

The decomposition of starch through phosphorolysis by Streptoccus bovis
by Richard N Ushijima

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

The possibility of starch utilization by Streptococcus bovis through a starch phosphorylase was investigated.

Large quantities of cells were grown on a starch medium, and the cells were harvested by centrifugation. The cell-free enzyme extract was prepared by grinding the cells with glass beads in a micro-Waring blendor.

Starch phosphorylase was detected in the reaction mixture containing soluble starch, inorganic phosphate, enzyme preparation, and sodium hydrosulfite. Since a maltose phosphorylase was also found, the formation of maltose was prevented by adjusting the mixture to pH 8.6, which inhibited alpha amylase activity.

The experiments also revealed the presence of a phosphoglucomutase, which converts glucose-1-phosphate to glucose-6-phosphate, a product utilizable through the glycolytic pathways.

INTRODUCTION

By virtue of radiant energy from the sun, plants containing chlorophyll are able to utilize carbon dioxide and synthesize complex carbohydrates. Carbohydrates are stored by all plants as reserve food, and starch is by far the most abundant and widely distributed.

Investigation of starch began in the early 1800's. According to Whistler and Smart (1953), in 1811 Kirchoff treated an aqueous solution of starch with sulfuric acid and obtained crystalline glucose. In the latter years of the 19th century, Emil Fischer concluded that starch consisted of pyranose forms of glucose.

The empirical formula of starch is written as $(C_6H_{10}O_5)_x$. However, starch consists of either amylose (straight chain glucose molecules) or amylopectin (branched chain glucose molecules). Some of the properties of starch are listed on Table I.

There are many microorganisms possessing enzymes which catalyze the hydrolysis of starch. These enzymes, known as amylases, enable the microorganisms to utilize starch as the primary source of carbohydrate. The enzymatic hydrolysis of starch by the alpha and the beta amylases was demonstrated in 1833 by Payen and Perez (Whistler and Smart, 1953). Both of these enzymes will readily hydrolyze the 1-4 linkages of the starch molecules but will not hydrolyze the 1-6 linkages, which occur in branching chains.

Alpha amylase, known as the liquefying enzyme, hydrolyzes amylose into glucose, maltose, and maltotriose. The enzyme hydrolyzes amylopectin into glucose, maltose, isomaltose, maltotriose, and a variety of small molecular weight dextrins containing alpha, 1-6 linkages. Beta amylase,

TABLE I

Properties of starch

AMYLOSE	AMYLOPECTIN
1) high digestibility with alpha and beta amylase (100 per cent)	low digestibility with beta amylase (50 per cent)
2) relatively low molecular weight--molecule contains 220 glucose residues with only one non-reducing end group per molecule	high molecular weight--molecule contains over 1,000 glucose residues with one non-reducing end group for every 20 to 30 glucose residues
3) deep blue with iodine	purple or reddish color with iodine
4) adsorption of large amounts of iodine isopotentially in potentiometer titration	does not adsorb iodine isopotentially
5) forms good films	forms poor films
6) poor pasting characteristics	makes strong pastes

known as the saccharifying enzyme, hydrolyzes amylose from the non-reducing end of the starch fraction to maltose. Amylopectin is similarly hydrolyzed, but further hydrolysis by beta amylase is blocked when a 1-6 linkage is encountered, leaving limit dextrins.

Some plants and animals possess the enzymes phosphorylases, which catalyze the breakdown or synthesis of starch-like materials. Cori et al (1938) were the first to discover this enzyme in the muscle tissues of animals. Hanes (1940) found starch phosphorylase in pea seeds and in potato tubers.

Hobson and MacPhearson (1952) claimed to have detected a trace of starch phosphorylase in a streptococcus that was isolated from the rumen

of sheep, but no definite evidence was given to substantiate their conclusions. Furthermore, it is questionable as to whether the phosphorolysis was due to a maltose phosphorylase or a starch phosphorylase in a system where starch is hydrolyzed to maltose in the presence of an amylase, since maltose phosphorylase has been shown to exist in microorganisms (Fitting and Doudoroff, 1952). There is also the possibility that the two enzymes mentioned are identical.

The reactions which are believed to occur in starch phosphorolysis and maltose phosphorolysis are as follows:

$$(\text{glucose})_n + H_3PO_4 \longrightarrow \text{glucose-1-phosphate} + (\text{glucose})_{n-1}$$

$$\text{maltose} + H_3PO_4 \longrightarrow \text{glucose-1- phosphate} + \text{glucose}$$

Like the hydrolysis of starch by amylase, phosphorolysis occurs at the 1-4 linkages but is blocked by the 1-6 linkages. In the presence of inorganic phosphate, the phosphorolysis of starch proceeds from the non-reducing end of the starch molecule releasing glucose-1-phosphate. Phosphoglucomutase converts the glucose-1-phosphate to glucose-6-phosphate, which can then enter into the glycolytic scheme through the action of established enzyme systems.

The purpose of this investigation was to search into the possibility of a starch phosphorylase being present in a bacterium. Streptococcus bovis was chosen as the experimental bacterium because of its rapid growth on starch. Furthermore, being a lactic acid bacterium which lives anaerobically in the rumen of cattle, Streptococcus bovis, through hydrolysis, is able to obtain only a small portion of the energy available from starch. Therefore, this organism might be expected to utilize starch in another manner that would yield more energy. One method whereby this could be done would be to break down starch by phosphorolysis.

Streptococcus bovis and other microorganisms living in the rumen of cattle break down the complex starch molecule by enzymatic action into simple compounds. The enzymatic degradation of complex carbohydrates is especially beneficial to the animal, since it does not have a ptyalin in the saliva or an amylase to secrete into the rumen. Hence, the animal relies on microorganisms or plant enzymes for rumen digestion.

