A new transglucosidase found in potatoes
by Clarence A Ryan

A THESIS Submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Chemistry at Montana State College
Montana State University

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A new transglucosidase, found in potatoes, is reported, The enzyme transfers the non-reducing glucose from isomaltose to acceptor molecules of maltose, maltotriose and1 higher molecular weight maltodextrins forming linear primers for the starch phosphorylase reaction, The enzyme was identified by activation of starch phosphorylase by forming primers for the latter enzyme. This activation was noted by both an increased formation of the amylose-I2 blue complex and increased release of inorganic phosphate when isomaltose was added to the partially purified enzyme system containing starch phosphorylase. The enzyme preparation also contained chromatographically detectable impurities of glucose, maltose and maltotriose.

The transglucosidase mechanism was identified by (1) it's role in furnishing substrates for the starch phosphorylase reaction, (2) incorporation of C14 from glucose-U-C14 into the isomaltose moiety after equilibration with the enzyme and isomaltose for 48 hours, and, (3) by the incorporation of Cl4 from isomaltose-U-Cl4 into maltodextrins with the subsequent release of glucose-Cl4 into the incubation media when the enzyme was incubated with isomaltose-U-C14, maltose and glucose-1-phosphate .

The proposed mechanism is as follows: Isomaltose + Enzy--------- Enzyme-Glucose + Glucose
Enzyme-Glucose + Maltose --------- Maltotriose + Enzyme / starch Maltotriose + Glucose-1-phosphate
phosphorylase amylose+ Pi

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Approved:

William L Cook
Head, Major Department

H J Loering
Chairman, Examining Committee

Leon D Johnson
Dean, Graduate Division

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ABSTRACT

A new transglucosidase, found in potatoes, is reported. The enzyme transfers the non-reducing glucose from isomaltose to acceptor molecules of maltose, maltotriose and higher molecular weight maltodextrins forming linear primers for the starch phosphorylase reaction.

The enzyme was identified by activation of starch phosphorylase by forming primers for the latter enzyme. This activation was noted by both an increased formation of the amylose-I_2 blue complex and increased release of inorganic phosphate when isomaltose was added to the partially purified enzyme system containing starch phosphorylase. The enzyme preparation also contained chromatographically detectable impurities of glucose, maltose and maltotriose.

The transglucosidase mechanism was identified by (1) it's role in furnishing substrates for the starch phosphorylase reaction, (2) incorporation of C^{14} from glucose-U-C^{14} into the isomaltose moiety after equilibration with the enzyme and isomaltose for 48 hours, and, (3) by the incorporation of C^{14} from isomaltose-U-C^{14} into maltodextrins with the subsequent release of glucose-C^{14} into the incubation media when the enzyme was incubated with isomaltose-U-C^{14}, maltose and glucose-1-phosphate.

The proposed mechanism is as follows:

$$\text{Isomaltose} + \text{Enzyme} \longleftrightarrow \text{Enzyme-Glucose} + \text{Glucose}$$

$$\text{Enzyme-Glucose} + \text{Maltose} \longleftrightarrow \text{Maltotriose} + \text{Enzyme}$$

$$\text{Maltotriose} + \text{Glucose-1-phosphate} \xleftrightarrow[\text{phosphorylase}]{\text{starch}} \text{Amylose} + P_i$$

I. INTRODUCTION

The normal metabolism of a large number of plants involves the storage of carbohydrate material as a readily available energy source. Many of these plants store this carbohydrate material in the form of starch, a large polymer of alpha-D-glucose, which can be fractionated into two molecular types called amylose and amylopectin. The amylose fraction is linear and only alpha 1-4 bonds between the glucose units have been identified in the molecule. There is however an anomalous bond present which can be detected by Z-enzyme (1). The nature of this bond is unknown due to the inactivity of the enzyme on synthetic substrates (2). The molecular size of amylose may vary somewhat from an average of about 300 glucose units (3,4) although no length limiting factor has yet been discovered. The lower limit of the molecular size is difficult to define. Amylose and amylopectin both form colored complexes with iodine due to the quantitative binding of I_2 inside of the helix of the starch molecule (5). One full turn of the helix involves about six glucose units and binds approximately 0.21 g. of iodine per complete turn. The color of this complex is brown for amylopectin and blue for amylose. The blue complex of amylose persists down to about a 16 glucose unit chain called a maltodextrin. As the chain length decreases the color changes, becoming light red at approximately 8 glucose units (6). It would seem logical to consider this 8 unit chain the lower limit of the size of the amylose molecule although lower molecular weight straight chain oligosaccharides are usually found associated with this fraction.

Amylopectin possesses a branched, treelike structure. The straight chained portions of the molecule between branches are bonded alpha 1-4 and the branching points involve an alpha 1-6 bond. The average distance between branching points is about 10-12 glucose units (7). The average amylopectin molecule is much larger than the average molecule of amylose, having a D.P.[1] of about 1300 glucose units (4,7,8).

The percentages of the two fractions, amylose and amylopectin, vary with the source of the starch. Starches generally contain a larger percentage of amylopectin than amylose, usually from 70% to 90% of the total starch.

The synthesis of starch in the plant is not well understood. In 1940, Hayne discovered an enzyme in the potato (9) that required alpha-D-glucose-1-phosphate and suitable maltodextrins as substrates for the synthesis of amylose. It utilizes the energy present in the hemiacetal phosphate bond of glucose-1-phosphate to synthesize an alpha 1-4 bond. This enzyme catalyzes the transfer of the glucose of glucose-1-phosphate to suitable acceptor molecules, the maltodextrins, to form longer linear amylose type molecules with the simultaneous release of inorganic phosphate into the medium. The enzyme, starch phosphorylase, requires maltotriose or a larger maltodextrin as the acceptor molecule for the glucose moiety. Neither maltose nor free glucose will act as a substrate for this enzyme (10). The equilibrium of the starch phosphorylase reaction is 75.6 to 24.4 in favor

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1. D. P. - Degree of polymerization

of synthesis of polysaccharide at a pH of 6.96 (11).

The phosphorylase reaction is, in fact, a transglucosidase reaction, that is, a reaction that involves the transfer of glucose from one moiety to another, conserving the energy in the original bond. The energy of the bond in the original substrate, e.g. glucose-1-phosphate, or between two sugar moieties, is conserved in an enzyme-substrate compound and this energy again conserved in an acceptor-glucose bond. This is a double displacement reaction and the configuration in the glucose-acceptor molecule is the same as in the original compound.

An enzyme from the potato has been reported that transfers two or more units from maltodextrins to suitable acceptors to give straight chained dextrins (12). The substrate appears to be maltotriose or larger maltodextrins and only alpha 1-4 bonds are synthesized. The enzyme has been called the disproportionating or D enzyme. Since glucose and maltose will act as acceptors, degradation of starch proceeds when these acceptors are present in appreciable amounts. It is probable that this enzyme synthesizes amylose only in the young plant when large amounts of small maltodextrins are present or when hexokinase reduces the glucose concentration to a minimum (13).

A third starch synthesizing enzyme, Q enzyme, is also a transglucosidase (14). It transfers straight chained amylose fragments to the 6 position of acceptor maltodextrins forming branched components or amylopectin like molecules. This enzyme requires a maltodextrin of at least 42 glucose units in length as the substrate (15,16).

A recent paper has suggested that the formation of starch involves glycogen as a precursor to both amylose and amylopectin but no experimental evidence of the enzyme systems has been offered (17).

To the present time no evidence regarding the origin of the primers for phosphorylase or D enzyme has been found. It has been suggested that transglucosidases may be involved. Enzymes of this type have been found in bacteria. An enzyme found in Pseudomonas saccharophila (18) catalyzes the formation of an amylose-like molecule from sucrose. The reaction is as follows:

$$n \text{ Sucrose} \longleftrightarrow (\text{Glucose})_n + n \text{ Fructose}$$

A similar enzyme from Escherichia coli (19) catalyzes the formation of primers from maltose.

$$n \text{ Maltose} \longleftrightarrow (\text{Glucose})_n + n \text{ Glucose}$$

Sucrose and maltose are both known constituents of plants. The synthesis of sucrose is known to occur in wheat germ by three pathways(20).

(1) UDPG[2] + Fructose $\longleftrightarrow$ Sucrose-phosphate + UMP[3]

(2) UDPG + Fructose-6-phosphate $\longleftrightarrow$ Sucrose-phosphate + UDP

(3) UDPG + Fructose $\longleftrightarrow$ Sucrose + UDP

Maltose is a common constituent of plants and is generally believed to be a result of alpha and beta amylase action on starch. No enzymes have been reported which synthesize maltose in the plant. However, it has been

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2. UDPG -- Uridine Diphosphoglucose
3. UMP -- Uridine Monophosphate

reported that maltose-U-C^{14} is utilized in the tobacco leaf for starch synthesis (21). The hypothesis suggested a conversion to sucrose, and then to starch.

Maltose is known to be synthesized by a maltose phosphorylase found in Neisseria meningitidis (22). The enzyme utilizes beta-D-glucose-1-phosphate and glucose as substrates.

$$\text{Beta-D-Glucose-1-phosphate} + \text{Glucose} \rightleftharpoons \text{Maltose} + P_i$$

Both sucrose and maltose require an activated intermediate for their synthesis. This leaves the investigator many paths to investigate. The problems in elucidation of the origin of starch primers can be seen to be very complex.

In the present paper the presence of a transglucosidase in the potato is reported which catalyzes the transfer of the non-reducing terminal glucose of isomaltose to maltose and higher maltodextrins, thus building linear type molecules which can assume the role of primer for starch phosphorylase. A mechanism for this reaction has been proposed from the experimental evidence.

A synthesis of isomaltose from monosaccharides has not been reported at the present time; however, the action of the enzyme described in this paper would suggest the presence of an isomaltose synthesizing enzyme in the potato.

Isomaltose has not been previously considered as a course of the primers for starch phosphorylase. The only reports of isomaltose in plant metabolism has been from the breakdown of starch and this was in very low

yield i.e. 1.0 to 2.0%. The presence of sucrose in most young plants led workers to believe that this was probably the source of amylose via a transglucosidation reaction. This is still speculation and may occur in some plants. The absence of isomaltose does not rule the disaccharide out of a possible role in 'primer' synthesis since we know in a "steady state" a build up of an intermediate may not occur. Nevertheless isomaltose has been overlooked as an intermediate. This paper would suggest that a detailed investigation of 'primers', especially in the young plant, is in order.

II. EXPERIMENTAL

1. Preliminary Observations.

During an investigation of starch phosphorylase in a centrifuged, 60 hour dialyzed fraction of potato juice, it was found that the addition of C.P. D-glucose to a mixture of alpha-D-glucose-1-phosphate and an aliquot of the juice increased the rate of formation of the blue amylose-I_2 complex normally formed by the starch phosphorylase reaction (Fig. 1).

The enzyme was prepared by first thinly slicing peeled Russet potatoes into a sodium dithionite solution (7 g./1), and soaking them for 10 minutes at room temperature. The slices were pulped in a Waring blendor and the juice from the pulp was expressed through six thicknesses of cheese cloth into a cooled beaker. The juice was centrifuged in a refrigerated centrifuge at 5° C. at 10,000 x g. and the residue discarded. This juice was dialyzed against cold running tap water for 60 hours. This treatment destroyed much of the starch phosphorylase and precipitated large amounts of protein material. The dialyzed fraction was centrifuged at 10,000 x g. The supernatant was cooled and used as the crude enzyme preparation.

The enzyme assay contained the following reagents: 0.5 ml. of the enzyme preparation; 0.5 ml. of 0.25 M. citrate buffer pH 6.0; 10 μM. of glucose-1-phosphate; and 5 μM. of glucose. A similar assay was made without the addition of glucose. Final volumes were made to 2.5 ml. with distilled water. A 1.0 ml. aliquot was taken from the mixture after a 15 minute interval and added to 2.0 ml. of I_2 in KI solution (23). The I_2 - KI solution was made by diluting 1.0 ml. of a 0.5 M. KI, 0.05M. KCl solution containing

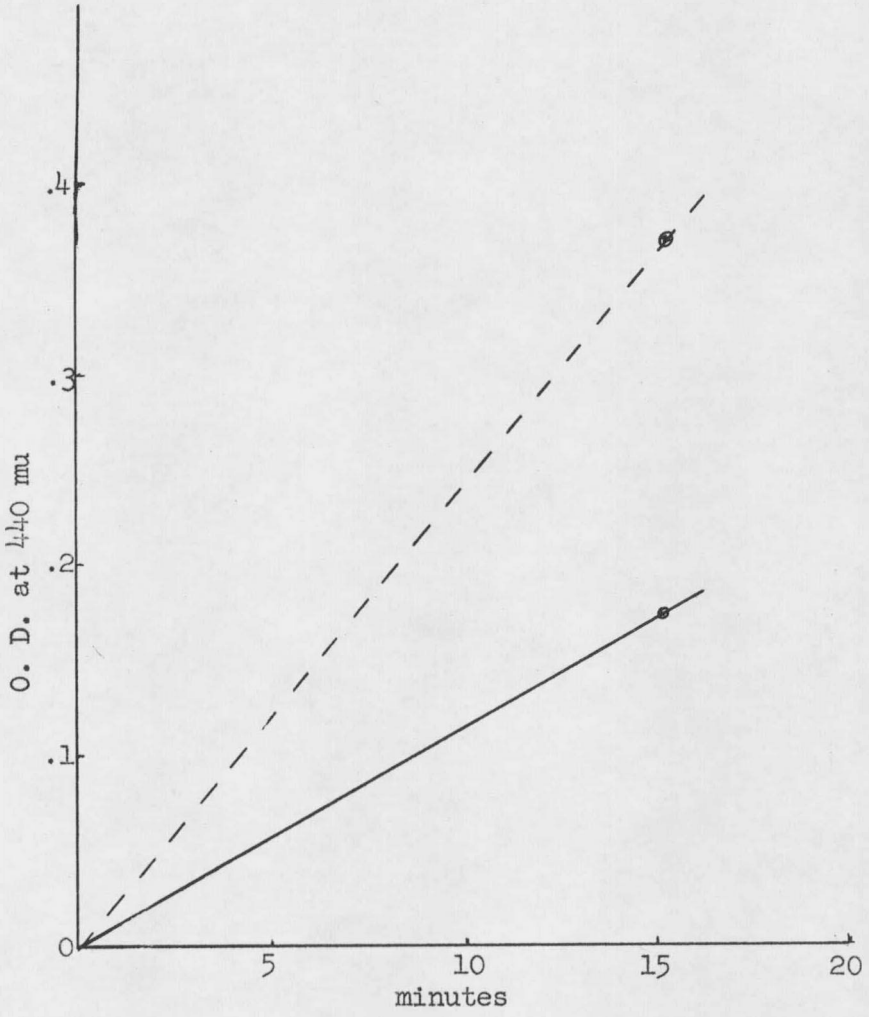


Figure 1. The effect of the addition of glucose on the formation of the blue I_2-amylose complex formed by the starch phosphorylase reaction. The enzyme preparation also contained primer molecules for the reaction as impurities.

Legend: - - - - - 5 μM. of glucose added to assay.
_____ no glucose added to assay.

2.00 g. of I_2 per liter to 50 ml. with water. The color, which is stable for 10 minutes, was recorded immediately at 440 mu on a Beckman Model B spectrophotometer. These results were reproducible. This assay is but a qualitative measurement of a rate of polysaccharide formation and tells little about the concentration. This is because one long amylose molecule will complex more I_2 than will two shorter molecules containing the same total number of glucose units. The only conclusion that could be drawn from the observed increased in the rate of formation of the blue I_2 complex was that more primer molecules must have been present in the assay mixture due to the addition of glucose. This suggested the incorporation of glucose into polymers. No known enzyme systems are capable of catalyzing this reaction under these conditions.

