This Thesis is dedicated to

A. L. A., A. M. R. and I. E.

Carbohydrate Metabolism in Potato Tubers as Influenced by Temperature

Thesis by

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In partial fulfillment of the Requirements for the Degree of Doctor of Philosophy

California Institute of Technology

Pasadena, California

1946

Acknowledgments

The author wishes to express his gratitude to Professor James Bonner for guidance and help throughout this work, to Professor F. W. Went for his ideas and interest in this research and to Professor George E. MacGinitie for his valuable advice.

The writer also wishes to thank Mr. Manuel Avila Camacho and Mr. Marte R. Gómez, President and Secretary of Agriculture of Mexico respectively and to Captain Roberto T. Amézaza for a grant which made this project possible.

He is also indebted to Mrs. Irene Young for most of the analysis of sugars.

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INTRODUCTION

Introduction. - It has long been known that plants and plant parts maintained at low temperature tend to convert stored starch to sucrose. Higher storage temperatures on the other hand result in accumulation of reserve carbohydrates in the form of starch. Although this sweetening of potatoes, sweet potatoes, and other tissues during cold storage is well known, the mechanisms responsible for the starch-sucrose conversion have been obscure. This is in part due to the fact that the enzymes and enzyme systems involved in carbohydrate metabolism which have been so extensively studied in microorganisms and animals, have been but little studied in the higher plants.

The present investigation was undertaken in the hope of clarifying in the light of the modern knowledge of carbohydrate metabolism the mechanism of sucrose formation in potato tubers stored under different temperature conditions.

Review of Literature. - Müller-Thurgau (54) in his classical investigation found an accumulation of sugars and a corresponding loss of starch in potatoes kept at temperatures between 0° and 6°C. However, no sugar was formed in potatoes which had been actually frozen. Chemical analysis showed that most of the sugar which appeared was reducing sugar and, to a lesser extent, sucrose. He interpreted his results to mean that the transformation of starch into sugars is an enzymatic process which is rapid at high temperature. The formation of sugar during drying of potatoes was first observed by Marcacci in 1891.

Watermann studied the formation of sugars both at low temperatures and by drying, and considered the two processes to be related to each other. He suggested that the two reactions:

1 Starch ——→ sucrose

2 Sucrose ——→ starch

are independent in the potato and that the velocity of reaction 1 is greatest between 35° and 40°C. By either an increase or a decrease of temperature this reaction is slowed down. The velocity of reaction 2 is greatest between 15° and 20°C. and falls rapidly on both sides of this maximum. Thus sucrose accumulation at low temperature would be due to the relative preponderance of 1 over 2 at low temperature.

Wolff (78) repeated the work of Müller-Thurgau and Watermann. He considers the amount of formation of sucrose to be dependent on the concentration of sucrose already present in the tissue. Below 10°C. sucrose formation goes on until a definite concentration is reached. He summarizes his results for normal potatoes in figure 1 in which the equilibrium sugar ⇌ starch is shifted

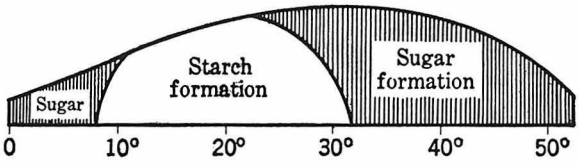


Fig. 1 Showing diagrammatically the temperature effect on the sugar ⇌ starch equilibrium in the potato. (After de Wolff.)

with temperature changes. He suggested that the area of sugar formation shown at high temperatures is governed by changes in water relationship.

Wolff also investigated sucrose formation during drying of potatoes at various temperatures and his results are in complete harmony with those of Watermann. Both found maximum sucrose formation between 30° and 40°C. The results of a typical drying experiment are given in Table 1.

Table 1 (after Wolff)

Drying of potatoes at various temperatures.

Expt. No.	Loss of wt. during expt. in %	Temps. of drying in °C.	Duration of drying in hours	Increase in sucrose content in %
1	71.9	16 - 18	280	1.39
2	71.1	30.5	32	1.33
3	71.6	35.0	24	1.26
4	71.5	40.8	22	1.13
5	71.4	45.5	20	.86
6	72.0	46.0	20	.85
7	70.3	59.0	14.	.55
8	69.4	98 - 100	6	.21

This table shows that sucrose formation is greatest at the lower temperatures of drying. At 98-100° the formation of sucrose is almost nil.

It would appear that drying at temperatures which kill the potato at once stops sucrose accumulation, owing perhaps to

destruction of the sucrose synthesizing mechanism. Wolff noticed that reducing sugar varied irregularly, and he looked upon the reaction of conversion of starch to sucrose as independent of the reducing sugar present. The age of potatoes had also some influence in their ability to form sucrose during drying, young potatoes being more active.

Wolff performed other experiments by wounding and drying and found that whereas wounding intensifies respiration, wounded potatoes form little sucrose. Thus chopped potatoes failed to form sucrose suggesting that the synthesis of sucrose needs the presence of organized tissue to carry out the reactions involved. He concluded from his experiments that the increase of sucrose during drying of potatoes is due primarily to a loss of water and not to temperature effects, since formation of sucrose did not occur in drying potatoes until the dry weight became greater than 53%.

Schroeder and Horn (66) studying the increase of sucrose in drying Tropaeolum leaves reached the conclusion that starch is converted into sucrose in a more direct way than through the reducing sugars as an intermediate stage.

Nelson and Auchincloss (56) dipped potato slices in aqueous solutions of glucose and fructose. The sections were then removed and incubated under moist conditions, after which they were washed and dried for analysis.

A larger increase in sucrose content resulted from this treatment than when the slices were dried without dipping into hexose solution. In a nitrogen atmosphere no sucrose was formed

by the slices. In this connection it is known that green bananas coated with paraffin or immersed in oil, fail to convert starch into sugars. Here again the dependence of sucrose synthesis on oxygen supply is evident. Nelson and Auchincloss believe that, contrary to the conclusions of the earlier workers, dehydration of potato slices is not necessary in order to affect marked increases of sucrose content. They contributed evidence that sucrose in the potato is synthesized from glucose or fructose rather than directly from starch. Oxygen is essential for conversion of reducing sugars in potato slices to sucrose.

The phenomenon of sugar increases in sweet potatoes during winter storage had been observed by Harrington in 1895. Hasselbring and Hawkins (33) in experiments with sweet potatoes also found that at 4°C. there is a rapid disappearance of starch accompanied by an increase in sucrose. This change does not reach equilibrium since sweet potatoes usually rot by the action of fungi before the maximum changes are attained.

Hopkins (41) made studies on respiration of potatoes placed in chambers at various temperatures and then measuring the carbon dioxide given off. His findings showed that potatoes at 0°C. respired faster than those at 4.4°C. where the respiration was in both cases measured at 22°C. Hopkins' experiments can be summarized in the following curve of respiration.

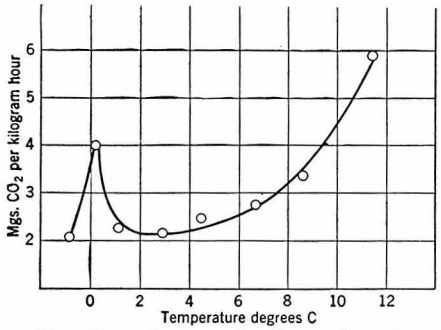


Fig. 2. The respiration of potato tubers at various temperatures. (Data of Hopkins.)

This curve indicates that the rate of respiration of potatoes does not decline consistently as the temperature decreases, but declines to a minimum at about 3°C. and then increases to a maximum at 0°C. below which it declines again. The author ascribes this to an accelerating effect of the low temperature, such as postulated by Palladin. The results of Hopkins can, however, be interpreted on the basis that something needed in respiration is produced in the tubers which regulates the rate of respiration at 22°C. This substance may be one of the intermediates of the carbohydrate breakdown.

Conclusion. - The survey of the literature on sucrose synthesis as affected by temperature shows that many tissues form sugar in the cold. Similar changes occur as a result of drying especially at elevated but not lethal temperatures.

The mechanisms of sucrose formation are obscure. The purpose of this investigation is to study, with the techniques of modern biochemistry, all of the reactions between starch and sucrose together with the reactions which may play some role or have a connection with the formation of sucrose in general.

General Methods. - Storage. - Potato tubers of the Russet and White Rose varieties were bought in the market for the experiments. Each batch of tubers was randomly divided into several portions. Each portion was stored at a controlled (±1°C.) temperature. The temperatures used were 0°, 10°, 20° and 30° or in other cases 0°, 9°, 16° and 25°C.

Fractional Precipitation. - In the work with isolated enzyme systems, the tubers were ground in a meat chopper and the juice then pressed out by means of a hand press. This potato juice was used in a few cases for crude tests. In most of the cases, however, it was fractionally precipitated with salts. Usually saturated ammonium sulphate solution was used as a precipitant, although in some cases magnesium sulphate was employed. The fractionations were done in the cold at 0°C. in order to avoid denaturation as far as possible. The fractions obtained were purified further by dialysis in cellulose bags in order to eliminate the precipitant. Alcohol fractionation of the juice or fractions resulted in the cases tried in decreased enzymatic activity.

Sugar Analysis. - Potato samples for analysis were usually dried in a ventilated oven at 70°C., although in other cases analysis on the juice was also used. Once dried the samples were ground in a Wiley mill to 20 mesh, aliquot portions were then weighed out and extracted with 80% ethyl alcohol which extracts all of

the simple sugars, leaving behind the starch. The alcohol extract is concentrated and cleared with neutral lead acetate as described by Hassid (34). Aliquots of the cleared extract are used for determinations of reducing sugars by the Ceric sulphate method. Sucrose is determined as the increase in reducing power after hydrolysis with invertase. Non-sugar reducing substances are determined by the reducing power in aliquots of the extracts which have been previously fermented with yeast. Fructose gives a characteristic color reaction with alcoholic resorcinol in strong acid solution (59) and was determined colorimetrically in a Klett-Summerson photoelectric colorimeter. Starch is analyzed in the residue of the alcohol extract by hydrolysis either with amylase or with acid and titrated as glucose.

Starch was also determined colorimetrically with Iodine and potassium iodide solution, and the color compared with a calibration curve made for potato starch.

Phosphorylated Intermediates. - A portion of the dry sample is extracted several times with 5% trichloroacetic acid in the cold by a slight modification of the method described in (74), until the acid solution gives a negative test for phosphorus. The combined extracts are further treated and separated into three fractions, on the basis of the solubilities of the barium salts in water and alcohol. Each fraction contains several of the intermediates which then can be determined in samples by characteristic and specific reactions.

Phosphorus Determinations. - Inorganic phosphate and 7-minutes hydrolyzable phosphate (or Cori ester) are determined by the Fiske and Subbarow (20) colorimetric method. The methods of King (46) and Allen (1) were also tried but showed no advantages over the former. Fructose-6-phosphate is first hydrolyzed into its components by a drastic hydrolysis, and then the phosphate liberated determined as inorganic phosphate.

Chemical Changes Caused in Potatoes at Different Temperatures. - The first experiment to be presented is one in which an attempt was made to obtain a picture of the changes induced in potatoes by storage at different temperatures, not only with respect to starch and sucrose but also with respect to all sugars and sugar derivatives in the tissue.

The storage temperatures selected were 0°, 9°, 16° and 25°C. An initial sample was taken and further samples were taken after two weeks, and analyzed by the procedures described under Methods.

The results of this experiment are given in Table 2. If we compare the values obtained after two weeks with the initial ones we get the following:

1. Starch decreases in all conditions, the reduction being most marked at 0°C.
2. Glucose increases slightly in concentration at 0° and 9°C. and decreases a little at the higher temperatures.

3. A remarkable change occurs with fructose which increases nearly nine fold at 0° and two fold at 9° but remains approximately constant in concentration at the two higher temperatures.
4. Glucose-1-phosphate is present in very small quantities at 0° and 9°C. and absent at the other temperatures.
5. Striking changes occur in glucose-6-phosphate and fructose-6-phosphate. The first compound exists in greater quantities at 16° and 25°C. than at temperatures of 0° and 9°C. Just the reverse is true for fructose-6-phosphate.

In the reaction:

Glucose-6-phosphate $\rightleftharpoons$ Fructose-6-phosphate

the equilibrium appears to be shifted to the right side at lower temperatures and to the left at higher temperatures.

6. Fructose-1,6-diphosphate did not accumulate under the conditions of this experiment.
7. Triose phosphates were higher in concentration at 0°C. than at higher temperatures.
8. Adenosine triphosphate did not occur in significant quantity.

This experiment shows that the formation of sucrose by the potato when at low temperatures is merely part of a far reaching metabolic change which occurs when the tubers are shifted from high to low temperatures. In particular, not only the sugars

Table 2

Components and intermediates in potato tubers
as influenced by temperature of storage.

Values given in % of dry weight.

Substance analyzed	Zero time	After 2 weeks of storage			
	Market	0°	9°	16°	25°
Starch	67	61.0	65.0	63.0	64.0
Glucose	.62	.79	.73	.49	.56
Fructose	.17	1.50	.34	.22	.15
Sucrose	1.07	6.65	1.25	.75	.84
Non-sugar red. substances	.00	.60	.09	.19	.09
Glucose-1-phosphate	.00	.17	.04	.00	.00
Glucose-6-phosphate	3.50	.70	.66	4.20	4.50
Fructose-6-phosphate	.17	2.50	1.05	.25	.35
Fructose-1,6-diphosphate	.00	.00	.00	.00	.00
Triose phosphates	.37	.94	.107	.57	.26
ATP	.04	.09	.12	.00	.04

Russet variety potatoes were used. For analysis 1 gm. of dry sample was employed.

but also the phosphorylated sugar derivatives are involved in the changes induced by low temperature treatment.

These results indicate at once that investigation of the problem of sugar formation in potato should include study of the metabolism of the phosphorylated sugars.

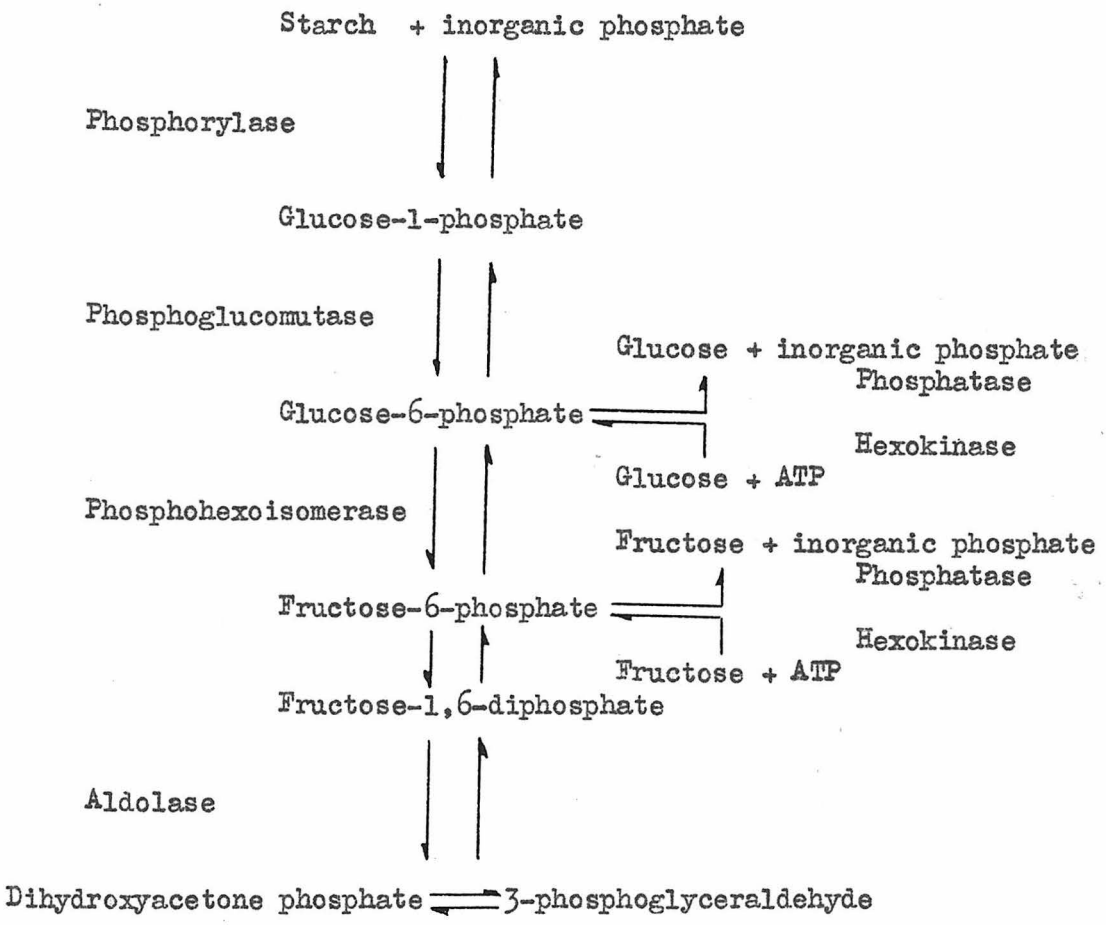
Carbohydrate Metabolism in Animal Tissues and in Yeast. - The mechanism of the carbohydrate metabolism has been unraveled with the discovery of phosphorylated compounds in sugar breakdown. The outstanding work of Harden and Young, Meyerhof, Neuberg, Embden, Parnas, Cori and Cori and others has revealed the series of reactions involved in the breakdown of carbohydrate to pyruvic acid.

A partial scheme of the glycolysis cycle as it is known today is given in figure 3 (26) and this scheme will be used in further discussions. All the reactions in the scheme are reversible except the dephosphorylation of pyruvic acid by adenosine diphosphate. By means of coupling the phosphate into esters the energy of oxidation is fixed into phosphate bond energy which then can be utilized in other coupled reactions.

Carbohydrate Metabolism in Higher Plants and What is Known About the Synthesis of Sucrose. - The stimulation of fermentation by seeds and seed meals of higher plants by inorganic phosphate suggested a role of phosphate in higher plants metabolism. This

Figure 3

Glycolysis Cycle



finding was later confirmed by many workers who found that phosphate affects not only the anaerobic but also the aerobic respiration.

Bodnar (6) demonstrated the disappearance of inorganic phosphate added to ungerminated ground peas. He was inclined to the view that this disappearance was due to esterification of phosphoric acid with sugars. This was substantiated by Tanko (72) who using the same material isolated the phosphorylated compounds, 90% of which were present as fructose-1,6-diphosphate (Harden and Young ester). Hexose monophosphates were also present.

In more recent work the breakdown of carbohydrate to pyruvic acid has been shown conclusively in barley plants by James and coworkers (42), (43), (44).

At the present time it is then known that phosphorylated hexoses do occur in higher plants. Some of the enzymes of the glycolysis cycle have also been identified in higher plant material, as will be discussed in more detail below. There is a general reason to believe that carbohydrate breakdown in higher plants may follow pathways generally similar to that followed in muscle and in yeast, although the systems have not been as yet worked out for any single plant.

Sucrose which does not appear in the glycolysis cycle of animals is present in all higher plants. Some workers have suggested that sucrose is the first product of photosynthesis.

Their assumption is based on the findings that sucrose follows the diurnal cycle of photosynthesis more closely than the other sugars. This view has been supported by Brown and Morris (7), Went (76) and others. At the present time the radioactive carbon experiments strongly indicate, however, that the first product of photosynthesis is a high molecular weight compound and that non-photosynthesizing organs such as potatoes form sucrose at the expense of other sugars already present if conditions are suitable for such synthesis and that sucrose is formed by mechanisms which do not bear any direct connection to photosynthesis. Thus, as shown above, sucrose production in the potato is at the expense of starch and involves the interconversion of glucose and fructose in the tuber. The interconversion of sugars in plants has been studied by McCready and Hassid (50) in barley. They found that sucrose was synthesized when barley leaves were vacuum infiltrated with solutions of other sugars. Glucose, fructose, mannose, galactose, lactose, and maltose were all effective, the disaccharides being probably first hydrolyzed into the component hexoses. By far the most effective sugar was glucose, followed by fructose and then galactose. Thus glucose can be converted to fructose and vice versa. This is in agreement with the findings of Husein (61) with carrot slices. McCready and Hassid showed also that sucrose synthesis requires oxygen but is independent of light and chlorophyll.