Streptococcus bovis was first isolated by Winslow and Palmer (1910) but was later described and named by Orla-Jensen (Sherman, 1937).

Some characteristics of Streptococcus bovis, as defined by Bergey's Manual of Determinative Bacteriology (Breed, et al, 1948) are as follows: gram positive cocci appearing in short chains; acid in milk at 3 to 5 days, followed by reduction of litmus; tolerant of 2 per cent NaCl but not 4 per cent NaCl; non-hemolytic, facultatively anaerobic, nitrites not produced from nitrates, starch hydrolyzed.

METHODS AND MATERIALS

CULTURE

A culture of Streptococcus bovis was isolated from the rumen fluid of an Angus bull. Serial dilutions of fresh rumen fluid were made in tubes of a sterilized culture medium consisting of 1.0 per cent commercial grade starch, 1.0 per cent yeast extract, 10 per cent by volume of the rumen fluid, 1.5 per cent agar, and tap water. The roll tube technique as suggested by Hungate (1950) was used. The tubes were incubated at 35 C for 20 hours.

Starch hydrolyzing colonies were surrounded by clear zones. These were picked with sterile pasteur pipettes and reinoculated into other sterilized culture media. Further serial dilutions were made to insure the isolation of a pure colony. A pure culture having most of the characteristics of Streptococcus bovis, as defined by the Bergey's Manual of Determinative Bacteriology, was transferred into stock culture agar containing 1.0 per cent added soluble starch. Stock cultures of Streptococcus bovis were viable for over three months on this medium.

PREPARATION OF LARGE CULTURES

Several grams of bacterial cells were required to prepare cell-free enzyme extracts of Streptococcus bovis. In order to obtain a sufficient quantity of bacterial cells, a large volume of culture fluid was necessary.

A 10 ml culture, which was inoculated with the organism picked from the stock culture agar medium, served as the inoculum for a two liter culture.

Two liters of medium consisting of 0.8 per cent commercial starch, 0.5 per cent yeast extract, 0.2 per cent peptone, 0.05 per cent K_2HPO_4,

0.05 per cent KH_2PO_4, 0.002 per cent $(NH_4)_2SO_4$, 0.002 per cent NaCl, 0.0005 per cent $MgSO_4$, 0.0001 per cent $CaCl_2$, 0.0001 per cent sodium thioglycollate, and 0.5 per cent $NaHCO_3$ yielded about 20 grams of wet cells. This quantity was sufficient for several experiments.

Since anaerobic conditions favored the growth of the organism, oxygen was removed from the medium after inoculation by flushing with carbon dioxide. The carbon dioxide in combination with the $NaHCO_3$ also served as a buffering system at about pH 7.0.

Occasionally, more than 20 grams of cells were needed. Instead of growing several two liter cultures, a twenty liter culture in a carboy was used. Cultures of this size required continuous mixing for the most rapid growth. This was done with a magnetic stirrer.

All of the cultures were incubated at 35 C. The average incubation time was thirty hours, at which time the culture was ready for harvesting. The cells were harvested in a Sharples super centrifuge at a relative centrifugal force of 62,000 G. The cell paste obtained was used for the preparation of enzyme extracts.

PREPARATION OF CELL-FREE BACTERIAL EXTRACTS

The cell-free enzyme extract was prepared by grinding the cell paste with glass beads. The method of Lamanna and Mallette (1954) was used. A micro-Waring blendor surrounded with an ice jacket was used for grinding the cells. Five ml of distilled water were mixed with 20 grams of wet cells for one minute. Forty-five grams of glass beads were then slowly added while continuously mixing. After 20 minutes, 20 ml of 0.1 M pH 7.5 Tris buffer (tris hydroxy aminomethane) were added, and the mixing was continued

for an additional five minutes. The mixture was centrifuged at 8-10,000 G for one and a half hours under refrigeration. The supernatant fluid contained the enzymes active on starch.

A protein determination on the cell-free enzyme preparation was done as suggested by Levin and Braur (1951) using the Biuret reagent, which was standardized by the kjeldahl method. The enzyme extract usually contained about 12 mg of protein per ml.

PHOSPHORUS DETERMINATION

Inorganic phosphate concentration was determined by the method of Lowry and Lopez (1946) without protein precipitation. Two normal HCl was used to stop the reaction instead of trichloroacetic acid. A blank containing the same concentration of enzyme preparation and the reagents, as in the other tubes but without added inorganic phosphate, served as a control for the presence of inorganic phosphate in the enzyme preparation. The blank also contained glucose instead of soluble starch. All readings were made with the Klett-Summerson colorimeter using filter 66 (red).

To assay for acid labile phosphate esters, such as glucose-1-phosphate, an equal volume of 2 N HCl was added to an aliquot of the reaction mixture, which was then boiled for seven minutes. A quantitative determination of the alkaline labile phosphate esters, as glucose-6-phosphate, was made by adding an equal volume of 2 N NaOH to the reaction mixture, which was then boiled for 30 minutes.

The phosphate released from the esters was measurable colorimetrically. Therefore, the increase in concentration of inorganic phosphate following acid or alkaline hydrolysis determined the concentration of organic phosphate esters formed in the reaction mixtures.

CHROMATOGRAPHY

The ascending paper chromatographic technique was used to identify the phosphate esters. Schleicher and Schuell No. 589 paper was utilized with the solvents of Bandurski and Axelrod (1951); 60 parts of methanol, 15 parts of 88 per cent formic acid, and 5 parts of water. The chromatograms were run at 9 C in glass battery jars sealed with scotch tape.

After approximately 24 hours, the chromatograms were dried and were then dipped through the indicator of Burrows, Grylls and Harrison (1952); 1 g ammonium molybdate, 8 ml water, 3 ml 12 N hydrochloric acid, 3 ml perchloric acid, and 86 ml acetone. After redrying completely, the chromatograms were placed under an ultraviolet lamp for 5 minutes to develop the blue color of the phosphomolybdic complexes formed.