The glucose, when chromatographed on paper, did not reveal the presence of primers of starch phosphorylase as impurities. A typical chromatogram was made by spotting 100 µg. of glucose one inch from one end of a strip of Whatman #1 filter paper, 1 1/2 inches wide by 12 inches long. After air drying the spot, the chromatogram was suspended inside a bell jar with the end containing the spot immersed to a depth of about 10 mm. into a n-butanol, pyridine, water mixture (6:4:3 v/v). This chromatogram was dried and re-ascended to get better separation of the sugars. The chromatogram, after drying, was sprayed with a glucose-1-phosphate-starch phosphorylase spray reagent (10) and incubated in a moist atmosphere for fifteen minutes. The chromatogram was then sprayed with the I_2 in KI, KCl solution that was used in the amylose-I_2 color assay mentioned above. Any primer molecules

present as impurities would have resulted in the formation of blue spots due to the action of the starch phosphorylase which forms amylose type molecules from the primers. Maltotriose is the smallest primer that can be utilized by starch phosphorylase for synthesis of the larger molecules (10). This method will detect microgram quantities of primers.

The phosphorylase was prepared from crude potato juice by a modification of the method of Baum and Gilbert (24). The crude juice was prepared in a manner similar to that of the new enzyme preparation except that the juice was not dialyzed. Instead, after centrifuging the cooled, crude juice at 10,000 x g, 0.28 ml. of 50% ethanol (w/v) in 0.01 M. sodium citrate buffer, pH 6.0, was added per ml. of the supernatant. This mixture was centrifuged at 10,000 x g and the precipitate discarded. The supernatant liquid contained active starch phosphorylase and was used in spraying the chromatograms. This preparation located primer impurities in C. P. maltose when used under the conditions of the previous experiment.

2. The effect of the citrate ion in phosphate analysis using a molybdate reagent.

The increase in the rate of formation of the blue I_2 complex due to glucose was not consistently accompanied by release of inorganic phosphate as measured by the method of Sumner (25). Any theory explaining the observed glucose stimulation of starch phosphorylase action requires the release of inorganic phosphate, (P_i), since polysaccharide formation could not be detected without the addition of glucose-1-phosphate to the incubation mixture.

The inconsistency of the P_i release was thought to be caused by the

reagents. However, when standard phosphate solutions were analyzed in the presence of increasing concentrations of the 0.25 M. citrate buffer, pH 6.0, which was used for the enzyme assays, it was found that the citrate ion quantitatively bound the molybdate ion in solution independently of the phosphate present (Fig. 2). It was shown that the addition of 0.3 ml. of 0.25 M citrate buffer to a phosphate assay barely inhibited the formation of the blue phosphomolybdate color. During the original enzyme assays, 0.5 ml. aliquots, when taken from the enzyme assay mixture, were equal to 0.125 ml. of the buffer (Fig. 2). This explained the wide variations in the phosphate assays.

To correct for this phenomenon the percent of ammonium molybdate was raised from 2.5% to 7.5%. A series of known phosphate solutions were assayed with increasing aliquots of the citrate buffer to find if the increased percent of ammonium molybdate would correct the situation (Fig. 3). No effect due to the buffer was observed when as much as 0.5 ml. of the buffer was added. This was four times the amount generally used in the enzyme assays for P_i.

The 7.5% ammonium molybdate solutions gave reproducible P_i analyses during the remainder of the laboratory work.

3. The enzyme preparations.

The early evidence of the new enzyme in potato juice had shown the enzyme to be stable toward prolonged dialysis. This treatment precipitated large volumes of protein material during the first 12 to 18 hours. It was decided to fractionate the 18 hour dialysate with ammonium sulfate in order

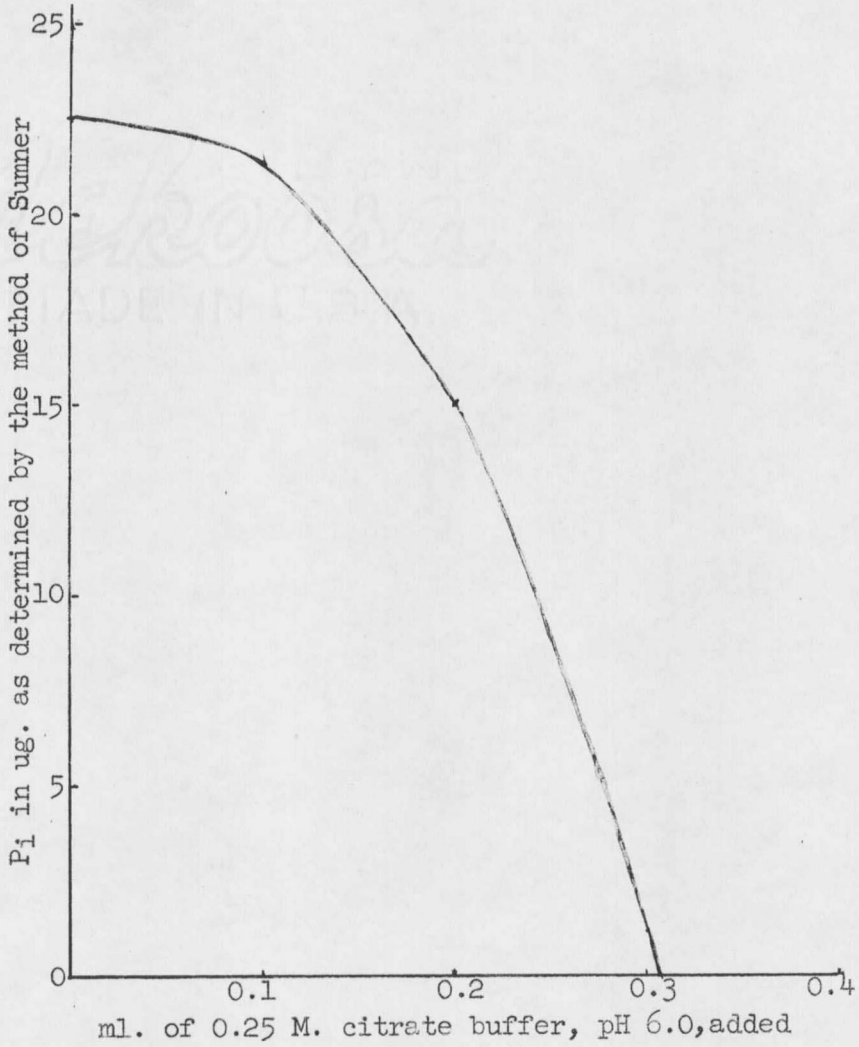


Figure 2. The effect of increasing concentrations of the citrate ion on the determination of inorganic phosphate by the method of Sumner using 2.5% ammonium molybdate in 7.5 N. H_2SO_4 as the color reagent.

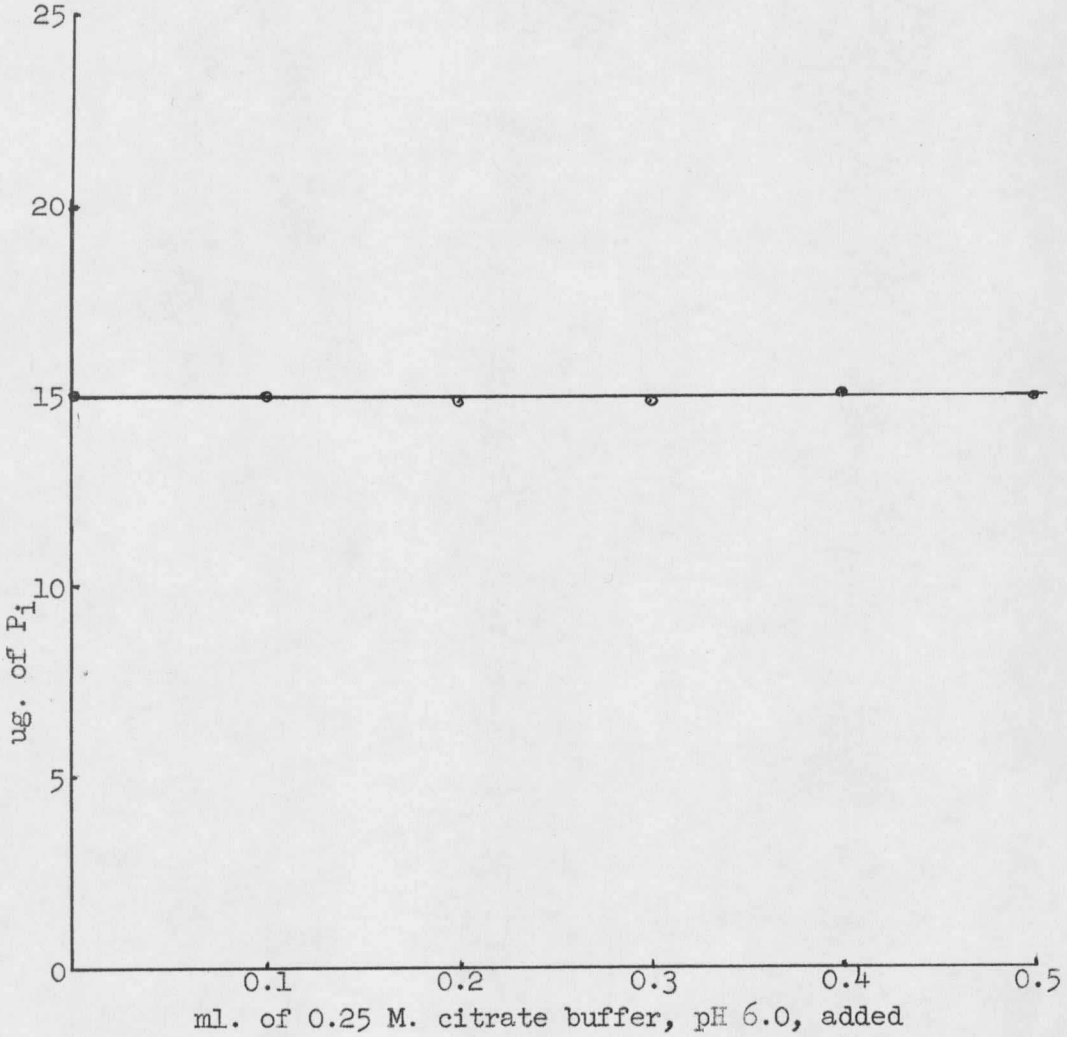


Figure 3. The effect of increasing concentrations of the citrate ion on the determinations of inorganic phosphate by the method of Sumner using 7.5% ammonium molybdate in 7.5 N. H_2SO_4 as the color reagent.

to concentrate the enzyme and possibly separate it from inactive protein material.

Green and Stumpf (26) published an account of the preparation of starch phosphorylase in which they report high yields of this enzyme in the 0-35% and 35-40% ammonium sulfate fractions (w/v). Some preliminary work suggested that the enzyme responsible for the early findings was precipitated at ammonium sulfate concentrations of less than 40%. It was decided to fractionate the 18 hour potato juice with increments of 10% ammonium sulfate (w/v). Preliminary experiments revealed that no protein precipitated at 10% ammonium sulfate and a small amount in the 10-20% fraction from the dialyzed juice. Since only a small amount of protein was present in the first two fractions, the 0-30% ammonium sulfate fraction was precipitated in a single operation.

The potato juice used for the preparation of the ammonium sulfate fraction was prepared in a manner similar to the juice in the "preliminary observations" except for the period of dialysis. In this preparation, the juice was dialyzed for 18 hours against cold running tap water. After dialysis the juice was again cooled to 5° C. and then 30 g. of ammonium sulfate was added per 100 ml. of juice. The precipitate was centrifuged at 10,000 x g for 10 minutes and the supernatant decanted off. The residue was dissolved in 20 ml. of 0.25 M. citrate buffer, pH 6.0 and this constituted the 0-30% fraction.

Ten grams of ammonium sulfate were added per 100 ml. of the supernatant. The resulting precipitate was centrifuged and dissolved in citrate buffer

as above. This was the 30-40% fraction.

The two fractions were assayed as before for stimulation of the amylose-I_2 complex. The stimulation was evident in only the 0-30% fraction. The analysis of P_i release showed but a small increase due to the addition of the glucose to this fraction.

4. Isomaltose as a substrate.

Two mechanisms explaining the priming activity by glucose were postulated at this time, (a) a phosphorylase, forming primers from glucose and glucose-1-phosphate, and (b) a transglucosidase reaction involving an intermediate present in the enzyme preparation.

If the first mechanism, (a) were operating it would have to have been present in very small amounts since P_i release from assays was very small. If (b) were the mechanism, it must have involved a branched intermediate(s). A third explanation, the production of new alpha 1-4 bonds from alpha 1-4 bonds already present as impurities in the potato juice, was possible but not logical in this case. An enzyme found in the potato called D-enzyme produces primers from longer chains, but in the presence of glucose it depresses blue I_2 complexing chains by forming shorter dextrins as mentioned in the introduction.

The second mechanism involving a branched intermediate seemed more logical from the data at hand, mainly because of the small increases in P_i due to the addition of glucose. This small increase would not be characteristic of a new phosphorylase.

An alpha amylase hydrolysate of amylopectin was made to secure isomaltose and other small branched fragments. These fragments will not act

as primers for starch phosphorylase (10), but could have been involved in primer synthesis by another mechanism involving glucose. One hundred mg. of clarase, a commercial preparation of alpha amylase, was added to a 1% gelatinized amylopectin mixture. The mixture was incubated at 37° C. for 3 hours. The reaction was terminated in a water bath at 97° C. for 10 minutes. The hydrolysate was deionized with Amberlite MB-3 mixed resin and dried in an air stream. An aliquot corresponding to 1 µM. of sugar was spotted one inch from the base of a strip of Whatman #1 filter paper, 1 1/2" x 10" and ascended two times with a n-butanol, pyridine, water mixture (6:4:3 v/v) to a height of 8". Maltose, maltotriose, maltotetraose and maltopentaose, which are present in commercial maltose as impurities, were used as references along with glucose. The chromatograms were sprayed with a mixture containing 0.5 ml. of 0-30% enzyme fraction and 50 µM. of glucose-1-phosphate in 2.5 ml. of 0.25 M. citrate buffer pH 6.0. The chromatograms were incubated at room temperature in an enclosed chamber containing a moist atmosphere for 30 minutes. The strips were dried in an oven at 100° C. The dried chromatograms were then sprayed with the I_2 in KI spray previously mentioned.

A pronounced priming activity was present at approximately the maltose R_f value (Fig. 4). Maltotriose and larger polysaccharides were also present in the amylopectin hydrolysate. This was the first evidence known of any disaccharides with priming activity toward a starch phosphorylase preparation from potatoes.

The amylopectin hydrolysate was chromatographed on a 10 cm. by 12 mm.

