Studies on the enzymic decomposition of cellulose by Clostridium cellobioparus
by James D Macmillan

A THESIS Submitted to the Graduate Faculty in partial fulfillment Of the requirements for the degree of Master of Science in Bacteriology
Montana State University

Abstract:
Clostridium cellobioparus was studied to find the enzymic mechanism responsible for the decomposition of cellulose. This organism was of interest because it was reported that no glucose was formed during cellulose degradation by old cultures of the organism. This was interpreted to indicate that the organism possessed no cellobiase and therefore utilized cellobiose in a manner different from that of the usual cellulose-decomposing organism.

Large quantities of bacterial cells were grown in a cellobiose medium and harvested by centrifugation. The enzymes were liberated from the cells by grinding them with alumina, A constitutive cellulase was found in cell-free bacterial extracts of Clostridium cellobioparus when it was grown on cellobiose.

An active cellobiase was found in the cell-free extract. This finding was contrary to what had been expected.

Further experiments also revealed the presence of a cellobiose-phosphorylase, an enzyme which causes the phosphorolysis of cellobiose to glucose-1-phosphate and glucose. The products of cellobiose decomposition by either the cellobiase or the cellobiose-phosphorylase could be used by the organism through the glycolytic pathway since both glucokinase and phosphoglucomutase were present. The kinase could form glucose-6- phosphate from glucose and the phosphoglucomutase could change glucose-1-phosphate to glucose-6-phosphate.

There was not sufficient evidence to confirm the presence of a cellobiokinase which had been postulated.

Neither glucose nor cellobiose was found in old cultures of the organism grown on cellulose as had been reported.

This work showed that the enzymes necessary for cellulose hydrolysis and for two different pathways of cellobiose utilization were present in cell-free extracts of Clostridium cellobioparus.

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ABSTRACT

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INTRODUCTION AND HISTORICAL REVIEW

The biological carbon compounds take part in a well organized system known as the carbon cycle. This cycle begins with the production of high molecular weight organic compounds from the CO_2 in the atmosphere. These compounds are produced by organisms which receive their energy from the sun. Other organisms may utilize these compounds in their own metabolism with the result that finally the CO_2 is returned again to the atmosphere. Although the enzymic means by which organisms can accomplish the synthesis or degradation of complex organic compounds has been studied by numerous workers, many of the basic considerations are but little understood.

An important part of this scheme is the degradation of cellulose by microbial action. This is a common enzymic process which has been extensively studied. It is, however, still incompletely understood. The early work of Globig (1888) was one of the first studies of cellulose decomposition. He found that microorganisms played a role in the decomposition of cellulose in manure piles. A variety of microorganisms were studied during the next 25 years with cultures which have usually been found to be impure. Aerobic cellulose decomposition was studied by van Iterson (1904), who concluded that soil-harbored aerobic bacteria were capable of this action. Despite the early interest in cellulose decomposition, pure cultures of cellulose decomposing bacteria were not obtained until the work of McBeth and Scales (1913) who isolated several aerobic species. It had been thought prior to this time, due to the difficulty in obtaining pure cultures, that cellulose could only be decomposed symbiotically.

The pure culture studies on anaerobic cellulolytic bacteria lagged behind those on aerobic bacteria by about ten years. Madame Khouvine's (1923)

isolation of Bacillus cellulosae dissolvens yielded the first pure culture of an anaerobic cellulolytic bacterium. Viljoen, Fred and Peterson (1926) described Clostridium thermocellum, which they obtained from manure. They found it to be an anaerobic cellulose fermenting thermophile. The organism was unable to ferment cellulose, however, after it had been grown in a cellulose-free medium. McBee (1948) questioned the purity of the culture of this organism and redescribed it on the basis of its pure culture characteristics (1954).

Cowles and Rettger (1931) studied Clostridium cellulosolvens, a mesophilic cellulolytic anaerobe from soil, and presented adequate data that their culture was pure, but it was not maintained and is no longer available for study. The greatest difficulty seemed to be an inability for workers to obtain pure cultures of anaerobic cellulose decomposing organisms.