They used also respiratory poisons as iodoacetic acid and found that sucrose synthesis was inhibited by this compound. Cyanide was not effective, but they attributed this failure to the possibility that the poison reacted with glucose. Thiocyanate did not affect sucrose synthesis, and the same was true for invertase.

Lissyzin found that in minced stems of sorghum a decomposition of sucrose occurred accompanied by an increase in the amounts of dextrins and starch. Total sugars decreased notably, the diminution not being compensated for by the increment of dextrins and starch. He concludes that the transformation of sucrose into dextrins and starch is connected with the formation of great amounts of hexoses, which are obviously the intermediate stages of this process as suggested by the following scheme:

sucrose $\rightleftharpoons$ hexoses $\rightleftharpoons$ dextrins $\rightleftharpoons$ starch

Lissyzin believes then that no direct transformation of sucrose into starch takes place, and that the conversion of starch into sucrose proceeds through hexose formation.

Virtanen and Nordlung (75) depleted clover leaves of starch by starving in the dark. The leaves were next placed with their petioles immersed in glucose solution for 24 hours in the dark and then analyzed after drying. Their results showed that reducing sugar and particularly sucrose, increased markedly as a result of glucose feeding. With wheat, the same workers, using the same technique but placing the stalk in glucose or fructose

solution, found similar increases of reducing sugar and sucrose. Glucose was more effective than fructose in inducing sucrose formation. Their findings also show that sucrose may actually originate from individual hexoses and in addition indicate the interconversion of glucose and fructose.

Leonard (48) studied sucrose synthesis in leaves of corn, sorghum, cotton and cabbage by dipping the petioles in the solution to be tested. The midribs were discarded after incubation, and the leaves then dried and analyzed. Sucrose increased in the dark when glucose was fed while reducing sugar changed only slightly. Fructose appeared only after fourteen hours. Cotton leaves in the dark formed sucrose when given glucose or fructose and to lesser extent when fed maltose or lactose. Leonard found also that drying of corn and cabbage leaves in air at 34°C. produced an increase in sucrose and reducing sugar. When the leaves were dried at the same temperature in an atmosphere of nitrogen, no sucrose was formed, which shows that the synthesis is aerobic.

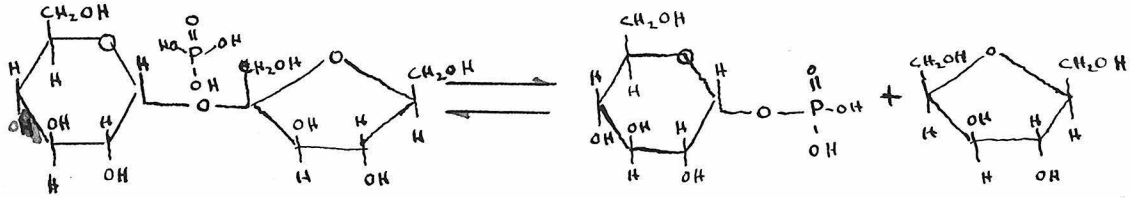
Hartt (29), (30) found that sucrose synthesis took place within detached sugar can leaf blades placed with their bases immersed in glucose or fructose solutions in the dark. Cane sugar increased up to 16% on a dry wt. basis after a week of incubation, a figure higher than is ever found in attached leaf blades. With plants growing in nutrient solution with and without phosphate, she found that those with phosphate formed more sucrose and also

converted more glucose into fructose than those which lacked phosphate. She found 30°C. to be the optimal temperature for sucrose synthesis. The interconversion of glucose and fructose did not take place at 6°C., but it kept pace with the formation of sucrose at 20°C. while at higher temperatures it was increased still further. The study of temperature coefficients suggested to her that the absorption of simple sugar is a purely physical process at low temperature, but that at high temperature the absorption was limited by a chemical reaction. The synthesis of sucrose, however, appeared to be limited by a chemical reaction at lower temperature but by a physical process at high temperature. She concludes that simple sugars are intermediates in sucrose synthesis.

Hartt studied the synthesis of sucrose in sugar cane blades as noted above and found that detached leaf blades immersed in solutions of glucose or fructose form sucrose. The shape of the curves relating increase in sucrose to time are similar to the fluctuations of sugars in attached blades during day and night. Hartt believes that the synthesis of sucrose takes place by the same mechanism regardless of whether glucose is supplied artificially or naturally by the process of photosynthesis.

The only case in which the synthesis of sucrose has been definitely established in vitro is the one reported by Doudoroff, Kaplan and Hassid (18) and by Doudoroff, Hassid and Coworkers (19), (38). They found that the bacterium Pseudomonas saccharophila

Doudoroff contains a sucrose synthesizing enzyme, which catalizes the following reversible reaction:



The product formed was characterized chemically, and also by comparison of the X-ray diffraction pattern of synthetic and natural sucrose, and rate of hydrolysis with hydrochloric acid. The dephosphorolytic condensation of α-glucose-1-phosphate and fructose results then in the formation of sucrose, a finding which supports the conclusion that flucose exists in the sucrose molecule in the α-form.

This survey of the literature shows that the information concerning sucrose synthesis in higher plants is extremely limited. It can, however, be said that the synthesis is aerobic, and is consequently linked with respiration directly or indirectly. The evidence presented suggests that sucrose is formed from hexoses rather than directly from starch.

Since the reactions of carbohydrate breakdown have been studied very little in higher plants, the obvious approach to the present problem is to study first of all the possible occurrence in higher plants of such reactions as have been reported to occur in yeast and muscle extracts.

EXPERIMENTS IN VITRO WITH ISOLATED ENZYME SYSTEMS

The purpose of these studies was to examine every enzyme system involved or possibly involved in carbohydrate metabolism between starch and sucrose. Study of the individual enzymes in vitro gave evidence as to their presence or absence in potato tubers and also supplied data concerning their kinetics in part, and their kinetics at various temperatures.

The enzymes studied in this section are:

1. - Amylase
2. - Phosphorylase
3. - Phosphoglucomutase
4. - Isomerase
5. - Phosphatase
6. - Hexokinase

Amylase. - Historical and description. - Amylase was the first enzyme system to be discovered and is present in nearly all plants and animals. This enzyme hydrolyzes starch to dextrins amd maltose.

Experimental. - Whole juice pressed out from potatoes was used. Attempts were made to enrich the enzyme by fractional precipitation of the juice with saturated salt solutions in the search for a good method of extraction and purification. The precipitant used with most success was a saturated solution of ammonium sulphate and in general the whole juice was divided into four fractions as follows:

Fraction No.	% of saturation in ammonium sulphate
1	30
2	35
3	40
4	50 (Most active fraction)

The fractionations took place in the cold at 0°C. The maximum amylase activity was present in fraction 4, but fraction 2 and 3 also contained some activity. Further dialysis of the fractions in the cold produced loss in activity, a fact also noted by Sherman (64). Dialysis against distilled water inactivates phosphorylase thus permitting a separation of amylase from this interfering enzyme. This step can be speeded up by frequent changes of water. Precipitation of the fractions with 95% ethyl alcohol as used by Klinkerberg and others (68) for the separation of α and β amylase from barley malt yielded an inactive product in the present case.

Activity. - Determinations of enzyme activity were done by a standard technique which permitted the making of quantitative comparisons between different preparations. This consisted in a modification of Wohlgemuth's (77) method which follows the fading in color of the iodine-starch complex as a function of time.

To control the acidity of the digest, acetate buffer of pH 5.0 was used. Previous workers (63) found an optimum pH for amylase to be in the neighborhood of this value. Blanks were run at the

same time and the temperature of incubation was kept constant at 25°C. by the use of a water bath.

The standard enzyme preparations were allowed to act upon a standard solution of starch and the hydrolysis of starch determined by the decrease in intensity of color of the iodine-starch complex.

In figure 4, milligrams of starch are plotted as ordinates against time as the absissae and shows the amylase activity of one enzyme fraction from the ammonium sulphate precipitation; and its activity after dialysis and alcohol precipitation.

The enzyme found in potato juice is a β-amylase which as has been shown previously for other amylases converts 60% of the starch into maltose and dextrins. This enzyme attacks the starch chain at the ends, leaving as final product of its action maltose and α-amylodextrins which are resistant to β-amylase. The color formed with iodine does not disappear completely due to the fact that not all of the starch is broken down.

It is known that in seeds a portion of the amylase may be bound and that the total amylase may be determined by setting free the α or bound amylase with papain (17). The bound amylase was not determined in the present case since the main interest was to know how much of the enzyme was active under the conditions of storage.

Tests for amylophosphatase or phosphate liberating enzyme

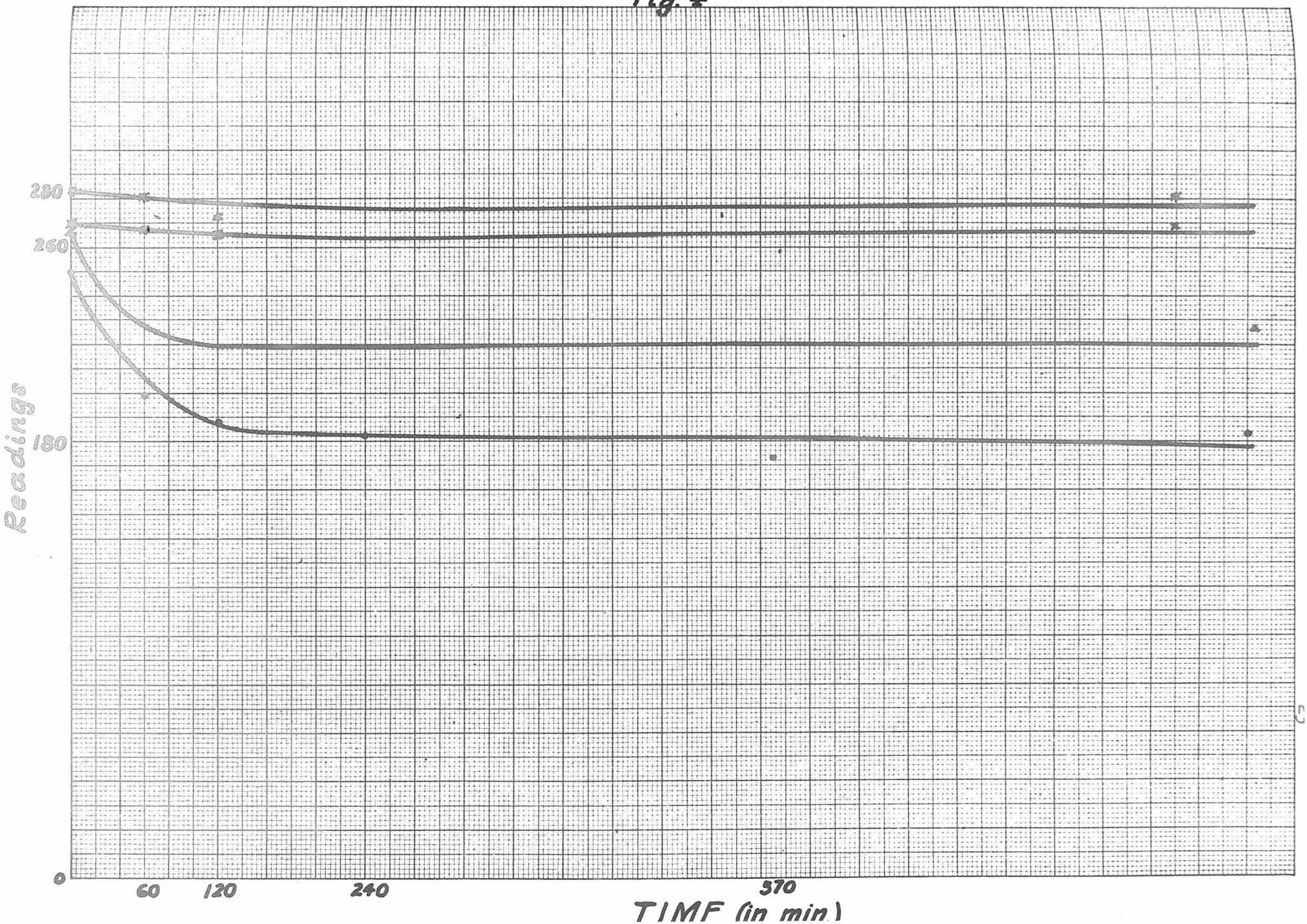
Legend for Fig. 4.

Expt. P-98. Russet potatoes from market. Enzyme concentration 2x, 5 cc. of enzyme in digest, 3 cc. of acetate buffer of pH 5.0, 40 mgs. of starch in digest. Total volume of digest 20 cc. Incubated at 25°C.

Amylase activity.

Digest No.	Protein precipitated with 35% ammonium sulphate saturation	Further treatment	
1	"		● ——
2	"	dialyzed	▲ ——
3	"	" and precip. with 60% ethyl alcohol	× ——
4	"	dialyzed and precip. with 65% ethyl alcohol	* ——

Fig. 4



gave negative results.

Potatoes from varying storage temperatures were next studied for their amylase activity. The tubers had been stored 5 weeks. The results are given in the following table 3 in which an aliquot of the protein fraction that precipitated with 50% saturated ammonium sulphate was used as enzyme.

Table 3

Effect of temperature of storage on the amylase content.

Average values of experiments P-108, P-109, P-110 and P-111	Temperature of storage 0°	10°	20°	30°
mgs. of starch degradated in 200 min./cc. of digest	0.37	0.29	0.27	0.43
% of starch degradated in 200 min.	12.0	8.5	9.6	17.2

The digest contained 2.1/2 mgrs./cc. of starch, 5 cc. of Enzyme preparation, plus 3 cc. of acetate buffer and incubation was at 25° C. Potatoes from 30°C. storage conditions contained more amylase per cc. of enzyme preparation than potatoes from any of the other conditions as can be seen in table 3.

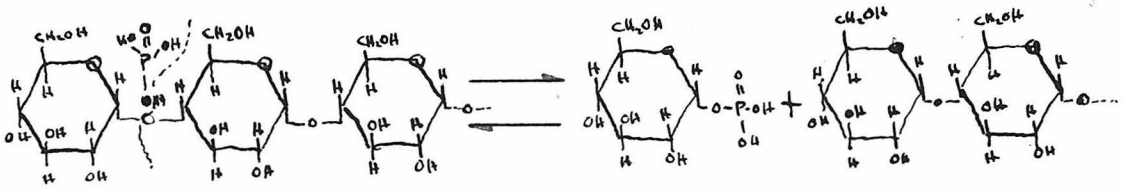
Digests containing amylase placed at 0°C. showed that at this low temperature amylase activity is completely supressed. If these results may be applied to the potato, it would suggest that the enzyme even if present in tubers would not be active at low temperatures and consequently could not participate actively in the cleavage of starch which occurs at such temperatures.

Phosphorylase. - Historical and Description. - The formation of glycogen and starch from monosaccharides has been extensively studied since the time of Claude Bernard, but an insight into the mechanism has been gained only recently. Robison (58) in a fundamental discovery showed that a mixture of glucose and fructose-6-phosphates is formed from hexoses by yeast preparations, and the isolation by Embden and Zimmerman of a similar mixture of esters from skeletal muscle, established that hexose monophosphates are intermediates in the polysaccharide hexoses transformation. Parnas showed that in animal tissues, glycogen undergoes esterification with phosphate to form hexose monophosphates, a process which he called phosphorolysis. One of the esters thus formed was later found to be Embden ester (fructose-6-phosphate). Cori and Cori (11) isolated from frog muscle glucose-1-phosphate or Cori ester as the first product of phosphorolysis of glycogen. Later they obtained the same ester from rabbit muscle (12) and proved that here also the presence of the enzyme phosphorylase is responsible for the reaction. They also prepared the ester synthetically by chemical means and showed the biological behavior of the synthetic and the natural Cori ester was identical. The ester is d-glucopyranose-1-phosphate. The corresponding acid is stronger than phosphoric acid. This ester has been prepared biologically by Hanes (28) and by McCready and Hassid (51). The enzyme phosphorylase has been found not only in muscle but also in yeast (45) in pea meal and potatoes (27), (28), in

heart and liver (12) and in kidney, heart, brain, skin, lungs, and tumor (62). The enzyme is absolutely specific and catalyzes the following reversible reaction (14):

$$\text{Starch} \underset{-\text{ inorganic phosphate}}{\overset{+\text{ inorganic phosphate}}{\rightleftharpoons}} \text{Glucose-1-phosphate}$$

The equilibrium is dependent on pH as will be discussed later. The formation of glucose-1-phosphate is a result of a phosphorolytic cleavage of the terminal units of the glucose residues which constitutes the starch while the reverse reaction is a dephosphorolytic condensation of Cori ester. This can be more clearly shown by using the structural formulas. The starch is esterfied at carbon atom 1 and the entire molecule is gradually



dismembered into uniform glucose-1-phosphate fragments.

Galactose-1-phosphate or mannose-1-phosphate which differ only in carbon atom 4 and carbon atom 2 respectively from glucose-1-phosphate are not converted into polysaccharides by the enzyme nor is phosphate liberated from them (8).

The synthesis of starch by phosphorylase is an autocatalytic reaction as can be seen by the shape of the reaction rate curve. When no polysaccharide is present in the reacting mixture

there is a lag period during which the reaction proceeds at a barely measurable rate, this rate increases exponentially as is expected for autocalytic reactions. This lag or induction period is prolonged with increasing purification of the enzyme. No lag period is observed when the enzyme is phosphorylating starch. The plant enzyme is primed by the addition of starch, amylase, amylopectin, dextrins, and even slightly by maltose (28), (70). All polyhexoses which are catalytically active in starch synthesis have two characteristics in common:

a/ They are formed by chains of α-glucose units in 1-4 linkages.

b/ They can be phosphorylated to glucose-1-phosphate by the enzyme.

Animal phosphorylase cannot be primed with amylose which shows that this enzyme differs somehow from plant phosphorylase (70). Adenylic acid acts as a coenzyme for animal phosphorylase (15) but is not needed for the plant enzyme. Green and Cori (25) recently obtained a completely pure phosphorylase in crystalline form from rabbit muscle. They separated (15) this enzyme into 2 portions namely:

1. Phosphorylase "a" an active enzyme (holoenzyme) which contains adenylic acid bound to the molecule. The adenylic acid can be dialyzed out leaving an amorphous preparation which they called
2. Phosphorylase "b" or apoenzyme which requires the

addition of adenylic acid to restore its activity. The conversion of phosphorylase "a" to "b" is also carried out by an enzyme present in the rabbit muscle (16) which they called P. R. (prosthetic-group removing) enzyme. This enzyme has a proteolytic activity but does not destroy the phosphorylase in several hours of incubation. The molecular weight of phosphorylase "a" was determined by ultracentrifuge and by diffusion and gave the values of 340,000 and 400,000. Liver, heart, and brain phosphorylase synthesize glycogen in vivo and in vitro while muscle phosphorylase synthesizes starch in vitro and glycogen in vivo. Plant phosphorylase forms only starch in both conditions. The starch synthesized by muscle phosphorylase in vitro and by plant phosphorylase resembles the amylase fraction of starch, this subject will be referred to later.