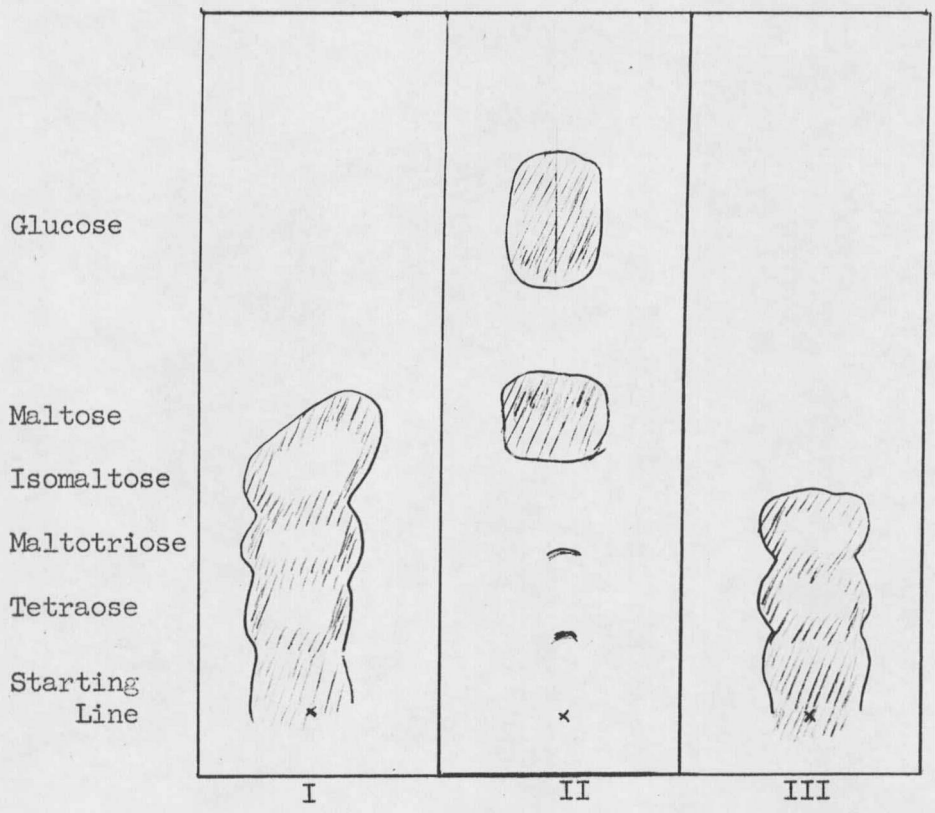


Figure 4. Evidence of a new enzyme found in the potato. Chromatogram I exhibits a primer for starch phosphorylase at the R_f of maltose-isomaltose from an alpha amylase digest of amylopectin. The chromatogram was sprayed with a starch phosphorylase, glucose-1-phosphate preparation followed by an I_2 in KI spray reagent. Chromatogram III was treated in an identical manner and contains only linear molecules. Chromatogram II is a reference chromatogram developed with CD 1 developer.

charcoal-celite column (1:1) as described by Whistler (27). This method fractionates sugar mixtures into mono-, di-, and trisaccharide fractions by eluting with varying percentages of an alcohol-water mixture. The entire hydrolysate was placed on the column and allowed to pass into the column. One hundred ml. of water was then passed through the column to elute monosaccharides. This was followed by 300 ml. of 5% ethanol to elute the disaccharides. The elution was followed colorimetrically to determine the total sugar content in each fraction by the method of Dubois and Gilles (28). The disaccharide fraction was dried in an air stream, dissolved in 5 ml. of distilled water and an aliquot chromatographed on Whatman #1 filter paper using maltose as a reference (Fig. 5). All of the paper chromatograms during the remainder of the thesis work were made with Whatman #1 filter paper. Two spots appeared when the chromatograms were developed by spraying with CD-1 developer (29), a solution of o aminodiphenyl, oxalic acid, glycerol and acetone that develops spots ranging from grey-brown for glucose to brown for maltose and grey for isomaltose. One spot was brown with an R_f identical to that of maltose. The other major spot was grey and migrated at the same R_f as isomaltose (30). Isomaltose is the only disaccharide recorded that develops a grey color (29) and the only disaccharide besides maltose that is present after amylolysis of amylopectin in relatively high concentrations (31). Only a small amount of glucose was detectable.

The disaccharide fraction was tested for priming activity with the 0-30% enzyme preparation. A 1.0 ml. aliquot of the disaccharide fraction was incubated with 0.5 ml. of the 0-30% enzyme preparation and 10 µM. of

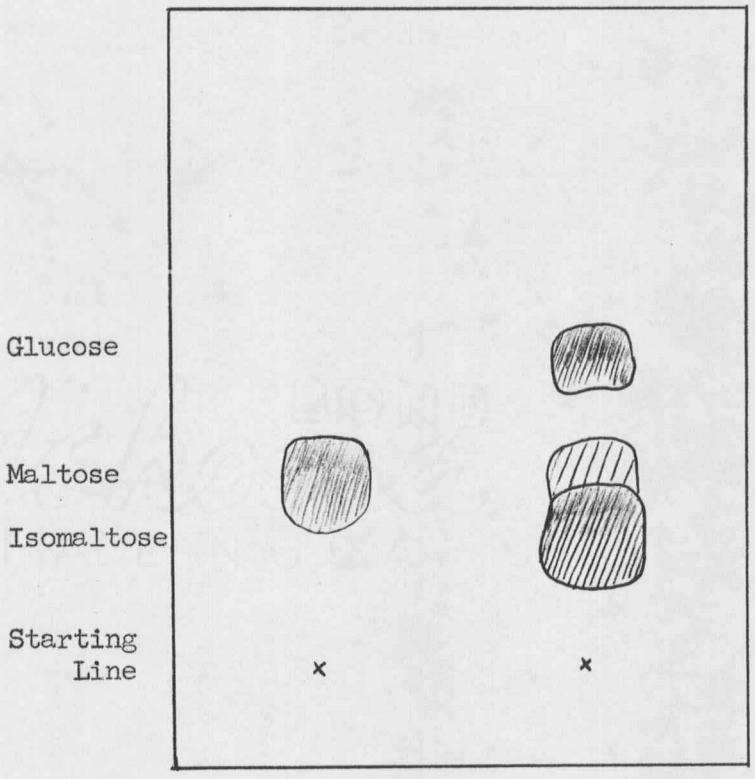


Figure 5. Chromatogram of the 5% ethanol eluate from a charcoal-celite column containing an alpha amylase hydrolysate of amylopectin. Chromatogram II was spotted with an aliquot of the hydrolysate. Chromatogram I is a maltose reference.

alpha-D-glucose-1-phosphate.

A control assay was made by omitting the disaccharide fraction. The increase in formation of the amylose-I_2 blue complex by the disaccharides was pronounced and the stimulation was much greater than that produced by glucose.

The C.P. glucose that contained the original priming action was analyzed chromatographically for disaccharide impurities. A 0.10 g sample of C.P. glucose was dissolved in 0.5 ml. of water, spotted on a strip of filter paper and chromatographed with the n-butanol, pyridine, water mixture. The chromatogram was developed with CD-1 developer. The only major impurity was a disaccharide, developing a grey spot, with an R_f value identical to that of isomaltose. This disaccharide was eluted from a chromatogram and incubated with 0.5 ml. of the enzyme in the usual manner. The disaccharide increased the rate of formation of the blue I_2 complex.

In order to purify the isomaltose from the clarase hydrolysate of amylopectin, the disaccharide fraction from the digest that had been collected from the charcoal-celite column was streaked uniformly over a line one inch from the base of a 7" x 18" strip of filter paper. The chromatogram was descended for 18 hours with the n-butanol, pyridine, water mixture. The positions of the disaccharides were located by cutting a 1/2" strip from the side of the chromatogram and developing it with CD-1 developer. The locations of the sugars were marked with a pencil and the strips cut out parallel to the starting line. These strips were then eluted with water and the purified disaccharide solutions dried in an air stream and

stored in a dessicator. The disaccharides were tested with the 0-30% enzyme preparation for the stimulation of the blue I_2 complex formation. The incubation mixtures contained 2.5 ml. of 0.25 M. citrate buffer, pH 6.0; 10 µM. of glucose-1-phosphate; 0.2 ml. of enzyme preparation; and 1 µM. of disaccharide to be assayed. A 0.5 ml. aliquot was taken for assay of blue I_2 color at 15, 30, 45, 60 and 75 minute intervals. The results are shown in Figure 6. The isomaltose appeared to effect an increase in the rate of blue I_2 color formation and P_i release. The I_2 color lag is due to the inability of the smaller amylose fragments to form the blue c complex with I_2. Maltose exhibited no priming action with this enzyme preparation.

It appeared that isomaltose was involved either in the synthesis of small linear chains by a transglucosidase reaction and these chains subsequently used by starch phosphorylase, or that a phosphorylase was present that utilized isomaltose and alpha-D-glucose-1-phosphate to synthesize singly branched molecules which starch phosphorylase would eventually use. When the 30-40 % enzyme fraction was used in identical assays, isomaltose would not stimulate formation of blue I_2 color (Fig. 7). This rather conconclusively eliminated the possibility of primer impurities being present with the isomaltose additions since both the 0-30% and 30-40% ammonium sulfate fractions exhibited starch phosphorylase activity using starch as a primer. This experiment revealed other important facts, the importance of which will be indicated later in this thesis.

Experiments to test the two proposed theories were planned. Both mechanisms involved isomaltose, so a convenient method of preparation of this

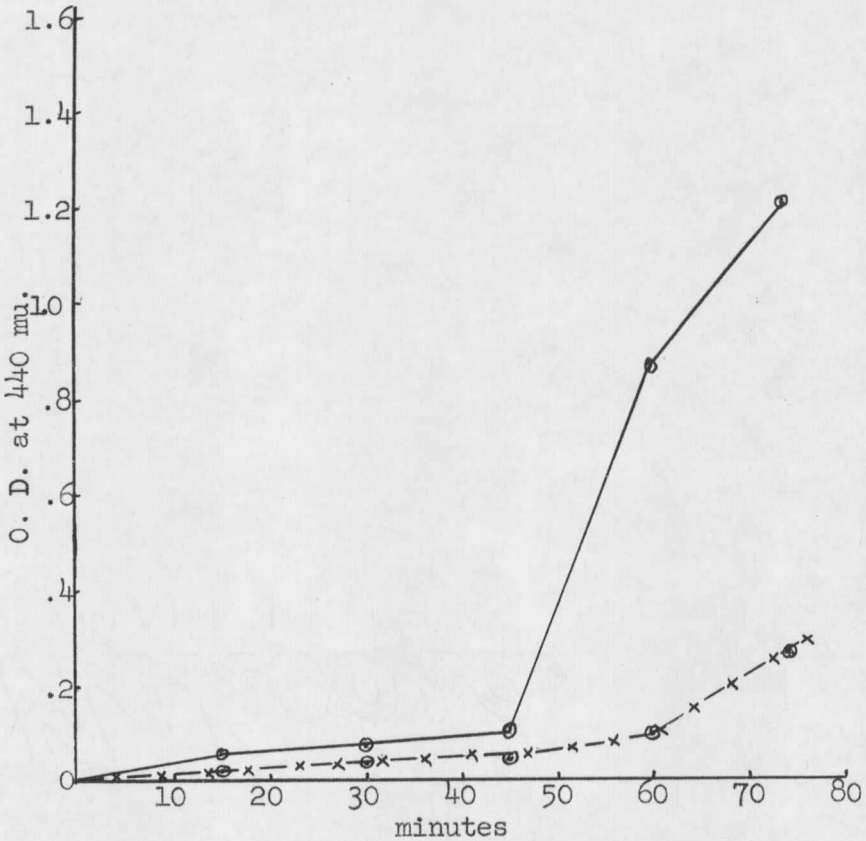


Figure 6. The effect of the addition of 1 μM. of maltose and 1 μM. of isomaltose on the rate of formation of iodine complexing molecules in assay mixtures of the 0-30% ammonium sulfate fraction from the juice of Russet potatoes. Other substrates present in all assays were glucose-1-phosphate, and primer impurities that were present in the preparation.

Legend: _____ 1 μM. of isomaltose added.
- - - - 1 μM. of maltose added.
x x x x no disaccharides added.

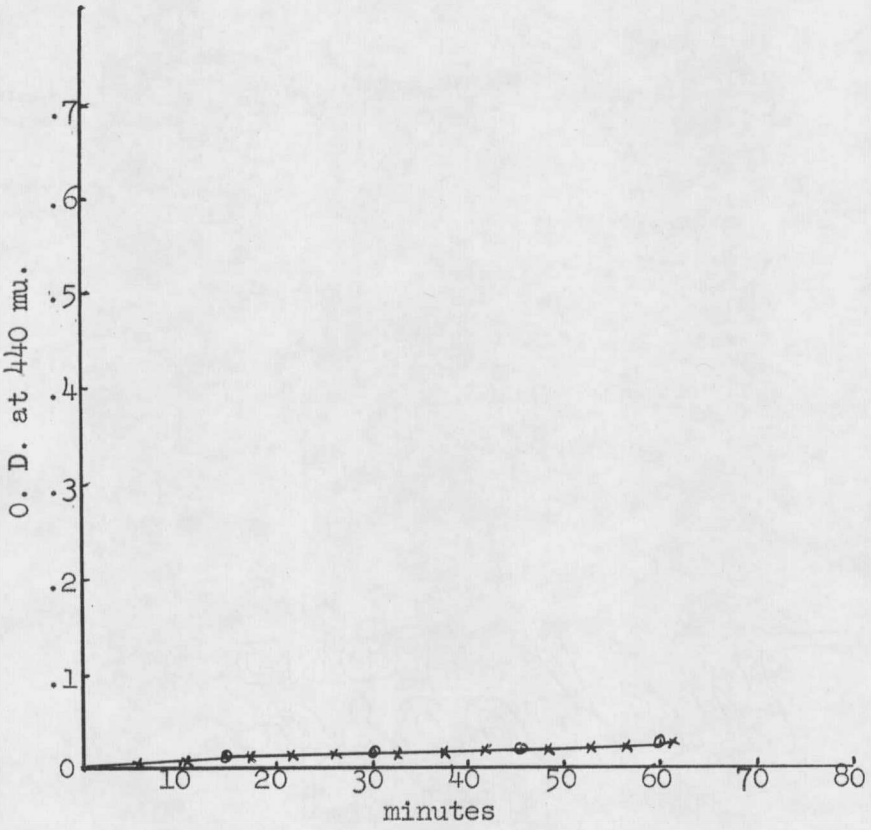


Figure 7. Assay of the 30-40% ammonium sulfate fraction from the juice of Russet potatoes using 1 µM. of isomaltose as substrate with glucose-1-phosphate and primers present as impurities in the fraction.

Legend: x x x x x 1 µM. of isomaltose added.
———— no isomaltose added.

substrate was sought.

5. Preparation of pure isomaltose.

The method of preparation of isomaltose using amylopectin and alpha amylase was time consuming and yielded only a very small quantity of isomaltose. A method of preparing the disaccharide by utilizing a bacterial dextran appeared feasible. Leuconostoc mesenteriodes synthesizes large amounts of a capsular polysaccharide of glucose consisting mainly of alpha 1-6 bonds when sucrose is used as the substrate (32). The polysaccharide, called bacterial dextran, is fragmented into isomaltose in yields above 50% by an exoenzyme of a mold, Penicillium finiculosum (33). L. mesenteroides B-512F and P. finiculosum NRRL 1132 strains were obtained from the Northern Regional Research Laboratory, Peoria, Illinois, through the courtesy of C. W. Hesselfine, Head, ARS Culture Collection Investigations and William C. Haynes, Bacteriologist. The dextran was prepared by the method of Jeanes et al. (34). The medium contained the following reagents:

10.00 g. sucrose
0.25 g. yeast extract
0.50 g. K_2HPO_4
0.02 g. $MgSO_4 \cdot 7H_2O$
0.06 g. $(NH_4)_2SO_4$
0.10 % NaCl
100 ml. water

The sterilized medium was inoculated with a loopful of an actively growing culture of L. mesenteriodes B-512F, grown from the dry culture received from the Northern Regional Research Laboratory. The inoculated medium was incubated for three days at 25°C. This culture was used to inoculate the 100 ml. of medium used for the dextran isolation.

