1943

A study of the phosphorylase of waxy maize

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NOTE TO USERS

A STUDY OF THE PHOSPHORYLASE OF WAXY MAIZE

by

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A Thesis Submitted to the Graduate Faculty
for the Degree of

DOCTOR OF PHILOSOPHY

Major Subject: Enzyme Chemistry

Approved:

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In Charge of Major Work

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Iowa State College
1943

TABLE OF CONTENTS

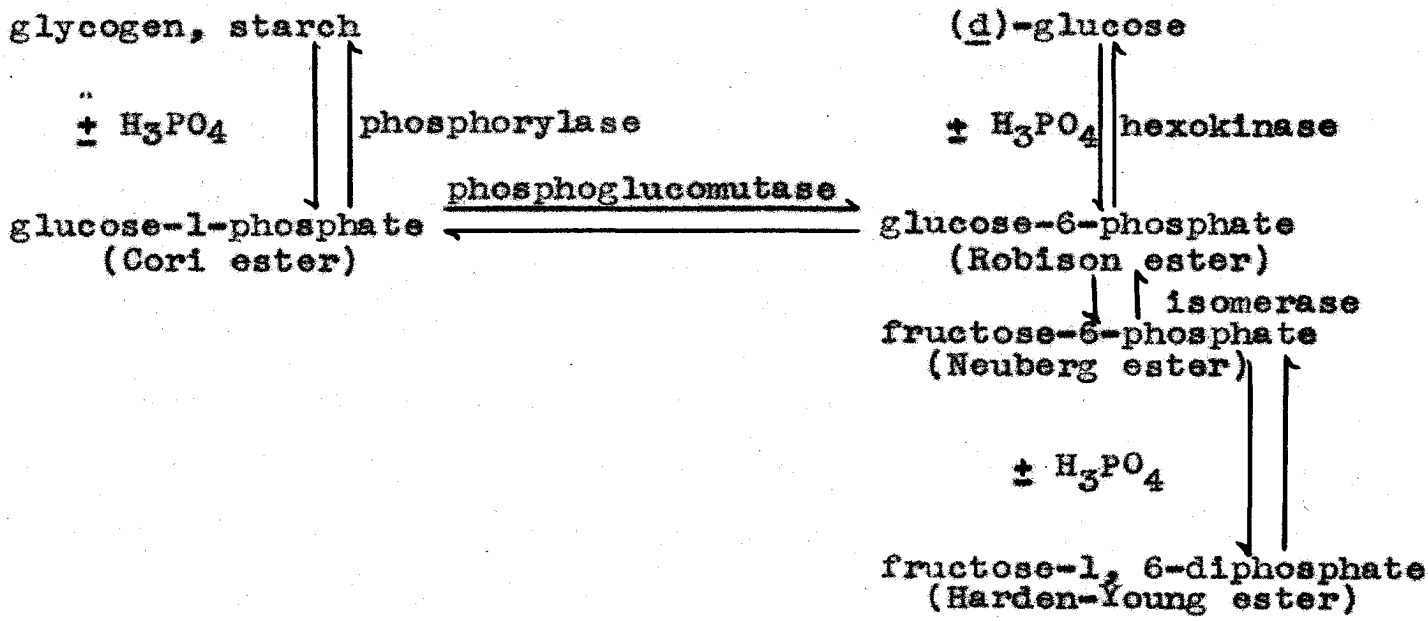
INTRODUCTION

From the time the studies of Meyerhof and co-workers were begun on the phosphorylation mechanism of the metabolism of carbohydrates, a great deal of research has been done in attempting to isolate the enzyme systems responsible for phosphorylation in plants and animals. It has been shown that these enzymes from various sources are closely related but not identical. The polysaccharides synthesized by these systems vary somewhat in iodine coloration and in x-ray diffraction patterns. Comparison of the synthetic products with the naturally occurring polysaccharides opens a new approach to the problem of determining the constitution of the glycogen and the starch molecules.

Emphasis on research relating to the structure of starch, interest in studies concerning the growth changes of corn, and the recent developments regarding the waxy species of maize have led to the initiation of this work, in the hope that the investigation of the phosphorylating enzyme from waxy maize may contribute toward the elucidation of the mechanism whereby starch is synthesized in growing corn.

REVIEW OF LITERATURE

In 1933 Meyerhof and co-workers (1) initiated a series of experiments which has led to the formulation of an extensive and now thoroughly established scheme for carbohydrate metabolism through phosphorylation. This phosphorylation mechanism may be shown in part as follows (2):



The development of this scheme has involved brilliant research on the part of numerous investigators. The immediate interest of the present study lies in the enzyme which catalyzes the reversible reaction between glycogen or starch and glucose-1-phosphate, constituting the first step of this mechanism.

In the past two years several excellent reviews of the

research on carbohydrate metabolism through phosphorylation have been published. The literature is adequately covered in its many phases in these papers by Cori (3), Cori and Cori (4), Kalckar (5), Lipmann (6), Meyer (7), Somogyi (8), Sowden and Fischer (9), and Werkman (10). Consequently, only a brief resume of the work particularly relevant to the problem at hand will be presented.

It has been shown by the experiments of numerous investigators that the enzyme phosphorylase is widely distributed in nature. Since its original discovery in the extracts of muscle (11), heart, liver, brain, and yeast (12), it has now been found in extracts of retina (13) and of a wide variety of plant tissues (14, 15). The behavior of the phosphorylases obtained from these various sources has been recorded in the literature and presents a foundation for further studies of the enzyme from other sources.

The extensive experiments of Cori and co-workers were suggested by their discovery and identification of a hexose-monophosphate in minced frog muscle extract (11). When this extract was incubated in phosphate buffer with adenylic acid a transfer of inorganic phosphate to carbohydrate was observed. This first phosphorylation product formed proved to be a new ester, which was isolated as the crystalline brucine salt and showed the properties of glucose-1-phosphoric acid. Further experiments (16) led to the conclusion that the ester was

d-glucopyranose-1-phosphoric acid. The behavior of the natural product was identical with that of the compound obtained synthetically by the interaction of acetobromoglucose and silver phosphate. Both were converted by muscle extract into hexose-6-phosphoric acid and then to the equilibrium, or Embden, ester (80% glucose-6-phosphate, 20% fructose-6-phosphate). Magnesium ions accelerated the conversion to the Embden ester. After these preliminary experiments, Cori et al. proceeded to the investigation of other sources of this enzyme system. They proved its presence in dialyzed rabbit muscle extract (17), in dialyzed liver extract (18), and in dialyzed extracts of rabbit brain and heart (12). Weak activity was shown by kidney extracts (12). Transformation to the 6-ester was less complete in all these extracts than in the muscle extract. Brewers' yeast also yielded an extract by which glucose-1- and glucose-6-phosphate were formed from glycogen and inorganic phosphate (12). Therefore it was concluded that the disruptive phosphorylation of glycogen is a widely occurring process.

Later studies by Cori and collaborators include experiments on the action of nucleotides in the phosphorylation of glycogen (19), the use of the enzyme system for synthesizing polysaccharides (20), the purification of phosphorylase (20), the breakdown of glycogen to glucose (21), the factors affecting the activity of phosphorylase (22), the kinetics of the

enzymatic synthesis of glycogen from glucose-1-phosphate (23), and the preparation of crystalline muscle phosphorylase (24). Recently from Cori's laboratory a complete synthesis of polysaccharide from glucose by means of purified enzymes has been reported by Colowick and Sutherland (25). The results of these experiments will be discussed in detail elsewhere in this thesis.

In 1940 Hanes (14) published the results of his experiments on the phosphorylase system in pea seeds. He reported that the phosphorylase of peas acts upon a much wider range of substrates than phosphorylase from muscle and yeast, which seems to show a rather strict specificity for undegraded glycogen and starch. The enzyme from peas acts upon almost any saccharide composed of α-glucopyranose units linked in positions 1 and 4 (as in maltose), regardless of chain length, whether in colloidal aggregation or in free form terminated by reducing groups. From his studies, Hanes concluded that the action of phosphorylase upon starch and dextrins consisted of an endwise attack at the non-aldehydic end of the chain structure, with the liberation of the individual glucose units in the form of glucose-1-phosphate.

Further experiments of Hanes (15) were carried out on the enzyme of potatoes. In addition to making an extensive investigation of the reversible formation of starch and the factors affecting the equilibrium of the reaction, he succeeded

in isolating glucose-1-phosphate in large quantities by the action of a partially purified phosphorylase preparation on starch.

The potato phosphorylase preparation reported by Hanes served as the basis for the experiments of Green and Stumpf (26). They succeeded in purifying the enzyme further, determined its activity in terms of phosphorylase units, and investigated some of the factors affecting starch formation by the enzyme.

Studies have been made recently on the properties of the synthetic polysaccharides. It is probable that these observations will contribute a great deal toward clarifying the picture of the starch molecule. Briefly, it can be stated that the polysaccharide synthesized by the enzymes prepared from brain, heart, liver, and yeast gives a brown color with iodine and resembles glycogen in every respect (27). The enzyme from muscle extract (20) and from potato extract (28), on the other hand, synthesizes a polysaccharide which gives a blue color with iodine and resembles starch. Very similar patterns are obtained on x-ray photographs of natural potato starch and of starch synthesized by the action of potato phosphorylase (29). X-ray diffraction patterns of the polysaccharide synthesized by muscle phosphorylase are almost identical with those of potato starch (30). Other investigations regarding the structure of the synthetic polysaccharides will be reviewed in a later section of this thesis.

STATEMENT OF THE PROBLEMS

The purpose of this investigation was three-fold:

1. To determine the phosphorylase content of waxy maize at different stages of growth.
2. To concentrate the waxy maize phosphorylase.
3. To characterize the product of phosphorylase action on glucose-1-phosphate.

In connection with these phases of the problem there was involved a study of the methods of measuring enzyme action, principally by the determination of phosphate. The construction of a photoelectric colorimeter and the preparation of glucose-1-phosphate were included in the work.

MATERIAL USED

Waxy Maize

Waxy maize is the particular variety of maize which contains waxy starch (31). This type of starch is distinguished from ordinary starch by its color reaction with iodine. Waxy starch gives a red color, whereas ordinary starch gives a blue color.

The waxy maize No. 939 used in these experiments was obtained from the Agronomy Department. As it was impossible to do all the work while the corn was fresh from the field, a quantity of each collection was frozen for later investigations. In 1940 the corn was sealed in Latex bags and frozen. In 1941 and 1942 the corn was wrapped in waxed paper. The corn was then put into a refrigerated room at -20° F. and kept until needed.

EXPERIMENTAL PART AND DISCUSSION

Experimental Methods

Determination of phosphorus

Investigators in this field have followed the progress of enzyme activity by phosphorus determinations. An examination of the reversible reaction,

starch or glycogen + inorganic phosphate $\rightleftharpoons$ glucose-1-phosphate,

shows how phosphorus determinations can be used as a direct measure of what is happening in the system. An increase in inorganic-P indicates that the reaction is proceeding toward the left, whereas a decrease in inorganic-P shows that glucose-1-phosphate is being formed. A further check on the progress of the reaction is made possible by acid hydrolysis of the glucose-1-phosphate ester and subsequent determination of the acid-labile ester-P plus inorganic-P. From this data the amount of ester-P can be calculated by subtracting the inorganic-P as determined in an unhydrolyzed aliquot.

It has been shown by Cori and co-workers (19) that the simple determination of inorganic-P is justified as a procedure for measuring formation or breakdown of glucose-1-phosphate. In a series of experiments these investigators

found that close checks were obtained when differences between initial inorganic-P and final inorganic-P were compared with ester-P determinations after acid hydrolysis.

Several procedures were employed by Hanes (14) to follow the reaction. Free- or inorganic-P and 7-minute-P determinations were considered adequate for the simple reaction between starch or glycogen and glucose-1-phosphate. In all cases aliquots of the enzymic digests were delivered into trichloracetic acid solution of such volume and strength as to give a final concentration of 7 to 8 per cent. The precipitated protein was then filtered off, and determinations of free-P were made on suitable volumes of the filtrates. The preliminary treatment used by Hanes for the 7-minute-P determination of ester-P + free-P was as follows: An aliquot of the trichloracetic acid filtrate was heated for 7 minutes at 100° C. in the presence of 1 N perchloric acid. Ester-P + free-P was then found by the same procedure as that employed for inorganic-P.

The method of phosphorus determination used by workers in this field is a modification of the Fiske and Subbarow colorimetric procedure (32). The original method consisted essentially of measuring the intensity of the blue color formed when a reducing agent acted upon phosphomolybdic acid. In making the test, the inorganic phosphate was treated with excess ammonium molybdate in sulfuric acid solution. A reducing agent was then added and the blue

color allowed to develop for a definite period of time. The intensity of the color was measured colorimetrically, either through a Duboscq colorimeter or by a photoelectric instrument.

The colorimetric method has several advantages: (1) it is exceedingly sensitive and thus is adapted to the estimation of very small amounts of phosphorus, of the magnitude of 0.01 mg.; (2) it is a rapid method of analysis; (3) it is inexpensive because of the small amounts of chemicals required.

Modifications of the original procedure of Fiske and Subbarow have been proposed to increase the accuracy of the determination for use in biological analyses. These modifications are concerned principally with the adjustment of the acidity of the solution, the choice of a reducing agent, and the optimum time for development of the blue color.

The influence of the acidity of the solution for the test was pointed out by King (33). The use of high acidity has the advantage of making allowable a much greater variation in the acidity without any appreciable variation in the amount of color produced. Therefore, no allowance is necessary for the presence of moderate amounts of trichloracetic acid in the test solutions. The use of low acidity for the determination would necessitate the addition of trichloracetic acid to the standard made up for comparison.

The disadvantage, however, of high acid concentration in the test lies in the fact that ester-P is somewhat labile in strong acid solution. Hanes (15) found the error from this source barely significant.

Both sulfuric and perchloric acids have been used by different investigators. Fiske and Subbarow employed sulfuric acid. For samples containing metallic ions which form insoluble sulfates, King recommended the use of perchloric acid, as the perchlorates are very soluble. However, for trichloracetic acid filtrates of biological materials (where ordinarily no barium salts are present) he found sulfuric acid preferable.

Probably most controversial is the subject of reducing agents for the test. Among those proposed have been hydroquinone, 1-amino-2-naphthol-6-sulfonic acid, 1-amino-2-naphthol-4-sulfonic acid, stannous chloride, amidol, and elon. The factors to be considered in the choice of the reducing agent are as follows: (1) speed of the reaction, (2) interference of other ions, (3) effect of temperature, (4) stability of the solution, (5) availability of the compound, and (6) expense. Fiske and Subbarow pointed out the disadvantages of using hydroquinone, which was originally suggested by Briggs (34), because of the slowness of the reaction and the large number of ions which interfere. Stannous chloride was recommended by Fontaine (35) on the

basis of its availability and cheapness. However, McCune and Weech (36) found by experiment that the 1-amino-2-naphthol-4-sulfonic acid was better than stannous chloride for this determination.

