrated to take place in a reaction mixture in which arsenate was substituted for phosphate. Beta-glucose-1-phosphate was unable to replace alpha-glucose-1-phosphate which indicates that a Walden inversion occurs during the synthesis of cellobiose. The following compounds were unable to act as glucose acceptors:

gluconate, glucuronate, glucose-6-phosphate, D-ribose, D-sorbitol, delta-gluconolactone, N-acetylglucosamine, L-arabinose, L-fructose, D-galactose and D-mannose.

Evidence was obtained which indicates that the exchange reactions between glucose-1-phosphate and arsenate and between cellobiose and D-xylose are not catalyzed by the enzyme. These results suggest that a cellobiose-enzyme-phosphate complex is formed in the reaction. A single displacement mechanism is proposed for the cellobiose phosphorylase reaction in which a cleavage occurs at the C-1 atom between the carbon and oxygen atoms of glucose-1-phosphate.

REFERENCES

Barr, S. 1954 Quantitative estimation of glucose by paper partition chromatography. Biochem. J., 58, 175-176.

Barron, E. S. G. and Singer, T. P. 1945 Studies on biological oxidations XIX Sulfhydryl enzymes in carbohydrate metabolism. J. Biol. Chem., 157, 221-240.

Boyer, P. D. 1954 Spectrophotometric study of the reaction of protein sulfhydryl groups with organic mercuricals. J. Am. Chem. Soc., 76, 4331-4337.

Breed, R. S., Murray, E. G. D. and Smith, N. R. 1957 Bergey's manual of determinative bacteriology. 7th Ed. The Williams & Wilkins Co., Baltimore.

Burton, K. and Krebs, H. A. 1953 The free-energy changes associated with the individual steps of the tricarboxylic acid cycle, glycolysis, and alcoholic fermentation and with the hydrolysis of the pyrophosphate groups of adenosinetriphosphate. Biochem. J., 54, 94-107.

Caputto, R., Leloir, L. F., Trucco, R. E., Cardini, C. E. and Paladini, A. 1948 A coenzyme for phosphoglucomutase. Arch. Biochem., 18, 201-203.

Chargaff, E., Levine, C. and Green, C. 1948 Techniques for the demonstration by chromatography of nitrogen lipide constituents, sulfur-containing amino acids, and reducing sugars. J. Biol. Chem., 175 67-71.

Cohn, M. 1949 Mechanisms of cleavage of glucose-1-phosphate. J. Biol. Chem., 180, 771-781.

Cohn, M. and Cori, G. T. 1948 On the mechanism of action of muscle and potato phosphorylase. J. Biol. Chem., 175, 89-93.

Colowick, S. P. and Kalckar, H. M. 1943 The role of myokinase in transphosphorylations. I. The enzymatic phosphorylations of hexoses by adenyl pyrophosphate. J. Biol. Chem., 148, 117-126.

Colowick, S. P. and Sutherland, E. W. 1942 Polysaccharide synthesis from glucose by means of purified enzymes. J. Biol. Chem., 144, 423-437.

Cori, C. F., Colowick, S. P. and Cori, G. T. 1937 The isolation and synthesis of glucose-1-phosphoric acid. J. Biol Chem., 121, 465-477.

Cori, C. F., Schmidt, G. and Cori, G. T. 1939 The synthesis of a polysaccharide from glucose-1-phosphate in muscle extract. Science, 89, 464-465.

Cori, C. F., Cori, G. T. and Green, A. A. 1943 Crystalline muscle phosphorylase. III. Kinetics. J. Biol. Chem., 151, 39-55.

Cori, G. T., Colowick, S. P. and Cori, C. F. 1939 The activity of the phosphorylating enzyme in muscle extract. J. Biol. Chem., 127, 771-782.

Cori, G. T. and Cori, C. F. 1939 The activating effect of glycogen on the enzymatic synthesis of glycogen from glucose-1-phosphate. J. Biol. Chem., 131, 397-398.