Experimental. - The standard procedure developed for obtaining the enzyme from potato consists of a modification of those used by Green and Stumpf (23) and by Hanes (28) both of which make use of a fractional precipitation of the proteins present in potato juice with ammonium sulphate. A comprehensive study of the method showed that the proteins should not remain in contact with ammonium sulphate during precipitations any longer than

absolutely essential since long contact caused losses in enzyme activity. Allowing the protein fraction containing the enzyme to dry in the Buchner funnel during manipulation also caused decreases in activity. A layer of toluene over the crude juice not only served as a bacteria repellant but also prevented oxidation which was shown to cause some inactivation of phosphorylase. It is best in extracting the enzyme to use low temperature and to do the various steps as fast as possible in order to decrease possible inactivating factors and keeping of the enzyme wet or otherwise in solution was also part of the technique. This method yields a very active enzyme free from amylase and phosphoglucomutase.

Activity. - Standard digests consisting of definite quantities of enzyme, substrate and priming substance were used in the course of the experiments for determinations of relative phosphorylase activity and only where stated were the conditions varied. When Cori ester is used as substrate for phosphorylase activity, the activity can be followed by the liberation of inorganic phosphate as shown graphically in figure 5 in which colorimeter readings (proportional to phosphate) are plotted against time in the abscissa. Likewise, activity can be determined colorimetrically as the amount of starch synthesized as shown graphically in figure 6 where the colorimeter readings (proportional to starch) of the iodine-starch complex are plotted against time. However, the last method gave rise to a complication since the values for

Legend for Fig. 5.

Expt. P-43. White Rose potatoes from the market. Digest contained 5 cc. of 5x concentrated enzyme, 3 cc. of maleate buffer pH 5.5, 30 mgs. of Cori ester and 2 mgs. of starch. Total volume 20 cc. Incubated at 25°C.

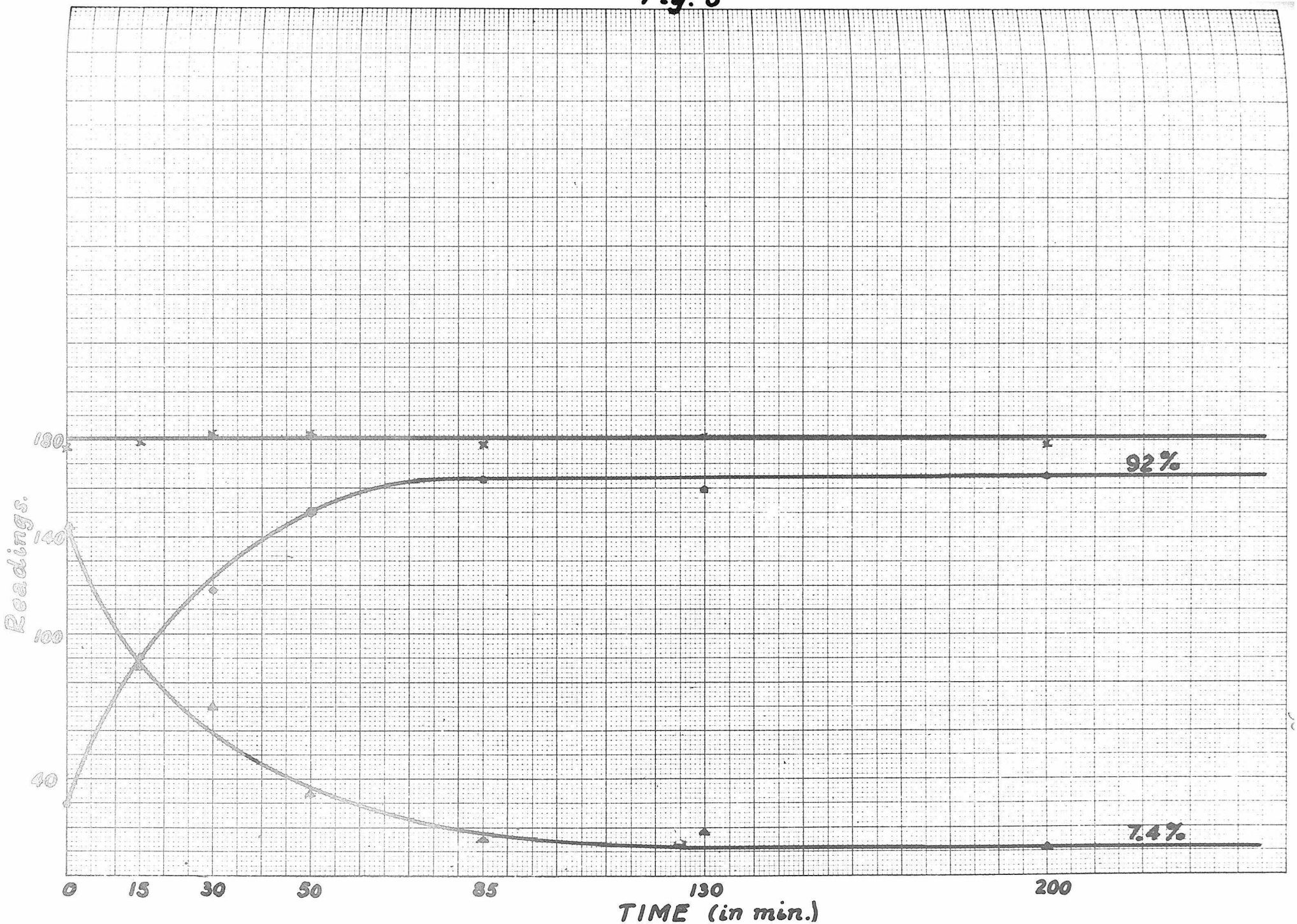
Determination of phosphorylase equilibrium.

Inorganic phosphate • ———

Organic " ▲ ———

Total " × ———

Fig. 5



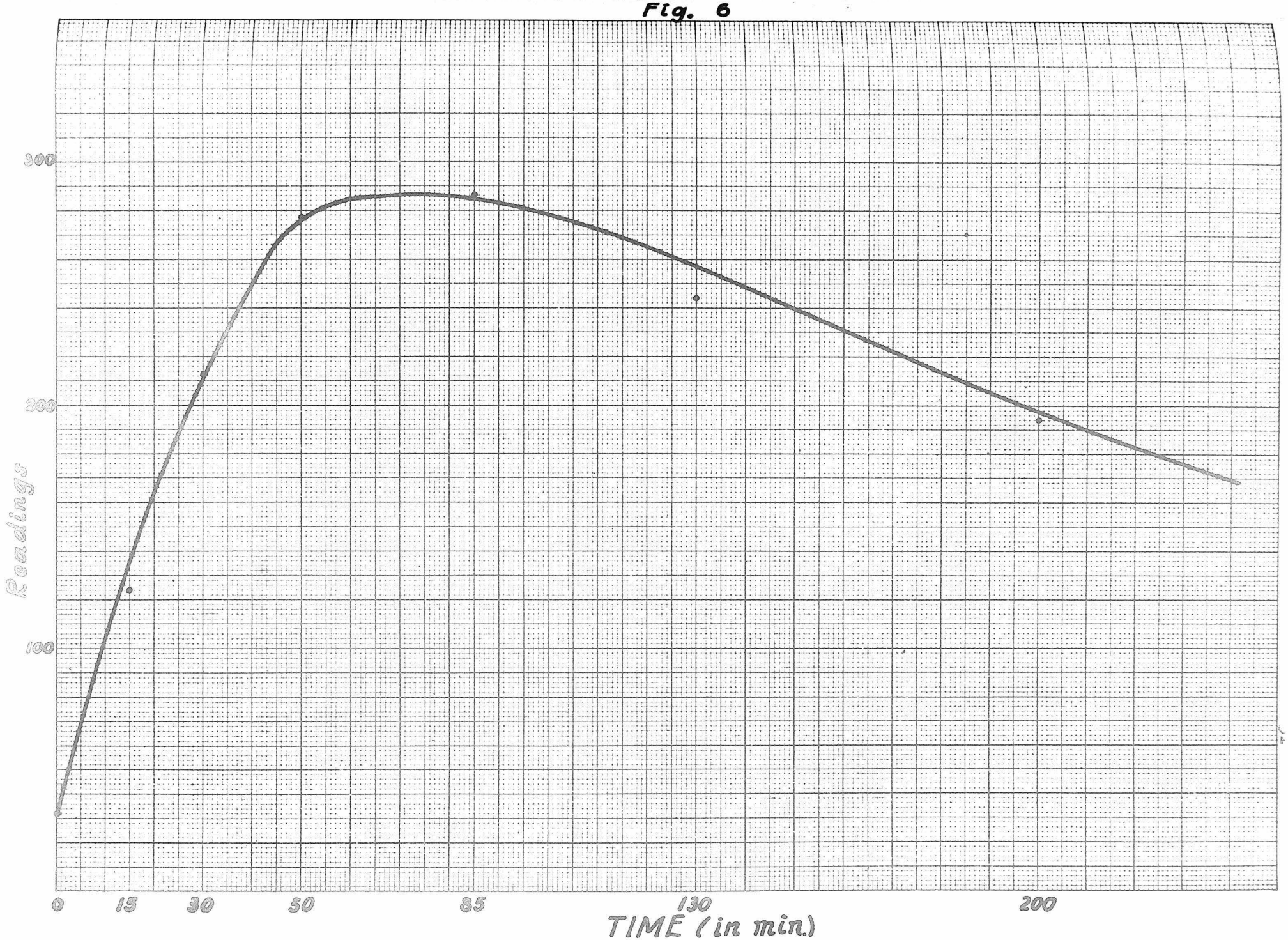
Legend for Fig. 6.

Expt. P-43. Conditions identical with those of Fig. 5.

Determination of phosphorylase equilibrium.

Color of the starch-iodine complex • ——

Fig. 6



starch rise to a maximum and then decrease again.

In all experiments whenever the starch synthesis was determined by the increase in optical density of the iodine-starch complex in the reaction mixture it was found that at the beginning the color increased with the amount of starch synthesized, but that as soon as the equilibrium was attained as judged by attainment of constant phosphate concentration, the color density began to decline. At the same time the color changed from a deep blue to a violet blue and violet reddish suggesting that either:

a/ Starch had been hydrolyzed by some non-phosphorolytic system

b/ Starch had undergone some structural changes resulting in decreased absorption of light by the starch-iodine complex.

Since the enzyme preparation had been freed of amylase by the fractional precipitation the fading in color could not be due to the action of amylase.

If during the period of declining iodine-starch color phosphorylase activity was tested by adding more Cori ester, an immediate synthesis of starch, at a rate similar to the initial resulted. Phosphorylase was therefore fully active in the equilibrium solution.

The second possibility above appears then to be the more likely especially since no re-esterification of starch to Cori

ester took place, pH of the digest remained unchanged and the equilibrium in terms of the phosphate fractions remained the same. Internal changes in the molecule of synthetic starch would appear to be taking place.

The reaction of iodine with starch has been long used as a method for determining starch, but recently it has been used in attempts to demonstrate the helical structure of starch (60), (21), (22), (3) and has yielded some positive evidence for such a structure.

Natural starch consists of two fractions which are designated as amylose and amylopectin or β-amylose and α-amylose respectively (37). The former is easily soluble in water and forms a slightly viscous solution, retrogrades from solution, is completely hydrolyzed by β-amylase and consists of straight unbranched chains of glucose molecules joined in 1 - 4 linkages. Amylose produces a color with iodine which is deep blue and six times as intense as that produced by amylopectin.

The amylopectin fraction is less soluble and gives a relatively opalescent and highly viscous solution, produces a violet-red or reddish color with iodine and is formed of chains of glycopyranose residues linked through 1 - 4 linkages but with frequent branches at position 6 of the gluco-pyranose residues.

The molecular structure of synthetic starch has been determined by Hassid (36) and by Haworth and coworkers (39). They found by methylation experiments and the end group assay

that it resembles the amylose fraction of starch in having a continuous chain length of eighty to ninety glucose members.

While these investigations were under way a paper appeared by Haworth and coworkers (40) who reported a separation from potato tubers of an amylase free enzyme system which catalyzes the conversion of glucose-1-phosphate into a polysaccharide which is not amylose, but which probably is identical with the amylopectin components of starch. The polysaccharide is composed of d-glucose units and gives a purple red color with iodine. It is soluble in water and does not retrograde from solution, is hydrolyzed by β-amylase with liberation of maltose but as with the natural amylopectin the hydrolysis is arrested before the conversion to maltose is complete, approximately 49% and 45% of these polysaccharides being converted by the enzyme in four hours. This behavior is characteristic of a branched chain sugar. They suggested that in phosphorylase there is a "Q" factor which modifies the normal synthetic activity of phosphorylase and enables 1,6 glycosidic linkages to be formed. As pointed out earlier the change in light absorption of the starch-iodine complex suggests and gives some support to the idea that such "Q" factor might be present in the enzyme preparations. Such a factor could cause the slow structural changes from straight to branched chain polysaccharide, from an amylose to an amylopectin structure. Critical evidence on this point could be obtained by hydrolysis with β-amylase of the

pre and post equilibrium products.

Absorption spectra of the iodine-starch complex before and after the equilibrium were made and are given in figure 7. The absorption spectra are different and show that:

a/ The maximum may shift from 340 to 345-350 mμ .

b/ The maximum at 580 mμ disappears.

c/ The absorption in the longer wave lengths is much decreased.

The changes in color intensity are due primarily to the change in the peak at 580 mμ .

In summary of the methods for determination of phosphorylase activity it can be said that by the starch-iodine method it is possible to measure relative amounts of enzyme very conveniently. The method is approximately quantitative up to the time when the maximum amount of starch has been synthesized. However, when equilibria are to be determined it is best to use the phosphate method.

Variability due to Manipulation and Sampling. - The variability in enzyme extraction and determination of the activity was investigated.

A standard lot of potatoes was cut into halves and the halves placed in two piles. Phosphorylase was extracted separately from each pile, and the activity of each measured. This experiment was repeated many times giving the results represented in figure 8 which shows that the curves of rates of the enzymes extracted from each pile of potatoes may almost be superimposed, either for

Legend for Fig. 7.

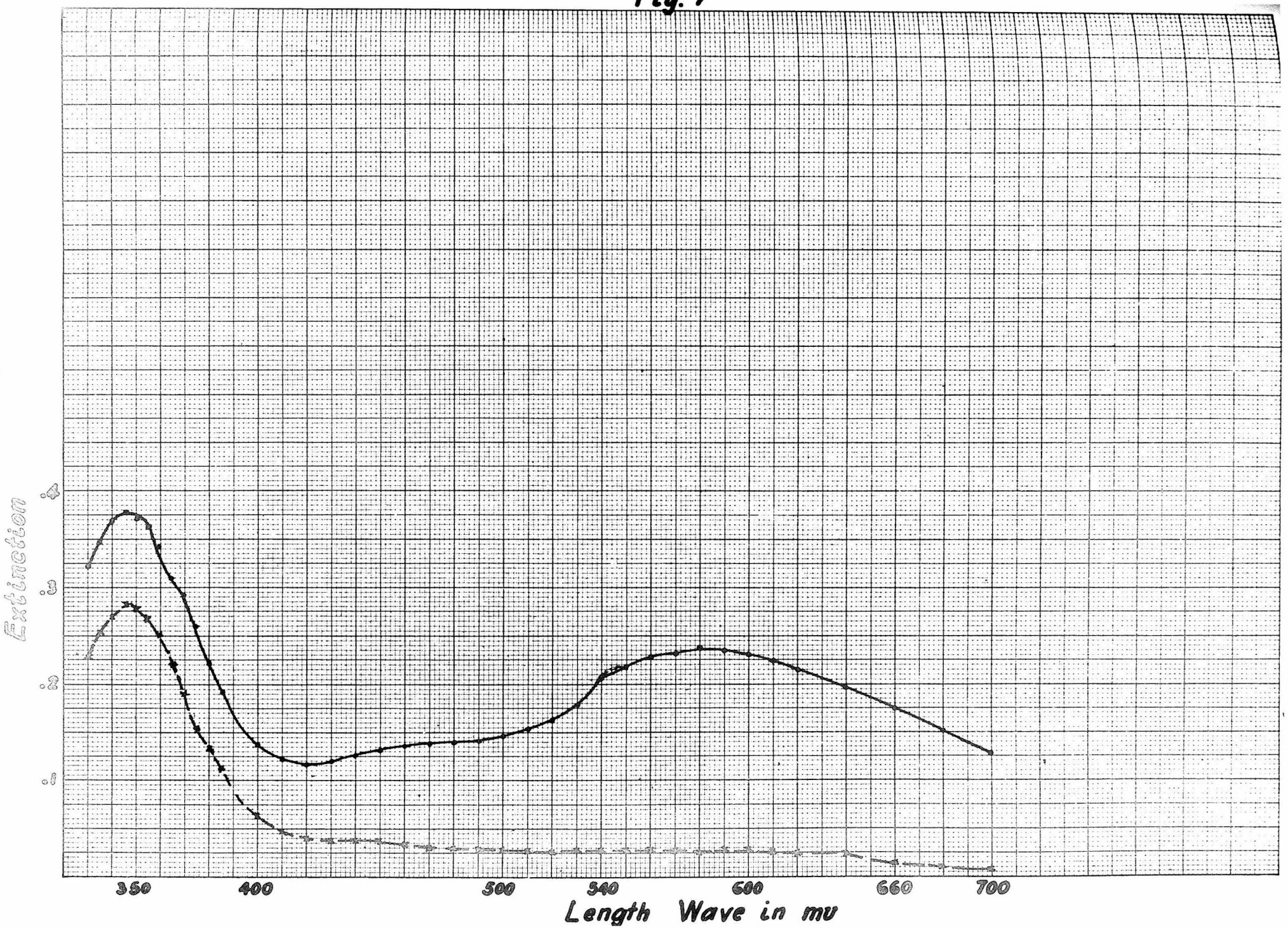
Expt. P-128. White Rose potatoes from 0° storage. Digest contained 5 cc. of 5x concentrated enzyme, 3 cc. of maleate buffer pH 5.5, 30 mgs. of Cori ester and 2 mgs. of starch. Total volume 20 cc. Incubated at 25°C.

Absorption spectra of the starch-iodine complex.