Fiske and Subbarow first suggested the use of the 1-amino-2-naphthol-6-sulfonic acid as the reducing agent because of the rapidity of the color development and the independence of the reaction on the presence of other ions. After further investigation, however, they discovered that the 1,2,4-isomer was more easily prepared and had approximately the same reducing value as the 1,2,6-compound. The 1,2,4-acid is commercially available. King and McCune and Weech recommended the use of the 1,2,4-compound. Its principal disadvantage is its relative instability. Fresh solutions must be prepared every two weeks.

Gomori (37) recently proposed elon (methyl-p-aminophenol sulfate) as a reducing agent because of its stability and low cost. The latest modification of the method was suggested by Allen (38), who claimed to have eliminated variations in color in the determination as a factor of time and temperature by employing a 1% amidol (2,4-diaminophenol hydrochloride) solution in 20% $NaHSO_3$.

As has been noted, in general the original Fiske and Subbarow method (using 1,2,4-aminonaphtholsulfonic acid) with minor modifications, has been adopted by most

investigators working with phosphorylase. Hanes (15) has used Allen's modified procedure (with amidol) in his more recent studies. In this laboratory King's modification (33), with sulfuric acid and 1-amino-2-naphthol-4-sulfonic acid, has been found very satisfactory. The application of this determination to the estimation of phosphorus in enzymic digests with the help of the photoelectric colorimeter has been described by Roman (39).

It has been observed in regard to the choice of acid and of reducing agent that the time factor plays an important part in the accuracy of the determination. General disagreement is found in the literature as to the optimal time of development of the blue color. King claimed that 5 minutes was the optimum, whereas Fiske and Subbarow recommended 20 minutes. Slight variations in the procedure or in the instrument used for readings may account for these differences in optimal time. Consequently, studies on the standardization of the method employed in this laboratory were made and will be reported following a detailed description of the procedure.

Procedure. The solutions required for the determination were prepared as follows:

1. 10% trichloracetic acid. One hundred grams of trichloracetic acid was dissolved in H_2O and made up to 1 liter in a volumetric flask.

2. 5% ammonium molybdate. To a mixture of 325 ml. distilled

water and 75 ml. conc. H_2SO_4 in a 500-ml. volumetric flask was added 22.52 g. $(NH_4)_6Mo_7O_{24}.4H_2O$. The mixture was shaken until solution was complete, cooled to room temperature, made up to volume, and mixed.

3. 0.2% 1-amino-2-naphthol-4-sulfonic acid. Two-tenths gram of 1-amino-2-naphthol-4-sulfonic acid (Eastman Kodak), 15.7 g. $Na_2S_2O_5$, and 2.4 g. crystalline Na_2SO_3 were dissolved in water and made up to 100 ml. The solution was allowed to stand overnight and filtered the following day.

4. Standard phosphate. Pure KH_2PO_4 was prepared according to the directions of Kolthoff and Rosenblum (40) by recrystallization three times from water and drying at 110°. A stock solution was made up by dissolving 2.1935 g. of the recrystallized KH_2PO_4 in 500 ml. H_2O. This solution contained 1.0 mg. P per ml. A less concentrated standard solution was prepared by diluting 25 ml. of the stock solution to 500 ml., giving a concentration of 0.05 mg. P per ml. Both solutions were kept saturated with chloroform to prevent bacterial growth, which would result in a loss of inorganic phosphate.

In making a phosphorus determination, the following procedure was used:

An aliquot was removed from the enzymic digest and added to 5 ml. of the 10% trichloracetic acid, to precipitate the protein. Dilution to 25 ml. was sometimes found convenient at this point. The precipitate was centrifuged

off in a Wilkens-Anderson semi-micro centrifuge. An aliquot (containing preferably 0.05-0.5 mg. P) was taken from the centrifugate and run into a 50-ml. volumetric flask. To this sample were added 5 ml. of the acidic ammonium molybdate solution, 2 ml. of 1-amino-2-naphthol-4-sulfonic acid, and water to volume. The reading against a standard solution was taken between 20 and 25 minutes after the addition of the reducing agent.

Construction of a photoelectric colorimeter. For the final reading of the phosphate concentration either a visual colorimeter of the Duboscq type or some kind of photoelectric instrument may be employed. The latter kind of instrument was chosen as being more convenient to use and less influenced by the error from visual fatigue. As no instrument of this kind was available, a colorimeter was constructed in the chemistry shop, according ti the circuit plan given by Müller (41).

The parts used in the instrument were as follows:

400-ohm potentiometer
15-ohm potentiometer
pushbutton switch and knobs
4-prong tube socket
200-ohm rheostat-potentiometer
dial with 3/8-inch shaft, 270° calibration, 200 divisions
2 matched Weston photronic photoelectric cells, type 2
2 lenses, 3.75 cm. diameter, focal length 5 cm.
115-volt, 100-watt flat-filament Mazda lamp

With the instrument a Leeds and Northrup portable lamp-and-scale type galvanometer of sensitivity 0.023 A/mm. was used.

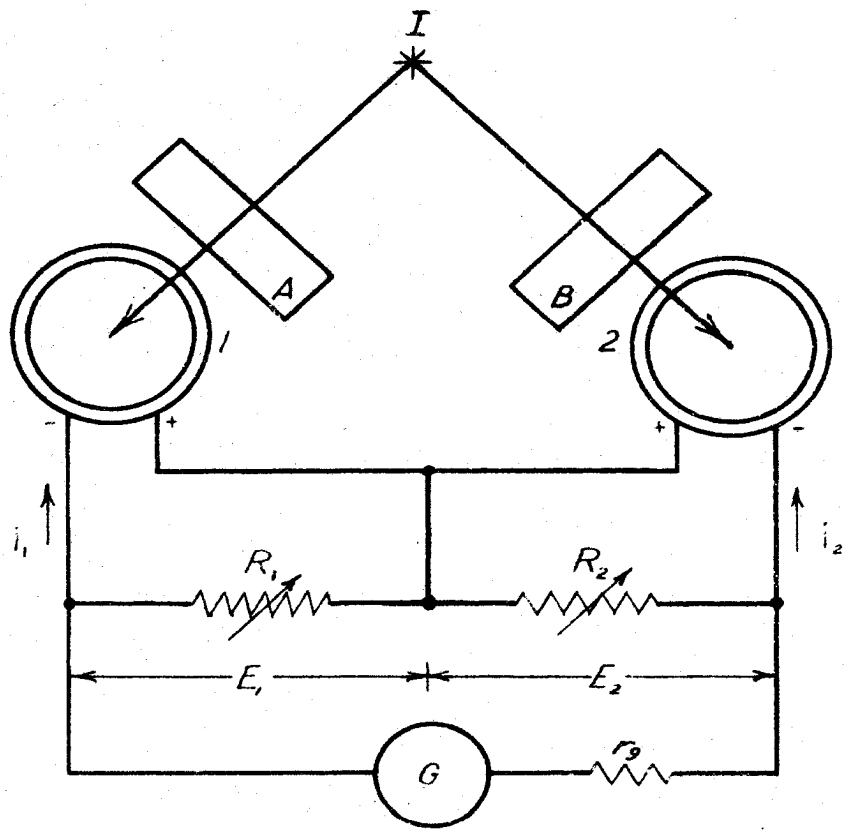


Fig. 1. Circuit of
Photoelectric Colorimeter

Corning Glass filters selected for the phosphate determinations were No. 396, Light Shade Aklo (for heat absorption), and No. 246, Red (4 mm.), which transmits light in the region of 600-650 mμ. Two 10-mm. fused glass absorption cells obtained from the American Instrument Company held the test solutions.

The following procedure was adopted for making a reading: The absorption cell containing the standard phosphate solution was put in place (A in Fig. 1). The dial was then set at 100 and the resistances adjusted so that the galvanometer read zero. The unknown solution was next put into place (B in Fig. 1) and the dial adjusted until the galvanometer came to zero again. The dial reading was converted by means of a calibration curve into the amount of inorganic-P present in 50 ml. of the solution.

Calibration of the instrument. In all the colorimetric phosphorus determinations the unknown solution was compared with a standard solution, made up at the same time. The advisability of this procedure lies in the variability of the color intensity development with the factors of time and temperature. The standard solution contained 5 ml. of the dilute standard KH_2PO_4 solution and thus represented 0.25 mg. of inorganic-P.

A curve was constructed from data obtained by comparing solutions containing increasing amounts of the standard solution with a 5-ml. sample of the standard. Table 1 shows the

data upon which the calibration curve, fig. 2, was con - structed.

Table 1

Calibration of the Colorimeter

Ml. KH_2PO_4 in 50 ml.	Mg. P in 50 ml.	Colorimeter reading
0.0	0.000	91.1
0.5	0.025	80.7
1.0	0.050	72.4
1.5	0.075	64.7
2.0	0.100	58.6
2.5	0.125	51.3
3.0	0.150	45.9
3.5	0.175	41.0
4.0	0.200	37.4
4.5	0.225	33.5
5.0	0.250	30.6
5.5	0.275	26.1
6.0	0.300	24.2
6.5	0.325	21.7
7.0	0.350	19.1
7.5	0.375	17.2
8.0	0.400	15.4
8.5	0.425	13.8
9.0	0.450	12.7
10.0	0.500	10.1
11.0	0.550	8.8
12.0	0.600	7.4
13.0	0.650	6.1

Investigation and standardization of the method. Recent publications by several investigators led to the criticism and close examination of the method of determining phosphorus with the photoelectric colorimeter described. Some of these studies

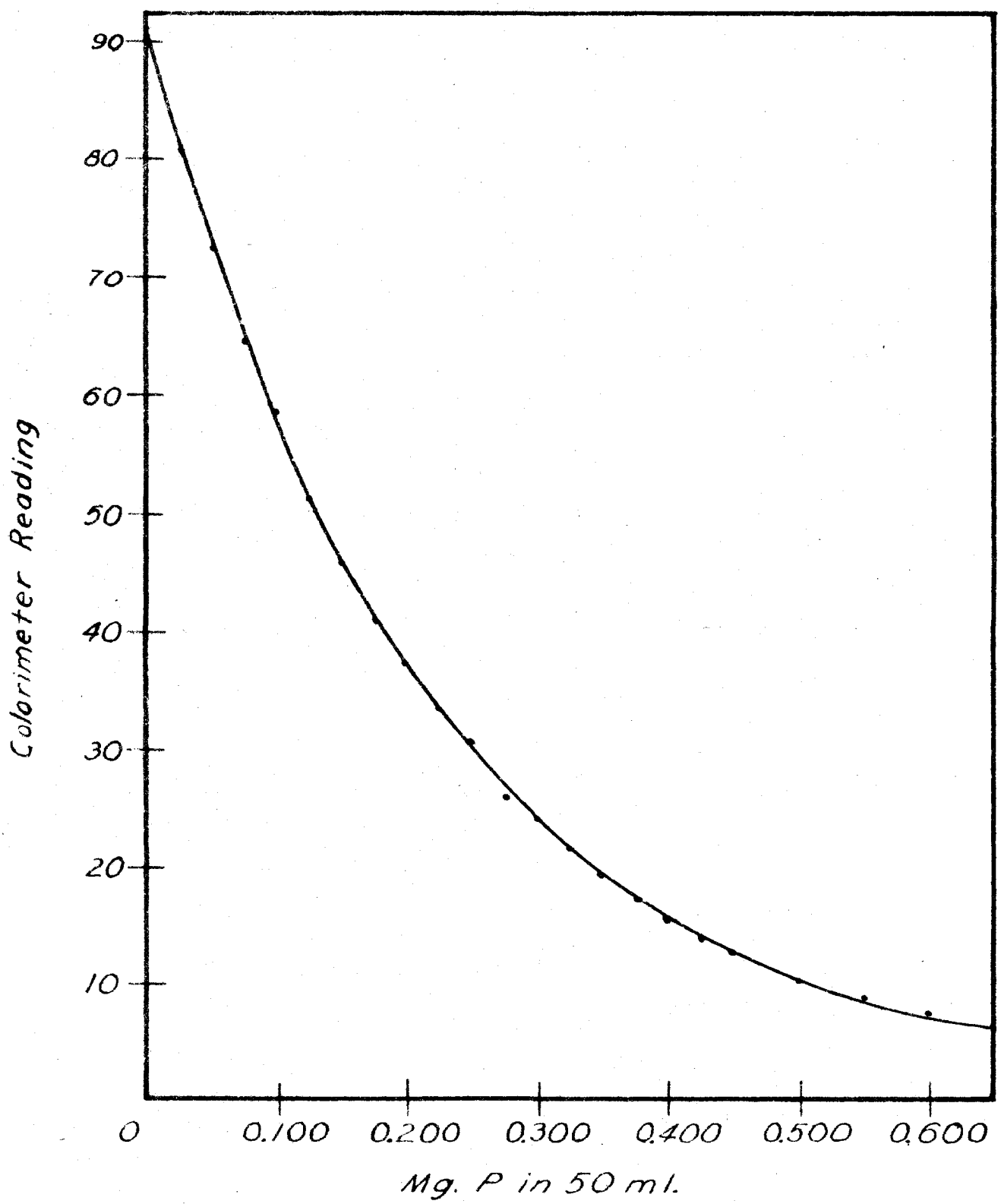


Fig. 2. Curve for Calibration of Colorimeter

pertained to the error caused by turbid solutions; others were concerned with the best filter system for detecting small differences in the blue color; and still others dealt mainly with the change of intensity and of quality of color with time.

With regard to the first critical study, the error caused by turbid solutions, it was found possible to avoid their formation in most cases by adequate centrifuging of the sample after addition of trichloracetic acid. In several instances, however, turbid solutions were formed upon addition of the molybdate reagent. In such cases the procedure was to prepare a compensating blank exactly as the test solution was prepared, with the exception that the reducing agent was omitted. This turbidity blank was then introduced into another 10-mm. absorption cell in front of the standard solution after adjustment of the resistances for the standard. Green and Stumpf (26) solved the problem of turbid solutions by addition of the molybdate reagent before removal of the protein precipitate.

The investigation of the filter system was suggested by the work of McCune and Weech (36). They found that for the phosphorus test the amount of absorption in the violet portion of the spectrum, between 400 and 420 mμ, remained considerably more constant than in any other region of the spectrum. Therefore, they recommended the isolation of the region between 400 and 430 mμ by the use of Corning filters Light Shade Aklo, No. 396, 2.0 mm.; Violet, No. 511, 3.0 mm.; Red Purple Ultra, No. 597, 3.3 mm.