Cowles, P. B. and Rettger, L. F. 1931 Isolation and study of an apparently widespread cellulose-fermenting anaerobe, C. cellulosolvens (N. sp.?)., J. Bacteriol., 21, 161-182.

Douderoff, M. 1943 Studies on the phosphorolysis of sucrose. J. Biol. Chem., 151, 351-361.

Douderoff, M. 1951 The problem of the "direct utilization" of disaccharides by certain microorganisms. In Phosphorus metabolism, 1 pp. 42-48. Edited by W. D. McElroy and B. Glass. The Johns Hopkins Press, Baltimore.

Douderoff, M., Kaplan, N. and Hassid, W. Z. 1943 Phosphorolysis and synthesis of sucrose with a bacterial preparation. J. Biol. Chem., 148, 67-75.

Douderoff, M., Barker, H. A. and Hassid, W. Z. 1949 Studies with bacterial sucrose phosphorylase. I. The mechanism of action of sucrose phosphorylase as a glucose-transferring enzyme (transglucosidase). J. Biol. Chem., 168, 725-732.

Douderoff, M., Barker, H. A. and Hassid, W. Z. 1949 Studies with bacterial phosphorylase. III. Arsenolytic decomposition of sucrose and of glucose-1-phosphate. J. Biol. Chem., 180, 147-150.

Dryer, R. L., Tammes, A. R. and Routh, J. I. 1957 The determination of phosphorus and phosphatase with N-phenyl-p-phenylenediamine. J. Biol. Chem., 225, 177-183.

Eneboe, L. 1951 On three bacteria connected with thermophilic cellulose fermentation. Physiol. Plantarum, 4, 652-666.

Fiske, C. H. and SubbaRow, Y. 1925 The colorimetric determination of phosphorus. J. Biol. Chem., 66, 375-400.

Fitting, C. and Douderoff, M. 1952a Enzymatic phosphorolysis of maltose with production of beta-glucose-1-phosphate. Federation Proc., 11, 212.

Fitting, C. and Douderoff, M. 1952b Phosphorolysis of maltose by enzyme preparations from Neisseria meningitidis. J. Biol. Chem., 199, 153-163.

Fitting, C. and Scherp, H. W. 1952 Observation on a strain of Neisseria meningitidis in the presence of glucose and maltose. III. Cell-free extracts and phosphorolysis of maltose. J. Bacteriol. 64, 287-298.

Green, D. E. and Stumpf, P. K. 1942 Starch phosphorylase of potato. J. Biol. Chem., 142, 355-366.

Hanes, C. S. 1940a The breakdown and synthesis of starch by an enzyme system from pea seeds. Proc. Roy. Soc. (London), 128B, 421-450.

Hanes, C. S. 1940b The reversible formation of starch from glucose-1-phosphate catalyzed by potato phosphorylase. Proc. Roy. Soc. (London), 129B, 174-208.

Hassid, W. Z. and Douderoff, M. 1950 Synthesis of disaccharides with bacterial enzymes. Advances in Enzymology, 10, 123-143.

Hoppe-Seylor, F. 1883 Gärung der Zellulose. Ber. Deutsch Chem. Gesellsch., 16, 122-123.

Hungate, R. E. 1944 Studies on cellulose fermentation. I. The culture and physiology of an anaerobic cellulose-digesting bacterium. J. Bacteriol., 48, 499-513.

Hungate, R. E. 1950 The anaerobic mesophilic cellulolytic bacteria. Bacteriol. Revs., 14, 1-49.

Kalckar, H. M. 1954 The mechanism of transglycosidation. In Mechanism of enzyme action, pp. 675-739. Edited by W. D. McElroy and B. Glass. The Johns Hopkins Press, Baltimore.

Katz, J. and Hassid, W. Z. 1948 Arsenolysis and phosphorolysis of the amylose and amylopectin fractions of starch. Nature, 161, 96.