The first successful use of classical methods to obtain pure cultures of anaerobic cellulose-decomposing bacteria was made by Hungate (1944) in the isolation of Clostridium cellobioparus. He later divided the anaerobic cellulolytic bacteria which had been studied into five categories: actinomycetes, thermophilic sporeformers, nonsporeforming rods and cocci, and mesophilic sporeformers (Hungate 1950).

The enzymic action of cellulose-decomposing bacteria has not been studied extensively. According to Siu (1951), Pringsheim was the first to suggest a hydrolytic path of cellulose breakdown. Pringsheim used cultures of cellulose bacteria which had been arrested through heat or antiseptics such as toluene, chloroform, or acetone. From these arrested cultures he was able to identify both glucose and cellobiose as products of cellulose

decomposition by bacteria. This led him in 1912 to propose a cellulose decomposition pathway as follows:

Cellulose ——— Cellobiose ——— Glucose

The enzyme carrying out the first reaction was named cellulase and that catalyzing the second cellobiase. This concept of cellulose hydrolysis is in common use today since enzymes from bacteria capable of splitting both cellulose and cellobiose have been demonstrated many times.

Levinson, Mandels, and Reese (1951) concluded that there were two enzymes involved in cellulose decomposition by fungi and Cytophaga. They determined that one enzyme degraded cellulose into soluble cellulose derivatives, mainly cellobiose, and that another is involved in the further degradation of the cellobiose into glucose.

Hammerstrom, Claus, Coghlan, and McBee (1954) reported the constitutive nature of bacterial cellulases for Clostridium thermocellum and some members of the genus Cellulomonas. These enzymes had formerly been assumed to be adaptive.

Another method of cellobiose-splitting was reported by Sih (1955) who obtained evidence of phosphorylation of the cellobiose with the production of glucose and glucose-1-phosphate. The phosphorolysis of disaccharides was described by Kagan, Latker, and Zfasman (1942) and Doudoroff, Kaplan, and Hassid (1943) during the study of sucrose utilization.

The products of cellobiose phosphorolysis can enter into the glycolytic scheme through the action of established enzyme systems. Phosphoglucomutase converts glucose-1-phosphate to glucose-6-phosphate and glucokinase transfers phosphates from adenosine triphosphate (ATP) to glucose to form glucose-6-phosphate.

Another method of cellobiose utilization has been postulated by Sih (1955). It is possible that one phosphate group from ATP could be transferred directly to a molecule of cellobiose through the action of a cellobiokinase, to form a cellobiose phosphate ester. By the action of a cellobiose-phosphorylase an additional phosphate could then be attached with a simultaneous splitting of the cellobiose molecule to form glucose-1-phosphate and one other glucose phosphate ester. Such a mechanism is, however, entirely hypothetical.

The present work deals with the enzyme systems by which the bacterium Clostridium cellobioparus utilizes cellulose and cellobiose. This organism was isolated by Hungate (1944) from the rumen of cattle. The organism is an anaerobic mesophilic cellulose-digesting bacterium which forms terminal spherical spores. The vegetative cells are slightly curved, medium sized gram negative rods possessing peritrichic flagella.

Hungate (1944) observed an interesting phenomenon in old cultures of this organism grown on a liquid cellulose medium containing more cellulose than could be fermented. Although the organisms were no longer growing, hydrolysis of the remaining cellulose continued and the culture fluid showed an increase in the concentration of reducing sugars. Cellobiose but not glucose was demonstrated in the culture fluid. This was unexpected in view of the work of others, since glucose had been assumed always to be a product of cellulose decomposition under such conditions.

This failure to obtain glucose as a product of cellulose decomposition indicated an ability of the organism to utilize enzymic pathways other than those recognized at the time it was isolated.

In this study an effort was made to find evidence for cellobiose utilization by routes other than those involving cellobiase activity. If the organism possessed a cellobiase or a cellobiose-phosphorylase, glucose should have accumulated in Hungate's old cultures.