Sample taken before equilibrium is reached •———

Sample taken after equilibrium is reached ×— — —

Fig. 7



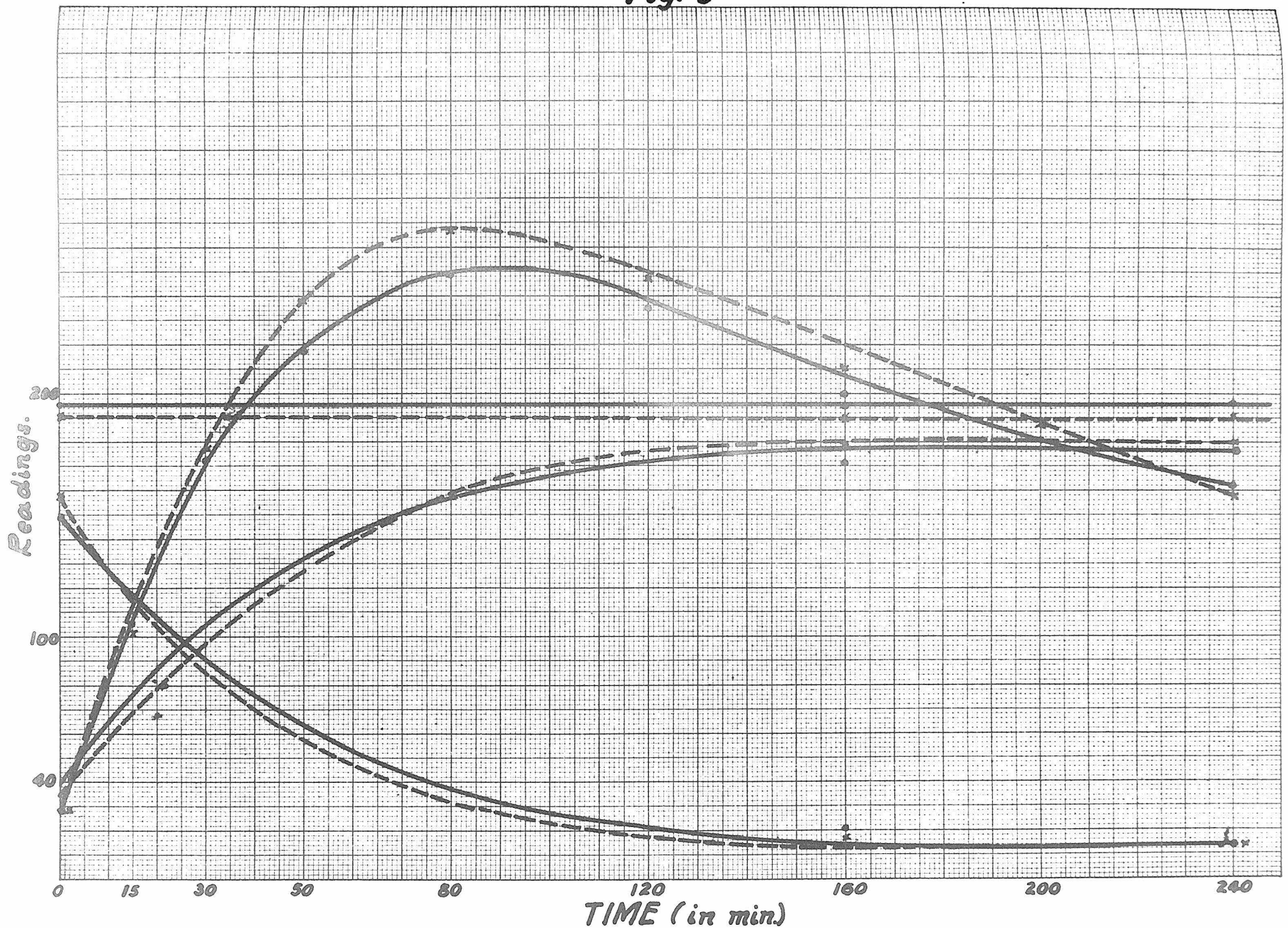
Legend for Fig. 8.

Expts. P-71 and P-72. Russet potatoes stored at 25°C. Digests contained 5 cc. of 5x concentrated enzyme, 3 cc. of maleate buffer pH 5.5, 30 mgs. of Cori ester and 2 mgs. of starch. Total volume 20 cc. Incubated at 25°C.

Variability due to manipulation and sampling.

Expt. No.		
P-71	Starch	• ———
	Inorganic phosphate	• ———
	Organic "	• ———
	Total "	• ———
P-72	Starch	× — — —
	Inorganic phosphate	× — — —
	Organic "	× — — —
	Total "	× — — —

Fig. 8



starch synthesis or inorganic phosphate liberation. Extraction and analytical variability is therefore small.

Another experiment was carried out to find out the variability of the potatoes themselves as to phosphorylase content. Two standard sized groups of potatoes from the same batch were extracted for phosphorylase in the standard way and again here the enzymatic activity determined. The curves of enzymatic synthesis are represented graphically in figure 9 which shows that the rates of enzyme action are very close for the two preparations and that errors due to sampling and variability of potato tubers themselves are small. Not only the rate of polysaccharide synthesis but also the position of the final equilibrium reached was the same (fig. 8).

Equilibrium attained in fig. 8 in terms of % of phosphates

	At equilibrium in a digest of pH 5.5	
Curve	inorganic phosphate in % of the total phosphate	organic phosphate in % of the total phosphate
1	93	7.0
2	95	6.0

This position of the equilibrium may also be determined reproducibly.

Activity of Various Concentrations of Phosphorylase. - A highly concentrated enzyme was prepared and then diluted to give a range of concentrations. These concentrations were related to the original volume of the potato juice used in the fractionation. Thus a

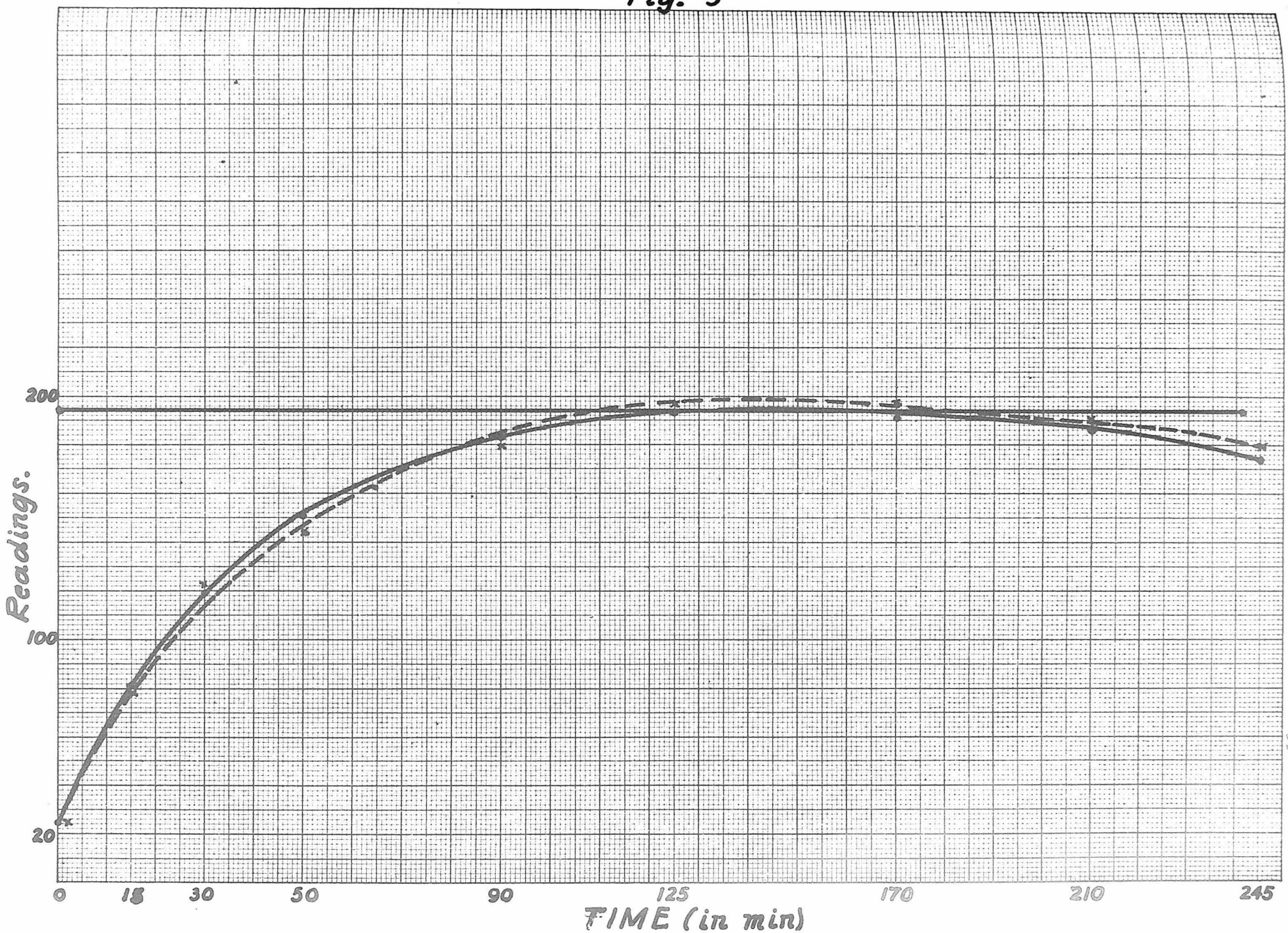
Legend for Fig. 9.

Expts. P-73 and P-74. Conditions similar to those of Fig. 8.

Errors due to sampling and variability of potatoes.

Expt. No.		
P-73	Starch	• ———
	Total phosphate	◦ ———
P-74	Starch	× — — —
	Total phosphate	• ———

Fig. 9



twenty times concentrated enzyme means one which after the purification steps was taken up in 1/20 of the original volume. This evaluation is based on the assumption that all the phosphorylase present in the juice passed through all the purification stages with no loss in quantity and activity or that the losses in each case were the same when working under exactly the same conditions. The enzyme concentrations studied were: 20x, 10x, 5x and 2.5x.

Results of such an experiment are represented graphically in figure 10, in which time is plotted in the abscissae against colorimeter readings of the starch-iodine complex on the ordinate. Colorimeter readings are directly proportional to the concentration of starch. In figure 10 there are represented the curves for each concentration of enzyme. It can be deduced that the rate of starch synthesis is proportional to the enzyme concentration within the range of concentrations studied here. In figure 10 it can be seen that there is a good proportionality between enzyme concentration and activity as shown for the following values of K when 50% of the substrate (glucose-1-phosphate) has been used up. $K = ET$, where T is time in minutes and E enzyme concentration.

Legend for Fig. 10.

Expt. P-17. White Rose potatoes from the market. Digest contained 5 cc. of enzyme, 3 cc. of maleate buffer pH 5.5, 30 mgs. of Cori ester and 2 mgs. of starch. Total volume 20 cc. Incubated at 25°C.

Activity of various concentrations of phosphorylase.

Enzyme concentration

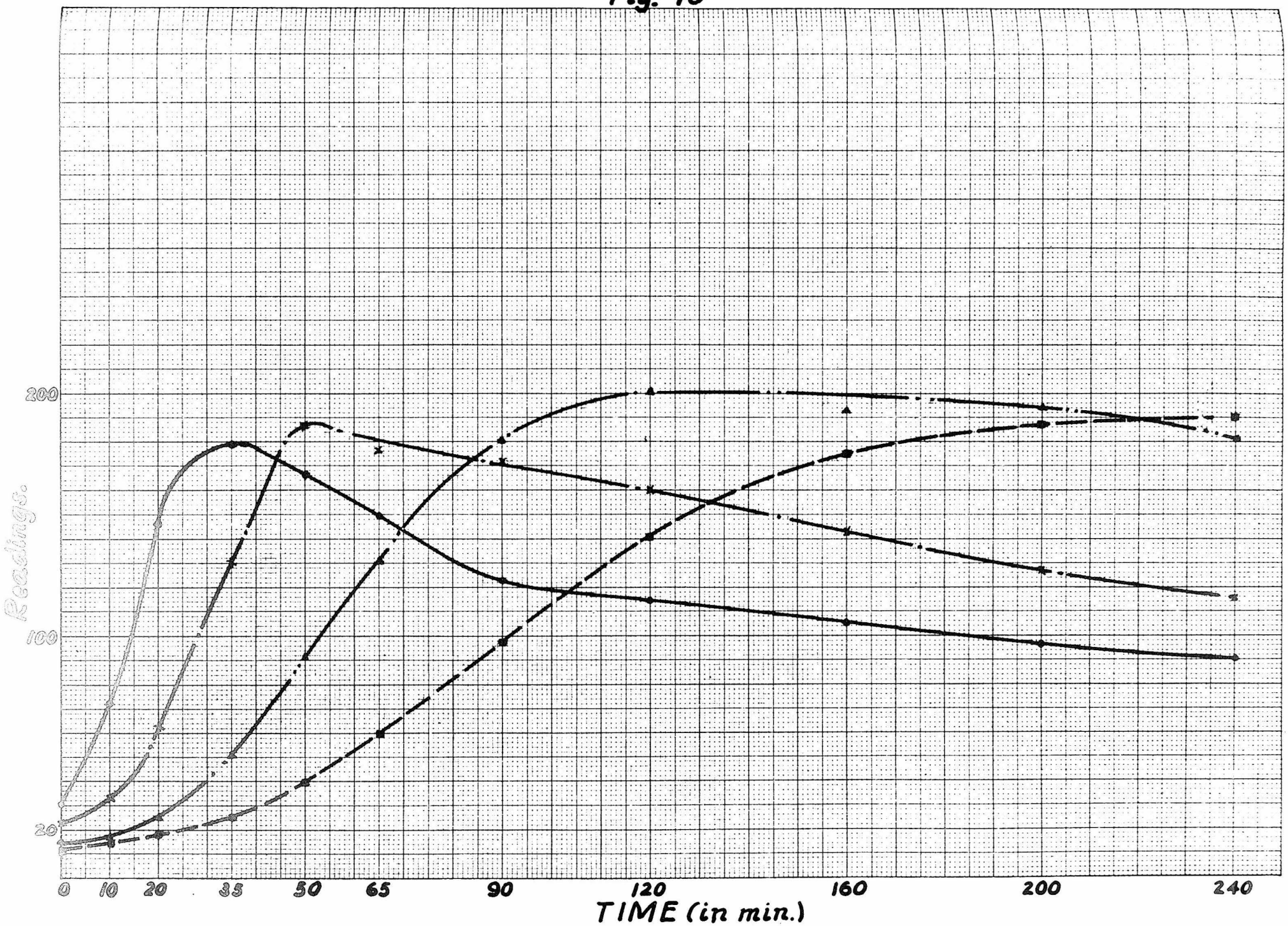
20x ● ————

10x × ———·———

5x △ ———··———

2.5x ■ — — —

Fig. 10


200
100
20
Readings.
0
10
20
35
50
65
90
120
160
200
240
TIME (in min.)

Curve	Concentration of enzyme	Values of K (= Enz. conc. x Time)
A	20 x	14.0
B	10 x	14.0
C	5 x	14.1
D	2.5 x	12.8

The final equilibria attained and the total amounts of starch synthesized from equal amounts of Cori ester were the same for all enzyme concentrations.

If the mgs. of starch synthesized per hour during the linear portion of each curve are plotted against the concentration of the enzyme figure 11 is obtained which summarizes the data and clearly establishes that the initial rate of reaction is approximately a linear function of the enzyme concentration. However, it seems that at higher concentrations the substrate may be a limiting factor.

Effect of pH on the Activity and Equilibrium. - The pH in the digest was adjusted by means of maleate (71) and borate buffers. In these experiments all other factors were kept constant and the pH was varied from 4.5 to 7.5.

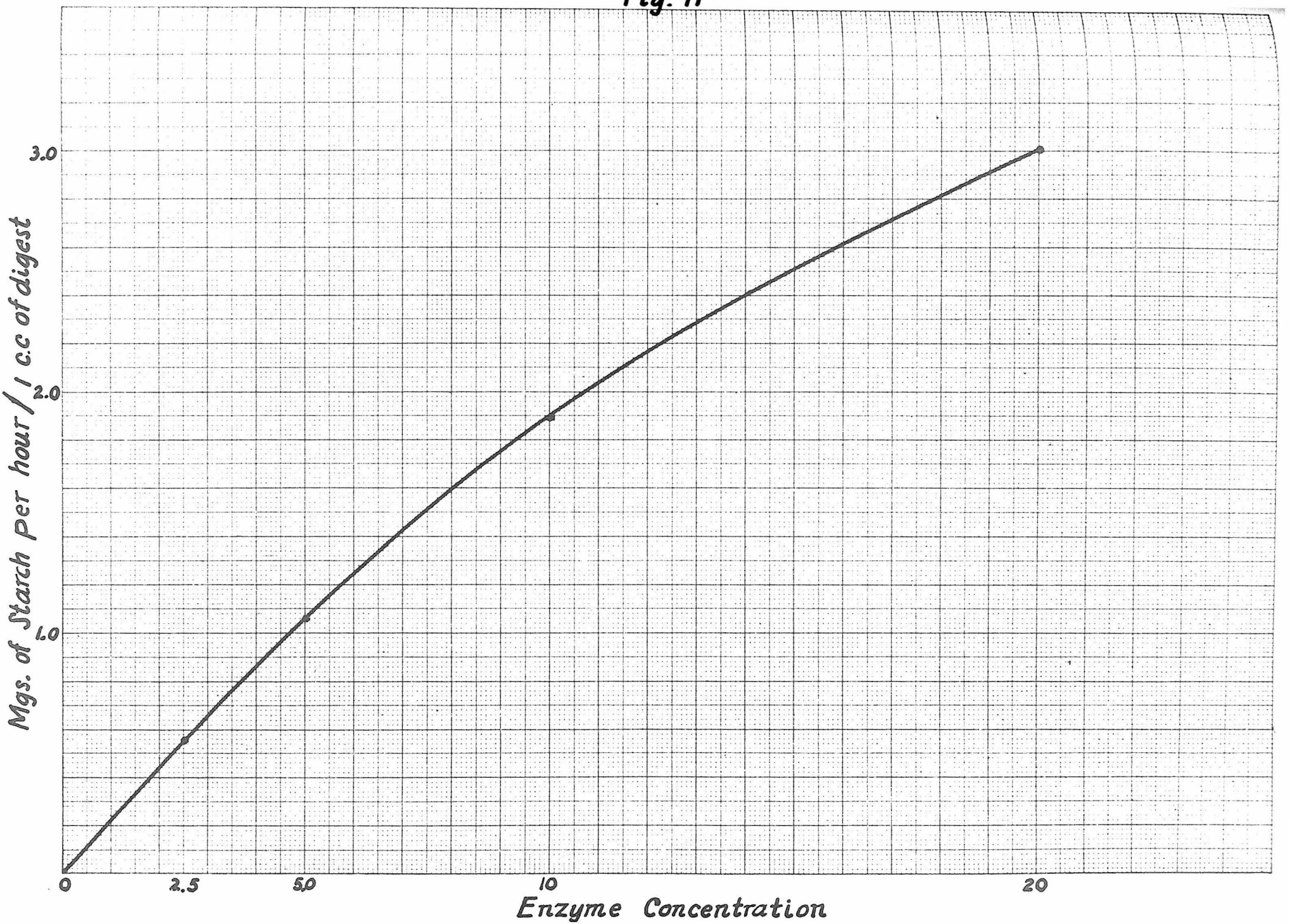
In figure 12 the results are depicted graphically with starch formation plotted against reaction time. Hydrogen ion concentration affected the rate of reaction markedly, the maximum rate corresponding to pH 5.5. Above or below this value there is a decrease in rate which is more noticeable towards the less acid side.

Legemd for Fig. 11.

Expt. P-17. Conditions identical with those of Fig. 10.