Reducing sugars were chromatogrammed by the descending paper chromatographic technique. The solvent of Jeanes et al (1951), butanol 5 parts, pyridine 3 parts, water 1.5 parts and the indicator of Baar (1955), 99 ml 85 per cent isopropanol, 1.66 g phthalic acid, and 1 ml aniline, were used. The chromatograms were developed at 100 C for ten minutes. Reducing sugars produced brown colored spots on the paper.

GLUCOSE DETERMINATION

In several instances it was necessary to measure glucose in the presence of other reducing sugars. This can be done manometrically by using glucose oxidase (Keilin and Hartree, 1948). The source of glucose oxidase was a commercial enzyme, Takamine Dee-O.

Glucose oxidase oxidizes glucose to gluconic acid and hydrogen peroxide as follows:

$$C_6H_{12}O_6 + H_2O + O_2 \text{ ---------- } C_6H_{12}O_7 + H_2O_2$$

In the presence of catalase, present in the commercial glucose oxidase, and added ethyl alcohol, the hydrogen peroxide is decomposed to water and oxygen with the simultaneous coupled oxidation of the alcohol. The oxygen uptake can be measured manometrically in a Warburg respirometer and is proportional to the amount of glucose oxidized. This method is highly specific for glucose.

EXPERIMENTAL RESULTS

PHOSPHORYLASE

The possibility of a starch phosphorylase being part of the enzyme system of Streptococcus bovis was investigated. The phosphorylase activity was measured by a reduction of inorganic phosphate in the reaction mixtures containing a cell-free enzyme preparation, inorganic phosphate, and either maltose or soluble starch (Table II).

TABLE II

Test for phosphorylase activity

TUBE NUMBER	1	2	3	4	5	6
Tris buffer pH 7.5	1.0	1.0	1.0	1.0	1.0	1.0
Enzyme	.5	.5	.5*	.5*	.5	.5
$MgSO_4$ (.1 M)	.05	.05	.05	.05	.05	.05
NaF (.5 M)	.2	.2	.2	.2	.2	.2
K_2HPO_4 (.1 M)		.2	.2	.2	.2	.2
Soluble starch (.1 M)				.5		.5
Maltose (.1 M)			.5		.5	
Glucose (.1 M)	.5	.5				
Distilled water	.25	.05	.05	.05	.05	.05
Total volume in ml	2.5	2.5	2.5	2.5	2.5	2.5

* Boiled enzyme

The $MgSO_4$ was added to activate any phosphorylase present and the NaF was added to inhibit any phosphatase activity which might interfere with the

accumulation of organic phosphate esters. The reaction tubes containing glucose served as a blank and a control. The temperature of the reaction mixture was maintained at 37 C throughout the experiment. The concentration of the inorganic phosphate and the acid labile phosphate esters were determined at 0, 30, 60, and 120 minute intervals.

As shown in figure 1 and figure 2, approximately 1.6 micromoles/ml of inorganic phosphate was converted to organic phosphate esters at 120 minutes with both maltose and soluble starch as substrates. The determination of acid labile phosphate esters indicated that 1.2 micromoles/ml of the inorganic phosphate had been incorporated into glucose-1-phosphate. Although no determination of the alkaline labile phosphate ester concentration was made, it was assumed that 0.4 micromole/ml of the phosphate was in the form of glucose-6-phosphate.

An aliquot of the reaction mixture chromatogrammed for reducing sugars showed that maltose was being formed as a result of amylase action on soluble starch. Consequently, no assumption can be made as to whether the phosphorylase was acting on maltose or starch.

Therefore, to demonstrate unequivocally the presence of a starch phosphorylase, it was necessary that the alpha amylase activity be inhibited without inhibiting the phosphorylase activity. This required further investigations of amylase activity.

AMYLASE

The majority of the starch hydrolyzing microorganisms found in the rumen have been shown to have an alpha amylase (Hobson and MacPherson, 1952). A preliminary experiment was made to determine whether Streptococcus bovis had an alpha or a beta amylase. This was done with the cell-free enzyme