MATERIALS AND METHODS

Culture. A culture of Clostridium cellobioparus was obtained from Dr. R. E. Hungate of the State College of Washington. The cultural and physiological characteristics of the organism were not studied in detail, but in general they agreed with those described by Hungate (1944). The organism was grown on a medium consisting of 0.3 per cent NaCl; 0.01 per cent $MgSO_4$; 0.1 per cent $(NH_4)_2SO_4$; 0.05 per cent KH_2PO_4; 0.05 per cent K_2HPO_4; 0.01 per cent $CaCl_2$; 0.15 per cent ball-milled cellulose; 0.05 per cent yeast extract; 0.01 per cent sodium thioglycollate; and 0.5 per cent $NaHCO_3$. A small amount of resazurin was added as an oxidation-reduction indicator.

The organism was carried in stock culture on a medium which contained 2.0 per cent agar in addition to the above ingredients. Hungate's (1950) anaerobic rolled tube technic was employed with all cultures in a solid medium. To insure anaerobic conditions CO_2 was bubbled through the melted agar medium in the culture tube prior to inoculation. The CO_2 in combination with the $NaHCO_3$ also served to set up a buffering system of about pH 7. The CO_2 was bubbled through the medium again after inoculation to replace any oxygen which might have been admitted by opening the tube.

Small colonies surrounded by clear areas appeared in the medium after about two weeks of incubation at 37 C. The clear areas surrounding the colonies were due to a disappearance of cellulose. Serial dilutions were usually prepared so that well isolated colonies were available for picking. The colonies were picked with sterile pasteur pipettes and inoculated again into rolled tubes, or transferred to a liquid medium.

The liquid cellulose medium contained no agar. The cellulose in liquid cultures settled to the bottom of the container and evidence of growth was first seen in the appearance of small gas bubbles arising from the cellulose layer. During growth of the organism, the cellulose gradually disappeared until none was left and the organisms were distributed throughout the medium. Prior to the disappearance of the cellulose, however, the organisms attached themselves to the cellulose, forming clots which appeared very stringy.

Preparation of Large Cultures. Several grams of bacterial cells were required for a study of cellulose enzymes. The production of these many cells required several gallons of culture fluid. Since growth was very slow in a cellulose medium, a large inoculum was always used to insure a high initial concentration of actively multiplying cells in the culture. A culture started with a single colony inoculated into 10 ml of liquid cellulose medium served as an inoculum for a 35 ml culture; this in turn served as an inoculum for a 100 ml culture. It was planned that this build up in size of inoculum was to be continued until an actively growing five-gallon culture was obtained. Although numerous attempts were made, no large actively growing cultures were obtained in a cellulose medium. Growth in the liter cultures usually seemed good at first, but always slowed down after two or three days of incubation. In some such cultures which had been kept incubated for over two months, a large amount of cellulose still remained in the bottom of the culture flask. It was thought that the lowering of the pH of the medium due to the production of acid by the bacteria was the cause of this slow rate of growth. Calcium carbonate was added to the medium to neutralize the acid formed but no better results were obtained in growing the organism.

Another medium was employed which utilized an atmosphere of sterile oxygen-free nitrogen containing five per cent CO_2 and a lower concentration of $NaHCO_3$. Good growth was even more difficult to maintain using this medium.

The poor growth of the organism suggests that further work is needed to determine growth requirements of large cultures. It is possible that the requirements for growth have changed slightly since the organism was originally isolated. It seems very likely that with the development of proper technics it will be possible to grow the organism on cellulose in large quantities. Such a study was not undertaken at this time since it was found that the organism could be produced in large quantities quite well by substituting cellobiose for cellulose in the culture medium.

Colonies from rolled tubes were picked and inoculated into tubes containing 10 ml of fluid thioglycollate medium (Difco Laboratories) which contained 0.5 per cent glucose. These tubes served as an inoculum for liter flasks of medium in which 0.2 per cent cellobiose was substituted for the cellulose. After two or three days' incubation at 37 C these flasks were suitable for inoculating a five-gallon carboy of the medium. The medium in the carboy was kept reduced by bubbling CO_2 through it for at least twelve hours after inoculation. Usually this flushing was continued until a rapid fermentation was evident and the organisms were producing sufficient CO_2 and H_2 to keep the medium in the reduced state. After the most active stage of the fermentation had ceased, usually three or four days, the culture was ready for harvesting. Cells were harvested in a Sharples super centrifuge at a relative centrifugal force of 62,000 G. During the harvesting, sufficient glacial acetic acid was added to the culture to maintain a pH of

about 7 in the supernatant fluid. The acid was added slowly while the culture was stirred with a magnetic mixer. The average five-gallon culture produced 15-20 grams wet weight of cells. This cell paste was used for the preparation of enzyme extracts.