Effect of Enzyme concentration on the rate of Phosphorylase

Fig. 11


3.0
2.0
1.0
Mgs. of Starch per hour / 1 c.c of digest
0
2.5
5.0
10
20
Enzyme Concentration

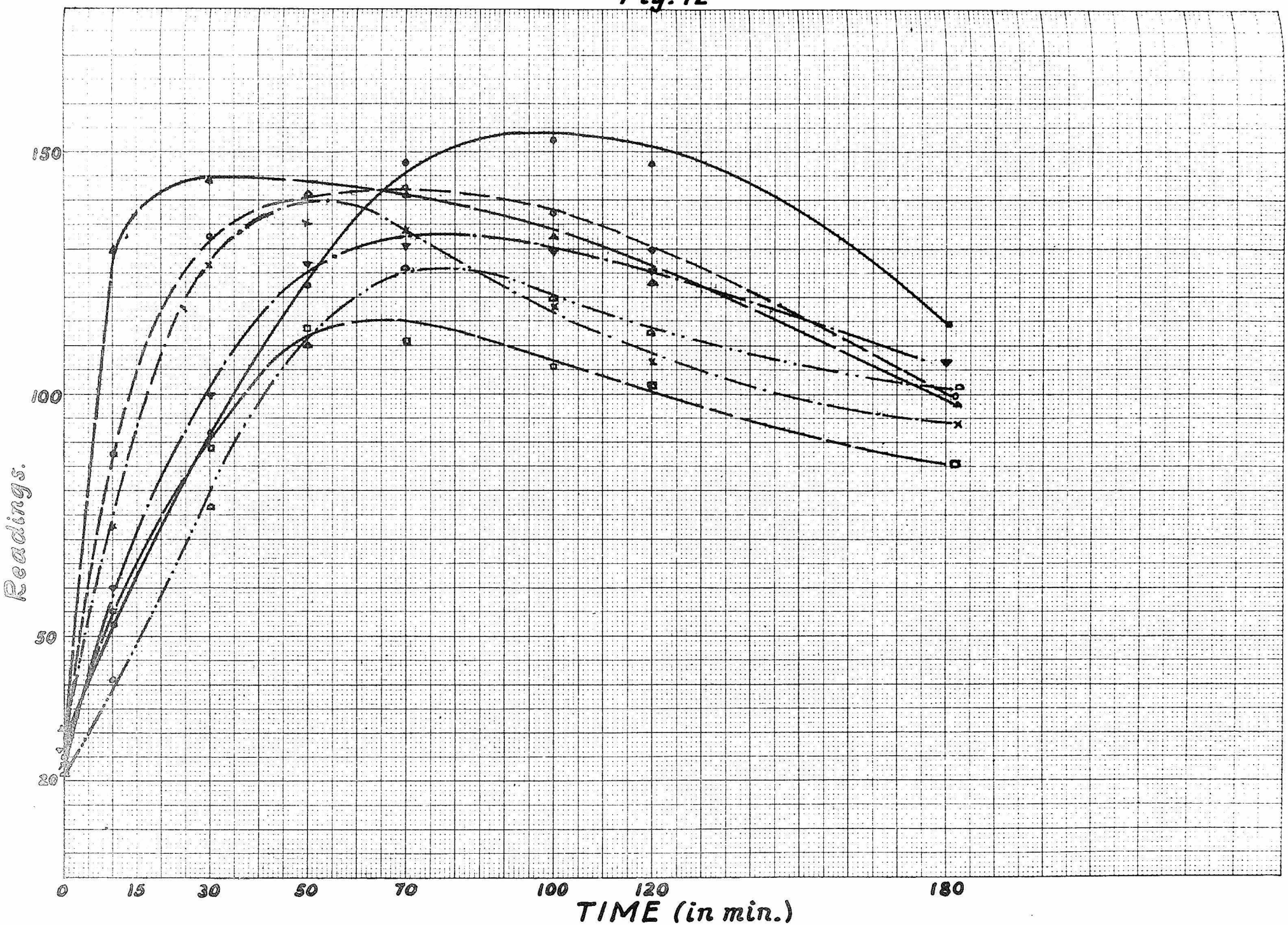
Legend for Fig. 12.

Expt. P-13. White Rose potatoes from the market. Digests contained 5 cc. of 10x concentrated enzyme, 3 cc. of buffer, 30 mgs. of Cori ester and 2 mgs. of starch. Total volume 20 cc. Incubated at 25°C.

Effect of pH on phosphorylase activity.

Digest No.	pH of digest	
1	4.5	● ———————
2	5.0	o — — — — —
3	5.5	▲ ——— ———
4	6.0	× —·—·—·—
5	6.5	▼ ———·———
6	7.0	○ —··—··—··
7	7.5	□ ——— — ———

Fig. 12



Plotting the slopes in the steep linear portions of the curves we get figure 13, in which starch synthesized in mgs. per cc. of digest per hour appears in the ordinate as function of pH in the abscissa. This figure shows that the velocity of starch synthesis is controlled in this case by the pH of the reacting mixture, and that there is a sharp optimum at pH 5.5.

The hydrogen ion concentration not only affects the rate of reaction but also the position of the final equilibrium. Under more acid conditions a larger proportion of the Cori ester in the digest is converted to starch and inorganic phosphate, as can be seen below. Previous workers found that this effect of

Table 4

Effect of pH on the equilibrium of phosphorylase.

Digests contained 30 mgs. of Cori, 5 cc. of enzyme preparation, 3 cc. of buffer and 2 mgs. of starch. The incubation temperature was 25°C.

pH of digests	4.5	5.0	5.5	6.0	6.5	7.0	7.5
mgs. of starch per cc. of digest at equilibrium	1.04	.99	.98	.95	.90	.85	.78

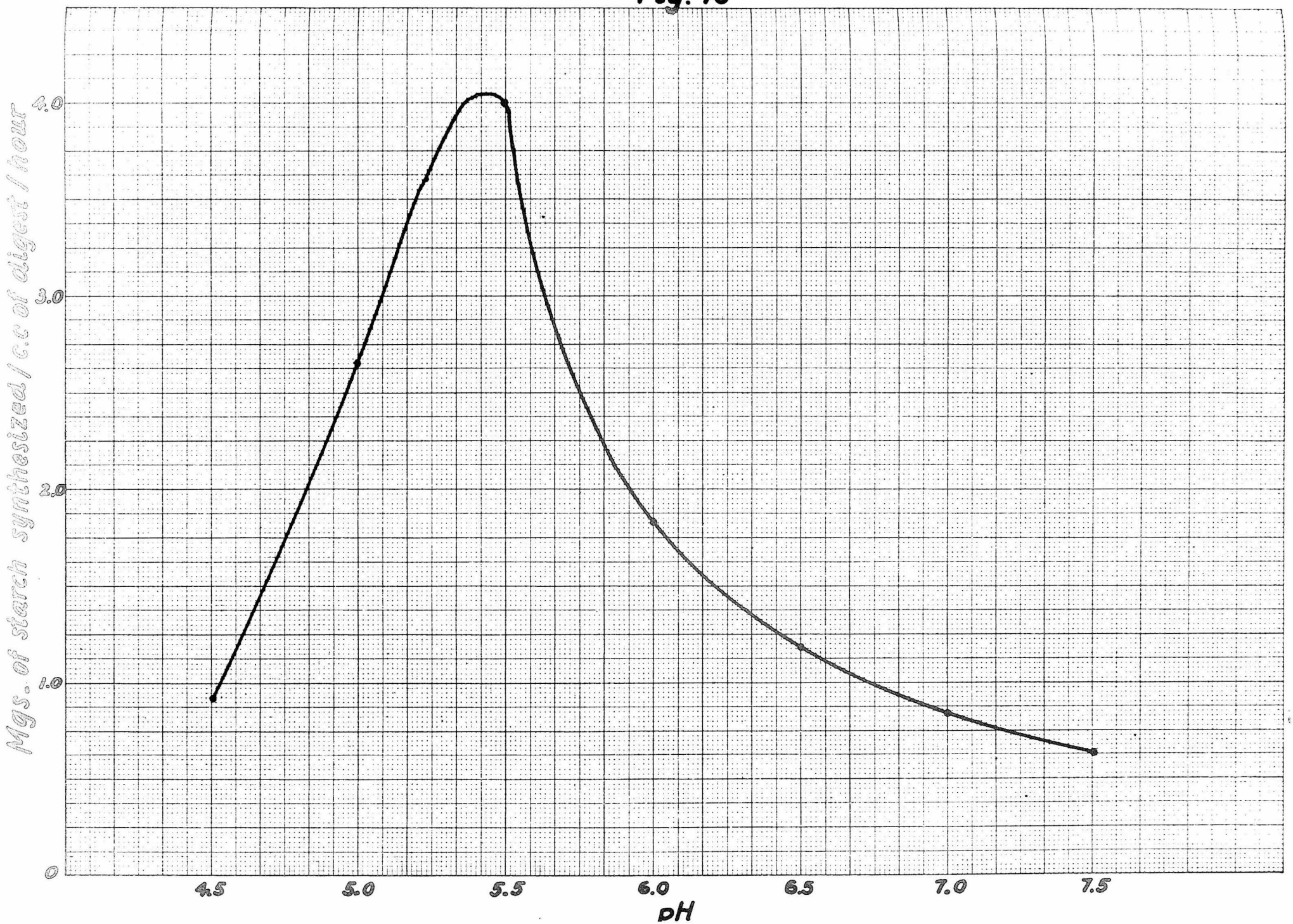
the pH on the equilibrium is due to the fact that inorganic phosphate and Cori ester possess different dissociation constants. Cori and Hanes have demonstrated that the equilibrium is determined exclusively by the divalent ions of phosphoric and glucose-1-phosphoric acids. Hanes determined the equilibrium constant of the reaction:

Legend for Fig. 13.

Expt. P-18. White Rose potatoes from the market. Digests contained 5 cc. of 5x concentrated enzyme, 3 cc. of buffer, 30 mgs. of Cori ester and 2 mgs. of starch. Total volume 20 cc. Incubated at 25°C.

Effect of pH on the initial rate of reaction.

Fig. 13


Mgs. of starch synthesized / c.c of digest / hour
4.0
3.0
2.0
1.0
0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
pH

$$\frac{HPO_4^{=}}{C_6H_{11}O_5OPO_3^{=}} = 2.2$$ within the range pH 4.0 to pH 7.0

He also determined at equilibrium the following values for the phosphate fractions in the digest.

23% of total phosphate as Cori ester

pH 7.0
77% " " " " inorganic phosphate
8% " " " " Cori ester

pH 5.0
92% " " " " inorganic phosphate

Plotting the data of table 4 graphically we obtain a series of points which roughly approach a straight line as shown in figure 14.

In the present investigation the equilibrium was determined many times at pH 5.5 both from the starch synthesized as well as by the inorganic phosphate liberated. The equilibrium was reached at the same time in the two cases as can be seen in figures 5 and 6. Figure 5 has three curves, one for the increases in inorganic phosphate, a second for the changes in concentration of Cori ester and a third for total phosphate.

In summary, hydrogen ion concentration affects the rate of phosphorylase action and determines the final equilibrium of the reaction.

Effect of Substrate Concentration. - Several experiments were carried out using fifteen, thirty and sixty mgs. of Cori ester in digests containing the same enzyme preparation and standard amounts of buffer and priming substance.

Legend for Fig. 14.

Expt. P-13. Conditions identical with those of Fig. 12.

Effect of pH on the amount of starch synthesized by phosphorylase.

Fig. 14

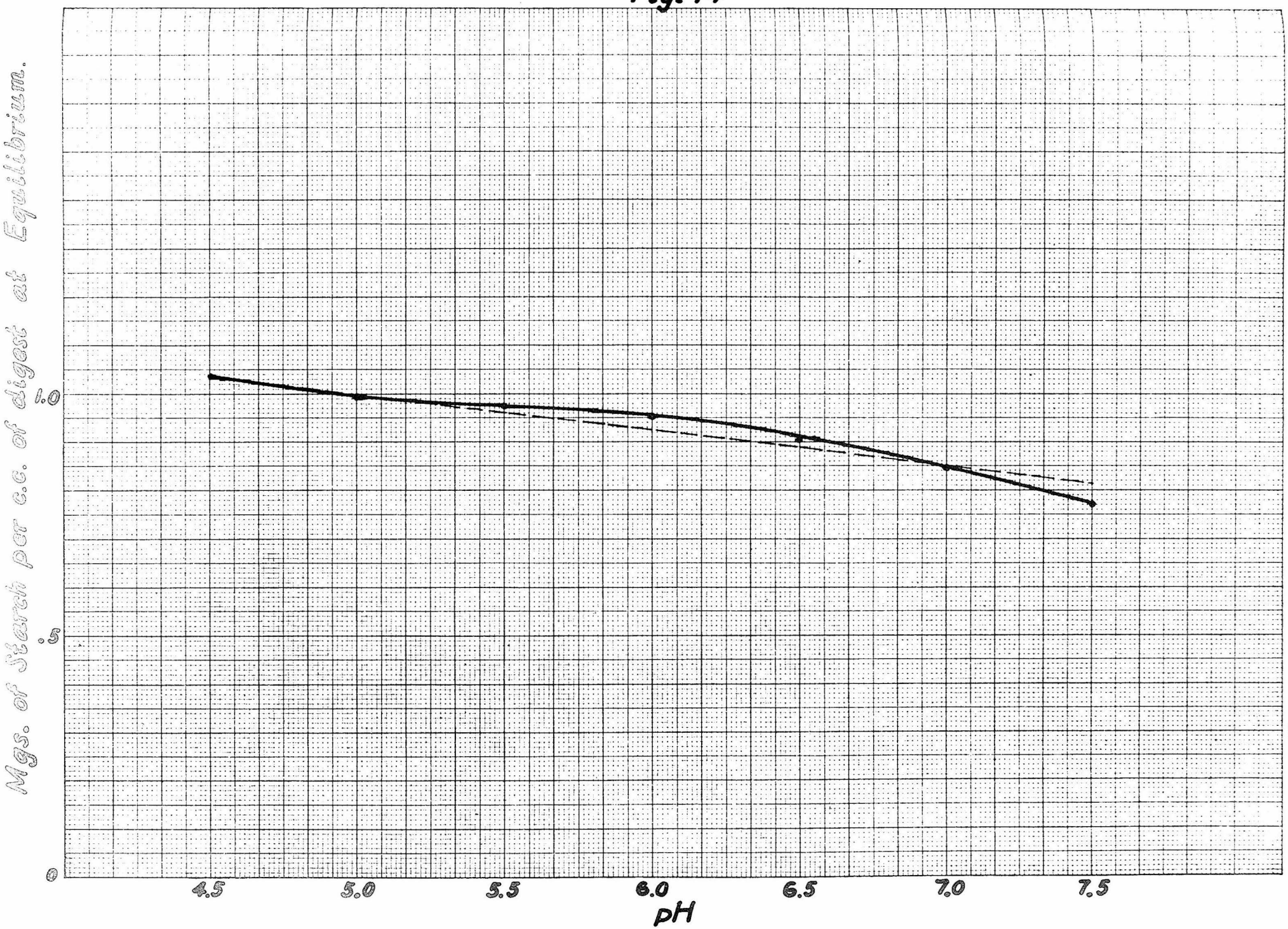

Mgs. of Starch per c.c. of digest at Equilibrium.
1.0
.5
0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
pH

Figure 15 shows the results of such an experiment in which all other conditions were held constant. The rate of starch synthesis was proportional to the amount of Cori ester added. The rate of inorganic phosphate liberation is also proportional to the substrate added as can be seen in figure 16.

The amounts of Cori ester do not change the position of the equilibrium markedly and only the rate of enzymatic action is proportional to the concentration of the substrate, this shows clearly that under the condition of the experiment the enzyme was not completely saturated with substrate.

Calculating the equilibrium for these 3 digests in terms of phosphates, we get Table 5.

Table 5

Equilibria attained in terms of % of phosphates in fig. 16.

Digests contained the same amount of substances as those of Table 4 and only Cori ester varied.

		At equilibrium	
Digest No.	Amount of Cori ester added to digest	Inorganic phosphate in % of the total	Organic phosphate in % of the total
1	15	11%	85%
2	30	9%	88%
3	60	9%	90%

This shows that as would be expected the effect of substrate concentration on the position of equilibrium is negligible.

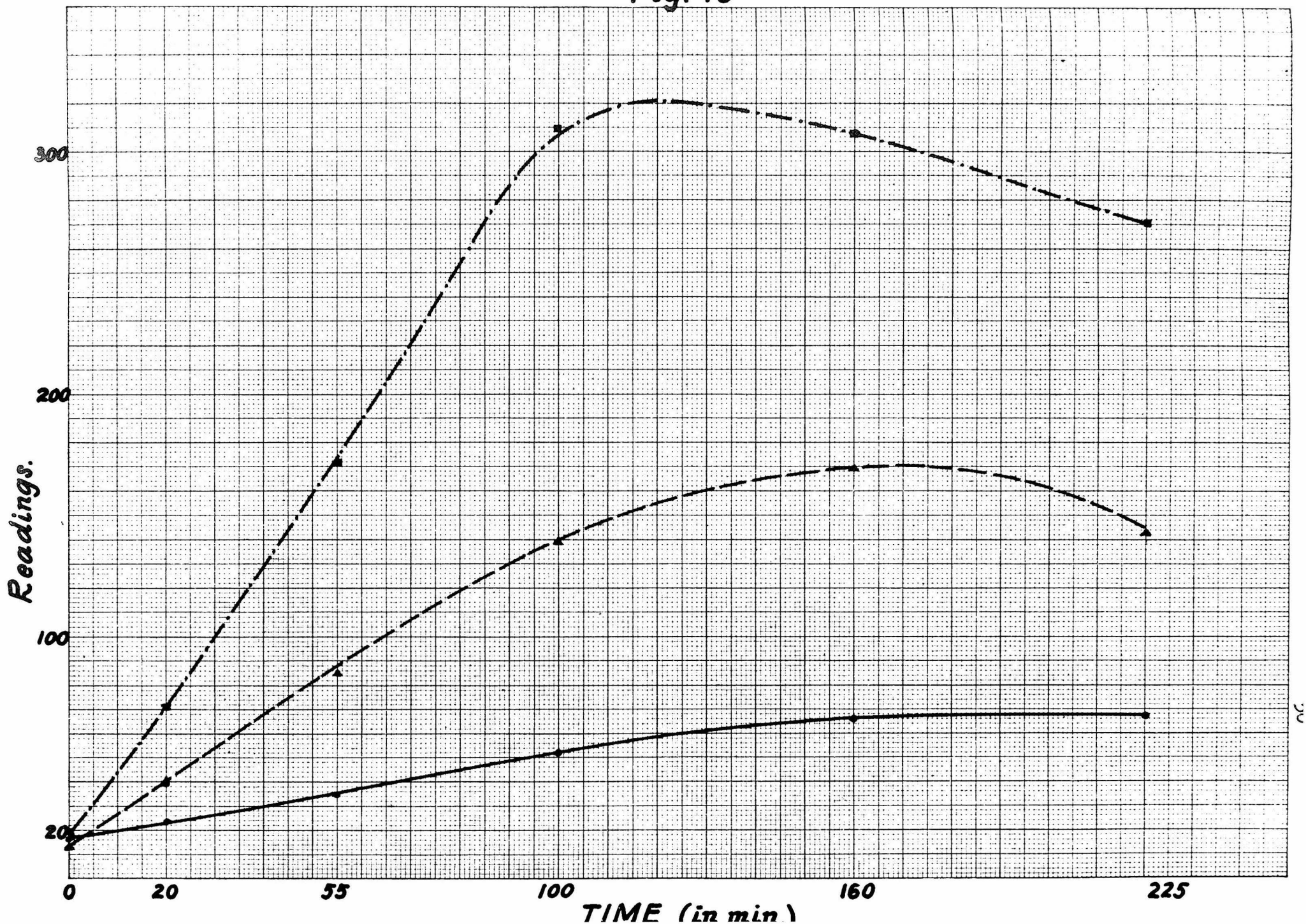
Legend for Fig. 15.

Expt. P-44. White Rose potatoes from 0°C. storage. Digests contained 5 cc. of 5x concentrated enzyme, 3 cc. of maleate buffer pH 5.5, Cori ester and 2 mgs. of starch. Total volume 20 cc. Incubated at 25°C.

Effect of substrate concentration on phosphorylase.