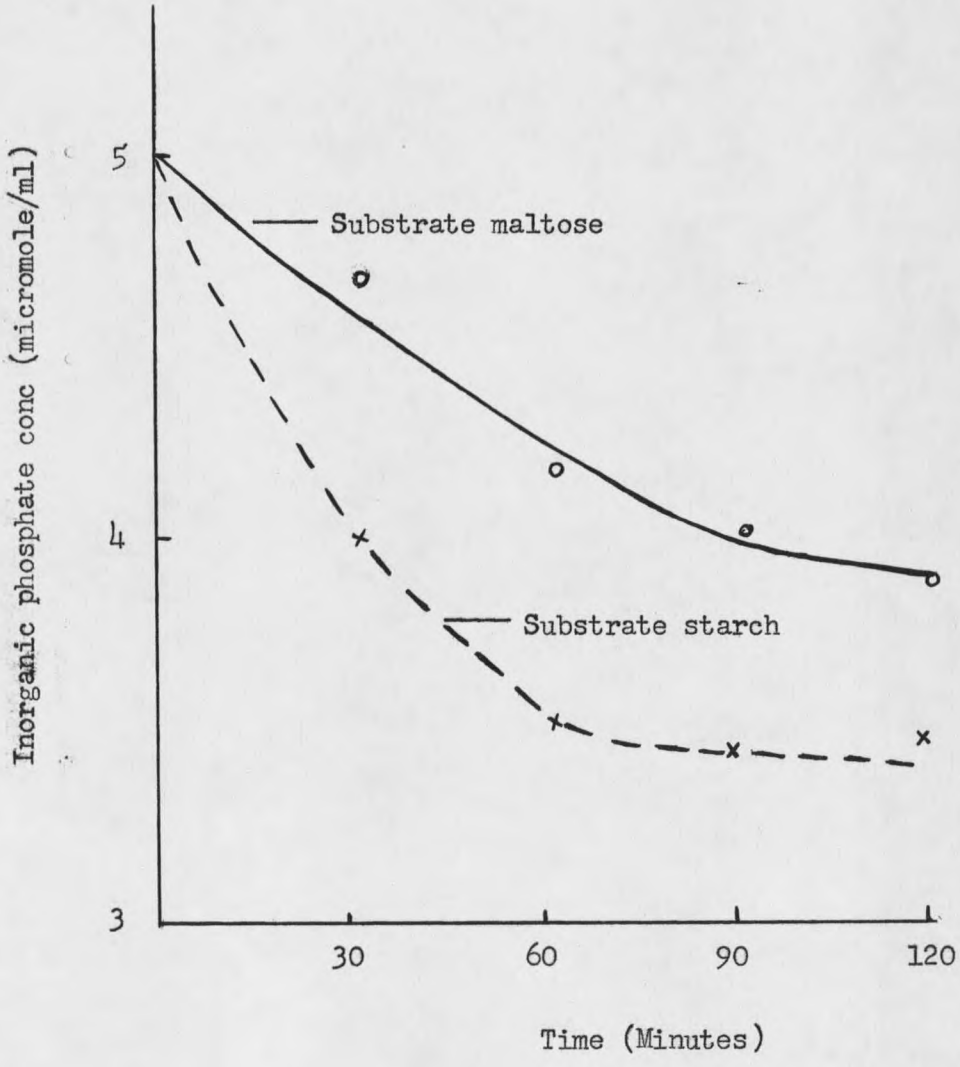


Figure 1 - Disappearance of inorganic phosphate during phosphorolysis of starch and maltose.

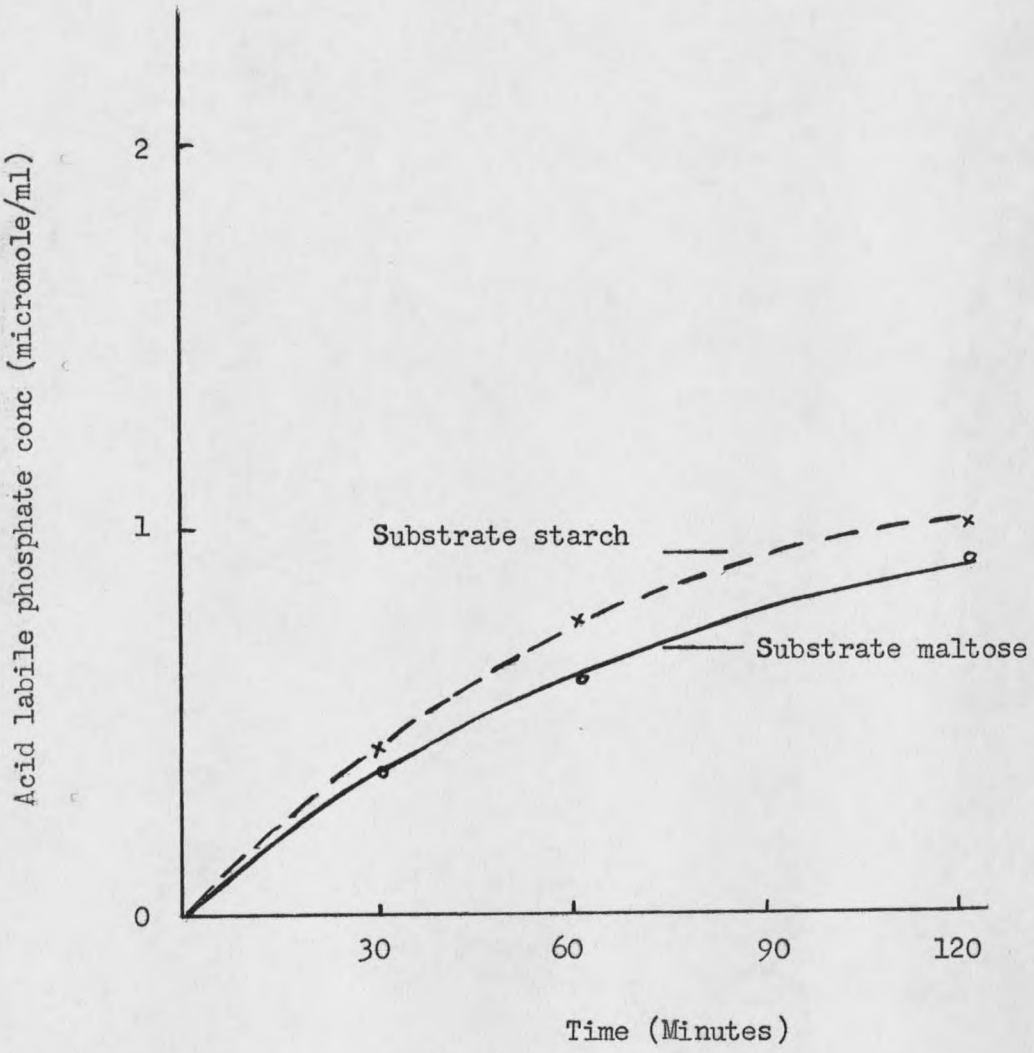


Figure 2 - Formation of acid labile phosphate esters during phosphorolysis of starch and maltose

preparation and also with the cell-free supernatant of the culture medium in which the bacteria had been grown.