Preparation of Cell-Free Bacterial Extracts. Cell-free extracts were obtained according to the method of McIlwain (1948) as recommended by Hayaishi and Stanier (1951). The cell paste was placed in a mortar cooled by an ice bath and a quantity of powdered alumina (Alcoa Chemicals Alumina A-301) twice the wet weight of the cells was added. The cells were broken by grinding with a pre-cooled pestle for at least ten minutes. The ground cells were then mixed with 2 ml of M/35 sodium acetate buffer pH 7 for each gram of wet cells. The alumina and cell debris were removed by centrifugation for one hour at 10 C using a relative centrifugal force of 2,000 G. The extract obtained in this manner was the enzyme source for most experiments and will be referred to hereafter as the enzyme preparation. No attempts were made to separate or purify any of the enzymes found in this extract.

Phosphorus Determination. The Fiske and SubbaRow (1925) method of inorganic phosphate determination was used without the precipitation of protein. It was found that protein interference was of minor importance and could be eliminated with the preparation of suitable blanks. The blank consisted of a tube containing the same concentration of enzyme preparation and reagents as in other tubes in the experiment without added inorganic phosphate. By setting the colorimeter to zero with the blank the effect of the protein and the trace of inorganic phosphate in the enzyme was cancelled. All readings were made with a Klett-Summerson colorimeter using filter 66 (red).

Acid-labile organic phosphates such as glucose-1-phosphate were determined by the same method following hydrolysis with 1 N HCl for seven minutes in a boiling water bath. No attempt was made to determine the concentration of any of the more stable phosphate esters.

Sugar Determinations. Reducing sugar concentration was determined either by the Folin-Malmros (1929) method or the Noetling and Bernfeld (1948) procedure. Glucose was sometimes measured more specifically using glucose oxidase (Takamine Dee-O) for a biological assay in a Warburg respirometer. In this case oxygen uptake was measured and the concentration of glucose calculated according to the method of Keilin and Hartree (1948).

EXPERIMENTAL RESULTS

Cellulase. A preliminary examination was undertaken to determine the nature of the cellulase from Clostridium cellobioparus. Liter cultures of the organism growing on glucose and cellobiose were prepared. After 4 days the cells were removed by centrifugation at 62,000 G and the supernatant was treated with chloroform to prevent any further growth of microorganisms. An examination was made of this culture fluid to determine any cellulase activity which might be present. Activity was measured on carboxymethyl-cellulose (CMC 50T) according to the method of Reese, Siu and Levinson (1950). The CMC was used in the cellulase tests because it is more easily hydrolyzed than cellulose. Correlations between tests using CMC and cellulose are extremely good (Levinson and Reese, 1950).

Sufficient CMC was added to 2 ml of these supernatants to give a final concentration of 0.5 per cent. The volume was adjusted to 5 ml with an acetate buffer of pH 5.5 and the mixture was incubated for 2 hours at 50 C. Since an increase in reducing sugar concentration would indicate the presence of cellulase activity, aliquot portions were checked for sugar concentration initially and after incubation. The Folin-Malmros (1929) method for reducing sugars was employed and all calculations are in terms of mg of glucose per ml.

Only an insignificant increase in sugar concentration was noted in this experiment, therefore, the supernatants were concentrated to one twentieth of their volume by vacuum distillation at 30 C. The experiment was then repeated using these concentrates. The increase in reducing sugar

concentration, calculated as glucose, was only 0.2 mg per ml of reaction mixture, indicating the presence of a weak cellulase.