A series of experiments was initiated in an attempt to verify the results of McCune and Weech. For these studies a Coleman Universal Spectrophotometer was used. A curve for the standard phosphate solution (0.25 mg. P in 50 ml.), with readings taken between 20 and 25 minutes after mixing, was plotted over the range of 300 mμ to 800 mμ. Results of this test showed maximum transmission at 450 mμ and a minimum at 360 mμ, as indicated by the data in table 2.

Table 2

Transmission of the Blue Solution at Different Wave-Lengths

mμ λ	% transmission	mμ λ	% transmission	mμ λ	% transmission
300	23.3	430	34.3	650	13.1
320	21.6	440	39.1	675	10.5
325	22.1	450	41.2	700	10.1
330	19.9	460	40.5	710	9.4
340	17.8	470	38.9	720	9.1
350	5.2	475	39.8	730	9.1
360	3.6	480	37.1	740	9.0
375	6.1	490	34.5	750	8.9
380	6.4	500	32.8	760	8.9
390	9.7	525	28.0	770	9.3
400	20.8	550	24.2	780	9.6
410	24.7	575	20.7	790	9.9
420	30.0	600	18.0	800	10.3
425	33.9	625	14.7		

The curve obtained from these data is shown in Fig. 3. Maximum transmission of 41% appeared at 450 mμ. At 360 mμ the minimum transmission of about 4% was reached.

To determine which filter system would give the most

accurate results in estimating the phosphorus content of the blue solution, a second test was run on two filter combinations to find the regions of their maximum absorption and transmission. System I consisted of Corning Glass filters No. 246 and 396, a red combination judged to be similar to that used by Roman (39) and other investigators. System II was composed of filters No. 511 (3 mm.), 597 (4 mm.), and 396, the blue combination specified by McCune and Weech. Table 3 contains the data obtained in this experiment.

Table 3

Transmission of Filter Combinations at Different Wave-Lengths

mμ λ	% transmission		mμ λ	% transmission	
	Blue	Red		Blue	Red
300	1.7	8.9	580	0.5	7.0
350	6.0	1.5	590	0.5	14.2
360	6.7		600	0.7	23.6
370	8.2		610	0.7	30.5
380	9.2		620	0.7	34.4
390	9.8		630	0.8	34.8
400	6.9	1.1	640	0.8	33.0
410	4.8		650	0.7	36.8
420	3.1		660	0.7	33.9
430	2.1		670	0.7	31.3
440	1.4		680	0.8	27.5
450	1.2	0.8	690	0.8	25.7
500	0.9	1.2	700	0.8	22.2
550	0.7	1.4	750	0.8	12.1
560	0.8	1.9	800	1.0	8.7
570	0.4	3.2			

Observation of the curves (Fig. 3) constructed from these data and comparison with the transmission curve of the phosphate solution (from the data in Table 2) showed that in the region recommended by McCune and Weech the net amount of transmission of the blue solution was very low. This fact may be illustrated by the following summary table.

Table 4

Net Transmission of Blue Light by the Blue Solution

mμ	% transmission		Net % transmission
	Blue filters	Blue solution	(calcd.)
390	9.8	9.7	1.0
400	6.9	20.8	1.4
410	4.8	24.7	1.2
420	3.1	30.0	0.93
430	2.1	34.2	0.72

From table 4 it is evident that the maximum net transmission for the blue solution and the blue filters occurred at 400 mμ, and that the minimum was at 430 mμ, showing a range of 0.72-1.4%. Consequently, small errors in reading or small variations with time would magnify the net error in the estimation of phosphorus. On the other hand, in the case of the red filter combination, at 650 mμ the net transmission reached a maximum of 36.8 for the solution and 13.1% for the filters, or a net of 4.82%.

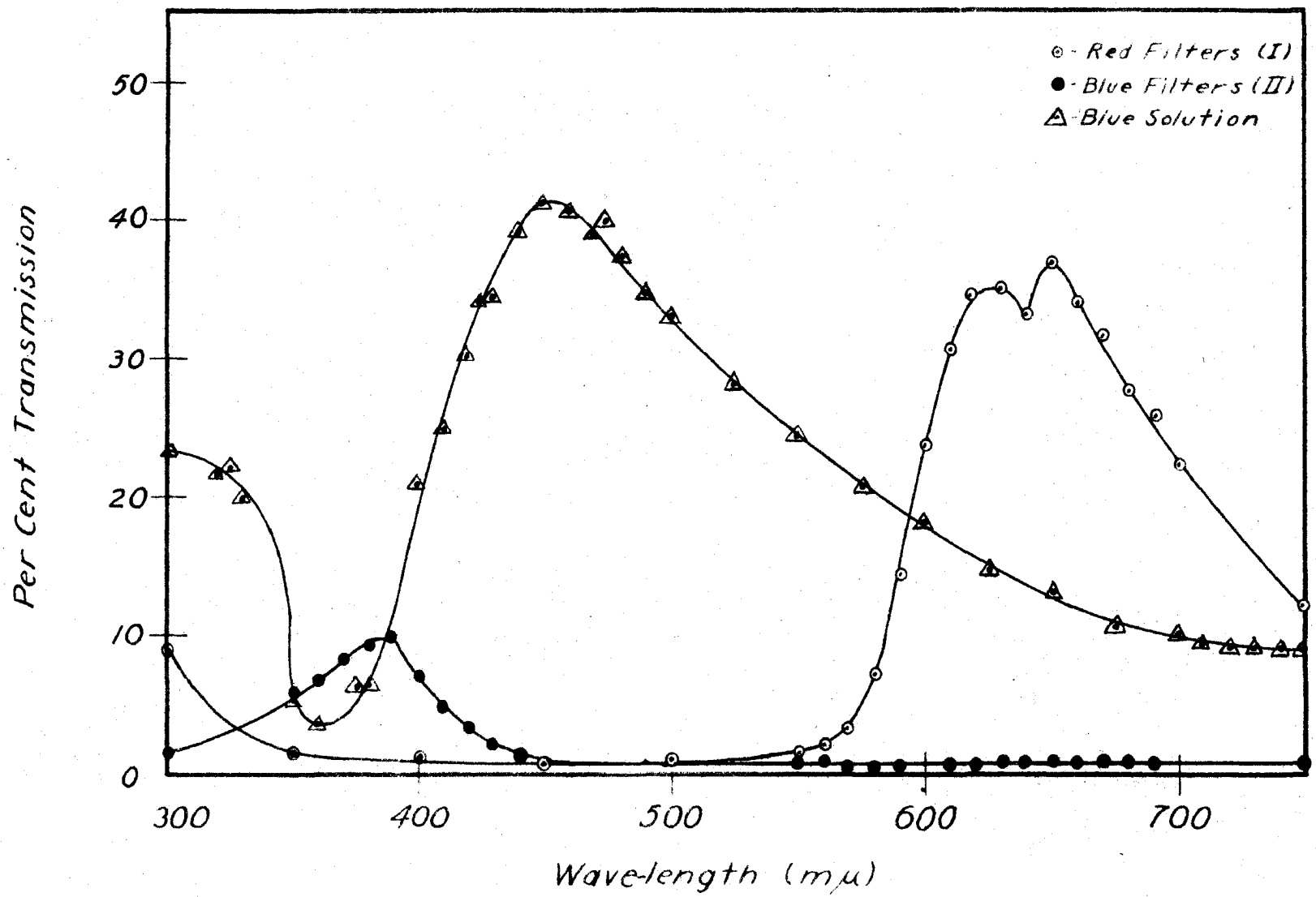


Fig. 3. Transmission of Filters and of Blue Solution

Tests with the blue filter combination in the photoelectric colorimeter as contrasted to the spectrophotometer showed that so small an amount of light was emitted by the light source in this region of the spectrum that readings were difficult to obtain.

It was concluded from the data of table 3 and the curves in fig. 3 that the use of the red filter combination was justified on the basis of the per cent transmission. On the basis of the tests with the blue filters in the photoelectric colorimeter, it was evident that the red combination enabled more accurate determination of the blue color with the instrument available than could be obtained by utilizing the blue combination recommended by McCune and Weech.

The choice of filters by McCune and Weech was made partially with regard to the time factor. They claimed that in the region of maximum transmission for their filter combination the color remained practically constant during the period between 30 and 60 minutes after mixing of the solutions and that little change took place during the next two hours. To test this time effect, three wave-lengths of light, representing regions of maximum transmission for the two filter combinations (380 mμ for the blue filters, 620 mμ for the red) and for the blue solution (455 mμ) were chosen for a series of experiments on the change in transmission with time. From the data in table 5 it is apparent that the variation of transmission with time at 620 mμ was considerably greater than that

at 380 mμ, in agreement with the findings of McCune and Weech. (The test solution contained 0.25 mg. P in 50 ml.)

Table 5

Transmission of the Blue Solution with Time of Development

Time (min.)	% transmission		
	380 mμ	455 mμ	620 mμ
2.5	7.5	42.5	16.7
5	7.7	41.8	16.2
10	7.7	40.9	15.8
15	7.6	40.5	15.0
20	7.4	39.8	14.8
25	7.7	39.6	14.2
30	7.3	39.2	13.6
35	7.6	38.4	13.0
40	7.1	38.0	12.0
45	7.0	37.2	11.4
50	6.8	36.5	10.8
55	6.6	35.6	10.1
60	6.4	34.9	9.3

The problem of handling the two factors, net transmission of light by the blue solution and change of transmission with time, to give the minimum per cent of error for small changes in phosphorus content at first appeared difficult. As has been noted, the instrument available for the phosphorus analyses showed very low sensitivity with the blue filter combination. It has also been found that the net transmission of light in the region of 400-430 mμ through the blue solution is about 1% in the spectrophotometer. The advantage of using light of such wave-length lies principally, then, in the fact that the

determination is relatively independent of the time factor, as shown in table 5.

Various investigators have pointed out that by choice of an optimal time for reading the color intensity, no error is involved through this factor. Because of the relatively large net transmission of light from the red filters through the blue solution, it was decided to use the red combination and to determine the optimal time for the readings.

Results of repeated tests in which a known amount of phosphorus was run against the usual standard in the photoelectric colorimeter showed that for the interval between 20 and 25 minutes after addition of the reducing agent the color exhibited the least change in transmission with time. On the basis of these results, a 20-minute period of color development was adopted as the standard procedure in making the phosphate tests.

The procedure given on page 15 was developed from the results of the experiments described in the investigation of the phosphorus determination. It was employed in all the present work on waxy maize phosphorylase. Its accuracy was limited by the order of sensitivity of the galvanometer, by the dial calibration, and by the necessity of reading phosphorus concentration from the calibration curve. It was calculated that the method was accurate for determinations to two significant figures.

Preliminary experiments

Introduction. The work reported by Hanes (14, 15) suggested the undertaking of a series of preliminary tests modeled after those which he carried out on peas and potatoes. In the present experiments Hanes' findings with regard to the effect of temperature, hydrogen-ion concentration, concentration of reactants, and preparation of the enzyme served as a guide for standardizing the procedure to be followed in the later work. The tests reported in this preliminary work were made on corn extracts treated in various ways and under varied conditions, in an attempt to estimate the amount of phosphorylase activity and to ascertain the factors which affect the activity. Field corn and waxy maize collected during the late summer and fall of 1940 and 1941 were used for the experiments.

As has been described, the work of Cori and Hanes indicated that the phosphorylase enzyme is concerned with a reversible reaction as follows:

starch + inorganic phosphate $\rightleftharpoons$ glucose-1-phosphate.

These experiments are concerned with the reversibility of this reaction. It must be borne in mind, however, that this reaction is a part of a complex system and not an isolated reaction. For an interpretation of the results of the experiments with crude extracts, a modification of the analysis outlined by Hanes (14) for pea extracts is helpful. Reference to the

scheme presented on page 2 of this thesis will be useful to show the relationship among the following principal reactions which may take place when glucose-1-phosphate is present originally in the system or when it is formed by the breakdown of starch by phosphorylase:

(1) Glucose-1-phosphate is transformed progressively into reducing hexose-6-phosphates.[1] This reaction itself involves no change in inorganic phosphate; but if conditions are such that it continues without reversal, eventually the equilibrium of the phosphorylase reaction will be affected and a decrease in inorganic phosphate will result, as will be clear from a study of the complete scheme.

(2) Glucose-1-phosphate is transformed into a polysaccharide, and inorganic phosphate is liberated. This reaction will be preceded by a short induction period if no polysaccharide is present in the original digest, as has been found by Cori and Cori (23, 43) and by Hanes.

(3) The polysaccharide (formed as a result of reaction 2) is progressively hydrolyzed by the action of amylase if present, with the formation of dextrins of varying complexity. (As this reaction proceeds, the decrease in concentration of polysaccharide causes the reversible phosphorylase reaction to continue to the left, liberating inorganic phosphate.)

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[1] It was formerly thought that this reaction was irreversible. However, Sutherland, Colowick, and Cori (42) have now shown that such is not the case.)

(4) Hexose-6-phosphate (formed in reaction 1) is transformed slowly into hexose-diphosphate by esterification at the expense of inorganic phosphate (present initially in the extract and formed in reaction 2).

(5) When the concentration of glucose-1-phosphate falls to a certain level (as a result of reactions 1 and 2) reaction 2 ceases. Further reduction in the amount of this ester (by continuance of reaction 1) causes the reversal of reaction 2; that is, it brings about the reconversion into glucose-1-phosphate of the polysaccharide previously formed in reaction 2, and dextrins formed from it (in reaction 3).

It is possible to vary the conditions of the reactions and the preliminary treatments of the extract and thus to alter very much the relative velocities of these different reactions. Hanes showed that a pH of 8 to 8.5 caused reaction 1 to proceed very rapidly at the expense of reaction 2, which became negligibly small. The opposite effect, speeding up of reaction 2 and slowing down of reaction 1, was noted at pH 6.4. Removal of the enzymes involved in competing reactions would, of course, affect the system. For example, several days' storage of an extract, according to Hanes, resulted in the inactivation of the phosphoglucose conversion system, whereas the activity of the phosphorylase was relatively unchanged. In such cases, only reactions 2 and 3 would occur when glucose-1-phosphate was present. Hanes observed that the presence or absence of amylase showed little effect on the

final level of conversion, although methods are available for removing this enzyme.

From the preceding survey of the reactions which may take place in crude or partially purified phosphorylase extracts, it is evident that the methods described for the determination of free- and 7-min.-P are adaptable to convenient and rapid analyses which are indicative of the course which the principal reaction, that between starch and glucose-1-phosphate, is taking. The concentration of free- or inorganic-P shows at all times which way the reaction is progressing (as was pointed out in the section on phosphorus determination).