The latter medium was incubated for 26 hours. It was found that this time period produced a 10% yield of dextran based on the available glucose moiety of sucrose. By increasing the duration of the incubation to 72 hours the yield was increased to 50%. The dextran isolated from the medium as follows: the medium was centrifuged at 25,000 x g for 10 minutes to remove the cells and absolute ethyl alcohol was added to give a 50% solution (v/v) which precipitated the dextran. The precipitate was centrifuged at 3000 x g and washed several times with 50% ethanol. The gummy mass was dried in vacuo for 5 days and weighed. The dry dextran was used for the preparation of isomaltose (33).

To 100 ml. of a 2% corn steep mixture was added two grams of dextran and the mixture was autoclaved at 15 lbs. pressure for 30 min. The mixture was cooled, inoculated with the spores of P. finiculosum NRRL 1132 and then placed in a shaker to incubate at 25°C. Usually after 5 days the liquid became clear except for the colonies of mold that were visible. The mixture was then heated to boiling to precipitate the protein, filtered and the filtrate deionized with MB-3 mixed resin. After drying the eluate in air, a portion of the dry residue was chromatographed on paper in the usual manner using maltose as a reference. The developed chromatogram (Fig. 8) showed the presence of glucose, fructose, isomaltose and a slower migrating spot which has an R_f value similar to that of the dextrotriose reported by Pazur (35). This trisaccharide is reported to have a structure of three glucose units linked alpha-1-6.

The purification of isomaltose and the dextrotriose from this mixture

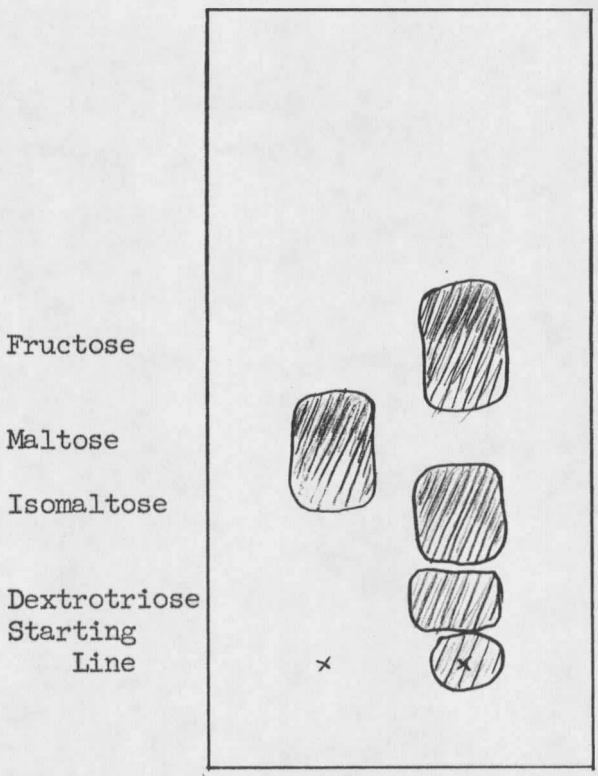


Figure 8. Chromatogram of a P. finiculosum exoenzyme hydrolysate of bacterial dextran using maltose as the reference.

was achieved by paper chromatography. A 17" x 18" sheet of filter paper was streaked several times along a line 5 inches from the top of the sheet with a solution of the sugar mixture. The sheet was then descended with the n-butanol, pyridine, water mixture mentioned earlier, for 18 hours. The sugars were identified and purified by the techniques used for the isolation of isomaltose from the C.P. glucose.

The purified sugars were identified chromatographically using the isomaltose formed from amylopectin as a reference. The dextran isomaltose did not show any chromatographically detectable impurities (Fig. 9). The sugar was further characterized by its optical rotation and infrared spectrum (36).

The optical rotation was measured with a Kern polarimeter. A 0.0555 g. sample of isomaltose was dissolved in 3.0 ml. of water at 25°C. This solution was transferred to a tube 6.35 cm. in length for measurement. The observed rotation was + 1.40°. The calculation of the optical rotation was as follows:

$$(\alpha)_D^{25^\circ} = \frac{\text{Observed rotation}}{l \times c}$$

l = length of tube in decimeters
c = concentration in g./cc.

$$(\alpha)_D^{25^\circ} = \frac{1.4}{0.635 \times 0.0185}$$

$$(\alpha)_D^{25^\circ} = + 119^\circ$$

The reported optical rotation for isomaltose is +120° (33,37).

The infrared spectra of isomaltose and maltose were both recorded from potassium bromide pellets. The pellets were prepared by drying solutions

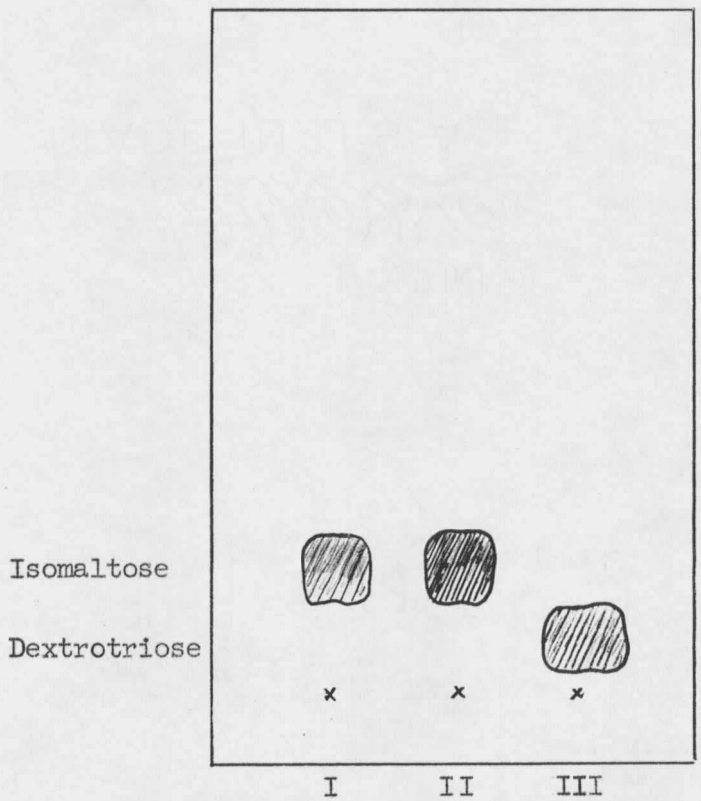


Figure 9. Chromatograms of chromatographically purified isomaltose (II) and dextrotriose (III) made from bacterial dextran. Isomaltose made from amylopectin was used as the reference (I).

containing 6 mg. of each sugar in 10 ml. of water containing 500 mg. of KBr. The solutions were dried in a desiccator for two weeks. This was followed by drying in a vacuum oven at 97°C. for 4 hours. The dry mixture was pulverized in a vibrating capsule that contained a small metal bar which moved freely in the capsule, grinding the sample. The pulverized material was then pressed in a standard die for 40 minutes under 9 tons pressure. The spectra of the two sugars were recorded with a Beckman IR 4 recording spectrophotometer. Both maltose and isomaltose exhibited spectra identical with the known spectra of the sugars (Fig. 10).

No attempt was made to calculate the yield of isomaltose from the dextran. The hydrolyzed dextran mixture was stored in a desiccator and the isomaltose was prepared chromatographically when needed for the following experiments.

6. The nature of the enzyme preparations.

New preparations of the 0-30% and 30-40% ammonium sulfate fractions were made for the following experiments. The enzyme preparations were made according to the flow sheet on the following page.

This preparation would not respond to the addition of isomaltose for 48 hours after the fractionation. After this time activation by the disaccharide could be detected. The reason for this phenomenon was not apparent at this time.

The 0-30% enzyme preparation was assayed for P_i release using isomaltose as substrate. The experiment involved recording any increase in inorganic phosphate in the assay mixture due to the additions of isomaltose

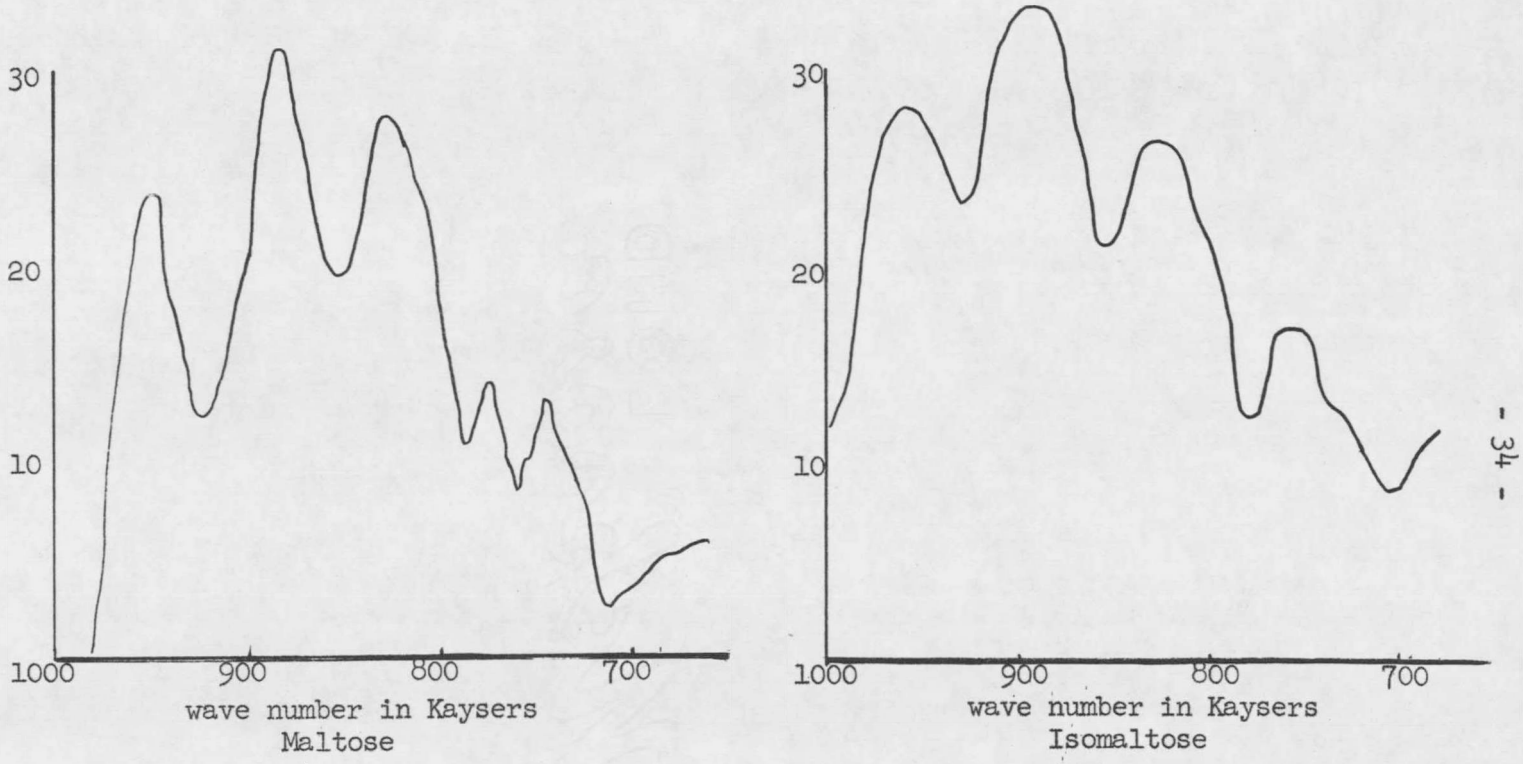
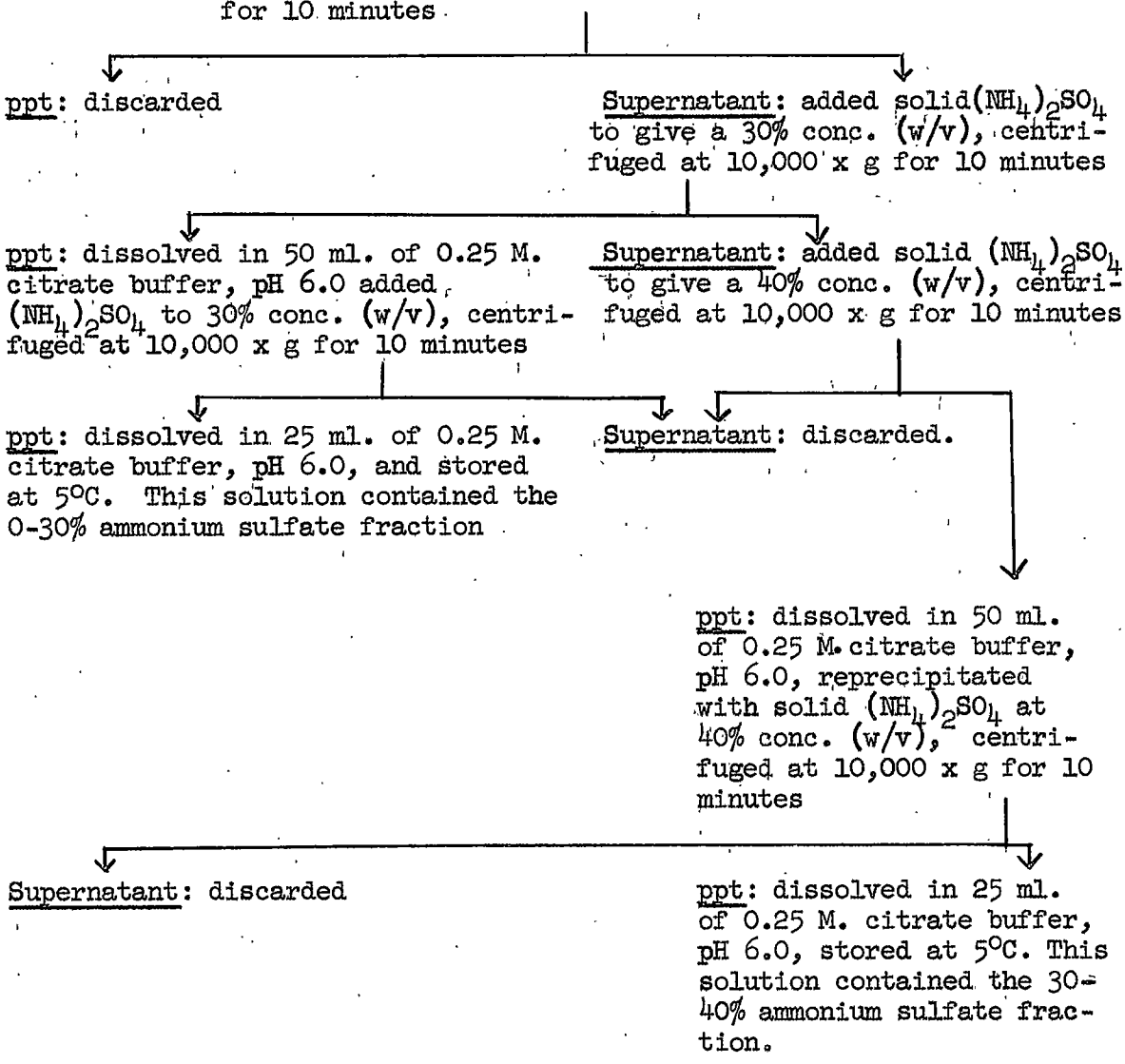


Figure 10. The infrared spectra of chromatographically purified C.P. maltose and of chromatographically purified isomaltose prepared from bacterial dextran.