A similar experiment with a fresh cell-free enzyme preparation gave greater cellulase activity. In this case 1 ml of the enzyme preparation was used as the source of cellulase. An average amount of 0.63 mg of glucose per ml was liberated after incubation with CMC at 50 C for 2 hours.

These experiments show that there is a constitutive cellulase produced by the organism, but that it is not secreted by the organism in large quantities. It is instead retained by the organism in its cell structure, possibly as a part of the cell wall. This might explain why the rapidly growing organism appears to attach itself to the cellulose forming stringy clumps. The constitutive nature of the cellulase is evident because no cellulose was used in growing the bacteria, yet the cellulase was still produced.

Cellobiase. Hungate (1944) reached the conclusion that there was no cellobiase in Clostridium cellobioparus. "Thus, in view of the inability to find even traces of glucose in the old cultures, it seems reasonable to conclude that no enzyme catalyzing the reaction (cellobiose ⟶ glucose) was present."[1] This reaction, however, was still not eliminated as a possible mechanism for cellobiose utilization by this organism. Therefore, the following experiments were performed to determine whether there was any cellobiase activity in the enzyme preparation.

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[1]Hungate, R. E. 1944 Studies on cellulose fermentation. I. The culture and physiology of an anaerobic cellulose-digesting bacterium. J. Bact., 48, 510.

Cellobiase activity was determined using glucose oxidase for a biological assay of glucose. This method was decided upon because a cellobiase would produce two molecules of glucose from one molecule of cellobiose. Thus a measure of the rate of production of glucose in an enzyme-cellobiose system would be proportional to the cellobiase activity.

Glucose oxidase oxidizes glucose to gluconic acid and H_2O_2. In the presence of catalase H_2O_2 is decomposed and the overall reaction is:

$$\text{Glucose} + H_2O + \tfrac{1}{2} O_2 \longrightarrow \text{Gluconic acid} + H_2O.$$

Catalase was present in the commercial glucose oxidase preparation. Oxygen uptake can be measured manometrically in a Warburg respirometer and is proportional to the amount of glucose oxidized.

Other methods for quantitative determinations of glucose in the presence of cellobiose could not be used since both are reducing sugars.

A 0.5 ml portion of the enzyme preparation was placed in each of two Warburg vessels along with .05 ml of the supernatant from a 12 per cent suspension of glucose oxidase. Twenty micromoles of $MgSO_4$ were added as an enzyme activator and 0.1 ml of 20 per cent ethanol was added to increase the rate of oxygen uptake as recommended by Keilin and Hartree (1948). Fifty micromoles of cellobiose dissolved in 0.5 ml of water were placed in the side arm of each vessel and the final volume in the vessel was adjusted to 2.0 ml with an acetate buffer of pH 5.5. Other vessels were prepared as suitable controls for the experiment.

At the start of the experiment the cellobiose was poured from the side arms into the main chambers of the vessels. The reaction was carried out with shaking in a water bath at 37 C. At the end of one hour

179 microliters of oxygen had been consumed, indicating the production and oxidation of approximately 16 micromoles of glucose in each vessel. At the end of this time the rate of the reaction was not diminished and it is assumed that it would go to completion. The rate of oxygen consumption for this experiment is as shown in figure 1.

The results of this experiment indicated that a cellobiase was present in the enzyme preparation. The action of a cellobiase could not be distinguished from that of a cellobiose-phosphorylase. Interference by a cellobiose phosphorylase would result from this enzyme producing one molecule of glucose and one of a glucose phosphate ester. Glucose produced in this way could then be oxidized by the glucose-oxidase giving a rate of oxygen uptake similar to that expected from the cellobiase. Since phosphorylase activity is dependent upon the presence of inorganic phosphate in the system, an attempt was made to remove the small amount of inorganic phosphate present in the enzyme preparation by dialysis against running tap water for 10 hours. The experiment was then repeated using this dialyzed enzyme preparation. In this case only about 9 micromoles of glucose were liberated in one hour (figure 2).