As has been observed, by removal of the enzymes which catalyze competing reactions, the reversible phosphorylase reaction can be isolated for study. Hanes (15) carried out extensive experiments on this isolated system with purified potato extracts. His studies included research on the effect of pH and of the concentrations of the three reactants (starch, glucose-1-phosphate, and inorganic phosphate) and enzyme with regard to the reversibility of the reaction. His observations may be summarized as follows:

(1) The final equilibrium state is dependent upon the pH of the digest. The equilibrium state may be directly measured by the ratio free-P/ free- + ester-P. This ratio increases with decreasing hydrogen-ion concentration over the range of pH 5 to 7. At initial pH 5.2 the ratio is 0.905; at pH 6.05 it reaches 0.855; and at 6.9 equilibrium occurs at

0.76. The hydrogen-ion concentration effect was shown to be due to the effects upon the extents of dissociation of the inorganic and esterified phosphate, with the divalent ions alone determining the equilibrium. The ratio

$$(HPO_4)^{=} / \ (C_6H_{11}O_5.O.PO_3)^{=}$$

remained practically constant at a value of 2.2 over a pH range of 5 to 7.[1]

(2) The final equilibrium state is independent of the concentrations of the reactants or the enzymes. However, the initial velocity of the reaction is greatly increased if a catalytic amount of starch is present at the beginning; the starch (or glycogen or maltose) abolishes the initial induction phase of the reaction toward the left. For this purpose the effective concentration of the starch is not proportional to the gross concentration.

From this review of Hanes' work on potato phosphorylase and from a study of similar experiments by Cori on animal enzyme, it was evident that certain phases of the examination of the phosphorylase system in corn would be unnecessary. However, differences inherent in the source of the enzyme led to an investigation of the methods of purification and to a study of the relative concentration and activity of the corn phosphorylase. These preliminary experiments, after the methods of Hanes, were carried out to acquaint the investigator

[1]Hanes and Maskell (44) recently published a paper which explained further the effect of hydrogen-ion concentration on the equilibrium.

with the behavior of the enzymic system, through which the enzyme itself could be studied with respect to purification procedures and activity determinations.

Methods and materials. The corn used as a source of enzyme for these experiments was collected from the field and sealed in Latex bags or wrapped in waxed paper, frozen, and kept at -20° F. until used. The method of preparing the corn for the enzyme extracts varied with the age of the corn. Very young corn was ground in a mortar with sea sand and a small amount of water. For corn with a lower moisture content a meat-grinder was used to macerate the kernels. Corn which was practically mature was run through a Wiley mill.

For extraction of the enzyme, the ground corn was mixed with twice its weight of distilled water, shaken well, and allowed to stand for 12 to 18 hours in the refrigerator. If it was necessary to keep the extract more than a few days, a small amount of powdered thymol was used to prevent bacterial action. Hanes (14) reported that water extraction was the most successful of all the methods tried for obtaining the enzyme from peas. In his laboratory the extracts were kept under toluene or thymol.

Treatment of the crude extracts differed somewhat. In some cases the extracts were dialyzed. For such experiments a collodion sac was prepared by coating the inside of a Kjeldahl flask with collodion, allowing the solvent to evaporate until the membrane became firm, and then removing the

sac from the flask with water.

In setting up the enzymic digests, the constituents used in addition to the enzyme preparation, were soluble starch (as substrate or catalyst), buffers (to adjust the pH and to act as a source of phosphate), and, in some cases, crystalline magnesium chloride.

The soluble starch used was a 2% solution prepared by making a paste containing the weighed amount of Baker and Adamson soluble starch, adding this paste to about 15 to 20 ml. of boiling water, boiling with stirring for two minutes, cooling, and making up to volume with water in a volumetric flask.

For these preliminary experiments inorganic phosphate was added in the form of 0.2 or 0.3 M Na_2HPO_4 or as 0.2 M buffer mixtures of the primary and secondary sodium phosphates, prepared in the usual manner.

In some of the experiments a trace of magnesium chloride was added to make the reaction go toward the right, forming glucose-1-phosphate. This salt functions in such a way that the phosphoglucose conversion system, if present, is activated. At the time these experiments were begun it was thought that the phosphoglucose conversion reaction was irreversible and thus that the phosphorylase reaction would tend to go to completion toward the right in the presence of magnesium chloride. It is now known through the work of Sutherland, Colowick, and

Cori (42) that the reaction is reversible and forms glucose-1-phosphate at pH 7 to the extent of 6 per cent. (See footnote, p. 29.)

The C, aluminum hydroxide used for purification of the enzyme in one case was prepared by the method of Willstätter (45). For another experiment a yeast extract was made by boiling 0.2 g. of dried bakers' yeast in 5 ml. of water for 10 minutes. The extract was then clarified by centrifugation.

All the digestions were run at room temperature. The course of the reaction was followed by determining free-P colorimetrically, as has been described. For the tests 1 ml. of the digest prepared as indicated in the experiments was run from a pipet into 5 ml. of 10% trichloracetic acid. The solution was mixed well and filtered. From the filtrate a 2-ml. aliquot was taken for the phosphate test.

Experimental. It has already been emphasized that in experiments involving enzymic action a large number of variable factors present themselves for investigation. In this series of preliminary tests certain of these factors (already studied by Hanes) were held constant, and the effects of changes in other factors were observed. From the possible variables the following were chosen for study with regard to their influence on the activity of the enzyme: substrate, age of corn used as a source of enzyme, treatment of the enzyme by dialysis, adsorption of the enzyme on $Al(OH)_3$, and the clarification of the extract by kaolin. Thus, these experiments were designed

essentially to determine conditions for obtaining maximum activity of the enzyme, as measured by the behavior of the enzyme in the phosphorylase reaction.

1. Effect of substrate. In the experiments with phosphorylase, one of two kinds of substrates may be included in the digestion mixture. Starch (or glycogen) and inorganic phosphate may be used, or glucose-1-phosphate may be added. The object of one experiment in this series was to determine what course the reaction would take if neither of these substrates was added. The experiment then became an "auto-digestive" reaction, dependent upon the balance of substrates which existed in the original extract.

The corn from which the extract was prepared was waxy maize collected 32 days after pollination. Following dialysis and centrifugation, digests with a pH of about 7.5 were set up at room temperature. Two 12-ml. digests containing only the enzyme extract, a trace of magnesium chloride, and secondary sodium phosphate, were prepared. To the third digest 5 ml. of 2% soluble starch solution was added, making a total volume of 17 ml. Table 6 shows the results, interpreted in terms of mg. P in 1 ml. of digest.

The data of table 6 indicated that at the end of almost 24 hours all the digests showed a definite increase in inorganic-P, or a trend toward starch synthesis, although equilibrium had not been reached. An approximate calculation to show the trend of the reaction could be based upon a ratio

of free-P/ free- + ester-P as obtained by using the highest value recorded for free-P in the denominator as free- + ester-P, and an estimated equilibrium value for free-P as the

Table 6

Effect of Substrate on Phosphorylase Activity

Time (hrs.)	Mg. P per ml. of digest		
	Digest 1	Digest 2	Digest 3
0	1.17	1.02	0.63
1	1.26	1.14	0.72
2	1.11	1.05	0.60
4	0.99	1.05	0.72
5	1.08	1.17	0.60
23	1.38	1.20	0.84

numerator. This calculation indicated that the ratio for the three digests was in the range of 0.7 to 0.9. Further study of table 6 revealed that the autodigestions (Digests 1 and 2) showed fewer changes in reversibility than did the experiment (Digest 3) to which soluble starch had been added. The observations that there were changes in the inorganic phosphorus concentration in all the digests and that addition of starch caused no noticeable modification in the nature of these changes were evidence that there was present in the "autodigestive" digest some substrate (starch or glucose-1-phosphate) to carry along phosphorylase action.

2. Effect of age of corn. Several experiments were set up with extracts of corn of varying ages. The extracts were prepared by grinding the corn in a meat-grinder or in a Wiley mill, extracting with two times its weight of distilled water for 24 hours in the refrigerator, and centrifuging. Each digest contained the enzyme extract, soluble starch as substrate, and Na_2HPO_4, making the pH about 7.5. Digestion was carried out at room temperature.

The data on experiments with waxy maize collected 14 days (Digest 4) and 18 days (Digest 5) after pollination are reported in table 7.

Table 7

Effect of Age of Corn on Phosphorylase Activity

Time (hrs.)	Mg. P per ml. of digest	
	Digest 4	Digest 5
0	0.84	0.75
1	0.75	0.75
3	0.66	0.69
5	0.69	0.69
7	0.69	0.69
23	0.63	0.66
25	0.63	0.66
29	0.60	0.66
47	0.63	0.66
53	0.66	0.69
71	0.66	0.72

In this comparison of 14- and 18-day old corn, the number of shifts in the direction of the reaction was greater for the young corn, and also the net change in inorganic-P covered a

wider range than for the 18-day corn.

Waxy maize about 7 weeks old was ground in a Wiley mill and extracted as previously described. Digests made up as for the 14- and 18-day corn were tested, and table 8 shows the results for two digestions.

Table 8

Phosphorylase Activity in Corn Collected 7 Weeks After Pollination

Time (hrs.)	Mg. P per ml. of digest	
	Digest 6	Digest 71
0	0.81	0.81
0.5	0.78	0.81
1	0.81	0.78
3	0.78	0.78
6	0.75	0.75
24	0.72	0.72
48	0.69	0.63

These data on 7-weeks old corn, compared with the data on 14-day corn, showed few reversals of the reaction but a definite trend toward the right, forming glucose-1-phosphate, and a comparatively small change in the fixation of phosphorus from the beginning to the end of the experiment. The experiment indicated less activity of the enzyme in the corn 7 weeks after pollination.

3. Effect of dialysis. Dialysis is a common procedure employed by various investigators for purifying phosphorylase extracts. The observations of Cori et al. have shown that

adenylic acid is necessary for the functioning of the animal phosphorylase system. In their experiments the mammalian extracts were dialyzed as a means of purification. By dialysis inorganic phosphate, is removed. Dialysis also aids in separating the enzyme from inactive material for, by this method, excess protein flocculates in the extract and can be filtered or centrifuged off. The removal of this protein is important because it adsorbs the enzyme, leaving the extract relatively inactive. Cori and co-workers found that the farther the purification procedure was carried, the less active the enzyme extracts became. However, when adenylic acid was added to these completely inactive extracts, they again became active. It was, therefore, assumed that the essential adenylic acid was removed by dialysis. Other nucleotides were studied and found ineffective in the capacity of activators, except in the case of adenosinetriphosphate in freshly prepared extracts. The activating effect of this compound, however, was attributed to the presence of pyrophosphatase, an enzyme which is capable of converting adenosinetriphosphate to adenylic acid. In confirmation of these findings, Green, Cori, and Cori (24) succeeded in obtaining muscle phosphorylase in crystalline form as an adenylic acid complex.

Hanes (14, 15) and Green and Stumpf (26), on the other hand, employed extensive dialysis for purification of the plant enzyme and could find no indication of inactivation

through the loss of adenylic acid. Cori and Cori (4) pointed out that there is not yet enough evidence to settle the question of whether adenylic acid is so closely bound to the plant enzyme protein that it is not removed by procedures which separate it from the animal enzyme or whether the plant enzyme is active without this coenzyme.

The possibility that dialysis might remove a factor important to the functioning of the phosphorylase system of corn was tested by several experiments in which the activity of raw, undialyzed extracts was compared with that of dialyzed extracts. The corn used for these enzyme preparations was collected two weeks after pollination. Each digest contained 10 ml. of enzyme extract, 1 ml. of 0.3 M Na_2HPO_4, and 4 ml. of 2% starch (pH about 7.5). Table 9 shows the results of these digestions.

A comparison of the activity of dialyzed (Digest 8) with undialyzed enzyme extract (Digest 9) indicated that dialysis had scarcely any effect upon the esterification reaction. The ratio of free-P/ free- - ester-P (as calculated by the approximation method previously described) was comparatively the same in both digests. It was apparent that the amount of initial-P in the dialyzed material was lower than that in the undialyzed extract, and that dialysis could be used as a means of removing inorganic-P from the enzyme material.

In the two following sections (experiments with yeast

Table 9

Effect of Dialysis on Phosphorylase Activity

Time (hrs.)	Mg. P per ml. of digest	
	Digest 8	Digest 9
0	0.72	0.84
0.5	0.72	
1	0.69	0.75
3	0.72	0.66
5		0.69
6	0.69	
7		0.69
11	0.66	
23		0.63
24	0.60	
25		0.63
29		0.60
30	0.57	
47		0.63
53		0.66
71		0.66

and experiments on adsorption by aluminum hydroxide and clarification by kaolin) are described tests in which both dialyzed and undialyzed extracts were used. The data were interpreted, therefore, in terms of their significance in relation to dialysis as well as in relation to the effect of the yeast and of the further treatments of the extracts upon the activity of the phosphorylase.

To test the possibility that dried yeast might contain some factor, such as nucleotides, which would influence the activity of the phosphorylase, digests of pH about 7.5 were

set up from undialyzed enzyme extract (Digest 10), from dialyzed but uncentrifuged extract (Digest 11), and from dialyzed, uncentrifuged extract with dried yeast extract added (Digest 12). In addition to the enzyme, soluble starch and secondary sodium phosphate were contained in the digests. The reaction was carried out in a water bath at 25° C. After 30 minutes a trace of magnesium chloride was added to each digest. Table 10 gives the data for this experiment.

Table 10

Effect of Addition of Yeast to Dialyzed Enzyme Extracts

Time (hrs.)	Mg. P per ml. of digest		
	Digest 10	Digest 11	Digest 12
0	0.81	0.72	0.33
0.5	0.78	0.72	0.33
1	0.75	0.69	0.33
3	0.72	0.72	0.33
6	0.66	0.69	0.30
11	0.60	0.66	0.24
24	0.57	0.60	0.21
30	0.63	0.57	0.27

From the data a slight difference in the ratio of free-P to free- + ester-P could be noted. The higher value for the dialyzed extract indicated that the reaction was tending slightly more toward the formation of starch than was the case with the undialyzed extract. It should be pointed out that the equilibrium value probably had not

been completely reached in these digests.

The effect of the addition of an inactivated yeast extract was insignificant, as the range and the direction of the reaction were unchanged. In all three types of digests tested for a period of 30 hours, the final results showed a decrease in free-P or a tendency toward the formation of glucose-1-phosphate.

4. Effect of adsorption by $Al(OH)_3$ and clarification by kaolin. Experiments previous to this series had been made with extracts which had received no purification other than dialysis. Investigations by Hanes (15) and by Cori (20) had indicated varying success in the use of such adsorbents as C_γ $Al(OH)_3$ and kaolin. $Al(OH)_3$ was found to adsorb the enzyme, whereas kaolin clarified the extract, leaving the phosphorylase in it. Consequently, these methods were examined for efficiency in purifying raw extracts.