Preparation of the 0-30% and 30-40% ammonium sulfate fractions from Russett potato juice

300 g. of Russett potatoes were peeled, and thinly sliced, soaked in $Na_2S_2O_4$ (7g./1.) for 20 minutes, washed well with tap water, and pulped in a Waring blendor. The juice was expressed through six thicknesses of cheesecloth, centrifuged at 10,000 x g, for 10 minutes, dialyzed for 18 hrs.vs. cold running tap water, cooled to 5°C, and centrifuged at 10,000 x g for 10 minutes



and glucose-1-phosphate.

Incubation mixtures contained the following reagents: 2.0 ml. of 0.25 M. citrate buffer, pH 6.0; 0.5 ml. of the 0-30% enzyme preparation; 20 μM. of glucose-1-phosphate; 0.5 mg., or 1.4 μM. of isomaltose; and distilled water to a total volume of 5.0 ml.

A zero time assay was made that contained all of the reagents except isomaltose.

The results which are recorded in Figure 11 indicate the rate of inorganic phosphate released was not a linear function of time. There was a rapid release of inorganic phosphate into the medium followed by a rapid decrease in the next few minutes. Then a second release of inorganic phosphate followed. A series of assays was made using varying concentrations of isomaltose as substrate to find the effect, if any, on this curve (Fig. 12). The assays were identical to those above except that 3 μM. and then 6 μM. of isomaltose were added. As is shown in Figure 12, the increase in the concentration of isomaltose affected a linear release of P_i. A previous article on a study of starch phosphorylase by Porter (38) explained this phenomenon. When sufficient primers are present to allow starch phosphorylase to work at maximum velocity, phosphatase activity present in the mixture is inhibited due to the starch phosphorylase effectively pulling the phosphatase reaction to the left. This is good evidence that when the isomaltose was present in concentrations of 6 μM. per 5 ml. primers were being furnished to starch phosphorylase at a rate equal to or exceeding that required by starch phosphorylase for maximum

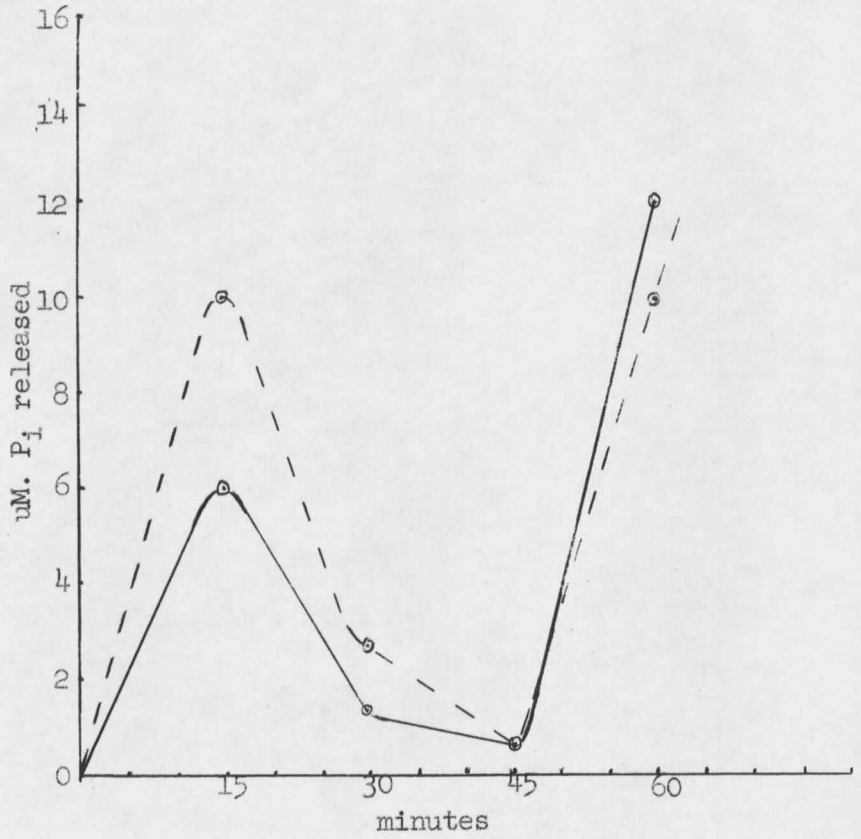


Figure 11. The effect of the addition of 1.4 μM. of isomaltose on the release of inorganic phosphate from 0.5 ml. of the 0-30% ammonium sulfate fraction from potato juice using 20 uM. of glucose-1-phosphate as a substrate.

Legend: _____1.4 μM. of isomaltose added
- - - - -no isomaltose added.

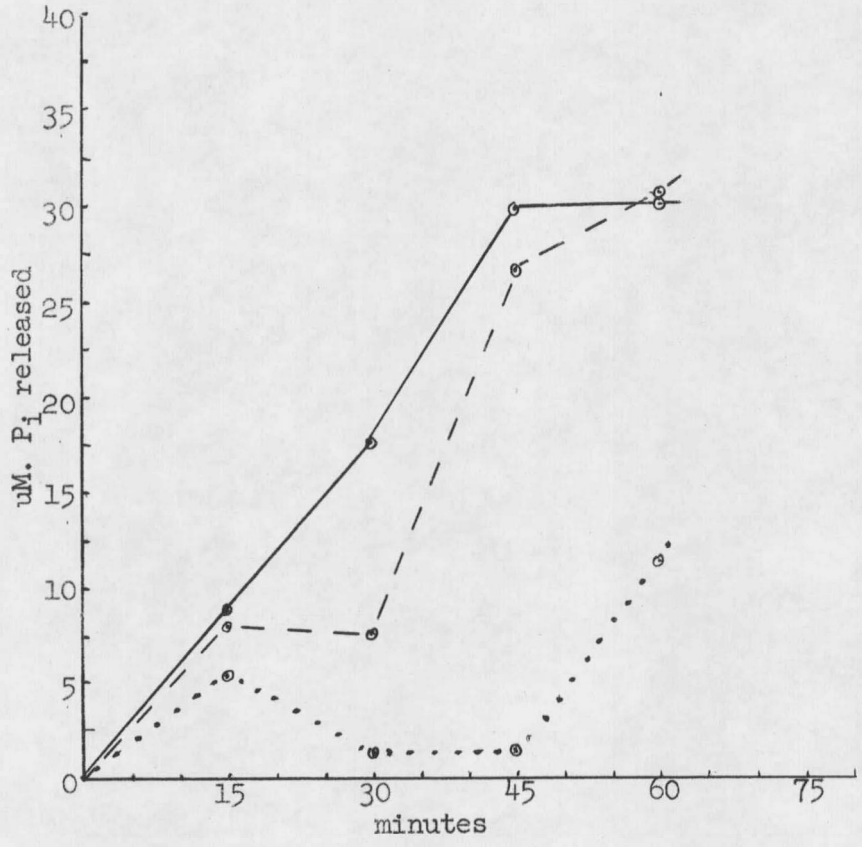


Figure 12. The effect of increasing concentrations of isomaltose on the release of inorganic phosphate in assays of the 0-30% ammonium sulfate fraction from potato juice containing 40 µM. of glucose-1-phosphate and primer impurities as substrates.

Legend: _____ 6 µM. of isomaltose added.
- - - - - 3 µM. of isomaltose added.
. . . . . 1.4 µM. of isomaltose added.

velocity.

The mixture was tested for phosphatase activity using fructose-6-phosphate as substrate. To 0.5 ml. of enzyme preparation was added 10 μM. of fructose-6-phosphate and the volume brought to 2.0 ml. with water. An aliquot was taken after one hour and assayed for inorganic phosphate. The enzyme released 128 μg. of inorganic phosphate into the assay mixture in one hour. This did not agree with the assays above, but no hemiacetal phosphate compounds were available that could be used in the presence of starch phosphorylase. This reaction is difficult to measure correctly since the true substrate of the phosphatase cannot be used due to the presence of primers of starch phosphorylase in the enzyme preparation. Therefore it was concluded that there was a phosphatase present that was more specific for glucose-1-phosphate than for ester phosphates. Only the presence of a phosphatase could explain the results that were found. Any other theory would have to include an intermediate of a type that would allow for the resynthesis of glucose-1-phosphate starting with the same compound. The fact that the presence of primer material at concentrations exceeding the maximum velocity requirements of starch phosphorylase will eliminate phosphatase activity is reported in the literature substantiates that the same problem has been encountered before in preparations containing starch phosphorylase.

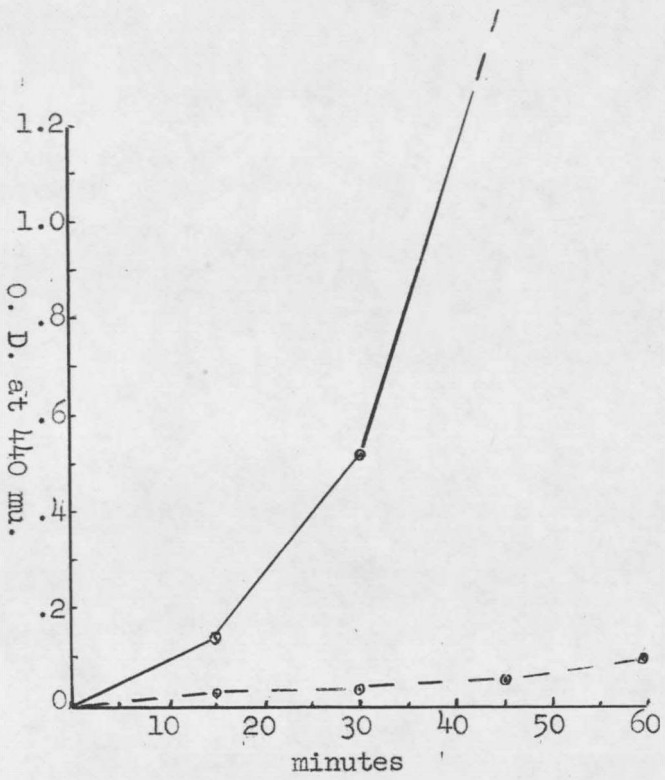
Since the addition of 6 μM of isomaltose apparently eliminated the phosphatase phenomena, it was evidently involved in synthesis of primers at a rate rapid enough to keep starch phosphorylase at maximum velocity.

Incubation mixtures were prepared using 6 μM of isomaltose with the 0-30% enzyme preparation and glucose-1-phosphate to determine blue amylose-I_2 formation and P_i release at this concentration. These assays were made similar to those of the previous assay involving the 0-30% enzyme preparation using isomaltose as a substrate. The results are shown in Figure 13.

A complete description of the 0-30% enzyme preparation in terms of the enzyme systems that were operating under the conditions of the assays of the new enzyme was desired. Assays for the following enzymes were made: (1) amylase, (2) D-enzyme, and (3) starch phosphorylase.

(1) Amylase: to two 0.5 ml. aliquots of the enzyme preparation were added 0.2 ml.of a 1% starch solution and 1.0 ml. of citrate buffer, pH 6.0. The total volumes were made to 3.2 ml. with water. One ml. of one assay was made up to 3.0 ml. with the I_2-KI solution used previously and read at 440 mμ using a blank of one ml. of water plus two ml. of the I_2-KI solution. The reading at zero time was 70 transmission units. The other assay mixture was allowed to incubate for 1 hour at 37° C. and then treated as the zero time assay and read also at 440 mμ. The reading was 72.5 transmission units. This very small difference indicated that only a small amount of amylase was present in the mixture. The test told nothing of the kind of amylase that was present, only that there was a trace of amylolytic activity.

(2) D-enzyme: a mixture identical to that of the 1 hour amylase assay was made with the addition of 10 μM. of glucose. The assay mixture



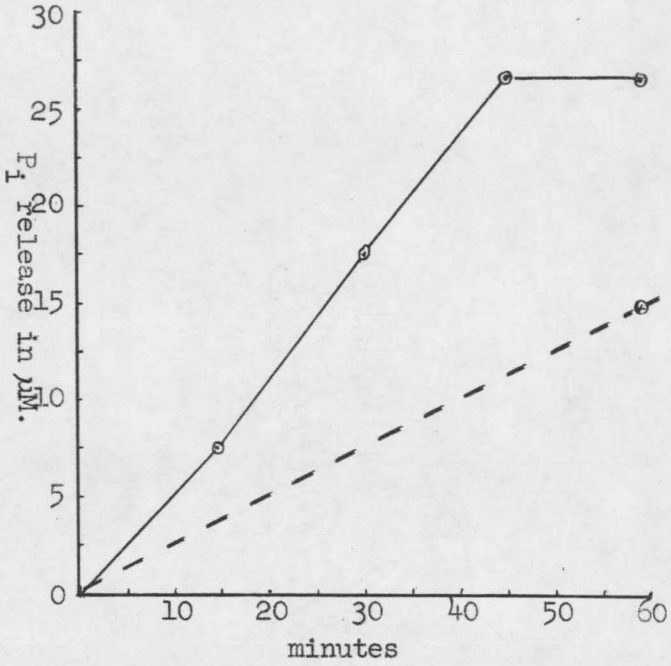


Figure 13. The effect of the addition of 6 μM. of isomaltose on the formation of iodine complexing molecules and on the release of inorganic phosphate from assays containing 0.5 ml. of the 0-30% ammonium sulfate fraction of potato juice, and 40 μM. of glucose-1-phosphate at a total volume of 5 ml.

Legend: _____ 6 μM. of isomaltose added.
- - - - - -no isomaltose added.

was also incubated for 1 hour as was the amylase assay and treated identically as the amylase assay. A value of 72.5 transmission units was recorded. This was the same as when no glucose was added. This meant that glucose did not affect the degradation of starch, thus ruling out D-enzyme activity from the preparation. Had any D-enzyme been present, a measurable decrease in the blue color would have been observed due to the breakdown of the long polymers with glucose acting as the acceptor molecule.

(3) Starch phosphorylase: 0.25 ml. of the enzyme was incubated with 0.2 ml. of a 5% starch solution; 1.0 ml. of a 0.25 M. citrate buffer, pH 6.0; and 20 μM. of glucose-1-phosphate at a final volume of 2.5 ml. Aliquots were assayed for inorganic phosphate at 15 min. intervals. These assays were made using the 0-30% enzyme preparation and the 30-40% enzyme preparation. Zero time assays were made in both cases. The results may be found in Figure 14. The 0-30% preparation had a much more active starch phosphorylase than did the 30-40% preparation, which had not reached equilibrium even after 1 hour.