The difference in the glucose production between dialyzed enzyme and non-dialyzed enzyme could not be attributed to the action of cellobiose-phosphorylase since inorganic phosphate determinations on both enzymes showed an insignificant amount of inorganic phosphate (Table I). The amount of glucose formed by phosphorolysis of cellobiose could not be in excess of the amount of inorganic phosphate available. The inorganic phosphate concentration was less than 10 per cent of the amount of glucose formed within

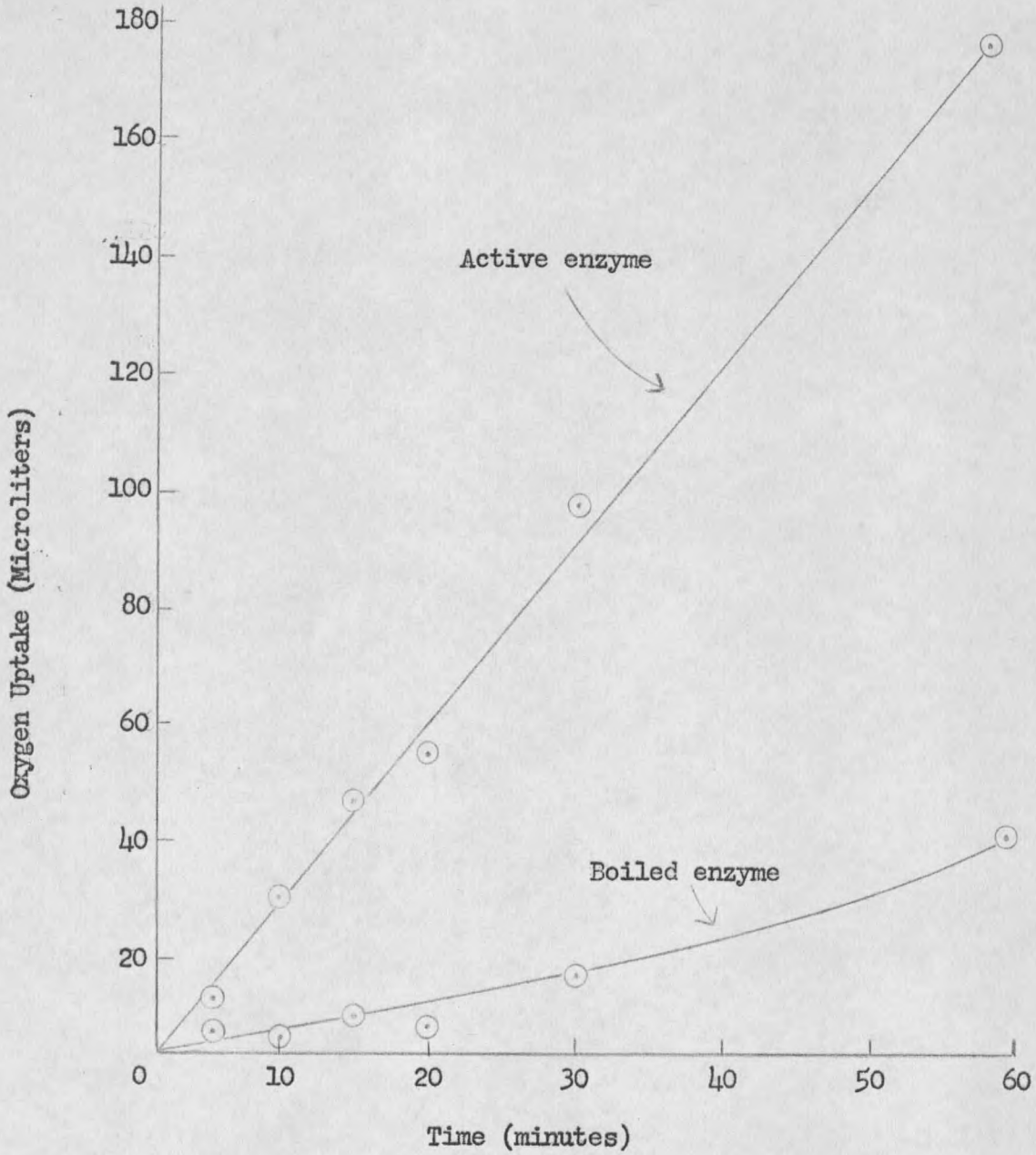

Figure 1 - Rate of oxygen uptake in a non-dialyzed enzyme-cellobiose system containing glucose oxidase.