Keilin, D. and Hartree, E. F. 1948 The use of glucose oxidase (notatin) for the determination of glucose in biological material and for the study of glucose-producing systems by manometric methods. Biochem. J., 42, 230-238.

Khouvine, Y. 1923 Digestion de la cellulose par la flore intestinale de l'homme. B. cellulosae dissolvens, n. sp. Ann. Inst. Pasteur, 37, 711-752. Seen in McBee, R. H. 1948 Studies on thermophilic cellulose decomposing bacteria. Ph. D. Thesis. The State College of Washington, Pullman.

Koshland, D. E. 1951 The mechanism of the hydrolysis of acetyl phosphate and its relation to some enzymatic reactions. In Phosphorus metabolism, 1, pp. 536-550. Edited by W. D. McElroy and B. Glass. The Johns Hopkins Press, Baltimore.

Koshland, D. E. 1954a Group transfer as a substitution mechanism. In mechanism of enzyme action, pp. 608-641. Edited by W. D. McElroy and B. Glass. The Johns Hopkins Press, Baltimore.

Koshland, D. E. 1954b A physical organic approach to enzymatic mechanisms. Trans. N. Y. Acad. Sci., 16, 110-113.

Lamanna, C. and Mallette, M. F. 1954 Use of glass beads for the mechanical rupture of microganisms in concentrated suspensions. J. Bacteriol., 67, 503-504.

Lardy, H. A. 1951 The influence of inorganic ions on phosphorylation reactions. In Phosphorus metabolism, 1, pp. 477-499. Edited by W. D. McElroy and B. Glass. The Johns Hopkins Press, Baltimore.

Levin, R. and Brauer, R. W. 1951 The biuret reaction for determination of proteins--an improved reagent and its application. J. Lab. Clin. Med., 38, 474-480.

Levinson, H. S., Mandels, G. R. and Reese, E. T. 1951 Products of enzymatic hydrolysis of cellulose and its derivatives. Arch. Biochem. Biophys., 31, 351-365.

Macfadyen, A. and Blaxall, F. R. 1899 Thermophilic bacteria. Trans. Jenner Inst. Prev. Med., 2, 162-187.

Macmillan, J. D. 1956 Studies on the enzymatic decomposition of cellulose by Clostridium cellobioparus. Masters Thesis. Montana State College, Bozeman.

McBee, R. H. 1948a Studies on thermophilic cellulose decomposing bacteria. Ph.D. Thesis. The State College of Washington, Pullman.

McBee, R. H. 1948b The culture and physiology of a thermophilic cellulose-fermenting bacterium. J. Bacteriol., 56, 653-663.

McBee, R. H. 1950 The anaerobic thermophilic cellulolytic bacteria. Bacteriol. Revs., 14, 51-63.

McBee, R. H. 1954 The characteristics of Clostridium thermocellum. J. Bacteriol., 67, 505-506.

Najjar, V. A. 1948 The isolation and properties of phosphoglucomutase. J. Biol. Chem., 175, 281-290.

Nelson, N. M. and McBee, R. H. 1957 A cellobiokinase from Clostridium thermocellum. Bacteriol. Proc., 1957, 121.

Neilands, J. B. and Stumpf, P. K. 1955 Outlines of enzyme chemistry. John Wiley & Sons, Inc., New York.

130563

Noetling, G. and Bernfeld, P. 1948 Sur les enzymes amylolytiques. III. La beta-amylase: dosage d'activite et controle de l'absence d'alpha-amylase. Helv. Chem. Acta., 31, 286-290.

Parnas, J. K. 1937 Der Mechanismus der Glykogenlyse im Muskel. Ergeb. Enzym., 6, 57-110.

Pochon, J. 1942 Fermentation de la cellulose par un anaerobie thermophile. Ann. Inst. Pasteur, 68, 353-354.