The 0-30% enzyme preparation was assayed for carbohydrate material present by the method of Dubois and Gillis (26), which utilizes concentrated sulfuric acid and phenol to form colored furfuraldehyde derivatives quantitatively from carbohydrate material. A 0.2 ml. aliquot of the enzyme mixture was used for the assay. To the enzyme was added a 0.2 ml. aliquot of an 80% phenol solution and then water to a volume of 2.0 ml. Concentrated sulfuric acid was added to a total volume of 7.0 ml. The tubes were cooled in a water bath at 25°C. for 15 minutes and the color

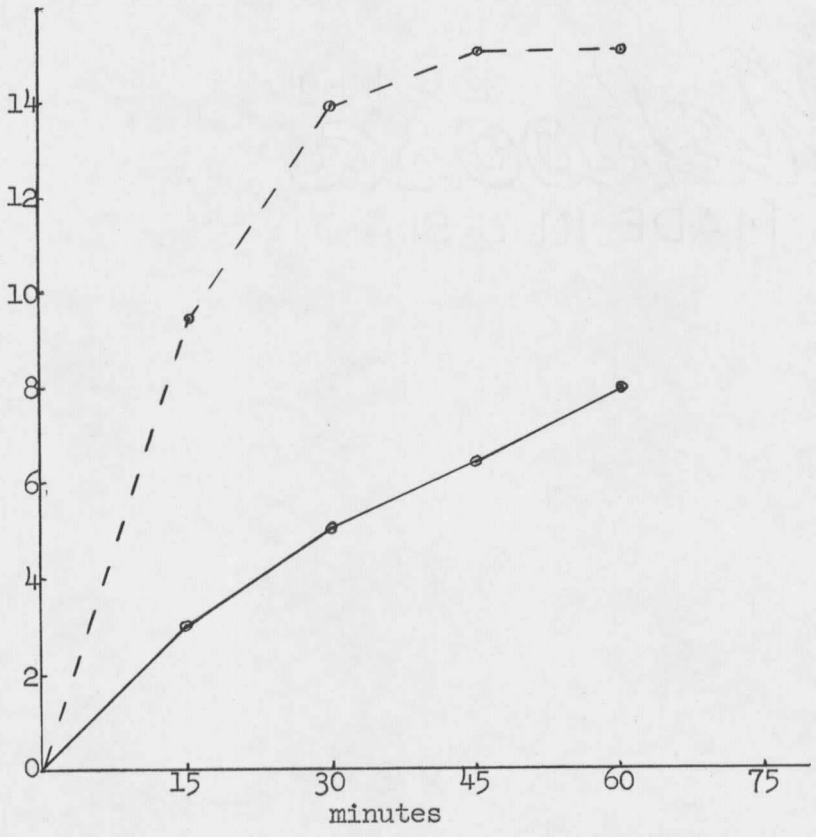


Figure 14. Assays of inorganic phosphate released by starch phosphorylase from the 0-30% and 30-40% ammonium sulfate fractions of potato juice. Assay mixtures contained 0.25 ml. of enzyme preparations; 0.2 ml. of a 1% starch solution; 20 uM. of glucose-1-phosphate; and 1.0 ml. of a 0.25 M. citrate buffer, pH 6.0. Final volumes were 2.5 ml.

Legend: - - - - - 0-30% fraction
_____ 30-40% fraction

read at 490 mμ. on the Model B spectrophotometer. The enzyme preparation contained 396 μg. of carbohydrate material per. ml. of enzyme preparation as impurities.

The carbohydrate material in the enzyme preparation was identified by paper chromatography. A 0.2 ml. aliquot of the enzyme preparation was placed in a test tube and the protein was precipitated by placing the tube in a boiling water bath at 97° for 10 minutes. The mixture was passed through a 1 cm. x 10 cm. column containing MB 3 mixed resin. The eluate from the column was collected and dried in air. The total carbohydrate residue was chromatographed on paper using the usual reagents and including glucose and maltose as references (Fig. 15). The major carbohydrate impurities were glucose, maltose and maltotriose.

The presence of glucose in the preparation suggested that the amylase present was alpha amylase. Beta amylase would not have formed any glucose from the breakdown of maltodextrins. The 18 hour dialysis would have removed any glucose from the potato juice along with the other small molecular weight sugars.

7. A starch phosphorylase or a transglucosidase.

The problem was now narrowed to the question of whether the action of the new enzyme involving isomaltose was a phosphorylase or a transglucosidase. Experiments were devised which would yield the maximum information about the nature of the reaction. It was decided to utilize carbon-14 compounds to try to clarify the situation.

Two isotope experiments were decided upon. The first was to incubate

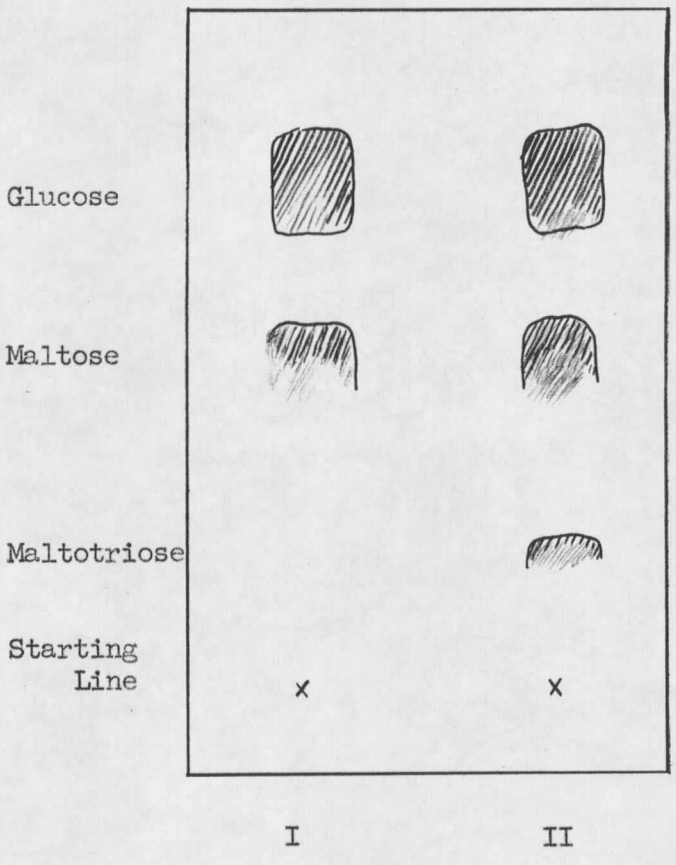


Figure 15. Chromatogram of the carbohydrate material that is present as impurities in the 0-30% ammonium sulfate fraction of potato juice after standing several days. II was spotted with the carbohydrate residue. I is a reference containing glucose and maltose.

the enzyme preparation with isomaltose and glucose-U-C^{14} for several hours. If a transglucosidase was involved, an exchange reaction involving an enzyme-glucose intermediate should incorporate glucose-U-C^{14} into isomaltose. This type of exchange had been observed earlier from an enzyme in Aspergillus oryzae (35). However, this latter enzyme used isomaltose and maltose to form panose, not maltotriose. A phosphorylase reaction would not result in an exchange, and no incorporation of glucose-U-C^{14} would occur.

The second experiment planned was to synthesize isomaltose-U-C^{14} from sucrose-U-C^{14}, through the production of dextran and the subsequent hydrolysis by microorganisms. This isomaltose-U-C^{14} was then to be incubated with maltose, glucose-1-phosphate and the 0-30% enzyme preparation. Any radioactive products that formed would be identified. If the enzyme was a phosphorylase, no free glucose-U-C^{14} would be released into the medium and the only tagging besides in the isomaltose would be in polysaccharides. If a transglucosidase was present, glucose-U-C^{14} would be released into the system and radioactivity would be found as glucose-U-C^{14} and also found incorporated into higher molecular weight maltodextrins. In the latter case the amount of radioactive incorporation into polymers should approximate that found in glucose.

A.) Experiment involving glucose-U-C^{14}

The first isotope experiment was initiated by placing 0.5 ml. of the 0-30% enzyme preparation into a test tube containing 0.7 mg. of

glucose-U-C^{14} which contained 1.4 μc[4] of carbon 14; 1.5 mg. of isomaltose; and the total volume was made to 1.0 ml. with water.

One half of the mixture was removed immediately after adding the enzyme, and to this mixture was added 0.2 ml. of 10% TCA[5]. This material was heated in a boiling water bath for 5 minutes. This mixture was considered the zero time incubation mixture.

The test tube containing the other half of the mixture was covered with a large marble to prevent evaporation and placed in a constant temperature oven at 37°C. for 48 hours.

The mixture that was denatured with TCA in the boiling water bath was cooled and deionized by passing through a column of MB-3 mixed resin and the column was washed with several ml. of distilled water. The combined eluates were dried in an air jet in a small collecting tube. The residue was taken up with a few drops of water and the entire solution spotted on a 7" x 12" sheet of filter paper on a line 1" from the bottom.

At the end of the 48 hour incubation period the other half of the mixture was treated as above and also spotted on the sheet of chromatography paper approximately one and one half inches away from the first spot on the same line. Glucose and isomaltose were spotted as references. The chromatogram was ascended two times to a height of 9" with the n-butanol, pyridine, water solvent used in preceeding chromatographic work. The twice ascended chromatogram was dried and the sugars identified with CD-1

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4. μc -- Microcurie
5. TCA -- Trichloroacetic acid

developer (Fig. 16). The chromatograms were placed on 'no screen' X ray film and allowed to expose for five days. The film, when developed (Fig. 17), revealed radioactivity at glucose, isomaltose and maltotriose at zero time. Radioactivity was observed at isomaltose and at the base line of the 48 hour reaction mixture. The activity in the zero time mixture of isomaltose and maltotriose was thought to be mechanical binding of glucose-U-C^{14} since such a short time of incubation was allowed. However, a mixture of isomaltose, maltose and small amounts of small primer molecules of approximately the same concentration as was present in the enzyme preparation along with glucose-U-C^{14} were chromatographed identically to that of the incubation mixtures. This revealed only a small mechanical binding of glucose-U-C^{14} by the maltotriose and no mechanical binding by any other polysaccharides. Isomaltose did not bind any glucose-U-C^{14} in this chromatogram. This indicated that the radioactivity in the zero time assay was a true incorporation and not mechanical binding and that the mixture was not of zero time but an approximate 5 minute incubation mixture. The experiment suggested the presence of a transglucosidase. The significance of the results will be treated fully in the discussion.

B.) Experiment involving isomaltose-U-C^{14}

The second experiment, involving isomaltose-U-C^{14}, required the preparation of this disaccharide since the compound is not commercially available. The preparation of this sugar was by the same method used for the preparation of non-radioactive isomaltose.

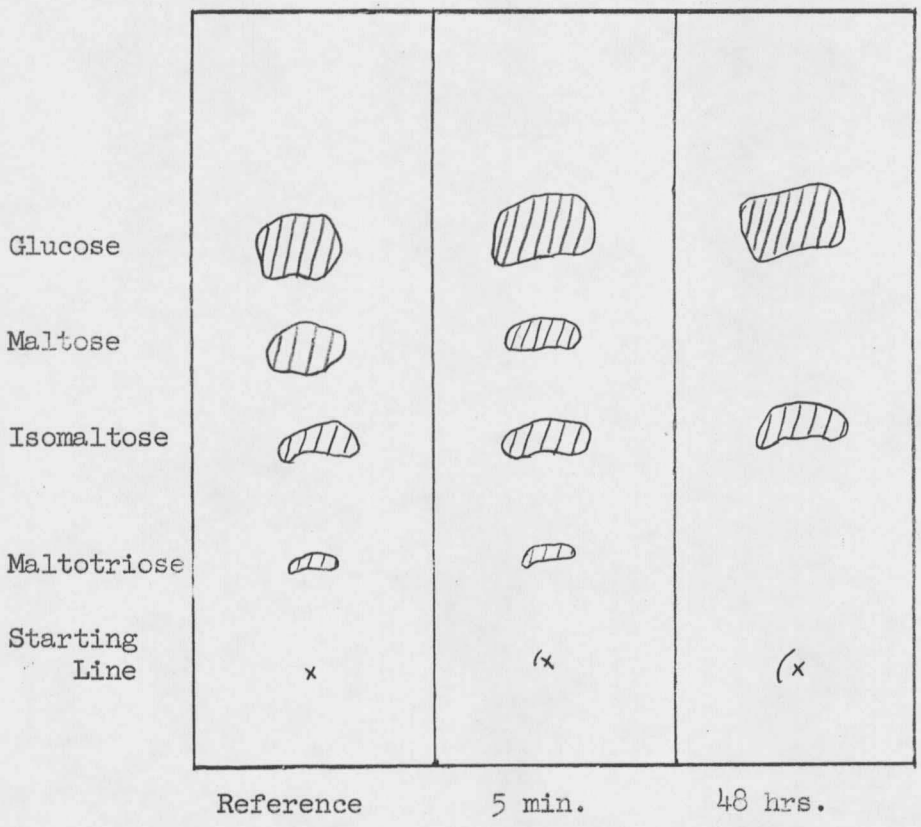

Figure 16. Chromatograms of an incubation mixture containing isomaltose, glucose-U-C^{14}, and the 0-30% enzyme preparation. The Chromatograms were ascended two times to a height of 9 inches and developed with CD 1 developer.

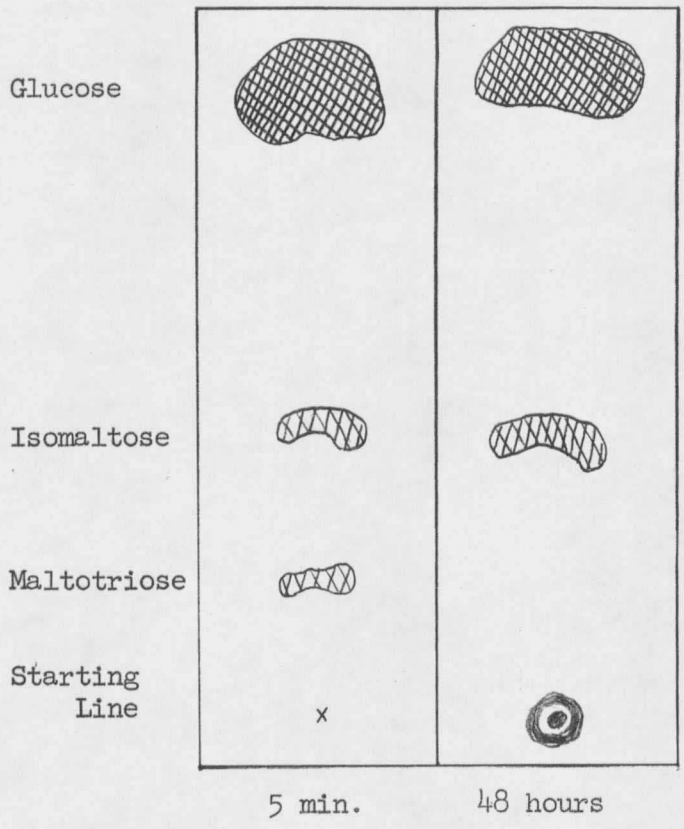

Figure 17. Radiochromatogram of an incubation mixture containing isomaltose, glucose-U-C^{14} and the 0-30% enzyme preparation.

Radioactive sucrose was utilized in the preparation of the dextran. The dextran was then degraded to isomaltose and recovered by paper chromatographic techniques. In order to achieve yields that could be recovered, considerably more sucrose had to be used than the radioactive material available. The sucrose was purchased in 0.29 mg. lots containing 0.05 mc[6]. Experiments were performed to establish the amount of sucrose that was needed to get a reasonable yield of isomaltose.

The medium used was identical to that on page 28 except that sucrose was omitted. Fifty ml. of this medium was placed in a 125 ml. Erlenmeyer flask and 1.0 g. of sucrose added. After the medium was autoclaved at 13 lbs. pressure for 30 minutes it was cooled and inoculated with a growing culture of L. mesenteroides. After incubating for 96 hours the mixture was centrifuged at 10,000 x g and the dextran precipitated by the addition of absolute ethanol to give a final concentration of 50% ethanol (v/v). The dextran was centrifuged and dried in an air stream over a steam bath. The yield was only 0.020 g. or 4% of the theoretical yield. The experiment was repeated and the incubation period extended to 5 days.

On isolation of the dextran, the yield was 0.145 g. of dry dextran representing 29% of the available glucose.

The theoretical radiochemical yield of dextran on the basis of a 5 day incubation period was calculated. The 0.05 mc of sucrose, if added to the media containing the 1.0 g. of sucrose, would yield theoretically

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6. mc -- Millicurie

0.29 x 0.05 x 0.5, or 0.00725 mc. of glucose-U-C^{14} incorporated into the dextran, or 0.05 uc. per mg. of dextran. This was considered an acceptable value in terms of the isotope experiment planned. That is, the isomaltose isolated from this dextran should have had enough activity in assay quantities to expose X ray film in approximately 14 days.