The reaction mixtures consisted of 0.5 ml of the enzyme preparation or the supernatant, 0.5 ml of 0.1 M amylopectin, and 1.0 ml of 0.05 M tris buffer pH 7.5 and were incubated at 37 C. A drop of iodine was added to an aliquot of each of the reaction mixtures at four minute intervals to determine the time required for hydrolysis of all of the starch.

Alpha amylase will completely hydrolyze amylopectin, destroying its ability to form a blue colored complex with iodine. Beta amylase, however, cannot hydrolyze amylopectin completely. When iodine is added at the end point of hydrolysis, a bluish-red color, which is characteristic of a beta amylase-amylopectin reaction, develops.

The reaction mixtures containing either the cell extract or the supernatant showed that the amylopectin was completely hydrolyzed within twenty minutes, demonstrating that starch hydrolysis was due to alpha amylase.

In order to demonstrate the presence of a starch phosphorylase in the presence of amylase, it was necessary that the amylase activity be inhibited without inhibiting the phosphorylase activity. Otherwise, no definite conclusion could be drawn as to whether phosphorylation was due to a maltose phosphorylase or a starch phosphorylase.

No published methods of separating amylase from phosphorylase were found. Therefore, the most practical methods for obtaining separate phosphorolytic activity appeared to be through the use of inhibitory chemicals and the influence of pH.

Many chemicals have been reported to have an affect on amylase inhibition. Among these are mercuric chloride (Ono and Hirom, 1954), ascorbic acid, copper sulfate (Ito and Abe, 1954), ammonium oxalate, and ethylenediaminetetraacetic acid (Yamamoto and Fukumoto, 1954).

These various chemicals in 0.1 M concentrations were added in 0.2, 0.5, and 1.0 ml amounts to separate tubes each containing 0.5 ml of enzyme preparation, 0.5 ml of 0.1 M soluble starch, and 1.0 ml of tris buffer pH 7.5. These mixtures were incubated at 37 C for two hours, and starch hydrolysis was determined with the iodine test (Table III).

TABLE III

Effects of various chemicals on amylase activity

	EXPERIMENTAL TUBES		
	0.2 ml	0.5 ml	1.0 ml
Mercuric chloride	✓*	-	-
Ammonium oxalate	✓	✓	-
Ethylenediaminetetraacetic acid	✓	✓	✓
Copper sulfate	-	-	-
Ascorbic acid	✓	✓	-

* (✓) Indicates amylase activity.

Some of these chemicals inhibited alpha amylase in the amounts used. However, when each of the chemicals was added to reaction mixtures prepared for the determination of phosphorolysis (Table II), phosphorylase activity was completely inhibited.

Since chemical means of inhibiting amylase activity without affecting the phosphorylase activity did not seem feasible, the effects of pH upon these two enzymes were determined. A series of tubes, each containing 0.5 ml of enzyme, 0.5 ml of 0.1 M soluble starch, 1.0 ml distilled water, and 1.0 ml of an appropriate buffer solution was set up. The mixtures were incubated at 37 C for two hours, and the amylase activity on starch was tested by the iodine test.

As shown in Table IV, amylase activity was not affected from pH 5.0 to pH 8.2 but was inhibited at pH 3.3 or below and at pH 8.6 and above.

TABLE IV

Tests for the effects of pH on amylase activity

pH	Buffer	Amylase	pH	Buffer	Amylase
3.0	.1 M citrate	-	8.0	.1 M tris	≠
3.2	.1 M citrate	-	8.2	.1 M tris	≠
3.3	.1 M citrate	-	8.4	.1 M tris	(?)
3.4	.1 M citrate	(?)*	8.5	.1 M tris	(?)
5.0	.1 M citrate	≠	8.6	.1 M tris	-
6.0	.1 M citrate	≠	8.8	.1 M tris	-
7.0	.03 M barbital	≠	8.9	.1 M tris	-

* Variable amylase activity.

STARCH PHOSPHORYLASE

Since it was shown that pH affected alpha amylase activity, tests for starch phosphorylase were made at pH values where amylase was not active, namely, 3.0, 3.2, 3.3, 8.6, 8.8 and 8.9. Citrate buffers were used for the

low pH values and tris buffer above pH 8. Inorganic phosphate tests indicated the absence of phosphorylase activity. If starch phosphorylase were present in the cell extract, the pH also affected starch phosphorylase activity.

Gill and Lehmann (1939) had found it necessary to add reducing agents to their reaction mixture of pH 9.0 to demonstrate the formation of the "Robinson ester," glucose-6-phosphate.

Therefore, the reaction mixtures, as shown in Table V, were prepared. Since it was found that a pH of 8.6-8.7 was sufficient to inhibit the alpha amylase activity, the reaction mixtures were adjusted to pH 8.7 and tested for phosphorylase activity. Sodium hydrosulfite was added as the reducing agent.

The reaction mixtures were incubated at 37 C. Quantitative analyses of acid and alkaline labile organic phosphate esters were made at 0, 30, 60 and 120 minute intervals.

The result, as shown in figure 3, indicated that there was a disappearance of two micromoles of inorganic phosphate per ml at the end of two hours of incubation. The assay for acid and alkaline labile phosphate esters showed that one micromole/ml was acid labile and another micromole/ml was alkaline labile. It was therefore assumed that the disappearance of inorganic phosphate in the absence of amylase activity was an indication of starch phosphorylase activity. This reasoning was followed by an examination of the reaction mixtures for glucose, since maltose phosphorylase action results in the formation of glucose-1-phosphate and glucose. The presence of glucose could not be detected in the reaction mixture with glucose oxidase. Therefore, the phosphorolysis must be due to a starch phosphorylase.