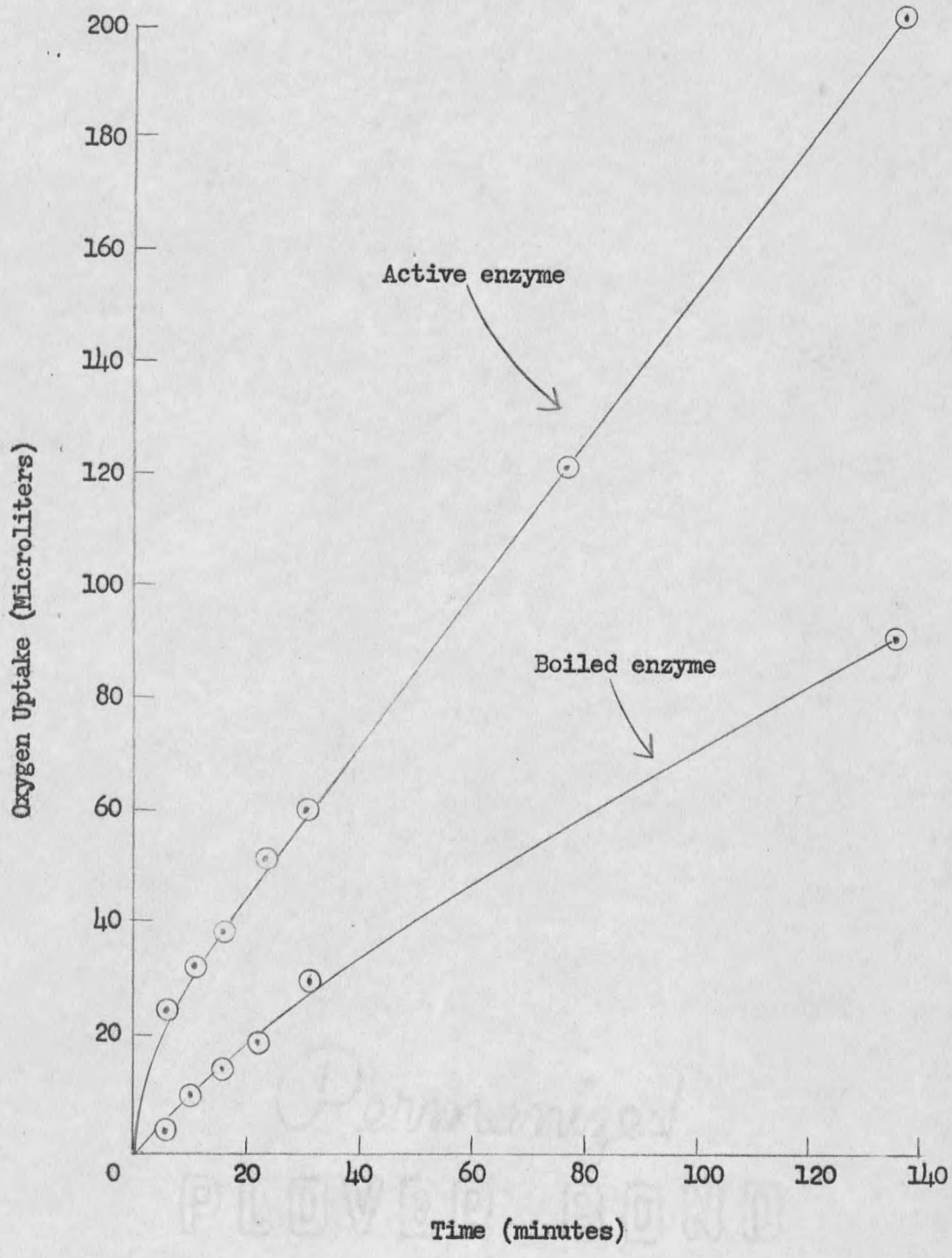

Figure 2 - Rate of oxygen uptake in a dialyzed enzyme-cellobiose system containing glucose oxidase.