Digest No.	Mgs. of Cori ester	Starch synthesis
1	15	● ————
2	30	▲ — — — —
3	60	■ — · — · — ·

Fig. 15



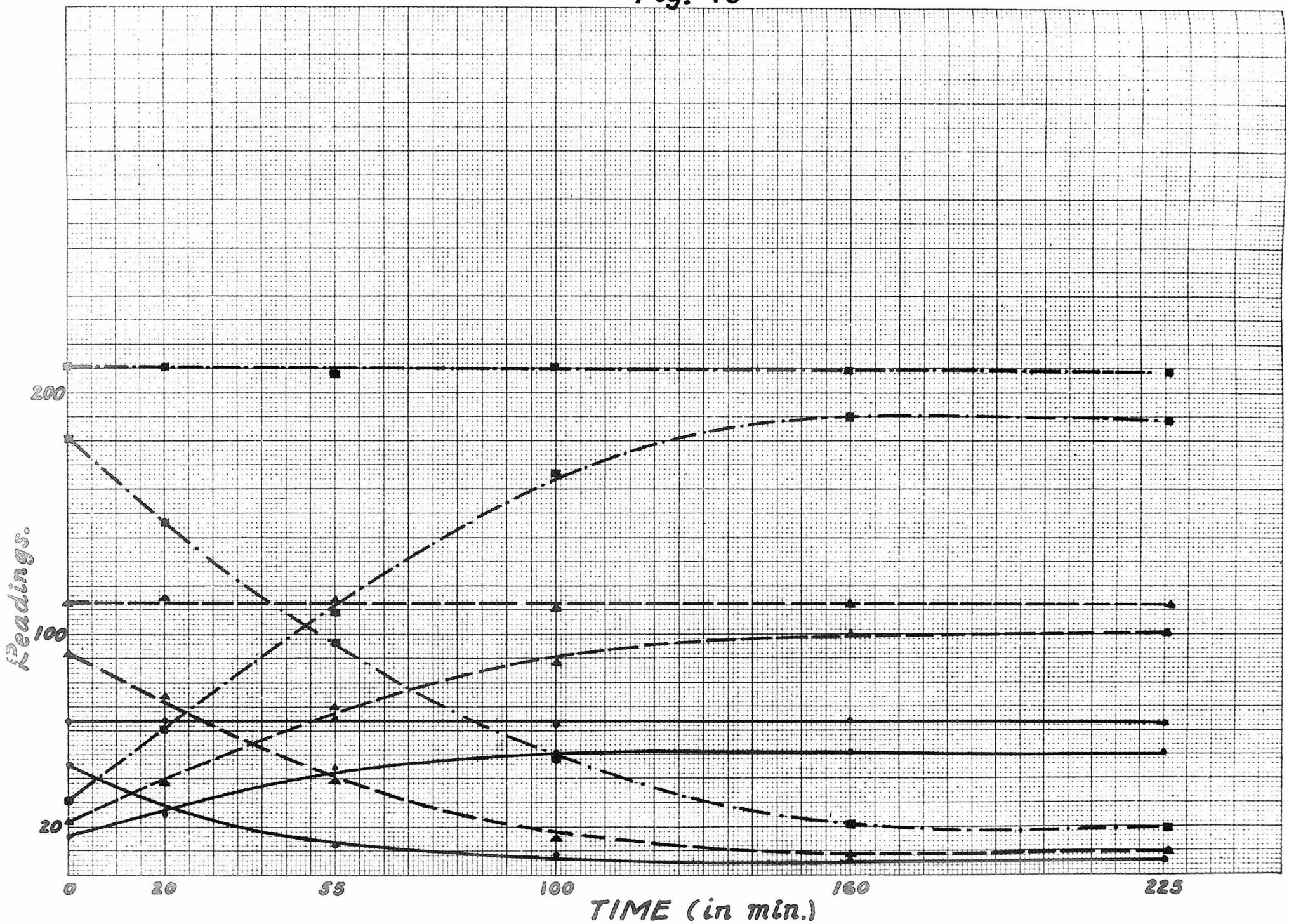
Legend for Fig. 16.

Expt. P-44. Conditions identical to those of Fig. 15.

Effect of substrate concentration on phosphorylase.

Digest No.	Mgs. of Cori ester	Phosphate changes in digests	
1	15	Inorganic phosphate	● ———
		Organic "	● ———
		Total "	● ———
2	30	Inorganic phosphate	▲ — — —
		Organic "	▲ — — —
		Total "	▲ — — —
3	60	Inorganic phosphate	■ — · — · —
		Organic "	■ — · — · —
		Total "	■ — · — · —

Fig. 16



Effect of Various Temperatures. - Experiments were carried out with all factors but temperature of digest constant. The temperatures used were 0°, 10°, 20°, and 30° and 40°C. ± 0.5°C.

Here again starch synthesis was the measure of enzyme activity as is shown in figure 17. It was found that the temperature affected the rate of reaction up to a certain limit. The equilibrium was not affected by temperature since the final amount of starch synthesized when equilibrium was attained was the same at all experimental temperatures used, as must be expected for an enzymatic process associated with an insignificant production of heat. The rate of reaction was however influenced by temperature. Calculating the temperature coefficient, or Q_{10}, for the rates plotted in figure 17 at three different points we obtain the following, where

$$Q_{10} = \frac{\text{Velocity at } T^\circ + 10^\circ}{\text{Velocity at } T^\circ}$$

	Q_{10} between			
Time in minutes	0-10°	10-20°	20-30°	30-40°
20	1.41	1.77	1.53	.94
35	2.00	2.17	1.50	.84
50	2.30	2.13	1.32	.74

The values for Q_{10} all lie in the range .7-2.3. At the higher temperature the Q_{10} is smaller than 1 and decreases consistently with time suggesting a more rapid destruction of

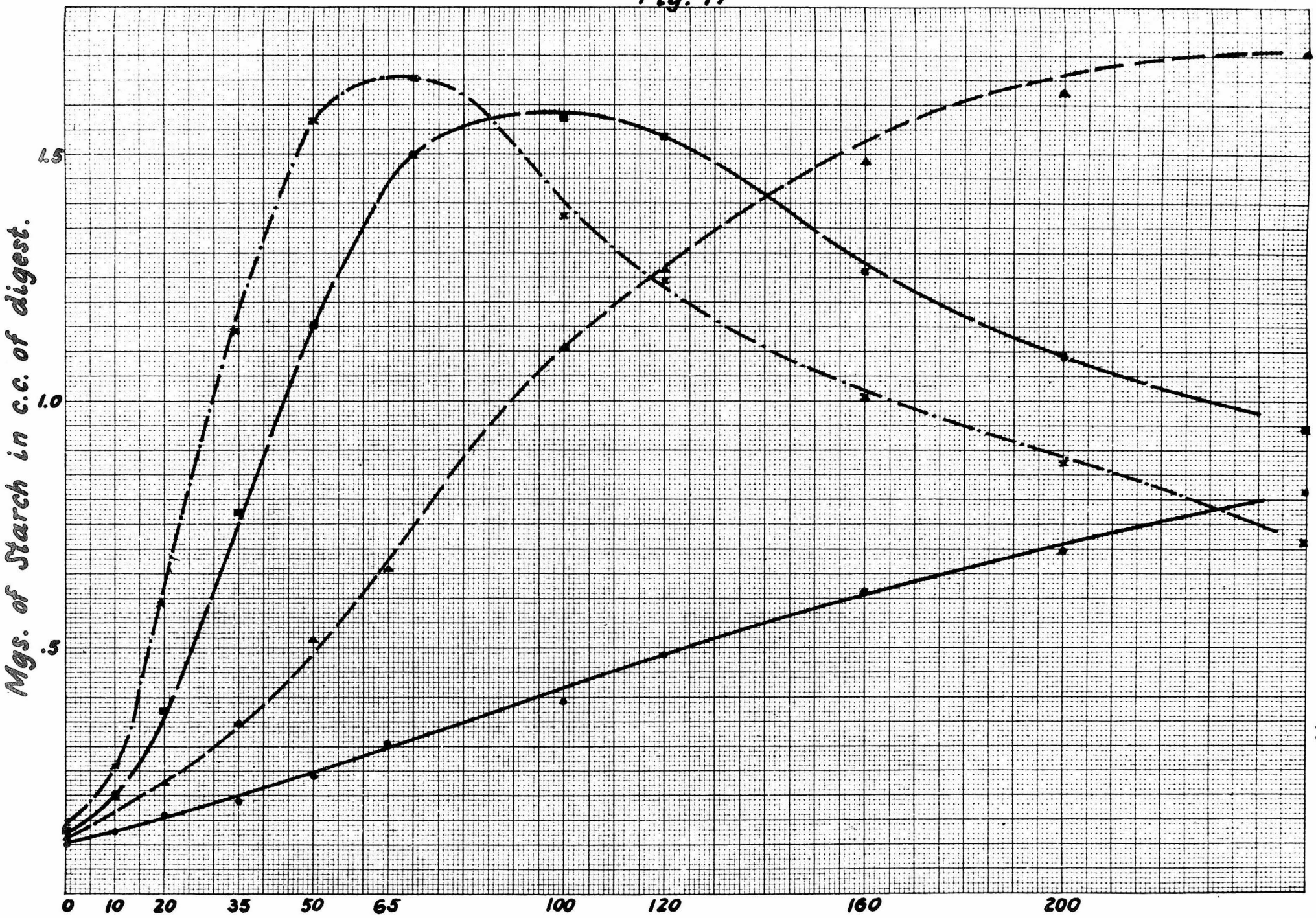
Legend for Fig. 17.

Expt. P-20. White Rose potatoes from the market. Digests contained 5 cc. of 5x concentrated enzyme, 3 cc. of maleate buffer pH 5.5, 30 mgs. of Cori ester and 2 mgs. of starch. Total volume 20 cc.

Effect of various temperatures on phosphorylase.

Digest No.	Incubated at °C.	
1	0	● ————
2	10	▲ — — — —
3	20	■ ——— ———
4	30	★ —·—·—·—

Fig. 17


Mgs. of Starch in c.c. of digest.
1.5
1.0
.5
0
10
20
35
50
65
100
120
160
200

the enzyme and a preponderance of this effect over the enhancing effect of higher temperature.

Early workers have shown that 61% of the activity of phosphorylase is lost by heating at 58°C. for three minutes while heating for the same time at 68°C. the loss in activity amounts to 97%.

Plotting the slopes of the steep linear parts of the curves of figure 17 against the corresponding temperatures we get figure 18 which shows that the optimum temperature lies at a little over 30°C. while at both sides of the maximum there is a rapid decrease in rate.

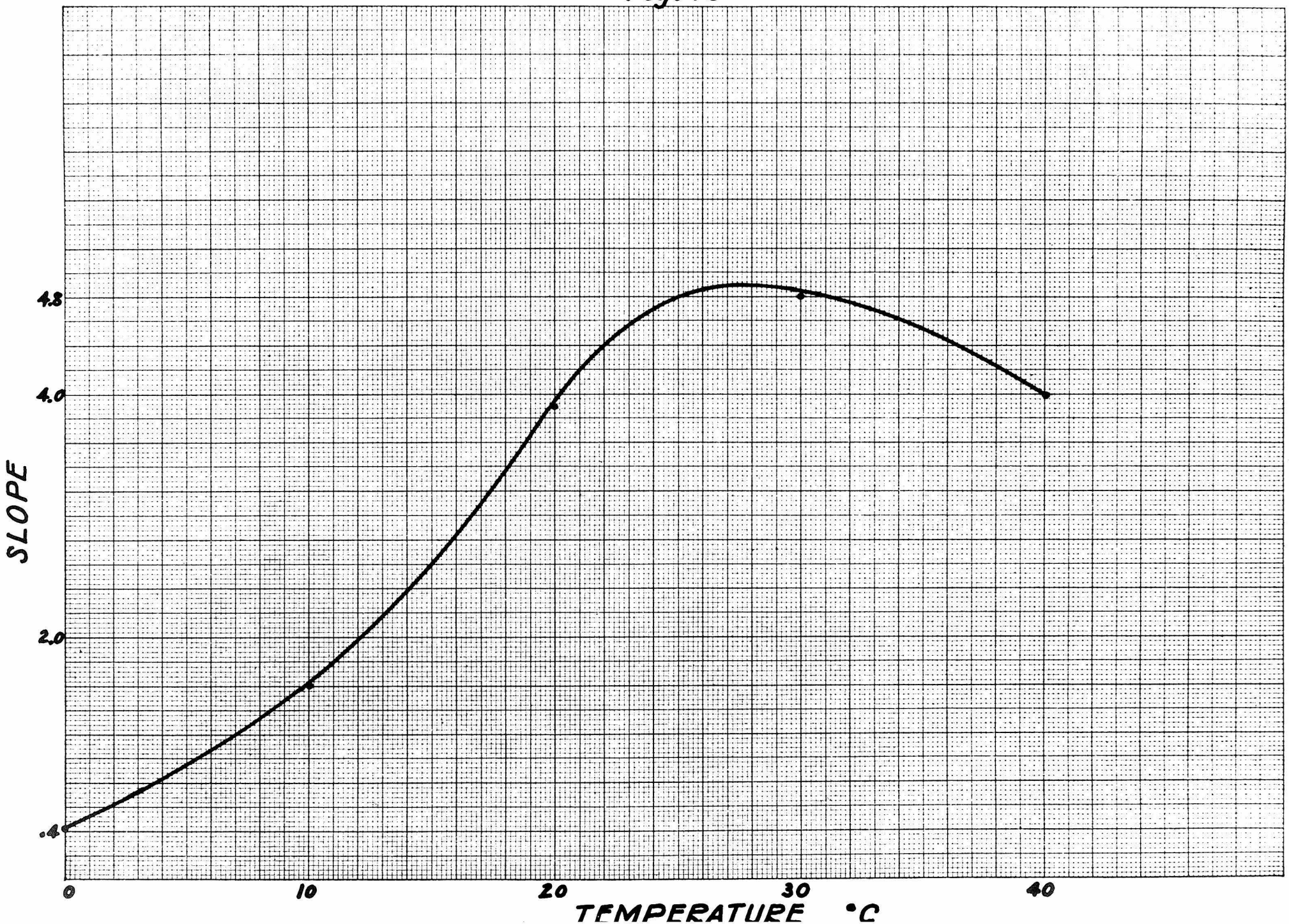
Effect of Various Temperatures of Storage in Phosphorylase. - Experiments were carried out to determine the effect of storage temperature on the phosphorylase activity of potato tubers. The purpose of these experiments was to determine quantitatively the changes in enzyme content and enzyme activity as a response to temperature and time of storage. This is particularly pertinent since the carbohydrate stored in potato tubers is mainly starch which is the source of sugars as was seen in Table 2, and consequently of the energy used in the various metabolic processes which take place in tubers. Potatoes from the market were randomized and then divided in four portions, the portions were stored at 0°, 10°, 20° and 30°C. Initial determinations of phosphorylase content and activity were made and after varying periods of storage more samples were taken for study of enzyme

Legend for Fig. 18.

Expts. P-20 and P-22. White Rose potatoes from the market were used. Digests contained 5 cc. of 5x concentrated enzyme, 3 cc. of maleate buffer, 30 mgs. of Cori ester and 2 mgs. of starch. Total volume 20 cc.

Effect of temperature on the initial rate.

Fig. 18



content. Comparisons of phosphorylase activity was based on the rate of starch formation from reacting mixtures prepared using standard amounts of components and standard enzyme preparations working under the same conditions at 25°C.

The results of one such storage experiment are given in Table 6.

Table 6

Storage Experiments

Temp. of storage in °C.	Time of storage in weeks	Temp. of reaction in °C.	Rate of starch formation in mgs./cc. digest/hr.	mgs. of starch/ cc. of digest at equilibrium
Market	0	25	2.0	1.5
0	3	25	2.1	1.3
10	3	25	1.6	1.2
20	3	25	2.0	1.4
30	3	25	2.0	1.5

This table represents average values of several enzyme extracts for each batch of potatoes. The results show that the activity of phosphorylase remained approximately constant under all conditions. The equilibrium was reached at the same time and the final amount of starch formed was roughly the same in all cases. However, when the digests were incubated at the same temperature as that of storage the rates of reaction as expected were a function of temperature of incubation as discussed above.

In a total of three large storage experiments results similar

to those just described were obtained. Thus the activity of phosphorylase is not affected by temperature of storage. The starch-Cori ester equilibrium is likewise little influenced by temperature.

Effect of temperature on phosphorylase hence cannot be the primary cause of sucrose formation in potato at low temperatures.

Phosphoglucomutase. - This enzyme is widely distributed in plants, animals and in microorganisms. It has been studied mostly in rabbit muscle, brain, heart, liver, also in frog muscle, and in yeast.

The enzyme causes the intramolecular migration of the phosphate group from the carbon atom 1 to the spacially adjacent carbon atom 6 in the glucose esters.

Glucose-1-phosphate (4%) $\rightleftharpoons$ glucose-6-phosphate (96%)

The reversibility of the reaction was proved using glucose-6-phosphate as substrate and also it was found that in the presence of phosphorylase the 1-ester was further used and made into glycogen.

Magnesium and manganese ions accelerate the rate of reaction very markedly (13) and without these ions the reaction proceeds slowly. The enzyme stands heating to a temperature of 50°C. for ten minutes with no loss in activity and is completely specific. Galactose-1-phosphate and mannose-1-phosphate cannot be used as substrates.

The activity of phosphoglucomutase can be followed by determining the unused Cori ester. This is based on the fact that glucose-1-phosphate is completely hydrolyzed in seven minutes at 100°C. with 1N sulfuric acid, while the glucose-6-phosphate is not appreciably hydrolyzed by this procedure.

Experimental. - Filtered whole potato juice was used for phosphoglucomutase determinations. The digest consisted of Cori ester, magnesium chloride, buffer of pH 7.5 and .006M phlorizin to poison the activity of phosphorylase, which otherwise converts part of the substrate into starch. The activity was determined by following the decrease in seven minutes phosphate which would account for the amount converted into non hydrolyzable glucose-6-phosphate.

The results of such experiments showed a small phosphoglucomutase activity which was larger in potatoes which had been stored at 25°C.

Phosphoglucomutase activity

Potatoes stored at (°C.)	% of decrease in seven min. hydrolyzable phosphate after 150 mins.	Decrease in seven mins. phosphate in mg./cc. of digest per hour
0	15.2	.009
25	23.7	.012

The data show only a very small phosphoglucomutase activity in the juice of potatoes.

Another indirect method for determination of phosphoglucomutase

should be possible adding fructose-6-phosphate to the filtered potato juice. This in the presence of the enzyme and of phosphorylase and isomerase should be converted to starch. Fructose-6-phosphate⇌Glucose-6-phosphate⇌glucose-1-phosphate⇌starch The results were however negative. This is to be partly attributed to the lability of isomerase as will be seen in the next section.

Summarizing phosphoglucomutase is present in potato tubers stored under both high and low temperatures. The activity of the enzyme in potato tubers is low.

Isomerase. - This enzyme could not be obtained in cell free preparations made from potato tubers. Whole juice and many fractional precipitations with various salts were used with no success. This enzyme will however be discussed under experiments in vivo.

Phosphatases. - In 1907 Japanese workers found that rice contained an enzyme which splits phosphoric acid from phytin. Later, phosphatases were found in bone, kidney, spleen, pancreas, blood, yeast, etc. They belong to the group of Esterases, the mono-esterases have been classified by some workers according with their optimum pH (5). They also differ among themselves in the specificity towards various substrates. Many phosphatases are activated by divalent metallic ions as magnesium, manganese, and cobalt. Early workers (57) have fractionated animal phosphatases.

Experimental. - Whole juice and fractions obtained with salt precipitations of the proteins were used as enzyme preparations. Precipitation with 50% saturated ammonium sulphate gave the most active fraction.

Fraction No.	% of ammonium sulphate saturation
1	30
2	40
3	50 (most active)
4	Remainder

The results show that the most active fraction is number 3. Whole juice however had still greater activity than any of the fractions as can be seen in figure 19. The activity was followed as the rate of inorganic phosphate liberated from the substrate, in this case fructose-6-phosphate. The optimum pH of potato phosphatase was determined and found to be near pH 8, the rate of phosphate liberated diminishing as the pH decreased below this value. Heating the enzyme one minute at 90°C. caused complete inactivation. The activity of potato phosphatase was also decreased 50% with a solution of 0.002% potassium fluoride, and the enzyme was also able to split phosphate from fructose-1,6-diphosphate.