Waxy maize collected 11 days after pollination was extracted in the refrigerator with water. The extract was centrifuged off. Part of it received no further treatment and was used in Digest 13. The remainder was dialyzed for 24 hours in 15 l. of distilled water in the refrigerator. Of the dialyzed extract, part was given no further treatment and was used in Digest 14. Another portion was treated with 0.5 volume of C_γ $Al(OH)_3$ in suspension, as directed by Cori et al. (27). The centrifuged adsorbate was washed

twice with water and eluted twice with 0.5 volume of 0.3 M Na_2HPO_4. The elutions were then treated with $(NH_4)_2SO_4$ to 0.3 saturation and the precipitate centrifuged off and suspended in 0.5% $(NH_4)_2SO_4$, comprising the extract used in Digest 15. The other part of the dialyzate was treated with kaolin, according to the directions of Hanes (14). Twenty grams of kaolin was added for 100 ml. of extract; the mixture was stirred for ten minutes, and then filtered by gravity. The yellowish filtrate became the enzyme extract for Digest 16.

Digests were set up for each of the four differently prepared extracts. Each digest contained enzyme extract, soluble starch, a trace of magnesium chloride, and phosphate buffer (pH 7.2). The data for the experiment are reported in table 11.

From these data it may be observed that there was no significant difference caused by the dialysis of the extract. Adsorption on $Al(OH)_3$ evidently increased the activity by concentrating the enzyme to some small extent. Removal of impurities by adsorption on kaolin showed no very definite effect.

It is interesting to note that the purified extracts (15 and 16) caused a decided increase in inorganic phosphate at the conclusion of the tests, or a trend toward starch synthesis.

Summary. As was pointed out, the object of the

Table 11

Effect of Purification Procedures on Phosphorylase Activity

Time (hrs.)	Mg. P per ml. of digest			
	Digest 13	Digest 14	Digest 15	Digest 16
0	1.26	1.20	1.29	1.20
0.5	1.20	1.20	1.32	1.20
1.0	1.23	1.20	1.29	1.38
3.0	1.29	1.23	1.29	1.20
6.0	1.26	1.20	1.32	1.20
9.0	1.26	1.23	1.29	1.20
24.0	1.26	1.26	1.20	1.23
48.0	1.20	1.20	1.32	1.23
72.0	1.20	1.17	1.38	1.32

preliminary experiments was to adapt the procedures worked out by Hanes to the present study of waxy maize phosphorylase. In this way a foundation could be laid for further investigation on the concentration and activity determinations of the enzyme.

From these experiments the following trends were indicated:

1. In the original extract, as used for the "auto-digestions", enough starch or glucose-1-phosphate was present to carry along some phosphorylase activity.

2. Experiments with extracts of corn at different stages of growth (varying from 14 days to 7 weeks after pollination) indicated that phosphorylase activity was greater in the early corn than in the more mature samples.

3. A comparison of phosphorylase activity in dialyzed

and undialyzed extracts indicated that the process of dialysis accomplished little that could not be brought about by other methods which had less deteriorating effect upon the enzyme.

4. Yeast contained no factor which favored the activity of phosphorylase in dialyzed corn extracts.

5. Treatment of dialyzed extracts with aluminum hydroxide showed a tendency toward purification or concentration of the phosphorylase, resulting in a product which favored the reaction of starch synthesis.

Preparation of glucose-1-phosphate

In the preliminary experiments on the phosphorylase of maize as described in the preceding section, the substrate employed was soluble starch, and thus the reaction at the beginning was that of starch + inorganic-P forming glucose-1-phosphate. However, as the work progressed, it became evident that it was necessary to study phosphorylase activity in relation to the reverse reaction--the synthesis of starch using glucose-1-phosphate as the substrate. For this purpose it was necessary to prepare, in quantities sufficient for enzyme studies, the Cori ester, glucose-1-phosphate.

Method. The procedure of Cori, Colowick, and Cori (16) was used in the chemical synthesis of the ester, starting with β-1,2,3,4,6-pentaacetylglucose. The following scheme

shows the reactions involved:

β-pentaacetylglucose $\xrightarrow{HBr + HAc}$ α-1-bromo-2,3,4,6-tetraacetyl-glucose

α-1-bromotetraacetylglucose $\xrightarrow{Ag_3PO_4}$ tri-(tetracetylglucose-1)-phosphate

tri-(tetraacetylglucose-1)-phosphate $\xrightarrow{HOH}$ glucose-1-phosphoric acid

glucose-1-phosphoric acid $\xrightarrow{Ba(OH)_2}$ Ba glucose-1-phosphate

Ba glucose-1-phosphate $\xrightarrow{K_2SO_4}$ K_2 glucose-1-phosphate

Cori used Fischer's method (46) for making the intermediate α-1-bromotetraacetylglucose (acetobromoglucose) from the pentaacetate. To prepare the acetobromoglucose, Fischer treated 150 g. of the pentaacetate with 300 g. of commercial glacial acetic adid-hydrogen bromide solution, dissolved the pentaacetate by vigorous shaking, and left it at room temperature for 2 hours. Then it was diluted with 600 ml. of $CHCl_3$, and the combined chloroform solutions washed with 1 liter of water.

The combined solutions were drawn off and dried by shaking with anhydrous $CaCl_2$. After concentration under diminished pressure, the solution was treated gradually with petroleum ether (Skelly D), from which the acetobromoglucose separated out in long needles. These were filtered off by suction and dissolved as quickly as possible in 75 ml. of amyl alcohol by warming on a water bath. Upon rapid cooling in ice, the colorless needles crystallized

and were separated by suction; these were washed carefully with petroleum ether. They were dried over soda-lime in a vacuum desiccator. Fischer obtained yields of 76% of the theoretical. He reported that the pure product kept for months, but impurities caused extensive decomposition in a few days.

The acetobromoglucose obtained by Fischer's procedure after crystallization from iso-amyl alcohol usually decomposed within a few days when put in a vacuum desiccator over soda-lime. However, Baker's (47) method of keeping the acetobromoglucose indefinitely by recrystallizing from anhydrous ether and storing in a vacuum desiccator over P_2O_5 and solid KOH was found successful.

Cori et al. made glucose-1-phosphate from acetobromoglucose in the following way:

A mixture of 3.5 g. of freshly prepared silver phosphate with a solution of 10 g. of dry acetobromoglucose in 30 ml. of sodium-dried benzene was refluxed for one hour with a calcium chloride-tube attached to the condenser. The silver salts were centrifuged off and washed once with dry benzene. The combined benzene solutions were then concentrated under diminished pressure at about 50° to a thick syrup. Upon further evacuation there was formed a foamy mass which usually crystallized when left under a high vacuum overnight.

A five per cent solution of this intermediate substance,

tri-(tetraacetylglucose-1)-phosphoric acid, in methanol was made to 0.2 N by the addition of 5 N HCl. The solution was allowed to hydrolyze at 25°.

The hydrolysis of the intermediate compound is the critical part of the procedure. Cori, Colowick, and Cori (16) suggested that during the hydrolysis the optimal rotation of the solution should decrease to about 4/10 its original value. In several preparations in this laboratory the optical rotation was used as a criterion for completion of the reaction. Colowick, in a personal communication to the writer (48), stated that the hydrolysis should be controlled rather by phosphate analyses. In his laboratory it was found that inorganic phosphate did not appear until hydrolysis had proceeded for 5 to 10 hours at 25°, after which time inorganic phosphate was formed at the rate of 4 to 6 per cent of the total phosphorus per hour. Colowick reported that the reaction should be stopped when the inorganic phosphate level had reached 15 to 20 per cent of the total phosphate. This method of phosphate analysis was found to be much more satisfactory than optical rotation for determining the course of the reaction and was used in later preparations of the synthetic compound in this laboratory. The time required for the process varied from 13 to 24 hours.

At the conclusion of the hydrolysis a hot saturated solution of $Ba(OH)_2$ was added to the hydrolyzate until the

reaction was distinctly alkaline to phenolphthalein. It was found in this laboratory that frequent additions were necessary in order to keep the solution alkaline. Colowick (48) suggested that the alkalinity be tested and $Ba(OH)_2$ added for a period of about one hour. At the end of this time the solution was cooled in the refrigerator for several hours. The precipitate was centrifuged off, drained, and extracted with water two or three times. Because of the small yields the extraction process is very important. Repeated extraction over extended periods was found to increase the yields.

The alcoholic mother liquor and the insoluble residue were discarded. Then the combined aqueous extracts were precipitated with 1 to 1.5 volumes of ethanol and the process repeated until a precipitate almost completely soluble in water was obtained. This product was dried in a vacuum desiccator. Cori (16) reported the formula as $C_6H_{11}O_5.O.PO_3.Ba.3H_2O$; $[\alpha]_D^{25} = +75^\circ$.

For use in phosphorylase tests it was necessary to convert the barium salt to a potassium, sodium, or ammonium salt. For this purpose Cori and Colowick (48) found it most convenient to use Kiessling's (49) method. The barium salt was dissolved in a small amount of water and treated with the calculated amount of 10% K_2SO_4. The barium sulfate was then centrifuged off. The remaining solution was heated and subsequently treated with about one and one-half volumes

of hot ethanol. Precipitation of the dipotassium salt as crystals took place when the solution was left overnight in the refrigerator. Small crystals could be obtained at once by rubbing the sides of the flask. However, the larger crystals required slow precipitation or seeding. Addition of excess alcohol resulted in the formation of the very fine crystals.

Kiessling (49) reported that several recrystallizations gave a very pure product containing two molecules of water of crystallization. It was found that this method of converting to the potassium salt was an effective means of purifying the barium salt, when recrystallization as the barium salt was unsatisfactory.

The structure of the ester was assumed by Cori (16) to be that of α-glucopyranose-1-phosphoric acid. Wolfrom and co-workers (50,51) proved this structure by a study of the chemical behavior of the dipotassium salt.

Experimental part. Because of the importance of the preparation of glucose-1-phosphate for subsequent experimental work on phosphorylase, the procedure was studied in detail. A typical preparation will be described.

For this work the β-pentaacetylglucose was obtained from three different sources. Some was the product of student syntheses in organic chemistry. A large quantity (600 g.) was bought from Eastman Kodak, and 2 kilograms were purchased from the Corn Products Refining Company.

No difficulty was encountered in making the acetobromoglucose by the action of hydrobromic acid (30-32% in acetic acid) on the pentaacetate. For the preparation of the intermediate product, 30 g. acetobromoglucose was dissolved in 90 ml. sodium-dried benzene. After refluxing and removal of the silver salts by centrifugation, the solution of the intermediate product in benzene was concentrated as much as possible by evacuation with a water-pump. Evacuation was continued with a Hyvac pump until the product when left in a vacuum desiccator remained completely friable (though not crystalline).[1]

Thirty and four-tenths grams of the intermediate product (obtained from several preparations of the intermediate product) was hydrolyzed at 25.5° C. in 760 ml. of methanol by the addition of 32.1 ml. of 5 N HCl. In accordance with Colowick's suggestion, the progress of hydrolysis was followed by tests for inorganic-P, using 2-ml. aliquots of the hydrolysis solution. Table 12 shows the progress of the hydrolysis.

The hydrolysis was halted at the end of 16 hours. It was calculated that in 30.4 g. (mol. wt. 1088) of the intermediate product, there should be 0.866 g. total P in the

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[1]In a private communication Colowick (48) stated that their product was friable, although he thought that the compound might be used in the subsequent step as a thick syrup if it was not obtainable in the other form. In this laboratory satisfactory results have been obtained by using the syrup form of the intermediate product.

Table 12

Hydrolysis of the Intermediate Product

Time (hrs.)	Mg. P from 2-ml. sample
0.5	0.070
11.5	0.208
12.5	0.320
14.0	0.350
15.3	0.400

800 ml. of the mixture. According to Colowick, the hydrolysis should be run until 15-20% of the total P, or 0.130-0.173 g., has been split off. Therefore, a 2-ml. sample should show 0.3 to 0.4 mg. of inorganic-P.

Hot saturated barium hydroxide[1] solution was added to the mixture until a distinctly alkaline test was given with phenolphthalein. The alkalinity was checked during the period of one hour, and frequent additions of barium hydroxide were made to maintain the proper pH.

After standing several hours in the refrigerator, the precipitate was removed by centrifugation. It was extracted with water twice over a period of 2 to 3 days. The 700 ml. of aqueous extract was then reprecipitated with 1050 ml. of 95% ethanol and allowed to stand in the refrigerator for

[1]Because of the high solubility of the barium salt in water, it was desirable to keep the solution as concentrated as possible. For this reason the barium hydroxide precipitating solution was saturated at a high temperature.

several hours. Centrifugation, extraction, and reprecipitation yielded a soluble salt weighing 1.89 g. and giving a light yellow clear aqueous solution.

The specific rotation for a 0.1 M solution of this barium salt was found to be +64.0°. Because the reading was quite low, it was decided to attempt purification through transformation to the potassium salt, by Kiessling's method. One and eight-tenths grams of the barium salt was dissolved in 50 ml. of H_2O and treated with 10 ml. of 10% K_2SO_4. The barium sulfate was centrifuged off. To the heated centrifugate was added about 120 ml. of hot absolute ethanol. Crystallization, which began immediately, was allowed to proceed overnight in the refrigerator. The crystals were then filtered off. The potassium salt was recrystallized once more from ethanol, and a micro-determination of the optical rotation was made. The specific rotation was found to be +78.8°. The value reported by Hanes (15) for the natural product was +78 - 79°.

Green and Stumpf (26) found that for making large quantities of glucose-1-phosphate the method of Hanes (15) was successful. In this laboratory the procedure was followed repeatedly. The yields, however, were uncertain and relatively low; the product obtained was very impure. The difficulty lay in the isolation of the ester rather than in the enzymic process of its formation, as free-P and ester-P determinations indicated that considerable

phosphorylation took place.

Because of the difficulties encountered in the isolation of the pure product, the enzymic method was abandoned and the chemical synthetic procedure used exclusively.

Determination of phosphorylase units

In the literature two different methods have been reported for determining the activity of the enzyme. The following is a summary of the procedure employed by Cori and Cori (23):

Neutralized glutathione was added to the enzyme 30 minutes before the activity was measured. The reaction mixture contained 0.016 M glucose-1-phosphate, 0.001 M adenylic acid, 0.5% glycogen, 0.02 M glutathione, and 0.05 M glycerophosphate buffer at pH 6.65. The temperature was kept at 25° C. After 5 to 10 minutes of incubation, the inorganic-P was determined in an aliquot part. Another aliquot was hydrolyzed for 5 minutes in N H_2SO_4 at 100° C.; the inorganic-P found after hydrolysis corresponded to the glucose-1-phosphate added. The inorganic-P formed was expressed in per cent of the glucose-1-phosphate added. The activity units were calculated by the equation: $K = \frac{1}{t} \log \frac{C}{C - x}$, where C is the amount of inorganic-P present when the reaction reaches equilibrium and x the amount of inorganic-P present at time t, both expressed in per cent of the glucose-1-phosphate present at time t_o. (The values were

multiplied by 1000.)