TABLE V

Test for starch phosphorylase at pH 8.6

TUBE NUMBERS	1	2	3	4
Tris Buffer pH 8.6 (0.1 M)	1.0	1.0	1.0	1.0
Glucose (0.1 M)	0.8	0.8		
Soluble starch (0.1 M)			0.8	0.8
$MgSO_4$ (0.1 M)	0.1	0.1	0.1	0.1
NaF (0.5 M)	0.2	0.2	0.2	0.2
K_2HPO_4 (0.1 M)		0.2	0.2	0.2
$Na_2S_2O_4$ (0.1 M)	0.5	0.5	0.5	0.5
H_2O	0.9	0.7	0.7	0.7
Enzyme	0.5	0.5	0.5*	0.5
Total volume in ml	4.0	4.0	4.0	4.0

* Boiled enzyme.

The identity of the unknown phosphate esters with known sugar phosphates was shown by chromatography. An ascending paper chromatogram was made with aliquots of the reaction mixtures at zero time and at two hours. The mixture at zero time showed no organic phosphate spots, but the mixture at two hours showed two spots with Rf values of 0.56 and 0.47, which corresponded with those of the control spots of glucose-1-phosphate and glucose-6-phosphate respectively. These results indicated that glucose-1-phosphate was formed by starch phosphorylase and that glucose-1-phosphate was further converted to glucose-6-phosphate by phosphoglucomutase.

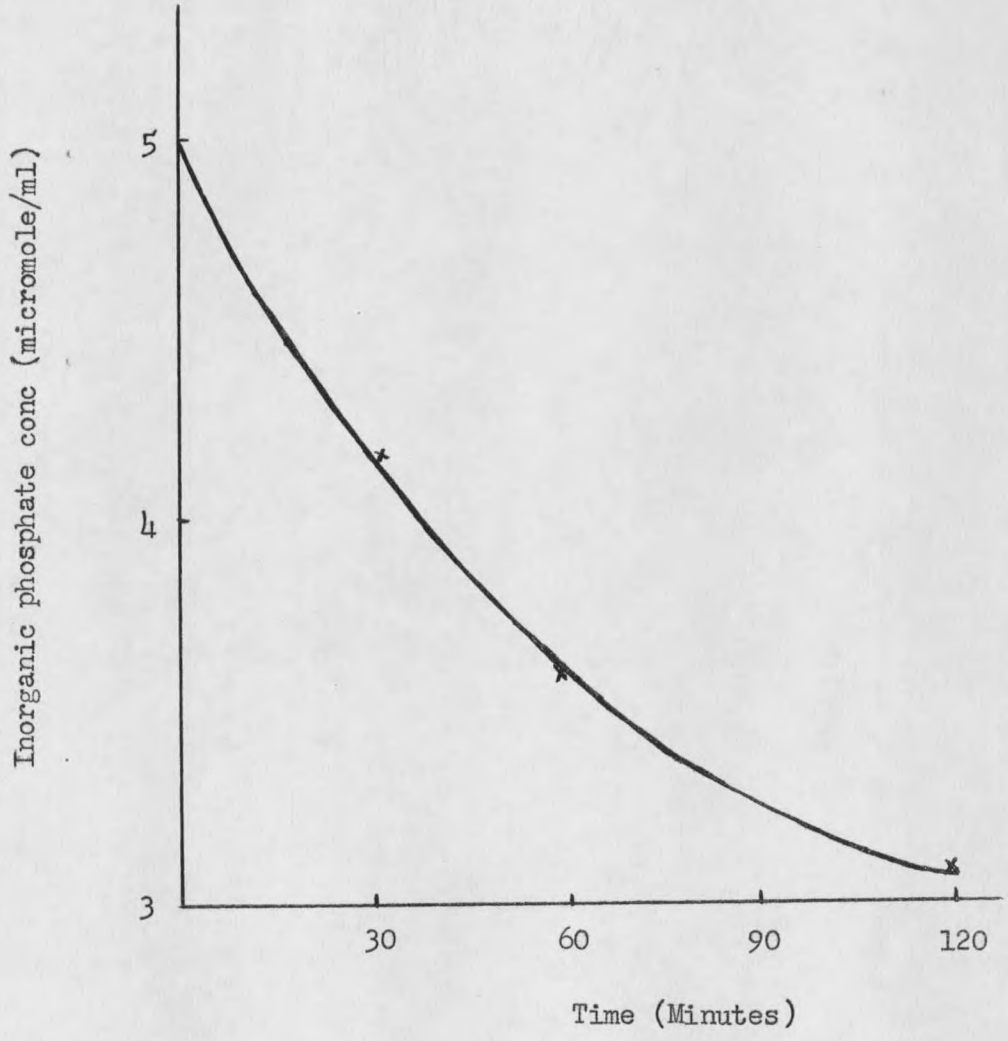


Figure 3 - Disappearance of inorganic phosphate in micromole/ml.

PHOSPHOGLUCOMUTASE

In order for an organism to utilize the glucose-1-phosphate formed by the phosphorylase, this ester must first be converted to glucose-6-phosphate by means of a phosphoglucomutase. This enzyme was demonstrated in a mixture of enzyme and glucose-1-phosphate by following the disappearance of acid labile phosphate at a pH of 7.5 (Table VI). Magnesium sulfate was added to activate phosphoglucomutase. Ethylenediaminetetraacetic acid, a chelating agent, was added to counteract interference by heavy metals, which may inhibit phosphoglucomutase activity.

TABLE VI

Test for phosphoglucomutase activity

TUBE NUMBERS	1	2	3
.08 M Tris buffer pH 7.5	1.0	1.0	1.0
0.1 M $MgSO_4$	0.1	0.1	0.1
.01 M ETDA*	0.2	0.2	0.0
H_2O	0.4	0.2	0.4
Enzyme	0.3	0.3	0.3
.05 M Glucose-1-phosphate		0.2	0.2
Total volume in ml	2.0	2.0	2.0

* Ethylenediaminetetraacetic acid

The enzyme preparation was found to contain phosphoglucomutase, which converted all of the glucose-1-phosphate to glucose-6-phosphate within eight minutes in the reaction tube containing ethylenediaminetetraacetic

acid (figure 4). The six-esters were formed at a slower rate in the absence of ethylenediaminetetraacetic acid.