Effect of Various Storage Temperatures on Phosphatase. - Russet potatoes were stored at 0° and 25°C., and phosphatase determinations made after three weeks of storage. The same phosphatase activity was found under both storage conditions as is graphically

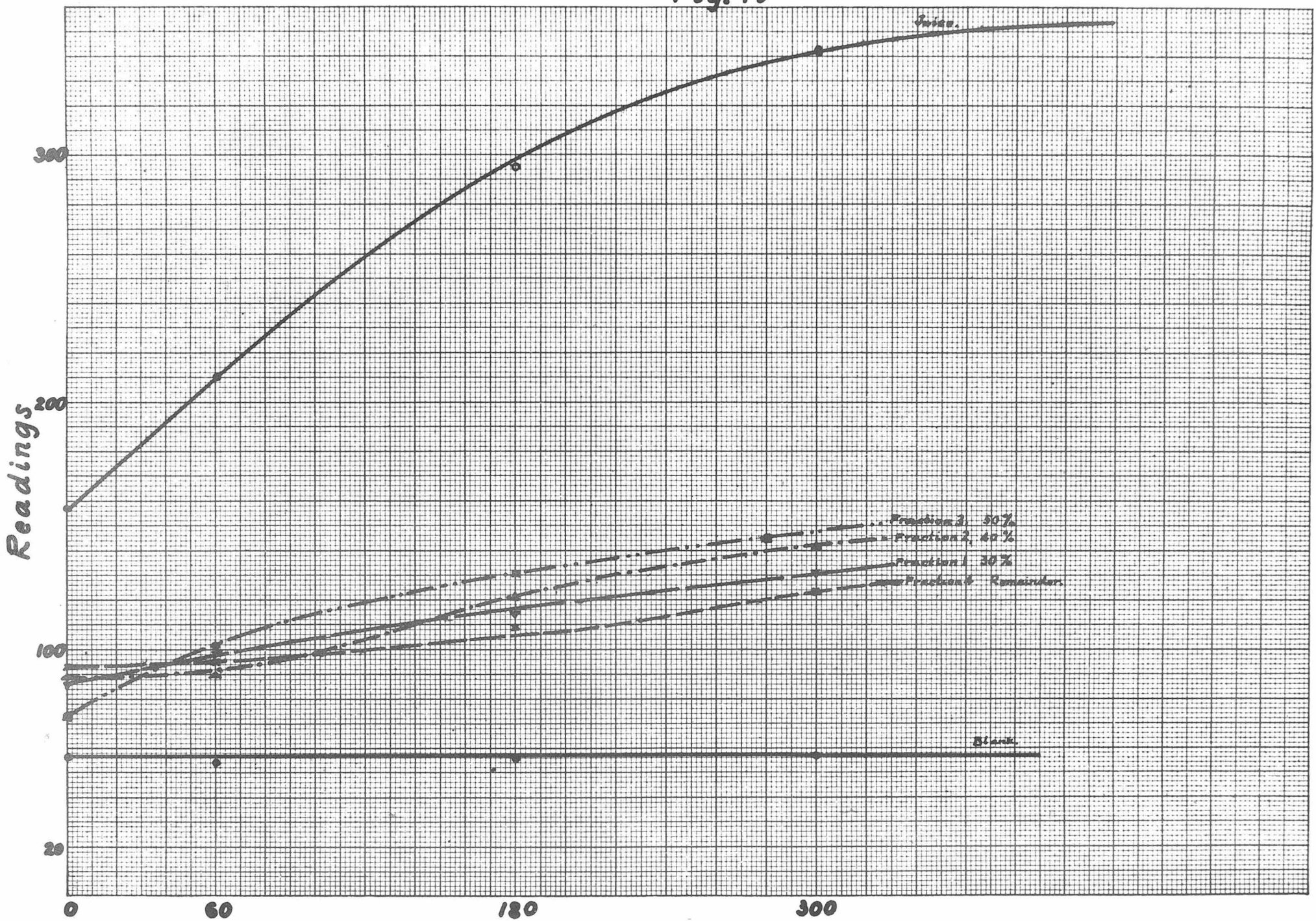
Legend for Fig. 19.

Expt. P-112. White Rose potatoes from 25°C. storage temperature. Digests contained 5 cc. of filtered juice or 5 cc. of protein solution, 3 cc. of borate buffer pH 8.0, 30 mgs. of fructose-6-phosphate as the sodium salt. Total volume 20 cc. Incubated at 25°C.

Phosphatase activity.

Digest No.	Enzyme used	Ammonium sulphate saturation of fractions	Inorganic phosphate liberated
1	juice		o ————
2	fraction 1	30%	▼ ————
3	" 2	40%	▲ — · — · — ·
4	" 3	50%	■ — · · — · · — · ·
5	" 4	remainder	× — — — — —

Fig. 19


Juice.
Fraction 3, 50%
Fraction 2, 60%
Fraction 1 30%
Fraction 4 Remainder.
Blank.
Readings
300
200
100
20
0
60
180
300

shown in figure 20 where the substrate used was fructose-6-phosphate. The average amounts of phosphate liberated by the preparation after 250 minutes are given in table 7.

Table 7

Effect of temperature of storage on potato phosphatase.

Digests contained: 10 cc. of saturated solution of fructose-6-phosphate, 2 cc. of 0.1M magnesium chloride, 3 cc. of borate buffer pH 8.0, and 5 cc. of filtered potato juice. Digests incubated at 25°C.

Temperature of storage in °C.	Time of storage	mgs., of inorganic phosphate liberated in 250 min./cc. of digest
Market	0	0.114
0	3 wks.	0.136
25	3 wks.	0.138

Other reaction mixtures containing phosphatase obtained from potatoes stored at 0° and at 25°C. were incubated at 0° and 25° respectively. It was found that the activity of phosphatase was completely suppressed by incubation at 0°C.

Hexokinase. - This enzyme was originally discovered in yeast and exists also in animal tissues. The enzyme catalyzes the transfer of phosphate from adenosine triphosphate to hexoses and is thus the first stage in glucose or fructose fermentation or respiration. According to Meyerhof the reaction is:

Adenosine triphosphate + 2 hexose → adenylic acid + 2 hexose-6-phosphate

Colowick and Kalckar (9) believe this reaction to be more complex.

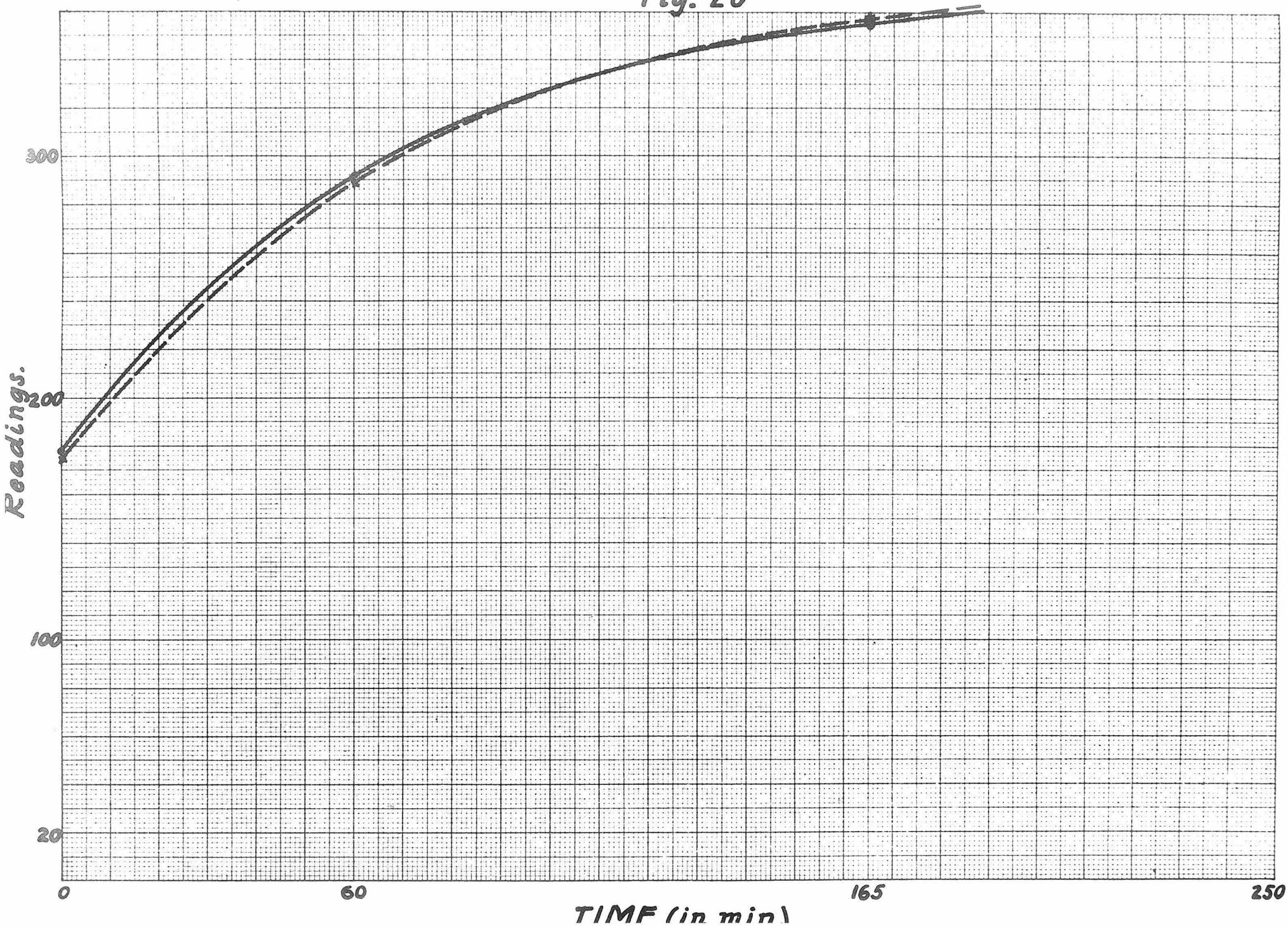
Legend for Fig. 20.

Expt. P-118. White Rose potatoes from 0° and 25° storage temperature tested after 3 weeks. Digests contained 5 cc. of filtered juice, 3 cc. of borate buffer pH 8.0, 30 mgs. of fructose-6-phosphate, and 2 cc. of 0.1M magnesium chloride. Total volume 20 cc. Incubated at 25°C.

Effect of storage temperature on phosphatase activity.

Digest No.	Storage temperature	Inorganic phosphate liberated
1	0°	● ———
2	25°	× — — — —

Fig. 20



They found that only one phosphate is transferred from ATP (adenosine triphosphate) to hexose in yeast preparation forming ADP (adenosine diphosphate). Two molecules of ADP may then react giving one molecule each of ATP and adenylic acid. The enzyme requires the presence of magnesium ions.

The activity of hexokinase can be followed by the disappearance of ATP or acid labil phosphate from the reaction mixture.

In some cases only one of the two labil phosphate groups in the nucleotide is transferable to sugar, an observation made by Meyerhof and Kiessling (65). The second labil phosphate can be transferred to the hexose provided that a heat stable protein present in muscle tissue is added to hexokinase. The heat stable protein has no activity by itself, in the presence of this protein and hexokinase the following reaction takes place:
Adenosine diphosphate + hexose → adenylic acid + hexose-6-phosphate
Experimental. - The presence of hexokinase was investigated in potato tubers in the following manner. Crude filtered potato juice was used as enzyme preparation. The digest consisted of potato juice, ATP, maleic buffer, glucose and magnesium ions. Fluoride was added to inhibit the breakdown of ATP by phosphatase. The activity was determined by the disappearance of acid labil phosphate which is converted to acid resistant glucose-6-phosphate. Thus any decrease in 7 minutes hydrolyzable

phosphate corresponded to the phosphate used in forming the glucose-6-phosphate. However the product thus formed could as well go to fructose-6-phosphate which is also highly resistant to acid treatment, (85% hydrolyzed with 1N hydrochloric acid in 180 minutes)

Inorganic phosphate appeared during the time of incubation which shows that not all the phosphatase activity was poisoned in this experiment.

The results of one experiment are given in table 8.

Table 8

Effect of temperature of storage on hexokinase activity.

Digest contained: 5 cc. of potato juice, .4 mgs. ATP/cc., .5 mgs./cc. glucose, buffer pH 6.5 and magnesium chloride .1 mg./cc., incubated at 25°C.

	Storage temperature 0	25°
Decrease in acid labil phosphate in mgs. P/cc. of digest in 250 minutes	.010	.012

These amounts are equivalent to .08 mgs. and .10 mgs. of glucose-6-phosphoric acid formed respectively per ml. of digest in 250 minutes.

The evidence presented here concerning the presence of hexokinase in potato tubers shows that it exists in small quantities and is equally active in potatoes stored at 0° and 25°C.

Summary of experiments in vitro. - The experiments in vitro with enzymes of the carbohydrate metabolism have shown that potatoes

contain the enzyme system responsible for phosphorolytic starch breakdown as well as systems that intervene in further transformation of sugars. The presence of these enzymes and the fact that phosphorylated sugars do occur in potato establishes the possibility that the initial carbohydrate breakdown in potato may follow pathways similar to those found in yeast and muscle. None of the enzyme systems studied were affected by temperature in any manner that could suggest that the system in question may be responsible for the accumulation of sugars by potatoes stored at low temperatures.

EXPERIMENTS IN VIVO

Experiments in vivo. - In the previous section isolated systems of the carbohydrate metabolism in potatoes were studied. Once the various enzyme systems are isolated their kinetics and equilibrium constants can be determined but it remains necessary to prove that these same systems are indeed active in living cells. When the cells are broken the components are mixed, producing a condition which does not exist in the intact tissue. It is quite possible that in organized tissue the interposition of membranes and interfaces might prohibit the occurrence of reactions which are found in extracts or in minced tissue. Even if the reactions that occur in vitro also occur in vivo they would not be the same. Usually the rate of reaction of an isolated enzyme system of the carbohydrate breakdown is extremely high as compared with the rates of respiration in living cells. Thus cells contain regulatory mechanisms such as the arrangement of enzymes inside of the cells, the interposition of interfaces, and perhaps the presence of other specific diffusible controlling substances as inhibitors, activators, etc.

In carbohydrate breakdown most of the reactions are reversible and in whole cells the concentration of reacting substances and of products, the concentration of ions which accelerate or retard the establishment of equilibrium, the hydrogen ion concentration, and the temperature are factors which determine the rate and the orientation of each reaction.

In the isolated system at constant temperature, and pH, the concentration of inorganic phosphate and glucose-1-phosphate

determines the equilibrium of the following reaction. Thus for

Starch + inorganic phosphate $\rightleftharpoons$ glucose-1-phosphate

25°C. and pH 7 this equilibrium is attained with 77% of inorganic phosphate and 23% of glucose-1-phosphate.

In reaction 2 of the glycolysis cycle the equilibrium is reached with 5% glucose-1-phosphate and 95% of glucose-6-phosphate. In reactions 1 and 2 of the glycolysis cycle the equilibrium is almost independent of temperature changes while in reaction 6, the conversion of hexose to triose phosphates, the equilibrium is greatly affected by temperature change. If, in reaction 1, glucose-1-phosphate is removed by other enzymatic reactions, the breakdown of starch will continue and must continue until the concentration of inorganic phosphate and glucose-1-phosphate has reached equilibrium conditions. When the concentration of inorganic phosphate is decreased reaction 1 will proceed in the direction of starch synthesis.

Results obtained with isolated enzyme systems give no complete picture of the behaviour of the enzymes of the carbohydrate metabolism when they are all present together as happens in potato tubers. Also synthesis of sucrose could not be obtained with whole potato juice. Experiments with intact potato discs were therefore carried out. This technique has been used by many workers for studies on salt uptake and is generally similar to the tissue slice technique used in animal tissues.

Method. - Potatoes from the market were stored at 0°, 9°, 16° and 25°C. temperature for various lengths of time. Cylinders were

cut from the tubers with a cork-borer, and then sliced into discs 1 to 2 mm. thick. The discs were washed in running tap water for 20 minutes and then immersed in beakers containing the various test solutions. They were next infiltrated by being transferred to desiccators, where vacuum was applied until no more air came out from the intercellular spaces of the discs. The vacuum was then released and the discs allowed to take up the circumambient liquid.

Controls were run simultaneously in each experiment by infiltrating the discs with distilled water under exactly similar conditions.

After the vacuum was released, the discs were transferred to Petri dishes provided with a sheet of filter paper wetted in the same infiltration solution, to keep the atmosphere inside of the dishes moist. They were then incubated at the desired temperature for varying lengths of time. At harvest the surface of the discs were washed thoroughly; they were then dried at 70°C. for analysis as described elsewhere.

The following substances were infiltrated in various concentrations: glucose, fructose, sucrose, Cori ester, glucose-6-phosphate, fructose-6-phosphate and fructose-1,6-diphosphate as their sodium salts. The following poisons were also used: potassium cyanide, iodoacetic,acid, sodium fluoride, arsenite and phlorizin.

Synthesis of sucrose by potato discs. - Sucrose was synthesized by potato discs which were infiltrated with aqueous solutions

of glucose or fructose, a result which strongly suggests that the two simple sugars are used as intermediates in sucrose synthesis.

In table 9 are summarized the results of a typical experiment in which the potatoes used had been stored at 25°C.

Table 9

Synthesis of sucrose by potato discs.

Infiltrated with	Incubation temperature in °C.	Time of incubation	Increase in sucrose content after incubation in % of dry weight
Water	0	24 hrs.	.34
	25	"	.04
1% Glucose	0	"	.59
	25	"	1.48
1% Fructose	0	"	1.38
	25	"	2.13
10% Fructose	0	"	.59
	25	"	4.29

In table 9 the values of sucrose have been corrected for hexoses and non-sugar reducing substances. Repeated experiments of this kind showed that the amount and rapidity of sucrose synthesis is influenced by the concentration of substance infiltrated, previous storage temperature and the incubation temperature. The storage temperature as seen in table 2 controls the kind and amount of sugars and sugar derivatives found in the tubers. Thus

potatoes at 25°C. storage were very high in glucose-6-phosphate but contained very little fructose or sucrose.

Comparing the figures of 25°C. incubation of table 8 with corresponding figures for incubation at 0° it can be seen that incubation at 25° always produced the larger increases in sucrose concentration. In addition, fructose was more effective in inducing sucrose synthesis than was the same concentration of glucose, which suggests that the lack of sucrose synthesis in potatoes stored at high temperature might perhaps be due to the absence of fructose.

Numerous experiments like the one described were carried out; all gave the same trends of sugar concentrations and relative quantities of sucrose synthesis.

Effect of temperature of storage on sucrose synthesis. - Infiltration experiments were carried out to find the effect of storage temperature upon the synthesis of sucrose by potato discs infiltrated with glucose or fructose. Potato tubers which had been stored during two weeks were used. The results of such an experiment appear in tables 10 and 11 which show the increase of sucrose during the incubation period in % of dry weight of the potato discs. Table 10 gives the sucrose synthesized when the discs were incubated at the previous storage temperature. Table 11 gives results when all discs were incubated at 25°C. which was used as a standard incubation temperature to compare the synthetic efficiency of tubers from the various storage temperatures.

Table 10

Effect of temperature of storage upon sucrose synthesis by potato discs.

The values in the four columns represent the increases in sucrose during the incubation period in % of dry weight.

Infiltrated with	Incubation temperature in °C.	Time of incubation in hours	Storage temperature 0°	9°	16°	25°
Water	Same as storage	24 hrs.	1.80	.38	.67	.46
1% Glucose	"	"	3.00	1.47	1.14	.60
1% Fructose	"	"	2.85	.70	.86	1.90
3% Glucose	"	"	4.00	1.66	----	----
3% Fructose	"	"	3.65	1.31	1.71	1.97

Table 11

Effect of temperature of storage upon the synthesis of sucrose by potato discs.