The method employed by Green and Stumpf (26) for expressing phosphorylase units was quite different from that of Cori. These investigators defined 1 unit of phosphorylase activity as "the amount of enzyme which catalyzes the liberation of 0.1 mg. of inorganic phosphate from glucose-1-phosphate in 3 minutes at 38° and pH 6.0." For the test mixture they used 5 to 10 units of enzyme (contained in 1.8 ml.), 0.5 ml. of 0.5 M citrate buffer[1] of pH 6.0, 0.2 ml. of 5% soluble starch, and 1 ml. of 0.1 M glucose-1-phosphate, making the final volume 3.5 ml. The glucose-1-phosphate was added after temperature equilibration. The reaction was stopped at a convenient time (5 to 10 minutes) by addition of 5 ml. of 5% trichloracetic acid and 2 ml. of 2.5% ammonium molybdate in 5 N H_2SO_4. The mixture was diluted to 25 ml. with water and an aliquot of the filtrate analyzed by the method of Fiske and Subbarow.

The method of Green and Stumpf was developed for use with extracts containing potato phosphorylase and has been found to be adaptable to the study of other plant phosphorylases. In the present work on the phosphorylase of waxy maize, the activity is reported in terms of the unit defined by Green and Stumpf.

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[1]The citrate buffer mixture employed for the present work was prepared according to the directions given by Kolthoff and Rosenblum (40). Monopotassium citrate was recrystallized from water. A 0.1 M solution of the citrate and a 0.05 M solution of borax were used in the proportions of 25 ml. to 44.1 ml. to obtain the required pH.

Measurement of Activity in the Development of Waxy Maize

It was thought that a study of the change in phosphorylase activity with the growth of the maize kernel might indicate the importance and the nature of the role played by this enzyme in the development and maturing of waxy maize. Also, it was necessary to have a source of phosphorylase for further study of the enzyme as to methods of concentration and reactivity. For this purpose, then, the maize should be collected at the time in its development when phosphorylase seemed most active or most abundant. The procedure adopted for a study of the phosphorylase activity at different stages of growth of the waxy maize was as follows:

The waxy maize was prepared for extraction by one of two methods. In the case of the very young corn, grinding in a mortar with a small amount of water and sea sand was decided upon as an effective method of rupturing the cells for extraction of the enzyme. For the older, harder corn grinding in a Wiley mill was used. In both cases the corn was then extracted for 18 hours with distilled water in the refrigerator. The weight of water added was equal to the weight of wet corn extracted. A small amount of powdered thymol was added to each extract to prevent the action of microörganisms.

At the end of the extraction period the extract was

removed from the corn residue by centrifugation in the case of the milled corn. However, for the early corn which had been ground in the mortar, it was found practicable to separate the residue by straining through cheesecloth.first.

The separation of the enzyme from the water extract was accomplished by a method similar to that reported by Green and Stumpf (26). To remove inactive material (protein and starch) the enzyme extract was heated rapidly to 50° C. and kept at that temperature for 5 minutes, after which solid ammonium sulfate (20 g. per 100 ml. of extract) was added while the solution was still warm. The precipitate was centrifuged off and discarded. From the centrifugate, another fractional precipitation with ammonium sulfate (16 additional g. per 100 ml. of extract) yielded active enzyme. This precipitate was dissolved in 0.1 M citrate buffer of pH 6, and the solution made up to 25 ml. The enzyme precipitate did not dissolve to form a clear solution, but gave a cloudy suspension which did not settle out on standing.

This citrate buffer suspension of the enzyme was used for determination of activity units according to the method outlined on p. 57. Results of this study were calculated on the basis of the number of phosphorylase units per gram of corn as collected, and also as the number of phosphorylase units per gram of total solid.

For the purpose of these calculations it was necessary

to know the moisture content of the corn. The Bidwell-Sterling method (52), by distillation of the water with toluene into especially calibrated pipets, was used in the moisture determinations.

From the reading for inorganic phosphorus obtained from the colorimeter and interpreted from the phosphorus curve for the instrument, it was found how many milligrams of inorganic-P were present in 2-ml. of the 25-ml. digest. This digest (made up as described on p.57) contained 1.8 ml. of enzyme suspension. Therefore, the set-up for calculating the amount of inorganic phosphate formed by all the enzyme suspension was as follows:

mg. P from curve $\times \frac{25}{2} \times \frac{25}{1.8} \times \frac{95}{31}$ = mg. total inorganic phosphate released by enzyme.

This amount was multiplied by ten to find the number of units present. When the total number of units was divided by the weight of corn used to make the extract, the number of units per g. of corn as collected was obtained; when the total number of units was divided by the weight of dry corn furnishing the enzyme, the number of units per g. of total solid was determined.

During the growing season, August and September, in 1941 and again in 1942, corn was collected from the field at intervals of a few days, and the extracts were prepared and tested for phosphorylase as described. Table 13 and fig. 4 show the data on a series of experiments made in

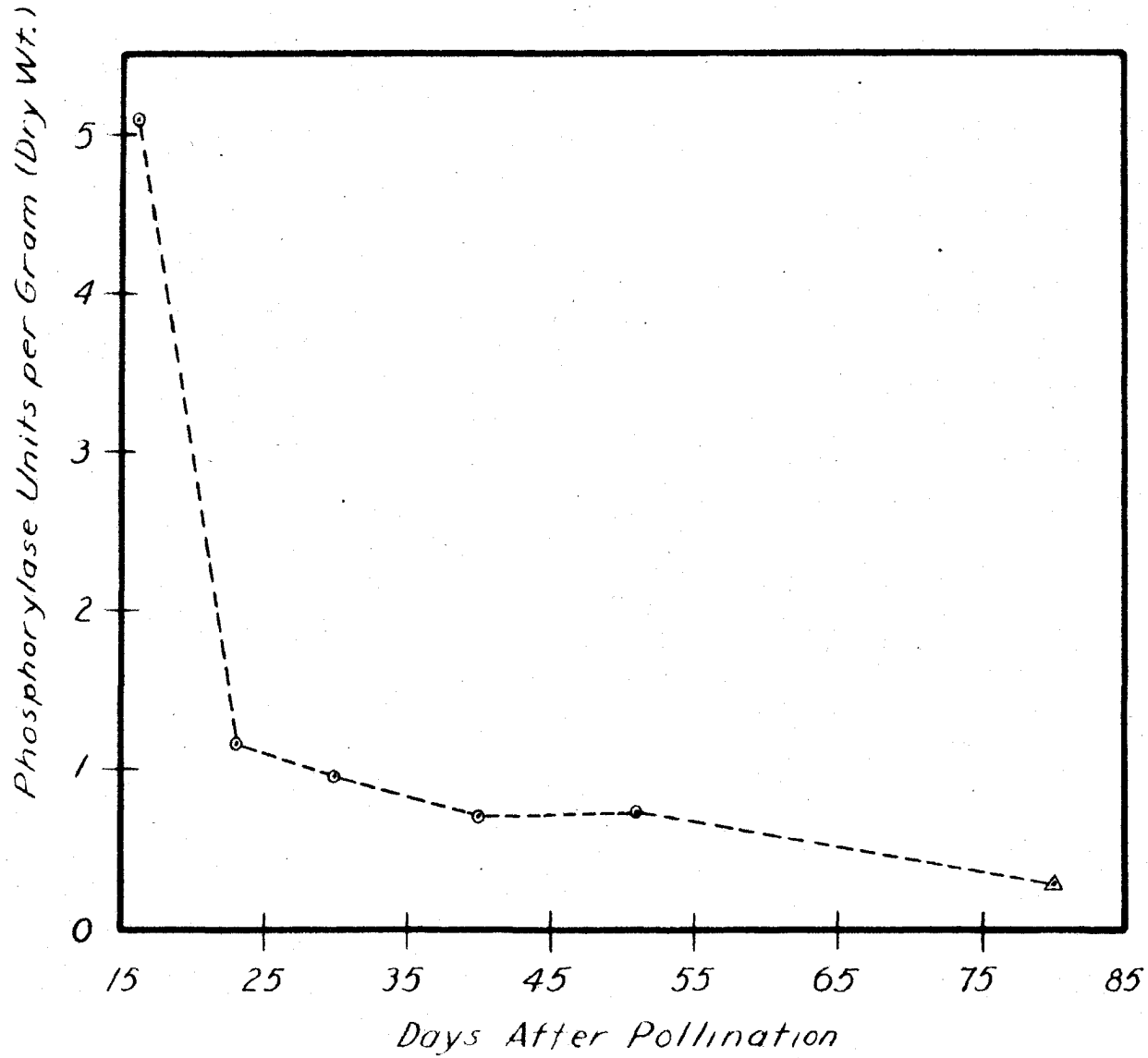

Fig. 4. Phosphorylose Activity in Corn at Different Stages of Growth

the fall of 1942 on waxy maize No. 939, pollinated August 1 and tested at intervals until maturity.

Table 13.

Phosphorylase Activity in Corn at Different Stages of Growth

Date of collection	Days after pollination	Moisture content %	Units/g. as collected	Units/g. dry weight
Aug. 17	16	83.2	0.85	5.09
Aug. 24	23	68.5	0.37	1.16
Aug. 31	30	51.5	0.45	0.95
Sept. 10	40	42.9	0.39	0.70
Sept. 21	51	35.0	0.48	0.74
mature		6.1	0.26	0.28

The corn collected 16 days after pollination consisted of kernels just in the process of formation, and the juice seemed like mere drops of water. Since the moisture content of this corn was 83%, the total solid was low; and whether the activity were calculated on the basis of units of phosphorylase per gram as collected, or units per gram of dry corn, the enzyme activity was high in this early corn.

During the period of greatest growth of the corn kernel, the per cent of moisture decreased and the amount of starch (and therefore, total solid) increased. The data on phosphorylase activity showed that a fairly constant amount

of enzyme could be precipitated from these extracts, as indicated by the units per gram of corn as collected. On the basis of dry weight, a more apparent decrease in phosphorylase was suggested, although the increase in total solids was a contributing factor in the calculation.

It is interesting to suggest that the function of phosphorylase as a catalyst in the synthesis of starch accounted for its pronounced activity in the early corn (16 days after pollination) and for the presence of the enzyme throughout the weeks of starch formation until maturity.

If these experiments are examined from the standpoint of deciding upon a source of active enzyme for concentration studies, it might seem that the early corn would best serve the purpose. However, in view of the fact that the kernels are so small and of such high moisture content, it would be necessary to have available a very large supply of corn to obtain enzyme from this source. It seemed desirable, therefore, to prepare enzyme extracts for further study from the corn in the later stages of growth (from 3 to 4 weeks after pollination).

Concentration of the Enzyme

Introduction

A systematic approach to the purification and concentration of the waxy maize enzyme seemed to be through the

investigation of the common methods employed for such purposes. In a general way, these techniques include precipitation, adsorption, and dialysis. Each of these procedures has been used by workers in the field of phosphorylase.

Investigators have tried with varying degrees of success to concentrate the enzyme phosphorylase from both plant and animal sources. Finally, in 1942 Green, Cori, and Cori (24) succeeded in preparing crystalline muscle phosphorylase. For this preparation a rabbit skeletal muscle extract was dialyzed, the globulins were filtered off after adjustment to pH 6, and the phosphorylase was precipitated by making to 1.68 M with ammonium sulfate at pH 7. Crystals separated after this precipitate were dissolved in glycerophosphate-cysteine buffer (pH 7) and the solution dialyzed at 0°. These crystals, in the shape of rodlets, proved to be an adenylic acid complex of phosphorylase.

For all their previous work Cori and co-workers used dialyzed extracts, although slightly different procedures, varying principally in the length of time of dialysis (12), were employed for the various kinds of animal tissues. In most cases, after dialysis, the extracts were filtered or centrifuged to remove insoluble protein and were stored under toluene in the refrigerator. It was found that, in general, such extracts did not form the 1-ester unless a

nucleotide was added (19). Thus it was evident that in animal extracts extensive dialysis removed the nucleotide necessary for phosphorylase action.

The method of dialysis was used to take out organic and inorganic phosphates, as well as for separation from other interfering enzymes in animal extracts. However, a loss of activity was always noted during the process, even when the operation took place at 5° C. Electrodialysis was also employed successfully for removal of phosphates by Cori and co-workers (53, 22).

Cori (27) obtained a partial separation of liver phosphorylase from other enzymes by adsorbing it on 0.5 volume of C_γ aluminum hydroxide, eluting with 0.3 M Na_2HPO_4, and precipitating by making the elutions to 0.3 saturation with ammonium sulfate. The precipitate was centrifuged off and dissolved in 0.5% KCl or K_2SO_4. The enzyme solution thus prepared was free of phosphoglucomutase.

In one paper Cori, Schmidt, and Cori (20) emphasized the usefulness of adsorption with aluminum hydroxide and elution with secondary sodium phosphate as a means of obtaining an enzyme solution rich in phosphorylase and relatively free from the conversion enzyme. They found that a second adsorption and elution resulted in almost complete separation of the two enzymes. This adsorption, however, should follow dialysis for removal of inorganic phosphate. An apparent difference between the behavior of animal tissue

preparations of the enzyme and the yeast enzyme was pointed out in this paper. Kiessling (54) reported that prolonged dialysis of the yeast phosphorylase extract against 0.3 saturated ammonium sulfate solution did not affect the activity of the enzyme. Whether the nucleotide is not removed by the process or whether the nucleotide is unnecessary for the action of the yeast enzyme has not been decided.

Hanes (14) in his experiments with pea seeds tried various methods of extraction. A 1% solution of Na_2HPO_4, and sodium carbonate at pH 7.5 were used but showed no advantage over simple extraction with water at the natural acidity of the tissue (about pH 6.2).

Dialysis was used by Hanes as a method of purification. The extract was dialyzed at 2° C. for 8 hours against 30 l. of running water and for 16 hours against running distilled water (saturated with thymol). A precipitate which formed during dialysis was centrifuged off. It was found, too, that protein was precipitated progressively on storage. Because the active enzyme was adsorbed on this protein precipitate, it was desirable to prevent the protein deposition. By treating dialyzed extracts with kaolin (15-25 g. of kaolin per 100 ml. of extract, followed by gravity filtration), clear yellowish filtrates were obtained, with almost the entire phosphorylase activity, and no sedimentation was formed during storage.

Green and Stumpf (26) used successive fractionations

with ammonium sulfate as a method of purification and concentration of potato phosphorylase. The success of their method could be measured by the fact that in their final preparation one unit was equivalent to 0.324 mg. of dry weight of potato, whereas in the original extract, one unit was obtained from 120 mg. of dry weight. The degree of concentration was, therefore, over 370-fold. These investigators tried other methods of purification without success. Alumina adsorption was not effective, as only about 35 to 40 per cent of the total enzyme adsorbed could be recovered with various eluting agents. They found that dialysis for 12 hours against running tap water led to small losses, and that the enzyme was inactivated after prolonged dialysis against distilled water, though not after prolonged dialysis against 0.2 M KCl. Complete inactivation of the enzyme was effected by precipitation in the cold with alcohol.