A problem in this experiment was in introducing the sucrose-U-C^{14} aseptically to the sterile incubation mixture. It has been shown that autoclaving destroys C^{14} containing sugars (39). It was necessary then to introduce the radioactive material after sterilization. This was accomplished by first preparing the sterile medium exactly as had been done just previously. The cotton plug was removed from the cooled, sterilized medium and the sealed glass vial containing the sucrose-U-C^{14} was gripped with two pairs of heavy tweezers. The tweezers and vial were washed well with absolute ethanol. The vial was then lowered into the opening of the flask and one end crushed into the flask by one pair of tweezers. The vial was then dropped into the medium. Inoculation was made from a stock culture and the cotton plug was replaced. This solution was placed in the shaker at room temperature. The shaking action dispersed the sugar from the open vial into the medium.

This medium was incubated for 5 days in the shaker. At the end of this time the contents of the flask were centrifuged at 10,000 x g for 10 minutes. The dextran was precipitated by adding absolute ethanol to a concentration of 50% ethanol (v/v) and the precipitate was centrifuged at 3000 x g for 10 minutes. The yield of dry dextran was 0.057 grams.

The degradation of the dextran was accomplished as before with NRRL 1132, P. finiculosum. As mentioned previously, the highest yield of isomaltose was achieved when the corn steep medium became clear. A 50 ml. portion of a 2% corn steep solution was employed for the preparation of the isomaltose. After sterilization at 13 lbs. for 30 minutes, the medium was inoculated and incubated at room temperature until the filtrate became clear except for visible colonies of mold. A 0.06 g. sample of untagged dextran was added to this mixture and incubation was continued for 24 hours. The purpose of this incubation mixture was to estimate the activity of the isomaltose producing enzyme which was present in the mixture.

The mixture was filtered through glass wool and the precipitate was washed with 50 ml. of distilled water. The filtrates were combined and deionized with MB-3 mixed resin and dried in an air stream. A portion of the combined filtrate was chromatographed in the usual manner and found to contain appreciable quantities of isomaltose. A quantitative recovery was not attempted.

The experiment was repeated as above except in this case the radioactive dextran was utilized.

The deionized, dry filtrate from the radioactive dextran hydrolysis was dissolved in 5 ml. of water. This total solution was streaked onto two 8" x 18" paper chromatograms, 3" from the top. These chromatograms were descended for 18 hours with the n-butanol, pyridine, water mixture. A 1 cm. wide strip was cut from the edge of the chromatograms and the

carbohydrate material identified with CD-1 developer. The isomaltose was identified by its R_f value. The area of this disaccharide was cut out of the chromatograms and the strips eluted. The eluates were dried in air and placed in a desiccator. The product was chromatographed using maltose as a reference. The only spot appearing from this eluate was that of the isomaltose. The yield of isomaltose was 0.011 grams.

A 0.30 mg. sample was plated onto a planchette, dried in vacuo for 24 hours and counted with a thin window geiger tube. This sample contained 442 counts per minute over background, or 1473 counts per minute per mg.

The radioactive isomaltose was used in the following experiment to determine what happens to the molecule when incubated with the enzyme preparation, maltose and glucose-1-phosphate.

Five mg. of the radioactive isomaltose, equalling 7315 counts per minute, were incubated with a mixture containing 2.0 ml. of 0.25 M. citrate buffer, pH 6.0; 40 uM. of glucose-1-phosphate; and 2.0 mg. of maltose at a total volume of 5.0 ml. One ml. aliquots were taken from the mixture at zero time, 30 minutes, and the remainder of the mixture at 60 minutes. The aliquots were heated at 97° for 5 minutes in a boiling water bath, filtered through MB-3 mixed resin and dried in an air stream. The residues were dissolved in 0.1 ml. of water and all of the zero time and 30 minute mixtures were spotted on a sheet of filter paper. One third of the 60 minute mixture was spotted on the same sheet. Glucose, maltose and isomaltose were spotted as references. The chromatogram was ascended two times to a height of 9 inches with the usual solvent. The chromatogram

was dried and then cut into strips perpendicular to the starting line, thus separating the chromatogram into individual chromatograms of the different incubation mixtures. These strips were placed on 'no screen' X ray film and allowed to expose for 40 days. After exposure, the film was developed. While the isomaltose spot was well defined, two other spots were barely detectable. One was at the R_f of glucose, the other was at the starting line (Fig. 18). The chromatograms were then developed with CD-1 developer. The developed spots confirmed the spots found on the X ray film as isomaltose, glucose and longer polysaccharides at the starting line (Fig. 19). The only significant radioactivity besides isomaltose was found at the glucose spot in the one hour assay chromatogram. The count was only 4 counts per minute above background when the strip was counted to approximately a 10% error.

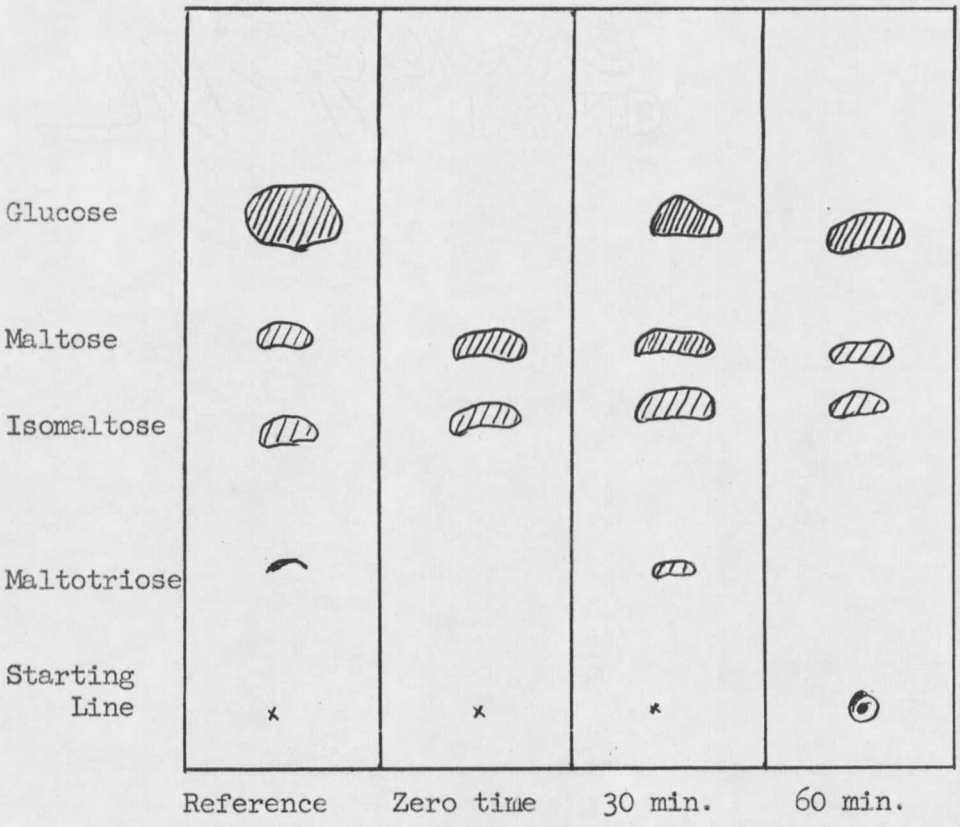

Figure 18. Chromatogram of an incubation mixture containing isomaltose-U-C^{14}, maltose, glucose-1-phosphate and the 0-30% enzyme preparation. The chromatograms were ascended two times to a height of 9 inches and developed with CD 1 developer.

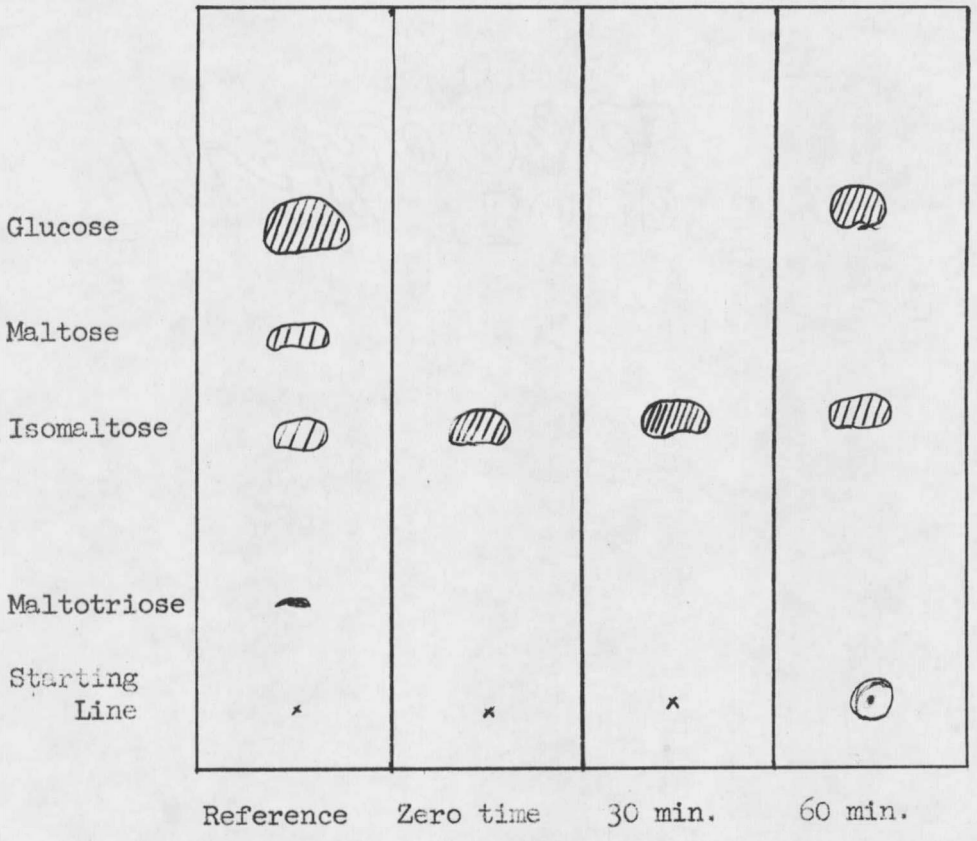

Figure 19. Radiochromatogram of an incubation mixture containing isomaltose-U-C^{14}, maltose, glucose-1-phosphate and the 0-30% enzyme preparation. The refence chromatogram was developed with CD 1 developer.

III. DISCUSSION

The ultimate aim of this research was to determine the type of reaction that was responsible for donating primers to the starch phosphorylase reaction. The only assays available for measuring this phenomenon were those for starch phosphorylase. The action of the new enzyme was not measured directly, but was detected by the increased rate of starch phosphorylase activity when isomaltose was added to assay mixtures containing both starch phosphorylase and the new enzyme with glucose-1-phosphate.

Several minor problems were encountered during the research. The most important of these was that of the inorganic phosphate analysis in which citrate ion in the assay mixtures bound the molybdate ion in the color reagent preventing the formation of the phosphomolybdate complex, thereby producing erratic results. Since the entire problem was based on inorganic phosphate release the inconsistency and unreliability of the phosphate analyses made the whole problem rather confusing. However, by increasing the concentration of molybdate in the color reagent to 7.5%, the inorganic phosphate could be assayed accurately in the presence of the citrate ion.

The early work on the enzyme systems suggested an activation of starch phosphorylase by the addition of C. P. glucose. The glucose additions increased the rate of formation of polysaccharide, but the increased rate was small in comparison with the amount of glucose added (Fig. 1). It was assumed that this was simply a case of very little of

the active enzyme being present. It was not until the isomaltose was found to prime starch phosphorylase in the amylopectin digest (Fig. 4) that it was considered as a possible impurity in C. P. glucose and the cause of the observed priming by the C.P. glucose. When isomaltose was found as an impurity in the glucose, it was considered probable that the small amount of activation by the glucose was due to the isomaltose impurity and not due to the presence of only a trace amount of the active enzyme.

The mechanism of how the isomaltose primed the starch phosphorylase reaction was unknown. Two main possibilities are:

(1) a phosphorylase, as follows:

$$\text{Isomaltose} + \text{Glucose-1-phosphate} \rightleftharpoons \text{Isomaltotriose} + P_i$$

$$\text{Isomaltotriose} + n\ \text{Glucose-1-phosphate} \rightleftharpoons \text{large molecules eventually acting as substrate for starch phosphorylase} + P_i$$

and:

(2) a transglucosidase:

$$n\ \text{Isomaltose} + \text{acceptors} \rightleftharpoons \text{primers for starch phosphorylase} + n\ \text{Glucose}$$

The formation of primer molecules was a direct result of the addition of isomaltose. This synthesis could have involved any of several molecular species in the assay mixtures. The phosphorylase would have involved the isomaltose with glucose-1-phosphate while the transglucosidase could have used isomaltose alone as the donor and acceptor or else utilized molecules present in the assay that were impurities in the enzyme preparation.

There were several facts that supported a transglucosidase mechanism. First, when a fresh preparation of the 0-30% ammonium sulfate fraction was made, isomaltose additions to the preparation did not increase the rate of the starch phosphorylase reaction. However, after standing for several hours, isomaltose did increase the rate of release of inorganic phosphate by starch phosphorylase (Fig. 6). Starch phosphorylase preparations made by ammonium sulfate fractionations always carry over carbohydrate material with the fractionated protein. In the case of the 0-30% fraction, these carbohydrates had to be rather long polymers, namely, the maltodextrins. The small dextrins and glucose would have been lost during the 18 hour dialysis. Therefore in a freshly made preparation no small maltodextrins could have been present to act as acceptor molecules. After several hours, the amylase present would have broken down the long maltodextrins to form acceptor molecules for a transglucosidase. This was evidenced on the chromatogram of the carbohydrate material in an aged preparation (Fig. 15).

A phosphorylase, utilizing isomaltose and glucose-1-phosphate, should have been as active, or more active in a freshly made preparation than in an older preparation.

Second, when the amylopectin hydrolysis by alpha amylase (page 20) was chromatographed and the chromatogram developed with the glucose-1-phosphate--starch phosphorylase spray followed by spraying with I_2 in KI (Fig. 4), the larger branched fragments, e.g. isomaltotriose, should have primed starch phosphorylase faster than isomaltose had a new phosphorylase

been present. These molecules should have been intermediates in the synthesis of singly branched dextrins that eventually would prime the starch phosphorylase reaction. They did not exhibit this priming activity on the chromatograms. The branched components migrate at a slower R_f than the linear equal molecular weight components (10). That is, isomaltose migrates just behind maltose, isomaltotriose just behind maltotriose, etc. These branched molecules will not prime starch phosphorylase until they are about six glucose units away from a branch. The chromatogram exhibited well defined priming spots at the R_fs equal to the linear components on the reference chromatogram, except at the maltose-isomaltose R_f. This minimized the possibility of a phosphorylase of the type mentioned.

Another possibility of a phosphorylase type of reaction would have been as follows:

(1) Isomaltose + Glucose-1-phosphate $\longleftrightarrow$ α 1-4 (isomaltosyl) D-glucose + P_i

(2) α 1-4 (isomaltosyl) D-glucose + n Glucose-1-phosphate $\longleftrightarrow$ 'primers' + P_i

thus building up another type of singly branched molecule. The trisaccharide shown would also have been present in the amylopectin digest and would have had an R_f approximately the same as isomaltotriose. This mechanism was also considered doubtful for the same reasons given for the preceeding mechanism involving singly branched molecules.