Pringsheim, H. 1912 Uber den fermentativen Abbau der Cellulose. Zsch. Physiol, Chem., 78, 266-291. Seen in Norman, A. G. and Fuller, W. H. 1942 Cellulose decomposition by microorganisms. Advances in Enzymology, 2, 239-264.

Pringsheim, H. 1913 Uber die Vergarung der Zellulose durch thermophilic Bakterien. Zent. Bakt. Parasitenk., II, 38, 513-516.

Reese, E. T., Siu, R. G. H. and Levinson, H. S. 1950 The biological degradation of soluble cellulose derivatives and its relationship to the mechanism of cellulose hydrolysis. J. Bacteriol., 59, 485-497.

Robbins, E. A. and Boyer, P. D. 1957 Determination of the equilibrium of the hexokinase reaction and free energy of hydrolysis of adenosine triphosphate. J. Biol. Chem., 224, 121-135.

Saifer, A., Valenstein, F. and Hughes, J. P. 1941 The photometric determination of sugar in biological fluids by ferricyanide reduction. J. Lab. Clin. Med., 26, 1969-1977.

Sih, C. J. 1955 A cellobiose-phosphorylase from Clostridium thermocellum. Masters Thesis. Montana State College, Bozeman.

Sih, C. J. and McBee, R. H. 1955a A phosphorylase active on cellobiose. Bacteriol. Proc., 1955, 126.

Sih, C. J. and McBee, R. H. 1955b A cellobiose-phosphorylase in Clostridium thermocellum. Proc. Mont. Acad. Sci., 15, 21-22.

Sih, C. J., Nelson, N. M. and McBee, R. H. 1957 Biological synthesis of cellobiose. Science, 126, 1116-1117.

Sizer, I. W. 1943 Effects of temperature on enzyme kinetics. Advances in Enzymology, 3, 35-62.

Slocum, D. S., and Varner, J. E. 1958 Oxygen transfer in enzymatic arsenolytic reactions. Federation Proc., 17, 312.

Sutherland, E. W. 1949 Activation of phosphoglucomutase by metal-binding agents. J. Biol. Chem., 180, 1270-1284.

Tauber, H. 1949 The chemistry and technology of enzymes. John Wiley & Sons, Inc., New York.

Umbreit, W. W., Burris, R. H. and Stauffer, J. F. 1957 Manometric techniques. Burgess Publishing Co., Minneapolis.

Viljoen, J. A., Fred, E. B. and Peterson, W. H. 1926 The fermentation of cellulose by thermophilic bacteria. J. Agr. Sci., 16, 1-17.

Warburg, O. and Christian, W. 1942 Isolierung und Kristallisation des Garungsferments Enolase. Biochem. Z., 310, 384-421. Seen in Lardy, H. A. 1951 The influence of inorganic ions on phosphorylation reactions. In Phosphorus metabolism, 1, pp. 477-499. Edited by W. D. McElroy and B. Glass. The Johns Hopkins Press, Baltimore.

Weimberg, R. and Doudoroff, M. 1954 Studies with three bacterial phosphorylases. J. Bacteriol., 68, 381-388.

Whitaker, D. R. 1953 Purification of Myrothecium verrucaria cellulase. Arch. Biochem. Biophys., 43, 253-268.

Whitaker, D. R. 1957 The mechanism of degradation of cellulose by Myrothecium cellulase. Can. J. Biochem. Physiol., 35, 733-742.

Wolochow, H., Putman, E. W., Douderoff, M., Hassid, W. Z. and Barker, H. A. 1949 Preparation of sucrose labelled with C14 in the glucose or fructose component. J. Biol. Chem., 180, 1237-1242.

MONTANA STATE UNIVERSITY LIBRARIES
3 1762 10005122 4

D378
A127s
cop.2
130563
Alexander, J. K.
Studies on a purified cellobiose phosphorylase.
NAME AND ADDRESS
OCT 1 1984
3 WKS USE
INTERLIBRARY
11-23-87
D378
A127s
cop. 2
130563

BOUND BY