Experimental

The methods here reported for waxy maize are based on those of Green and Stumpf (26) and of Hanes,(14,15) both of whom worked on potato phosphorylase and used ammonium sulfate as the precipitating agent. Certain modifications of these methods are described in the procedures.

In the potato the relationship of protein to carbohydrate is about 1:8 (1.8% to 13.8%), according to Whalley (55). In corn the ratio is approximately the same, 10%

protein to 80% starch and other carbohydrates (56). Because of this similarity, the findings of Green and Stumpf were used as a basis for the preliminary experimental work carried out with the waxy maize enzyme.

To determine the ammonium sulfate concentration which would give the most active precipitate, Green and Stumpf carried out a careful fractionation of potato phosphorylase. The results of their investigations indicated that the precipitate obtained by addition of ammonium sulfate to 0.26 saturation was relatively inactive and could be discarded. Their fractionation of enzyme was made on the precipitate which was formed between 0.26 and 0.45 ammonium sulfate saturation. These concentrations were used in the first work with waxy maize phosphorylase.

The precipitates were tested for activity by the procedure described in an earlier section of this thesis.[1] Two fractions representing 0 - 0.23 saturation and 0.23 - 0.52 saturation were taken for preliminary tests. Of the 0.23 - 0.52 fraction dissolved in citrate buffer the following precipitates were taken: 0.23 - 0.28, 0.28 - 0.34, 0.34 - 0.41, and 0.41 - 0.52 saturation. From data on the activity units contained in each fraction, it was evident

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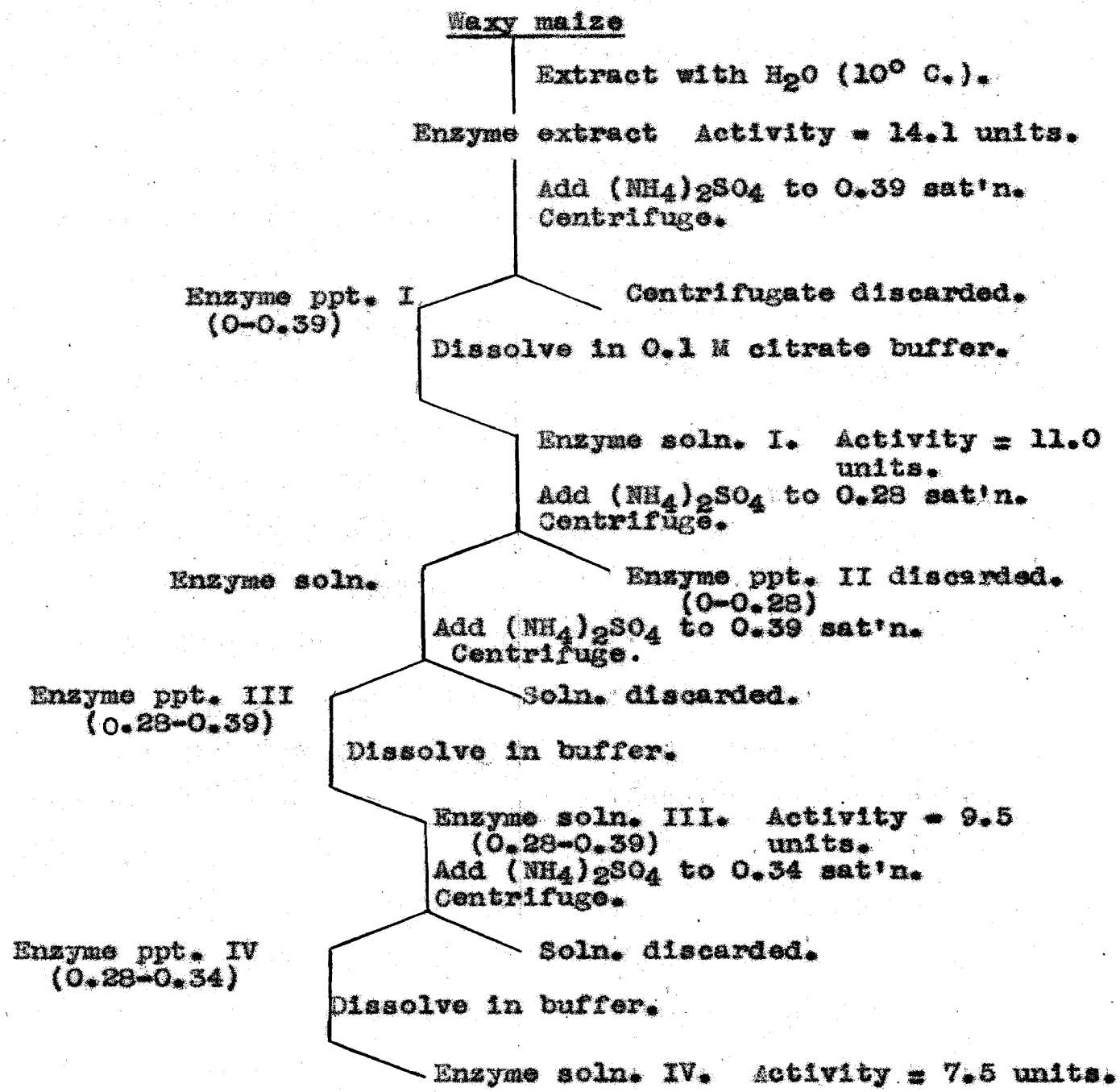
[1] In these studies it was found necessary to run "blank" determinations on the extracts used for activity tests because of the considerable amount of free phosphorus present in these extracts. Data obtained for the blanks showed that the amount of free phosphorus present decreased with each ammonium sulfate precipitation carried out. Blanks were also run on the glucose-1-phosphate and soluble starch as used in the digests for the activity determinations.

that the precipitates obtained with the lower concentrations of ammonium sulfate were the most active. However, it was also observed that in the higher concentrations of ammonium sulfate the enzyme lost some of its activity, probably from prolonged contact with ammonium sulphate during centrifuging and other operations. Therefore, a slightly different procedure was adopted for further work in concentration.

The method of concentration followed in this work can be explained by a description of a typical experiment on the concentration of phosphorylase from waxy maize with data on activity and per cent recovery.

An extract was made by treating 1260 g. of ground waxy maize (collected about one month after pollination) for 24 hours with 2520 g. of water in the refrigerator. The extract was strained through cheesecloth, centrifuged in the Sharples Supercentrifuge twice, and stored in the refrigerator. Activity tests were made on different fractions of the precipitate obtained from this extract by addition of ammonium sulfate. A test on the original extract indicated the presence of 14.1 phosphorylase units per 100 ml. of extract.

The following flow-sheet shows the steps in this fractionation procedure:



In the flow-sheet the activity was calculated on the basis of the total number of units recovered from 100 ml. of the original extract. Percentage recovery for the

fractions tested is shown in table 14.

Table 14

Concentration of Enzyme by $(NH_4)_2SO_4$ Precipitation

Fraction $(NH_4)_2SO_4$ sat'n	Units per 100 ml. original extract	% of total activity of original extract
0 (original)	14.1	100
I 0 - 0.39	11.0	78.0
II 0 - 0.28		10.6 (calcd.)
III 0.28 - 0.39	9.5	67.4
IV 0.28 - 0.34	7.5	53.2
0.34 - 0.39		14.2 (calcd.)

From these data it is apparent that fraction II (0 - 0.28) was comparatively inactive and could be discarded, whereas the most concentrated fraction was IV (0.28 - 0.34). Therefore, above 0.34 and below 0.28 saturation very little activity was obtained. Fraction IV gave the most concentrated suspension, which contained 4.5 units in 10 ml., or three times the concentration of the original extract.

The ammonium sulfate concentration which proved most effective for recovery of the enzyme was somewhat lower than that which gave the most active precipitate of potato phosphorylase. The fractionation was not carried beyond the concentration described because of the loss of activity

observed in the extracts as a result of the time-consuming procedure and because of the relatively low concentration of the enzyme in the original extracts of waxy maize. These preparations could be used satisfactorily for further studies on the properties of phosphorylase concentrates.

Potentiometric Iodine Titration

Review of literature

Natural starch is now known to consist of two fractions, amylose and amylopection (57). Amylose is the straight-chain fraction made up of glucose residues connected by 1,4-α-glucosidic linkages. Amylopectin has a branched chain structure containing both 1,4- and 1,6-linkages. With iodine, amylose gives a deep blue coloration, whereas amylopectin is blue-violet to reddish. Different varieties of starches from plant sources contain characteristic amounts of each of these two fractions (57). Therefore, in attempting to characterize the synthetic polysaccharides prepared by phosphorylase action, investigators naturally turned to a method of determining the amount of each fraction which was present in the synthetic product.

Preliminary observations regarding the differences between natural starch and the polysaccharides synthesized by potato phosphorylase *in vitro* were made by Hanes (15,28). He found the following points of contrast between the two

products: (1) the synthetic polysaccharide was only sparingly soluble in hot water and retrograded rapidly from solution; (2) the synthetic product gave a more brilliant blue color with iodine; (3) the two compounds differed in their action toward β-amylase. The last, most characteristic property of the synthetic polysaccharide, its ability to be completely converted to maltose by the action of β-amylase, distinguishes it definitely from natural starch, which is only 60 per cent degraded by this enzyme. Upon this distinctive behavior Hanes based his assumption that the synthetic product resembled most closely amyloamylose, a less pure form of amylose.

Haworth, Heath, and Peat (58) used as a basis for their experiments the synthetic starch prepared by Hanes through the action of potato phosphorylase. Only the insoluble part of the polysaccharide deposited during the enzyme action was studied; this fraction amounted to 85 per cent of the total synthetic product. From observations upon the behavior toward β-amylase, these investigators concluded, as did Hanes, that the polysaccharide resembled most the amyloamylose fraction of natural starch. They proposed, therefore, that this synthetic starch was composed only of glucose residues which were mutually united by 1,4-α-glucosidic linkages. From methylation studies they estimated that the average length of the unit chain was a minimum of 80 to 90 glucose units.

It is interesting to note in this connection that Hassid and McCready (59) carried out similar experiments on the methylation of starch synthesized by potato phosphorylase. They concluded, however, that there were no end-groups in the product and that either the chains formed continuous loops or the glucose units in the chains were too numerous to allow the isolation of tetramethylglucose under the conditions employed. Haworth, Heath, and Peat (56) pointed out that the sample used by Hassid and McCready was too small for end-group assay.

More recently Wolfrom, Smith, and Brown (60) applied the mercaptalation molecular size assay of Wolfrom et al. (61) to the synthetic starch prepared by the action of potato phosphorylase. This mercaptalation assay has the advantages of giving results which are independent of branching, whereas the methylation assay measures only the length of straight-chain molecules or of the straight-chain portions of branched molecules. The results of the experiments indicated a value of 32 ± 1 glucose units as the initial average degree of polymerization of the synthetic potato starch.

The question easily arises as to which fraction of natural starch is more readily attacked by phosphorylase in the synthesis of glucose-1-phosphate. In 1938 Euler, Bauer, and Lundberg (62) reported the results of their investigations as an answer to this question. They found

that the amylopectin fraction was phosphorylated to a much higher degree than was the amylose fraction. Dialyzed muscle extract was used for this work. It may be pointed out, however, that the several methods of isolating the two starch fractions (used as substrates in these experiments) have not yet been standardized so that they all yield identical products.

Kurt Meyer (7) has recently suggested the existence of two phosphorylases, one of which will attack the 1,4-bonds of a polysaccharide and the other which is specific for 1,6-bonds. Amylopectin and glycogen contain both α-1,4- and α-1,6-bonds. Through the use of β-amylase, which attacks only 1,4-bonds, it can be determined whether or not the enzyme in question contains both types of phosphorylase. If β-amylase is allowed to act upon amylopectin or glycogen, a residual dextrin is formed by the removal of all exterior straight-chain, or 1,4-linkage, portions up to the branch-points. The phosphorylase is then allowed to act on the residual dextrin and will break off the terminal groups of the branch-points if 1,6-phosphorylase is present. The branches will thus be exposed to further attack by β-amylase. If, however, the dextrin after the phosphorylase action still resists β-amylase, it can be concluded that the branch-points (α-1,6-bonds) remain and that a 1,6-phosphorylase was not present.

By this method Meyer and Bernfeld (63) reached the

conclusion that potato phosphorylase is a 1,4-phosphorylase. Yeast phosphorylase, however, has been shown to be a mixture of 1,4- and 1,6-phosphorylase (7). The muscle enzyme undoubtedly contains both types of phosphorylase, although a purified preparation obtained by Cori was of the 1,4-type only. Kiessling's preparation showed both types of action. Meyer pointed out that the stability of the residual dextrin toward 1,4-phosphorylase proves that the enzyme attacks the non-aldehydic end of the chain. In this respect it is similar to β-amylase.

It has been noted that in all the experiments described only α-linkages have been involved. Investigations by Wolfrom, Smith, and Brown (60) have proved that polysaccharides cannot be synthesized by potato phosphorylase from β-d-glucopyranose-1-phosphate, thereby suggesting that β-linkages are not subject to the attack of phosphorylases.

From these observations several interpretations and conclusions may be derived. It would seem logical to assume that a 1,4-phosphorylase would also synthesize 1,4-bonds. With this theory, the findings of Hanes (15,28) and of Haworth, Heath, and Peat (58) regarding the polysaccharide synthesized by the 1,4-potato phosphorylase are consistent, for the starch synthesized resembled amylose. However, it was pointed out by Bates, French, and Rundle (64) that natural potato starch contains both the amylose and amylopectin fractions. In spite of the difference of

external conditions *in vivo* and *in vitro*, it is scarcely probable that the 1,4-enzyme would synthesize 1,6-bonds *in vivo*. The obvious conclusion is, then, that the 1,6-phosphorylase is lost during the process of extraction.

Further analysis of the difference between the behavior of the enzyme system *in vivo* and *in vitro* was offered by Bates *et al.* (64). According to their studies, as well as those of Hassid and McCready (59), synthetic starch appeared to be essentially straight-chain. All starches produced *in vivo* have been found to contain much less amylose than amylopectin (64). The possibility that external conditions cause the difference in the degree of branching of starch produced *in vitro* and *in vivo* can be eliminated for two reasons: (1) External conditions in waxy maize and in ordinary maize cannot be greatly different, although starch free of the straight-chain component, amylose, is produced in waxy maize as a genetic characteristic; (2) it is unlikely that within the same starch granule some molecules should remain unbranched whereas others are very branched, if external conditions determine the degree of branching.