A third experimental evidence of a transglucosidase action was the starch phosphorylase primer at the R_f of the disaccharides on the

chromatogram of the amylopectin digest (Fig. 4). This priming activity on the chromatogram occurred at the R_f where maltose and isomaltose overlapped. This would place a donor (isomaltose) and an acceptor (maltose) together at this spot, thus resulting in the buildup of the triose concentrated at the spot shown. The triose then would act as a primer for the starch phosphorylase reaction.

Another contribution to the transglucosidase theory was the slight lag in inorganic phosphate release in the first 15 minutes of an assay when isomaltose was added along with glucose-1-phosphate to the 0-30% enzyme preparation (Fig. 13). This would indicate a slow release of inorganic phosphate but still a donation of primers at a high rate during this time interval. If a phosphorylase was donating primers at a rate to keep starch phosphorylase at maximum velocity then there should have been a rapid release of inorganic phosphate in the first several minutes, slowing down as the isomaltose disappeared. This was not observed.

If a phosphorylase was present that utilized glucose-1-phosphate and isomaltose to form starch primers at a slow rate, then the first portion of the curve could have been explained. However, calculations from data in Figures 13 and 14 minimized the possibility of a phosphorylase besides starch phosphorylase in the enzyme preparation. If two phosphorylases were operating, then the maximum velocity of the combined reactions should have exceeded the velocity of the starch phosphorylase reaction alone. A graph was made of the velocity of the reactions when using isomaltose as a substrate and using starch as a substrate. The velocity for

the starch phosphorylase reaction with starch as the substrate for the 0-30% enzyme preparation was 0.31 μM of P_i released/min./ 0.5 ml. of enzyme in the 15 to 30 minute time interval. The reaction using isomaltose as substrate for the same enzyme preparation, during the same time interval, exhibited a velocity of 0.64 μM of P_i released/min./0.25 ml. of enzyme preparation. These values were placed on a graph, plotting velocity vs. ml. of enzyme present in 5 ml. of the assay mixtures. The values were almost identical (Fig. 20). A line was drawn from the interaction of the two axes through the point. It can be seen that if two phosphorylases were operating simultaneously when isomaltose was used as a substrate, the value of the velocity of the combined reactions would have been above the line; or, the velocity of release of P_i from two reactions would have been greater than that of starch phosphorylase alone, per ml. of enzyme solution. This kinetic data greatly reduced any possibility of the presence of a new phosphorylase in the preparation.

The possibility that the enzyme was starch phosphorylase was ruled out when the 30-40% enzyme preparation containing active starch phosphorylase (Fig. 14) was not activated by isomaltose.

The new enzyme, in its role as a donor of primers for starch phosphorylase, was probably a transglucosidase, since the only energy source in the assay mixtures besides glucose-1-phosphate was that of the bonds present between sugar moieties. It was toward this theory that further work was directed.

Two transglucosidases are known to exist in the potato, D enzyme and

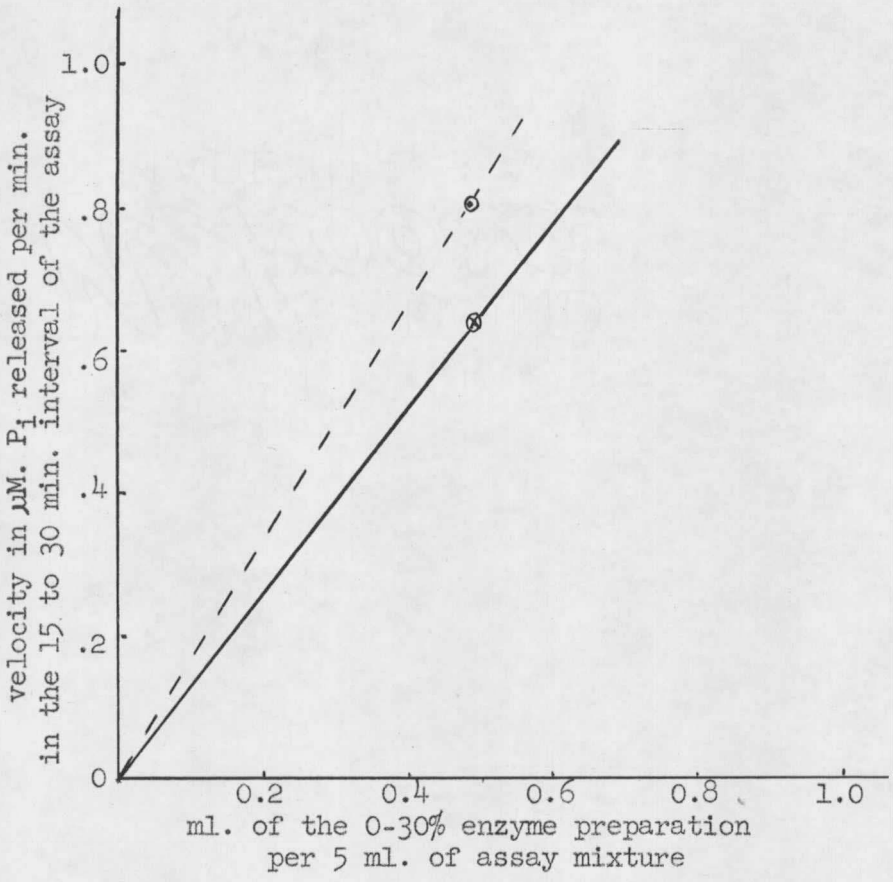

Figure 20. The plot of enzyme concentration vs. velocity of the starch phosphorylase reaction using both starch and isomaltose as substrates. Both plots lie on the same point. The dotted line represents the hypothetical case of an additional phosphorylase in the preparation.

Q enzyme. Both of these enzymes were discussed in the introduction. Q enzyme action was eliminated from consideration on the basis that it requires linear chains of at least 42 glucose units in length. D enzyme has been shown to have no action when isomaltose was used as the substrate (12). The latter enzyme was shown to be absent from the preparation containing the new enzyme. Therefore, any transglucosidase present was a new enzyme or at least new to plant metabolism. The enzyme that Pazur reported from A. oryzae (35) utilized isomaltose to form a dextrotriose molecule of three glucose units bonded alpha 1-6. This trisaccharide was a byproduct of isomaltose production from dextran by the exoenzyme of P. finiculosum. The dextrotriose had no effect on the release of P_i from the 0-30% enzyme preparation when incubated with the enzyme and glucose-1-phosphate. The enzyme was evidently not of the same type that Pazur reported. The lack of activity of the enzyme on this substrate was understandable in view of the primer role of the products of the new enzyme. Dextrotriose, being a doubly 'branched' molecule could not act as a primer for starch phosphorylase, nor could any polysaccharide containing completely alpha 1-6 bonds. This point was strengthened by the additional salivary amylase to incubation mixtures. This enzyme removed all I_2 staining properties of the mixture, indicating the presence of only α 1-4 bonds.

The problem of the acceptor molecule for a transglucosidase reaction was considered. Chromatography of the 0-30% enzyme preparation had previously revealed the presence of glucose, maltose and maltotriose. These

sugars were presumably present as a result of amylase action on long maltodextrins that were not removed by dialysis of the preparation. The earlier data showed that no new phosphorylase nor starch phosphorylase was utilizing the isomaltose and that the new enzyme was not using isomaltose as both the donor and acceptor molecule. Thus, the carbohydrate material present in the preparation as impurities must have assumed some type of role in the reaction.

The isotope experiments were designed to offer proof of the transglucosidase reaction and to offer knowledge of the role of the acceptor molecules in the preparation.

A transglucosidase mechanism involves an enzyme-glucose compound as an intermediate and requires acceptor molecules to complete the reaction. A reaction of this type involving isomaltose would require the first step as:

(1) Isomaltose + Enzyme $\rightleftharpoons$ Enzyme-glucose + Glucose

If glucose-U-C^{14} was incubated with the enzyme and isomaltose, some incorporation of radioactive glucose into isomaltose would occur due to the equilibrium of the reaction. When glucose-U-C^{14} was used with isomaltose as substrates for the new enzyme, the glucose was incorporated into isomaltose and also into the maltodextrins. After a 5 minute incubation period (Fig. 17), glucose-U-C^{14} was incorporated into both isomaltose and maltotriose. The first step (1) shown above cannot explain the incorporation of glucose-U-C^{14} into maltotriose unless an exchange reaction between the enzyme-glucose compound and glucose-U-C^{14} occurred.

This may be exemplified in the reaction as follows:

(2) Enzyme-glucose + Glucose-U-C^{14} $\rightleftharpoons$ Enzyme-glucose-U-C^{14} + Glucose

If glucose-U-C^{14} was substituted in the back reaction of (1) and (2) was not operating, it could never end up in the enzyme-glucose compound, and thus never be incorporated into the maltodextrins. Because of the incorporation of glucose-U-C^{14} into the maltotriose an exchange reaction of this type (2) must have occurred. The tagged enzyme-glucose-U-C^{14} could have then donated the glucose-U-C^{14} to maltose and formed the tagged maltotriose molecules observed in the 5 minute assay mixture as follows:

(3) Enzyme-glucose-U-C^{14} + maltose $\rightleftharpoons$ maltotriose-C^{14} + Enzyme

Maltose was a known constituent of the incubation mixture. The above mechanism was feasible since an enzyme is saturated with substrate in the first few milliseconds of the reaction. The enzyme-glucose formed would, in a very short time, begin the exchange reaction and incorporate C^{14} into both isomaltose and maltotriose.

The 48 hour incubation period (Fig. 17) exhibited incorporation of glucose-U-C^{14} into polysaccharides at the starting line and into isomaltose. This was consistent with the proposed mechanism involving an exchange reaction. No radioactivity was found at any oligosaccharides such as maltotriose, maltotetraose, etc. Since acceptor molecules were present in the enzyme preparation itself, the forward reaction of building up the maltodextrins would have continued as long as the acceptors were

of suitable size, slowing down as the acceptors became larger and less mobile. This action would have eliminated the acceptors of small size after an appreciable length of time, e.g. 48 hours. The lack of radioactivity in the smaller dextrins in the 48 hour incubation mixture had another important aspect. The glucose-U-C^{14} could not act as an acceptor for the glucose of the enzyme-glucose intermediate that is formed as the first step of the transglucosidase reaction to form maltose. The following reaction, then, did not take place:

$$\text{Enzyme-glucose} + \text{glucose} \rightleftharpoons \text{Maltose} + \text{Enzyme}$$

The glucose-U-C^{14} would have certainly been incorporated into maltose at a high rate and have resulted in highly tagged small dextrins had this reaction occurred. The chromatogram revealed no such tagging after 48 hours. It was concluded that maltose was the smallest acceptor molecule involved to form maltodextrins and that glucose may only act as an acceptor in the back reaction of the initial step involving isomaltose, that is:

(1) $\text{Isomaltose} + \text{Enzyme} \rightleftharpoons \text{Enzyme-glucose} + \text{Glucose}$

incorporating glucose-U-C^{14} into isomaltose. In the 48 hour incubation mixture isomaltose was highly radioactive.

From the isotope experiment and earlier evidence of the new transglucosidase the following mechanism is suggested:

(1) $\text{Isomaltose} + \text{Enzyme} \rightleftharpoons \text{Enzyme-glucose} + \text{Glucose}$

(2) $\text{Enzyme-glucose} + \text{Maltose} \rightleftharpoons \text{Maltotriose} + \text{Enzyme}$

(3) $n\ \text{Enzyme-glucose} + \text{Maltotriose} \rightleftharpoons \text{Higher molecular weight linear dextrins} + n\ \text{Enzyme}$

In a system containing starch phosphorylase and glucose-1-phosphate the small dextrins would immediately become substrates or 'primers' for starch phosphorylase so that reaction (3) above would be:

(3) Maltotriose + Glucose-1-phosphate $\xleftarrow[\text{phosphorylase}]{\text{starch}}$ Amylose + P_i

The second isotope experiment, involving isomaltose-U-C^{14} and glucose-1-phosphate as substrates for the 0-30% enzyme preparation supported the proposed mechanism. These results were not conclusive due to difficulties in detecting the radioactivity in the products formed during the reaction when they were chromatographed on paper. Several factors contributed to this difficulty. The amount of isomaltose-U-C^{14} added to the assay mixture contained enough radioactivity to be detected by X ray film in a short exposure time, but the amount that was to be used for a chromatogram was limited to a low number of counts. This limitation arises from (a) the relatively small amount of carbohydrate material that can be separated cleanly on a chromatogram, (b) absorption of weak beta particles by the chromatographic paper and (c) the distribution of the carbon-14 in the various products formed by the reaction. After 40 days of exposure on X ray film, the chromatogram exposed only light spots at the R_f of glucose and at the higher molecular weight dextrins at the starting line. These spots agreed with the proposed mechanism in that the transglucosidase releases one mole of glucose from isomaltose for every one transferred to suitable acceptors. For each molecule of isomaltose-U-C^{14} utilized, one molecule of glucose-U-C^{14} would have been released into the medium and one molecule of glucose-U-C^{14} transferred

to an acceptor. Starch phosphorylase would have utilized the small tagged dextrins as substrates to synthesize long polysaccharides which would not migrate on the paper chromatogram, thus concentrating these tagged polysaccharides at the starting line.

The transglucosidase involving isomaltose strongly suggests a mechanism for the synthesis of both maltose and isomaltose in the potato. When these problems are elucidated then a total synthesis of starch may be possible starting from glucose. The mechanism of the formation of primers for starch phosphorylase by the transglucosidase reported here brings us one glucose unit closer to the net synthesis of starch in the potato.

The production of small amounts of primers in the young potato plant would eventually result in the formation of large amounts of starch due to the starch phosphorylase present. The formation of several long polymers would supply substrates to D enzyme and alpha amylase, thus in turn forming more substrates for starch phosphorylase. The Q enzyme would eventually begin the branching process. This type of a system requires an original primer source which has remained hidden to the present time. The role of the enzyme reported in this thesis may be in supplying the original primers to starch phosphorylase.

IV. SUMMARY

1. A new transglucosidase, found in potatoes, which catalyzes the transfer of the non-reducing terminal glucose from isomaltose to suitable acceptors such as maltose, maltotriose and higher molecular weight maltodextrins forming the next higher linear homologue of the series is reported. The enzyme was found concentrated in the 0-30% ammonium sulfate fraction of dialyzed potato juice. The 30-40% ammonium sulfate fraction contained no measurable amounts of the enzyme.

2. The reaction involves an enzyme-glucose compound as an intermediate for the transfer. This intermediate will exchange the glucose moiety for glucose-U-C^{14} when the latter is used as a substrate with isomaltose. Glucose will act only as an acceptor molecule in the capacity to form isomaltose in the reverse reaction.

3. The enzyme will not utilize dextrotriose (three glucose units bonded by two alpha 1-6 bonds) as the donor molecule.

4. A study was made of the enzyme systems in the preparation containing the new transglucosidase under the same conditions which were used to assay the activity of the new enzyme. The preparation contained traces of alpha amylase, active starch phosphorylase and no measurable D enzyme activity.

5. The linear maltodextrins formed when isomaltose and maltose are substrates will act as primer for the starch phosphorylase present in the enzyme preparation. The enzyme reported is the only known enzyme from a plant source that can furnish 'primers' for starch synthesis

utilizing disaccharides as the substrates.

6. Kinetic data on the starch phosphorylase reaction in the preparation minimized the possibility of the presence of any other phosphorylases utilizing alpha-D-glucose-1-phosphate.

7. The starch phosphorylase present in the potato will not utilize isomaltose or dextrotriose as substrates for its' action.

V. ACKNOWLEDGEMENTS

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