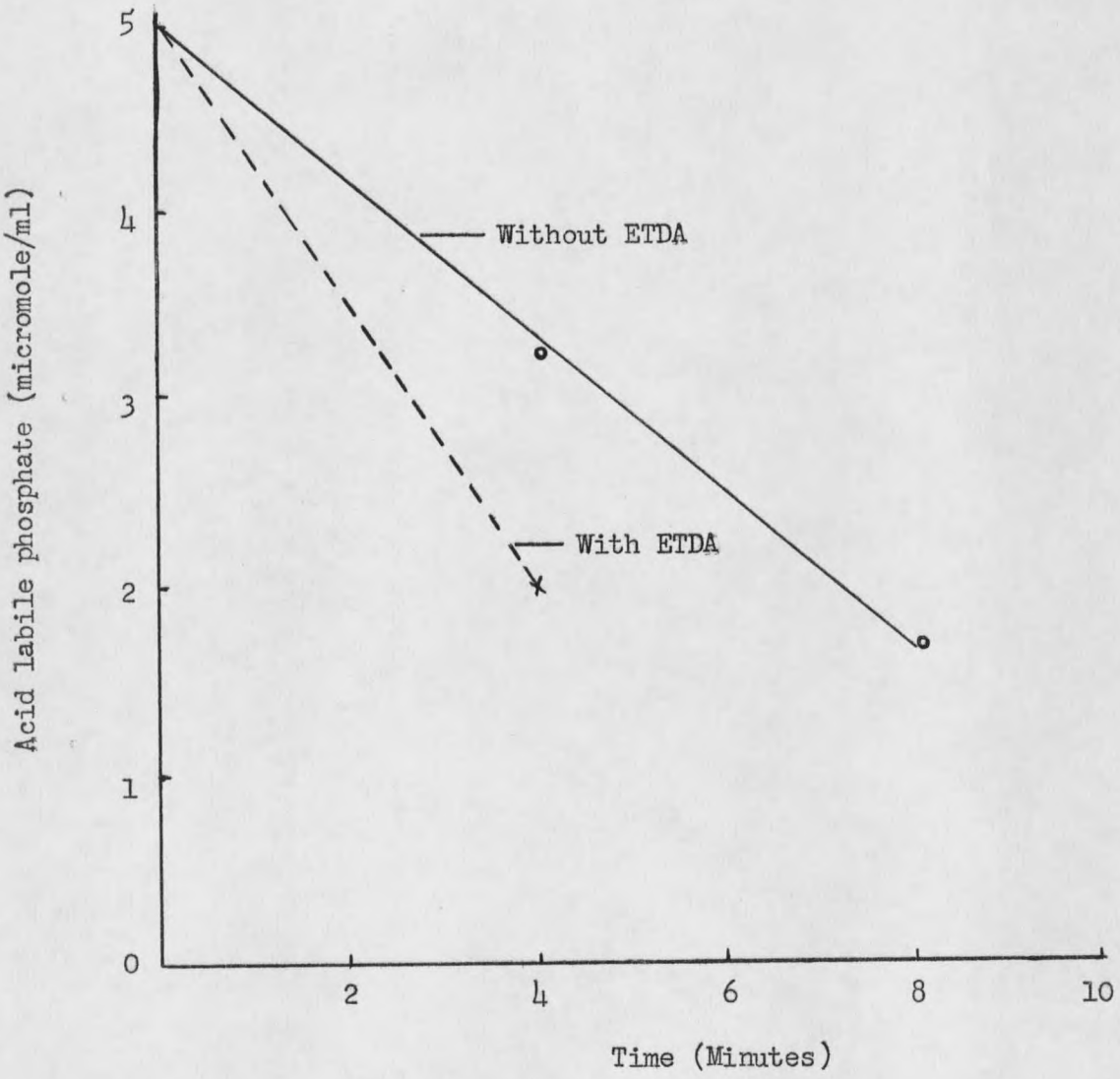


Figure 4 - Disappearance of acid labile phosphate during conversion of glucose-1-phosphate to glucose-6-phosphate by phosphoglucomutase.

DISCUSSION

The mechanism of hydrolysis and phosphorolysis is similar, except that the latter process involves the addition of phosphoric acid instead of water. Glucose-6-phosphate is derived as the end product of both processes, and presumably, is further metabolized through glycolysis. The amount of energy involved in the formation of glucose-6-phosphate through the two methods is different, however.

Starch is broken down by hydrolysis as follows:

starch ------→ maltose -------→ glucose

Glucose is then phosphorylated by glucokinase:

glucose + ATP ------→ glucose-6-phosphate + ADP

The formation of glucose-6-phosphate in the above reaction requires a high energy phosphate bond, which is donated by ATP. The phosphate bond of glucose-6-phosphate is at a relatively low energy level. Therefore, a considerable amount of energy has been expended by the organism through this reaction. The conversion of ADP back to ATP so that it can participate in another reaction will required energy that might otherwise be used for growth processes.

In contrast, starch and maltose can be cleaved phosphorolytically without the expenditure of a high energy phosphate bond as follows:

$(\text{glucose})_n + H_3PO_4$ ------→ glucose-1-phosphate + $(\text{glucose})_{n-1}$

maltose + H_3PO_4 ------→ glucose-1-phosphate + glucose

When glucose-1-phosphate is converted to glucose-6-phosphate by phosphoglucomutase, no expenditure of energy by the organism is required. Thus, when starch is cleaved by phosphorolysis, the organism has conserved one high energy phosphate per molecule of glucose, which can then be used for other purposes such as the synthesis of cellular materials.