TABLE I

Concentration of inorganic phosphate compared to protein concentration and the release of glucose in a reaction mixture consisting of enzyme and cellobiose.

	Total Inorganic Phosphate (micromoles)	Total Protein (mg)	Glucose After One Hour (micromoles)
Dialyzed Enzyme	0.7	7.25	9
Non-dialyzed Enzyme	1.3	12.50	16

one hour. Under these conditions it must be concluded that the glucose was formed by the action of a cellobiase.

The actual difference in rates between the two experiments was attributed to the difference in cellobiase concentration. This difference in cellobiase concentration was evidently caused by a dilution of the enzyme preparation during the dialysis. This is indicated by the decrease in protein concentration during dialysis. Since the ratio of inorganic phosphate to protein did not change during dialysis, it is concluded that the phosphate being measured was not inorganic.

Cellobiose-Phosphorylase. The preceding evidence for cellobiase did not rule out the possibility that a cellobiose-phosphorylase also existed as a part of the organism's enzymic system. Therefore, experiments were conducted to detect the presence of phosphorylase.

Phosphorylase activity was measured by a reduction in inorganic phosphate concentration in reaction tubes prepared as shown in Table II.

TABLE II

Reaction tubes prepared for the measurement of reduction in concentration of inorganic phosphate as an indication of cellobiose-phosphorylase activity. Numbers are ml of reagent added.

Tube Number	1	2	3	4	5	6
Enzyme	2.00	2.00	2.00*	2.00	2.00	2.00*
K_2HPO_4 (0.02M)	1.00	1.00	1.00	1.00	1.00	1.00
Acetate-barbital buffer pH 7	2.85	2.85	2.85	2.85	2.85	2.85
$MgSO_4$ (10%)	0.05	0.05	0.05	0.05	0.05	0.05
NaF (1 M)	0.05	0.05	0.05	0.05	0.05	0.05
Cellobiose (1%)	1.00	1.00	1.00			
Glucose (1%)				1.00	1.00	1.00

* enzyme boiled for 15 minutes for inactivation

The $MgSO_4$ was added as an enzyme activator and NaF was added to inhibit any phosphatases in the system which might interfere with the accumulation of organic phosphates. Inorganic phosphate and acid-labile phosphate concentrations were determined immediately after the addition of the sugars and at 1 and 2 hour intervals. The temperature of the reaction mixtures was maintained at 37 C during the experiment.

There was no significant change in concentration of phosphate in tubes to which glucose had been added as a substrate (Table III). In the tubes containing cellobiose, however, a decrease in inorganic phosphate concentration occurred indicating that phosphorolysis of the cellobiose had taken

TABLE III

Changes in phosphate concentration due to cellobiose phosphorylase in the presence of glucose and cellobiose. All values are in micromoles of phosphate per ml of reaction mixture.

		Cellobiose			Glucose		
		1	2	Control*	1	2	Control*
Pi **	0 hr	3.16	3.18	3.20	3.18	3.18	3.00
	1 hr	3.08	3.16	3.20	3.20	3.18	3.18
	2 hrs	2.68	2.64	3.18	3.24	3.08	3.18
△ Pi		-0.48	-0.54	-0.02	0.00	-0.10	0.00

* Control tubes were prepared with enzyme which had been inactivated by boiling for 10 minutes in water bath.

** Inorganic phosphate.

place. No corresponding increase in acid-labile organic phosphate was noted. If cellobiose were indeed phosphoralated such an increase would be expected unless the enzyme phosphoglucomutase were present to convert acid-labile glucose-1-phosphate to acid-stable glucose-6-phosphate. It was therefore suspected that phosphoglucomutase was present in the enzyme preparation. Evidence confirming this was found later.

The effect of pH on the activity of the cellobiose-phosphorylase was determined in the following experiment. A series of acetate-barbital buffers ranging in pH from 4.2 to 8.75 were prepared. An experiment was performed similar to the one described above for phosphorylase activity, using these buffers to maintain the pH in the reaction tubes. Each tube was at a different pH and incubated for two hours at 37 C. Activity of the cellobiose-