The values in the four columns represent the increase in sucrose during the incubation period in % of dry weight.

Infiltrated with	Incubation temperature in °C.	Time of incubation in hours	Storage temperature 0°	9°	16°	25°
Water	25°	24 hrs.	-.50	.24	.46	.46
1% Glucose	"	"	.65	.24	----	.60
1% Fructose	"	"	-.25 ?	.56	.92	1.90
3% Glucose	"	"	2.15	1.50	1.44	1.85
3% Fructose	"	"	2.25	.95	2.05	1.97

Storage temperature influenced the sucrose making mechanism of the discs since at the standard incubation temperature at 25°

the most efficient in synthesizing sucrose are discs from 0° storage in spite of the fact that they contained initially by far the greatest sucrose content.

It was also found that the effect of the substance infiltrated depended on the temperature of storage and consequently in the substances already present in the tubers. Thus it is known that potatoes stored at 0° contained the greatest quantities of fructose and sucrose. In discs from 0°, sucrose synthesis generally occurred at a slightly faster rate when glucose was given than when the same concentrations of fructose was given. This can be seen in the first columns of tables 10 and 11. On the contrary, potatoes stored at 25°C. contained only little sucrose as shown in table 2. Discs from these conditions responded more to fructose infiltration than to glucose, the differences being more striking for low concentrations of added sugars. The same can be said for potatoes from 16°C. At 9° some variability exists, in some cases glucose being more effective in producing sucrose formation and vice versa in others. It may also be seen that the sucrose mechanism is somehow controlled by temperature since more sucrose is synthesized at low temperatures of storage. This can be seen when the values obtained with potatoes stored at 0°, and incubated at 0°, are compared with those for potatoes stored at 25° as is shown in table 12.

Table 12

Figures represent increases of sucrose in % of dry wt. during incubation.

Infiltrated with	Temperature of incubation	Time of incubation	Storage temperature 0°	Storage temperature 25°
Water	0°	24 hrs.	1.80	.52
1% Glucose	"	"	3.00	.48
1% Fructose	"	"	2.85	.96
3% Glucose	"	"	4.00	.76
3% Fructose	"	"	3.65	1.26

Potato discs from high temperature storage, even when incubated at 0°, do not equal in activity those from potatoes stored at 0°. Low storage temperature most probably increases the amount of the mechanism responsible for sucrose formation or permits the accumulation of other intermediates formed by this system. It can be concluded from these experiments that both the enzymes and the intermediates, involved in sucrose synthesis, exist in smaller quantities in potatoes stored at high temperature than in potatoes stored at low temperatures.

Infiltration of intermediates. - The infiltration of phosphorylated hexoses into potato discs gave no reproducible and consistent results. This perhaps can be attributed to the difficulty which phosphorylated compounds experience in entering the cell. On account of this variability, the results of such experiments are not presented here.

Use of specific poisons. - The inhibition of sucrose synthesis by various poisons was investigated in potato discs. Discs were infiltrated with fructose only or with fructose plus the poisons, and the sucrose formed during incubation determined by analysis. The following substances were used: sodium arsenite, indoleacetic acid, malonate, iodoacetate, sodium fluoride and phlorizin. In each experiment controls were run which were infiltrated with fructose alone. The results of an experiment with potatoes from 25° storage are presented in table 13.

Table 13

Effect of poisons on sucrose synthesis by potato discs.

Discs infiltrated with	Incubated at	Time of incubation	Overall changes in sucrose in % of dry wt.	% of inhibition
Water	0°	6 hrs.	-.15	---
10% Fructose	"	"	1.21	0
10% Fructose + .1M sodium arsenite	"	"	-.03	100
10% Fructose + indolacetic acid .5 mgs./cc.	"	"	.78	36
10% Fructose + malonate 10 mgs./cc.	"	"	.23	81
10% Fructose + iodoacetate 10 mgs./cc.	"	"	.51	58
10% Fructose + sodium fluoride 1 mgs./cc.	"	"	.51	58

Negative figures mean that not only sucrose was not synthesized, but represent the decreases below the amount present initially at zero time. Inhibition is given in terms of decreased sucrose formation below that obtained with fructose alone. Discs infiltrated with fructose alone increased their sucrose content by 1.21% in six hours.

Arsenite is a potent inhibitor of respiratory processes but leaves fermentation almost unaffected, and it is believed that the use of this poison permits a separation of respiration and fermentation. Among other reactions, arsenite inhibits the formation of fructose-1,6-diphosphate. In table 13 it can be seen that arsenite gave 100% inhibition in the concentration used. A strong inhibition by arsenite was found by Hartt (32) in the synthesis of sucrose by sugar cane blades fed with hexose.

Indoleacetic acid, produced some inhibition, in accordance with Hartt's findings (32).

Malonate is a specific inhibitor for succinic dehydrogenase. When infiltrated as the sodium salt together with fructose, it depressed sucrose synthesis. A previous worker (32) reported only a small, if any, inhibition on sucrose synthesis by detached sugar cane blades.

Iodoacetate is said (73) to act selectively on fermentation at low concentrations and to act upon both fermentation and respiration at higher concentration. In the present case the concentration used was rather high and gave 58% inhibition of sucrose synthesis. Hartt also found this effect with sugar cane blades.

Sodium fluoride is known to hinder dephosphorylation and other steps in the glycolysis cycle such as the effect mentioned before on potato phosphatase. In the case of potato discs, fluoride produced a strong inhibition of sucrose formation, which is in agreement with Hartt's findings.

In other experiments, phlorizin and invertase were used together with fructose and the findings appear in table 14 in which potatoes from 25° storage and 24 hours of incubation were used.

Table 14

Storage temperature 25°C., incubation period 24 hours.

Infiltrated with	Incubated at	Changes in sucrose in % of dry wt.	% of inhibition
Water	25°	.13	—
3% Fructose	"	2.28	0
3% Fructose + invertase 1 mg./cc.	"	1.09	52
3% Fructose + .01M phlorizin	"	.39	83

Here again fructose alone caused a marked formation of sucrose. Phlorizin inhibits phosphorylation and dephosphorylation and especially depresses phosphorylase activity. It gave marked inhibition of sucrose synthesis, a fact observed by others (32). Kursanov claimed (47) that introduction of a very low concentration of invertase into leaves by means of vacuum infiltration stimulates

the formation of sucrose. However, in the present experiment, invertase apparently depressed the formation of sucrose from fructose in potato discs. Respiratory poisons inhibit the formation of sucrose in varying degrees, the most effective being arsenite.

The fact that malonate caused a marked inhibition suggests that there may be a coupling between succinic dehydrogenase and the reactions involved in sucrose synthesis either directly or through a chain of reactions. That indoleacetic acid exerts an inhibitory influence on sucrose synthesis may be related to the relation of this coupled to phosphatase. Increase in phosphatase activity by added indole acetate (as shown by Bonner in Avena coleoptiles, unpublished) might cause increased dephosphorylation of phosphorylated hexose intermediates.

Phosphohexoisomerase. - Lohmann (49) found in muscle extracts an enzyme which catalyzes an equilibrium reaction between glucose-6 and fructose-6-phosphate in the following manner.

Glucose-6-phosphate (70%) $\rightleftharpoons$ fructose-6-phosphate (30%)

According to Lohmann the transformation of Neuberg ester (fructose-6-phosphate) to Embden ester (glucose-6-phosphate) by rabbit muscle extracts occurs very rapidly, the equilibrium being reached in a few seconds. He found that the enzyme cannot be inhibited by addition of glucose-6-phosphate, nor poisoned by M/5000 iodacetate or N/100 sodium fluoride. He identified and

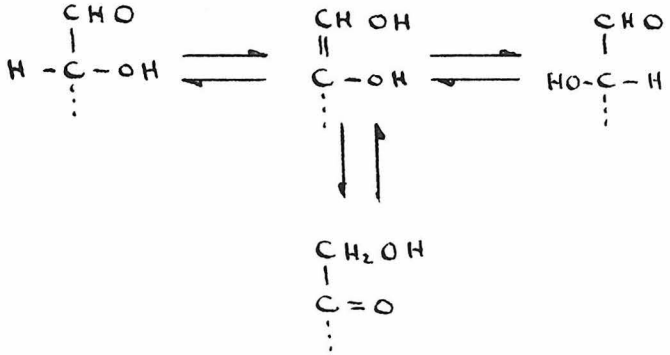
evaluated the quantities of the esters present at equilibrium.

In the case of higher plants Tanko reported (72) the conversion of fructose-6-phosphate to glucose-6-phosphate by a suspension of pea seed meal. More recently this was confirmed by Somers and Cosby (67) who used three independent methods of analysis. The equilibrium attained is in approximate agreement with that found by Lohmann for animal enzyme, but is reached more slowly. Hanes (27), (28) also states he found hexoisomerase activity in Laxton pea seeds and in potatoes.

In yeast the presence of hexoisomerase is well established. The great importance of this observation lies in the fact that it gave the first indication that reversible enzymatic equilibria play a role in lactic acid and alcohol fermentation.

The enzyme apparently does not require either coenzymes or specific ions.

In addition to hexoisomerase there is a non-enzymatic equilibrium which plays a role in transformation between glucose and fructose which occurs in alkaline conditions. This has been called the Lobry de Bruyn transformation. It can be explained on the basis that these sugars as well as mannose possess a common enol form as follows



In organisms however this equilibrium does not occur and the one based on the hexose-6-phosphates alone is found. Thus Robison ester is an equilibrium mixture of the 6-phosphate esters of the hexoses which are found in plants and animal tissues.

Experimental. - It was not possible to demonstrate hexoisomerase activity unambiguously in vitro. The presence of this enzyme can be inferred however by in vivo experiments in which potatoes were infiltrated with glucose or fructose and the concentrations of the respective hexoses followed. The potatoes used had been previously stored during three weeks at 0° and 25°C. Sections were made and glucose and fructose infiltrated as above. The amounts of the two hexoses were determined separately before and after the infiltration period. At the same time controls infiltrated with distilled water were carried out.

In table 15 the results of such an experiment are given. The potatoes were incubated at 0° for 24 hours after infiltration. The analytical results given represent the increase in the amount of each sugar formed as a result of infiltration, above the amount formed in the water infiltrated control. These results show clearly that with sections of potatoes stored at 0°, infiltration with glucose resulted in fructose formation and vice versa. At 25°C. however, a much smaller sugar conversion appears to have taken place.

Table 15

Figures represent increases of hexoses in % of dry wt. during incubation.

Infiltrated with	Incubated at	Time of incubation	Storage temperature 0° Glucose	0° Fructose	25° Glucose	25° Fructose
10% Glucose	0°	24 hrs.	1.14	.25	.01	.22
10% Fructose	0°	24 hrs.	1.17	.23	.01	.14
3% Glucose	0°	24 hrs.	1.10	.25	-.08	.15
3% Fructose	0°	24 hrs.	1.16	.09	.03	.17

In addition to the change in glucose and fructose sucrose was synthesized in each treatment. In table 16 the amounts of glucose and fructose are corrected for the amounts of the two sugars contained in the sucrose synthesized.

Table 16

Figures represent % of dry wt.

Infiltrated with	Incubated at	Time of incubation	Storage temperature 0° Glucose	0° Fructose	25° Glucose	25° Fructose
10% Glucose	0°	24 hrs.	2.04	1.15	.43	.64
10% Fructose	0°	24 hrs.	1.63	.69	.52	.69
3% Glucose	0°	24 hrs.	2.65	1.80	.52	.75
3% Fructose	0°	24 hrs.	1.30	.23	.63	.80

By this method of calculation also potatoes from low storage temperatures showed the greatest activity in converting glucose to fructose and conversely. They converted glucose into fructose in spite of the fact that they originally contained more fructose

than those from high storage temperature. Likewise, potatoes from 0° absorbed more glucose than fructose.

Potato discs from 25° storage absorbed slightly more glucose than fructose. Some glucose was converted to fructose showing that the mechanism for converting glucose into fructose exists in the tubers stored at high temperature. Another striking thing is that in potatoes from high temperature the amount of glucose and fructose found are approximately the same regardless of whether infiltration was with glucose or fructose.

The infiltration experiment of table 15 included treatments at the standard temperature of 25°C. Potato discs from the two storage conditions were infiltrated with glucose and fructose as before but incubated at 25°C. The results appear in table 17 in which figures represent increases in % of dry weight during incubation, corrected for the changes in the controls infiltrated with water.

Table 17

Figures represent % of dry wt. of hexoses.

Infiltrated with	Incubated at	Time of incubation	Storage temperature 0° Glucose	0° Fructose	25° Glucose	25° Fructose
10% Glucose	25°	24 hrs.	3.20	.38	.78	.29
10% Fructose	25°	24 hrs.	2.80	.34	.00	.18
3% Glucose	25°	24 hrs.	1.36	.24	.30	.06
3% Fructose	25°	24 hrs.	1.79	.25	-.28	.07

This table shows that potato discs from potatoes stored at 0° were much more active in interconverting glucose and fructose than potato discs from high storage temperature potatoes. Here again the concentrations of sugars found in the low temperature potatoes were approximately the same regardless of whether the infiltration was with glucose or fructose.

The amount of glucose formed from fructose was several fold higher in the potatoes at 0° than in those from 25°C.

Potato discs from 25°C. storage converted very little glucose into fructose and absorbed only small amounts of fructose. Glucose was absorbed in small quantities by discs from high storage temperature as compared with those from low temperatures. The absorption of fructose by the 25° tubers was very small and there was no conversion to glucose as the figures show in the last two columns of table 17.

Discussion. 1. From table 2 and other similar analyses of potatoes stored at various temperatures it can be concluded that the hydrolysis of starch and the accumulation of sugars at low temperatures are accompanied by changes in the phosphorylated sugars. The accumulation of sugars at low temperatures may hence be related to the metabolism of the phosphorous compounds.

2. In vitro experiments reveal no enzymatic system which is either specifically increased greatly in amount by storage at low temperature or whose rate is influenced

by temperature in such a manner as to suggest a relation to sucrose synthesis. The enzymes of potato studied *in vitro* and the effects of temperature upon these systems are given in the following table.

Table 18

Enzyme system	Effect of temperature on enzymatic reaction	Effect of storage temp. on enzyme amount
1-Amylase	very marked	none
2-Phosphorylase	"	"
3-Phosphoglucomutase	"	"
4-Phosphatase	"	"
5-Hexokinase	"	"

These systems include all but two of those known to be involved in carbohydrate metabolism in yeast muscle or higher plants which might be expected to intervene in the conversion of starch to simple sugars. Two systems, e.g., hexoisomerase and the system for condensation of hexose or hexose phosphate to sucrose could not be obtained in an active state *in vitro*.

3. In the experiments *in vivo* with discs cut from tubers which had been stored at various temperatures, it was found that sucrose is synthesized actively after infiltration of the discs with glucose or fructose.

    a. Glucose and fructose are equally effective in inducing sucrose synthesis in discs cut from

potatoes stored at low temperatures.

b. Fructose is more effective than glucose in inducing the formation of sucrose in discs cut from potatoes stored at high temperatures. This is related to the fact that potatoes stored at high temperatures contain but little fructose. It appears thus that fructose is a limiting factor in sucrose synthesis in potatoes stored at high temperatures.

4. Hexoisomerase activity could be studied only by determinations of the ability of potato discs infiltrated with glucose to produce fructose or vice versa. On this basis, potato tubers stored at low temperature contain much isomerase, those stored at high temperatures very little. This fact suggests that isomerase may play an important role in regulating sucrose formation in stored potatoes. From this point of view the failure of sugar accumulation in potatoes stored at high temperatures may may be due to the absence of available fructose induced by the low isomerase activity. This is not the only factor involved however as will be seen from 5 and 6.

5. Discs cut from tubers stored at low temperatures and infiltrated with hexose are more active in synthesizing sucrose than similar discs, similarly treated but cut from potatoes stored at high temperature. It would

appear that there is more of the sucrose synthesizing enzyme system in the potatoes stored at low temperatures.

6. Sucrose synthesis from hexose by potato discs differs from all of the other processes studied in that the rate of the reaction is as great at 0°C. as at 25°C.

The picture of sugar accumulation by potato tubers stored at low temperature suggested by this work is then as follows: at all temperatures starch is phosphorylitically transformed to glucose-1-phosphate. This is in part converted to glucose-6-phosphate. At high temperatures glucose-6-phosphate accumulates since it is only slowly transformed to fructose-6-phosphate. The small amounts thus formed are presumably used in respiration since fructose-6-phosphate does not accumulate. In the absence of fructose-6-phosphate and of fructose, sucrose synthesis does not take place. At low temperatures on the other hand glucose-6-phosphate is transformed to fructose-6-phosphate to a large extent. A portion of the fructose-6-phosphate thus produced is dephosphorylated with the production of fructose. In the presence of both fructose and glucose (and of fructose-6-phosphate and glucose-6-phosphate) sucrose synthesis takes place. This process takes place the more rapidly since the system responsible for sucrose synthesis from hexose is increased in amount by storage of the tubers at low temperature. The induction of sucrose synthesis at low temperatures appears thus to have a two fold origin, first, increase in activity of hexoisomerase

under conditions of low storage temperature, and second, increase in activity of the sucrose synthesizing system under the same conditions.

A second aspect of the problem is the reason for the absence of starch phosphorolysis in potatoes stored at high temperatures. Table 2 has shown that potatoes stored at 16° or 25° contain no detectable glucose-1-phosphate. Since they do contain abundant inorganic phosphate and since abundant phosphorylase may be extracted from them, it is evident that the phosphorylase is for some reason not active *in vivo*. The nature of this inhibition could constitute an extension of the present work.

This picture is necessarily incomplete since it has not been possible to study either of these two enzymes in cell free preparation.

Summary. - The present work has been concerned with attempts to discover the cause of the accumulation of sugars in potato tubers stored at low temperatures. To this end, enzymes of the carbohydrate metabolism were studied *in vivo* and *in vitro*.

1. The enzymes studied *in vitro* in potato tubers were the following: amylase, phosphorylase, phosphoglucomutase, phosphatase, and hexokinase. All of these enzymes were found to be present in tubers from the various storage temperatures used. In no case was the amount of enzyme activity affected by storage at various temperatures.

All of the enzymatic reactions have a positive and large temperature coefficient. In no case either was the equilibrium of the enzymatically catalyzed reaction found to be appreciably altered by temperatures over the range used. Results of the experiments in vitro did not lead to any conclusion as to the cause of the formation of sucrose in potato tubers stored at low temperature.

Neither sucrose synthesis nor the conversion of glucose to fructose could be carried out with cell free extracts of potato tubers.

2. Analysis of tubers from low and high temperatures show that marked changes take place in potatoes as a response to temperature of storage, not only in regard to starch, hexoses and sucrose but also with regard to hexose phosphates. This observation suggests that the phosphorylated hexoses may be intermediates in the conversion of starch to sucrose.
3. Experiments were carried out <u>in vivo</u> with discs cut from potatoes which had been held at various storage temperatures. Infiltration of the disc with glucose or fructose caused sucrose synthesis. Discs from potatoes stored at low temperature formed more sucrose under this condition than did discs from potatoes stored at high temperature.

The sucrose making mechanism would therefore seem to be enhanced by low temperatures of storage. The same holds true to a lesser extent of discs cut from potatoes stored

at high temperatures when infiltrated with hexose and incubated at low rather than high temperatures.

4. Fructose was more effective than glucose in inducing the production of sucrose in discs from potatoes stored at high temperatures.

Chemical analyses show that glucose infiltration of discs cut from potatoes stored at various temperatures resulted in fructose formation and vice versa. This change is probably due to isomerase activity together with other enzyme systems. Evidence to this point is found in the changes in hexose-6-phosphates.

5. Hexoisomerase activity is greater at low temperatures suggesting that this enzyme plays an important role in sucrose synthesis.

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