If it is assumed that glucose-6-phosphate is utilized through the glycolytic scheme and converted to lactic acid, then starch hydrolysis followed by glucose fermentation would result in a net yield of 2 high energy phosphate bonds per molecule of glucose fermented (20,000 calories per mole). On the other hand, phosphorolysis of starch would result in a net yield of 3 high energy phosphate bonds per molecule of glucose fermented (30,000 calories per mole). Therefore, it would be possible for the organism to obtain up to 50 per cent more energy by utilizing starch through phosphorolysis than through hydrolysis.

The bovine rumen is apparently a highly competitive environment. Besides containing several kinds of starch hydrolyzing bacteria, the bacterial population in rumen fluid often exceeds 30 billion cells per ml. It was found that Streptococcus bovis was the most numerous starch hydrolyzing microorganism in the rumen. Therefore, the presence of starch and maltose phosphorylase could possibly explain how Streptococcus bovis is able to compete so well with the other organisms in the bovine rumen.

It is believed that this investigation demonstrates the first conclusive evidence of a starch phosphorylase in a bacterium. Other workers as Hobson and MacPherson claim to have detected starch phosphorylase activity but they did not take into account the possibility that their results could be due to a combination of amylase and maltose phosphorylase activity. Unless amylase is completely inhibited or separated from phosphorylase, it is impossible to determine whether the observed phosphorolysis is due to a maltose or a starch phosphorylase, unless the phosphorylation of maltose cannot be observed in the reaction mixture.

It was found that adjusting the pH to 8.6 and the addition of a reducing agent were essential to the demonstration of starch phosphorylase. The influence of a reducing agent on the phosphorylase is not understood.

The possible pathways of starch utilization by Streptococcus bovis are shown in figure 5.

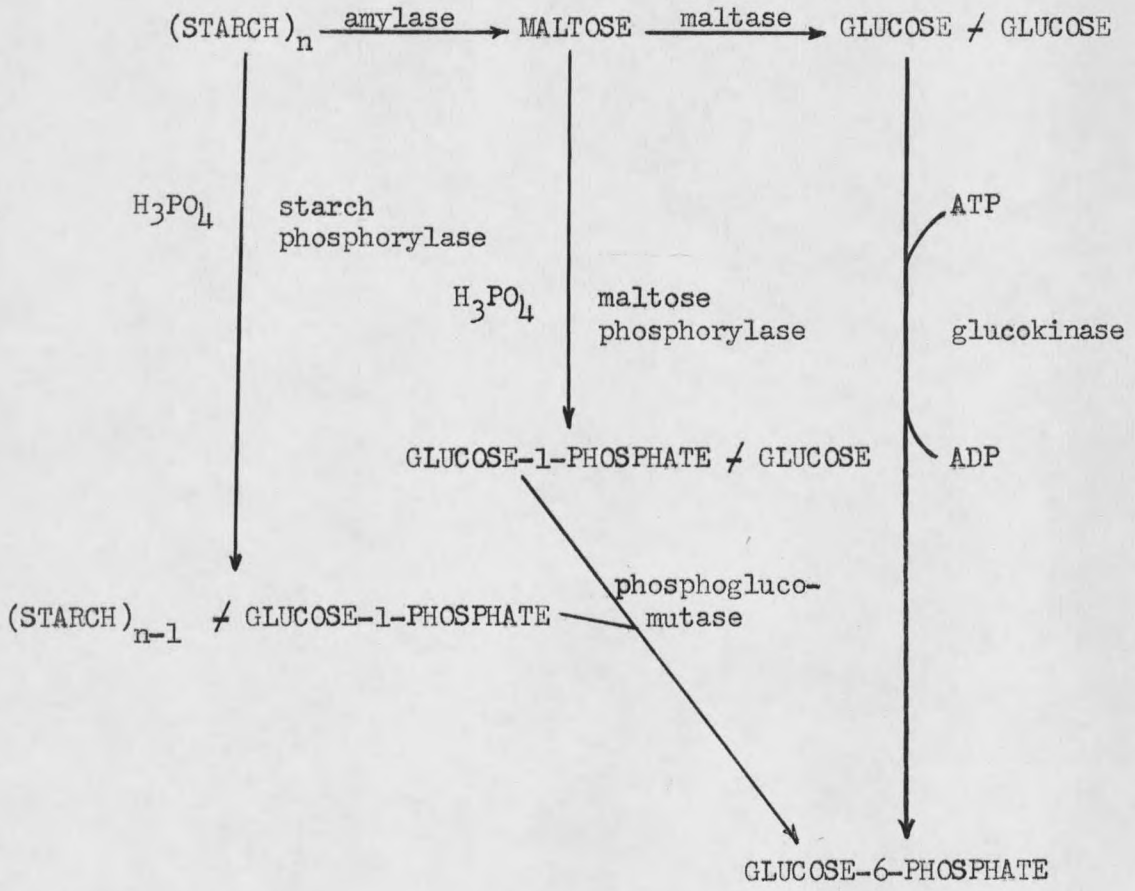


Figure 5 - Possible pathways by which Streptococcus bovis may use starch.

SUMMARY

The enzymatic degradation of starch through phosphorolysis was investigated. Starch phosphorylase was detected in the reaction mixture containing soluble starch, enzyme preparation, inorganic phosphate, and sodium hydrosulfite.

Since the phosphorolysis of maltose was noticed, maltose formation was prevented by inhibiting alpha amylase activity. This was done by adjusting the mixture to pH 8.6.

An equilibrium was noted at 2 hours, after the disappearance of 2 micromoles/ml of inorganic phosphate.

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