An investigation of the polysaccharide synthesized by waxy maize phosphorylase was suggested by the foregoing studies. Because of the very small activity of this phosphorylase, it was impossible to isolate a sufficient quantity of the polysaccharide for fractionation. However, a recently developed method for the estimation of amylose

and amylopectin content, both qualitatively and quantitatively, was reported by Bates, French, and Rundle (64). This method was suggested by the difference in the iodine color given by the two fractions. It was found by these investigators that the amylose component bound iodine in the form of a complex, whereas the amylopectin component bound little or none. Therefore, by a potentiometric titration of a given starch with iodine, the type of chain structure present could be determined through a study of the iodine activity during titration. While the complex was being formed with iodine (in the amylose fraction), the iodine activity remained practically constant; however, when the complex formation was completed, there was a continual rise in the iodine activity.

The potentiometric iodine titration offered a possible approach to a study of small amounts of synthetic polysaccharide, such as were available in the experiments with waxy maize phosphorylase. Further interest was added to the problem by the fact that the starch occurring in waxy maize itself is purely amylopectin and takes up a negligible amount of iodine. In view of the fact that all previous investigations of synthetic polysaccharides produced by the action of plant phosphorylases have shown only amylose to be present, a plant phosphorylase system which would synthesize amylopectin <u>in vitro</u> would be unique.

Experimental

The potentiometric iodine titration as developed by Bates, French, and Rundle (64) consisted of dispersing the starch in 0.5 N KOH, then making the solution 0.05 N with respect to KI (either by neutralization with hydriodic acid or by neutralization with hydrochloric acid and addition of 0.5 N KI), and titrating potentiometrically with about 0.001 N I_2 solution. The data were then plotted and the curve analyzed by comparison with the curves obtained for representative starches and starch fractions.

Bates and co-workers found that the amylose, or straight-chain, fraction of starch gave an almost flat curve as long as iodine was being taken up to form an iodine-complex. After this complex formation was completed, the potential began to rise. The amylopectin, or branched-chain fraction, on the other hand, showed a continual rise in iodine activity when iodine was added. Consequently, the curve for amylopectin had no flat portion, as the potential constantly increased with the addition of iodine. Analyses of a large number of amylose materials, amylopectin materials, and whole starches containing both fractions were carried out by these investigators.

From their analyses several general observations were made by Bates (65). It was found that the level[1] of the

[1]The level of the curve, as indicated by the potential, is a function of the iodine activity.

straight-line portion of the curve could be used as an indication of the length of the straight chains. The greater the potential, the shorter is the chain-length, in general. The slope of the curve is also a measure of the range of chain-length[1] of the amylose. Bates and co-workers concluded that the lower the potentiometric level of the flat portion, the longer is the chain of amylose. However, a given difference in potential indicates a smaller difference in chain-length at the high potentials than at the lower potentials.

It was discovered by Bates et al. that there is a direct proportionality between the amount of a given amylose present and the length of the straight-line portion of the curve. Therefore, these investigators were able to work out quantitative relations which made it possible for them to estimate the amount of each fraction present in the different starches.

The experiments with the iodine titration, as carried out in this laboratory, were necessarily of a preliminary nature because the method had not been thoroughly standardized. Interpretation was subject to the limitations in the present knowledge of the applications of this titration to starch analysis.

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[1] Range of chain-length refers to the presence of chains of varying lengths. The diversity of these chain-lengths is shown by the different potential levels at which the iodine-complex is formed; thus the slope of the straight-line portion of the curve is a function of this diversity.

The digestions investigated were of the type containing glucose-1-phosphate and a catalytic amount of starch, set up in an attempt to synthesize a polysaccharide which would give a characteristic curve by the iodine titration. The enzyme extracts used in the present experiments were prepared from waxy maize collected about one month after pollination. Extraction of the ground corn was carried out for 24 hours in the refrigerator with twice the weight of water.

For the first digest (Digest A) a concentrated enzyme preparation was made by suspending in 0.1 M citrate buffer the precipitate representing the fraction between 0.32 and 0.39 ammonium sulfate saturation of a waxy maize extract. The precipitate from 200 ml. of the original extract was contained in 10 ml. of buffer. With this enzyme preparation the following digest was made up:

5	ml. enzyme suspension
0.2	ml. 5% soluble starch
1	ml. 0.1 M K_2 glucose-1-phosphate
19.4	ml. 0.1 M citrate buffer, pH 6.0

Digestion was carried out for exactly 30 minutes in a 38° incubator. At the end of this time enzyme action was stopped by the addition of 10 ml. of 0.5 N KOH. The solution was then made 0.05 N with respect to KI, diluted with water to 100 ml., and titrated potentiometrically with iodine.

For the titration a micro-burette, graduated in 0.02-ml. divisions, was used. The 0.0009 N I_2 in 0.05 N KI solution

was added at the rate of 4 drops (0.28 ml.) at a time and potential readings taken after 1 minute, when equilibrium was reached. Thus, readings were made at 1-minute intervals throughout the titration.

The addition of a catalytic amount of soluble starch (to overcome the induction phase) at the beginning of the digestion made the running of a blank on this starch necessary. To increase the accuracy of the determination, 0.2 ml. of starch was measured as four drops from a pipet; therefore, the blank and the digest each contained this amount of starch. Fig. 5 and table 15 show the results of the iodine titration of Digest A.

Examination of fig. 5, which shows curves for the blank and for the digest, makes evident the fact that the soluble starch used as a catalyst contained no appreciable amylose fraction, as the curve is like that of amylopectin. After digestion, however, the shape of the curve changed considerably and resembled that of a starch containing a very small amount of amylose or, more likely, a very small amount of starch containing all amylose of short chain-length, as suggested by the high potential of the straight-line portion. An indication of the range of chain-length is found in the slope of the straight-line portion of the curve. Bates et al. discovered that a straight-line portion on such a curve was an indication of the presence of amylose. It is not unlikely that such a polysaccharide was being synthesized

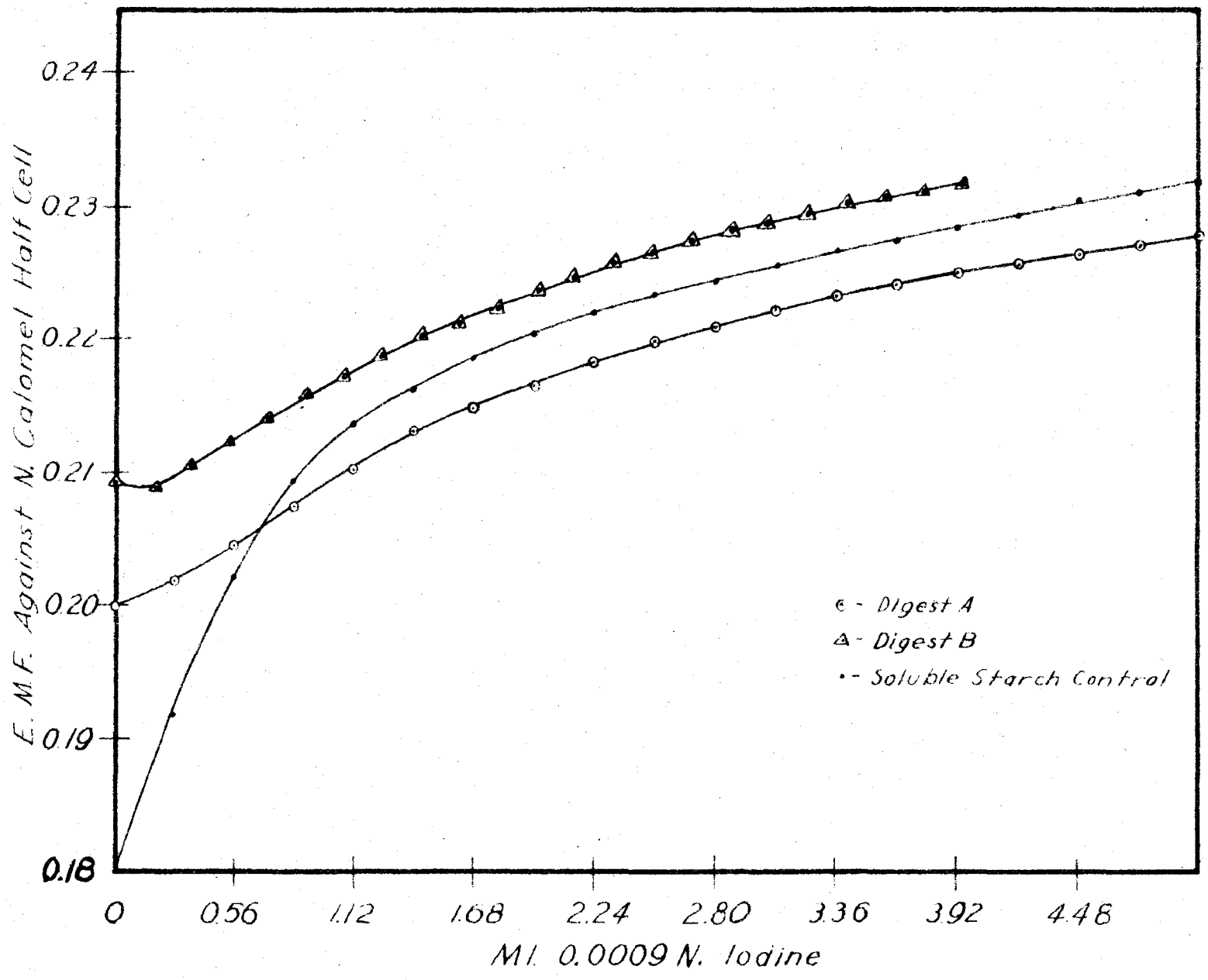

Fig. 5. Iodine Titration of Digests A and B

Table 15

Iodine Titration of Digest A

Ml. 0.0009 N I_2	E. M. F.	
	Starch blank	Digest A
0	0.1802	0.1999
0.28	0.1918	0.2018
0.56	0.2021	0.2045
0.84	0.2093	0.2075
1.12	0.2136	0.2102
1.40	0.2162	0.2132
1.68	0.2185	0.2147
1.96	0.2203	0.2166
2.24	0.2221	0.2183
2.52	0.2235	0.2198
2.80	0.2245	0.2210
3.08	0.2257	0.2222
3.36	0.2268	0.2232
3.64	0.2276	0.2241
3.92	0.2286	0.2250
4.20	0.2295	0.2258
4.48	0.2306	0.2266
4.76	0.2312	0.2272
5.04	0.2320	0.2280

by the waxy maize enzyme, inasmuch as no phosphorylase system could exist without the ability to produce 1,4-linkages. The theory of Meyer (7) implies the existence of a system containing only 1,4-phosphorylase or that of a system containing both the 1,4- and 1,6-enzyme. Although preparations have been made and found to contain only the 1,4-type (perhaps because of the loss of the 1,6-fraction in extraction of the enzyme), none has been reported to lack the 1,4-linkage property. It is obvious that such a

phosphorylase could not exhibit starch synthesis because 1,4-linkage ability is necessary for the formation of either amylose or amylopectin.

Another experiment was made with a slightly different enzyme preparation. The precipitate representing the fraction between 0.28 and 0.35 ammonium sulfate saturation was removed from 200 ml. of an aqueous extract (also used in Digest A) and suspended in 10 ml. of buffer. The digest (Digest B), which was run for 30 minutes at 38° in a water bath, contained 5 ml. of enzyme suspension, 0.2 ml. of 5% soluble starch, 1 ml. of 0.1 M K_2 glucose-1-phosphate, and 0.1 M citrate buffer to a total volume of 25 ml. The starch used was the same as that for Digest A. Therefore, the same blank could be applied. Table 16 and fig. 5 show the results of the iodine titration of this digest.

Curve B, representing Digest B, on fig. 5 is almost identical to that of Digest A, except for the level of the potential, indicating that the chain-lengths were probably shorter in Digest B than in Digest A. Data obtained by making activity tests on the enzyme preparation used in Digest B showed that 0.20 mg. of P was liberated from glucose-1-phosphate by 5 ml. of the enzyme in 3 minutes at 38°. This amount of phosphorus would correspond to the formation of 1.05 mg. of starch (in 3 minutes)[1].

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[1]The amount of polysaccharide synthesized was calculated on the basis that 1 mg. of P was liberated in the formation of 5.24 mg. of polysaccharide. Experiments by Hanes (15) showed that the actual yield approached very closely the theoretical.

The digest originally contained 10 mg. of the soluble (amylopectin) starch. The exact amount of polysaccharide synthesized in the 30-minute digestion could not be calculated from the data available. The fact that there is definitely a straight-line portion of the curve, which must have been due to some amylose content, indicates the presence of 1,4-phosphorylase. From the quantitative determinations of Bates et al., however, it was found that 1 mg. of amylose bound about 1.7 ml. of iodine solution. Curve B, however, shows only about 0.5 ml. of iodine bound by a minimum of 1.05 mg. of starch. Consequently, it is logical to suppose that the remainder of the synthetic product was amylopectin.

A third experiment was run at room temperature. The enzyme preparation contained the precipitate formed between 0.28 and 0.39 ammonium sulfate saturation from 500 ml. of aqueous extract. The precipitate was suspended in 0.1 M citrate buffer and made up to 25 ml. with buffer. The digest, Digest C, contained the entire 25 ml. of enzyme preparation, 0.2 ml. of 5% soluble starch, 1.0 ml. of 0.1 M K_2 glucose-1-phosphate, and 3.8 ml. of 0.1 M citrate buffer, making a total volume of 30 ml.

Five-milliliter aliquots were removed at the beginning of the digestion for tests on free-P and for the iodine titration. After 10 hours another sample was taken for titration with iodine. At the end of 24 hours a 5-ml. aliquot was removed for the free-P test, 4-ml. for 7-min.-P,

Table 16

Iodine Titration of Digest B

Ml. 0.0009 N I_2	E. M. F.	Ml. 0.0009 N I_2	E. M.F.
0	0.2091	2.16	0.2248
0.18	0.2089	2.34	0.2257
0.36	0.2105	2.52	0.2266
0.54	0.2123	2.70	0.2275
0.72	0.2140	2.88	0.2282
0.90	0.2158	3.06	0.2289
1.08	0.2173	3.24	0.2296
1.26	0.2188	3.42	0.2302
1.44	0.2203	3.60	0.2307
1.62	0.2214	3.78	0.2314
1.80	0.2224	3.96	0.2319
1.98	0.2237		

and 5-ml. for the iodine titration. Data for the iodine titration are given in table 17.

Fig. 6 illustrates how the curve became less steep as digestion continued, thus indicating a narrower range of chain-length as digestion progressed. Calculations of the initial free-P, final free-P, and final ester- + free-P showed the liberation of a total of 0.44 mg. of P during the 24 hours of digestion. This quantity of phosphorus would be equivalent to 2.3 mg. of starch, or 0.39 mg. per 5-ml. aliquot.

At the end of 24 hours there was evidence of the presence of amylose; as shown by the definite straight-line portion of the curve. A comparison of Digests A and B with Digest C

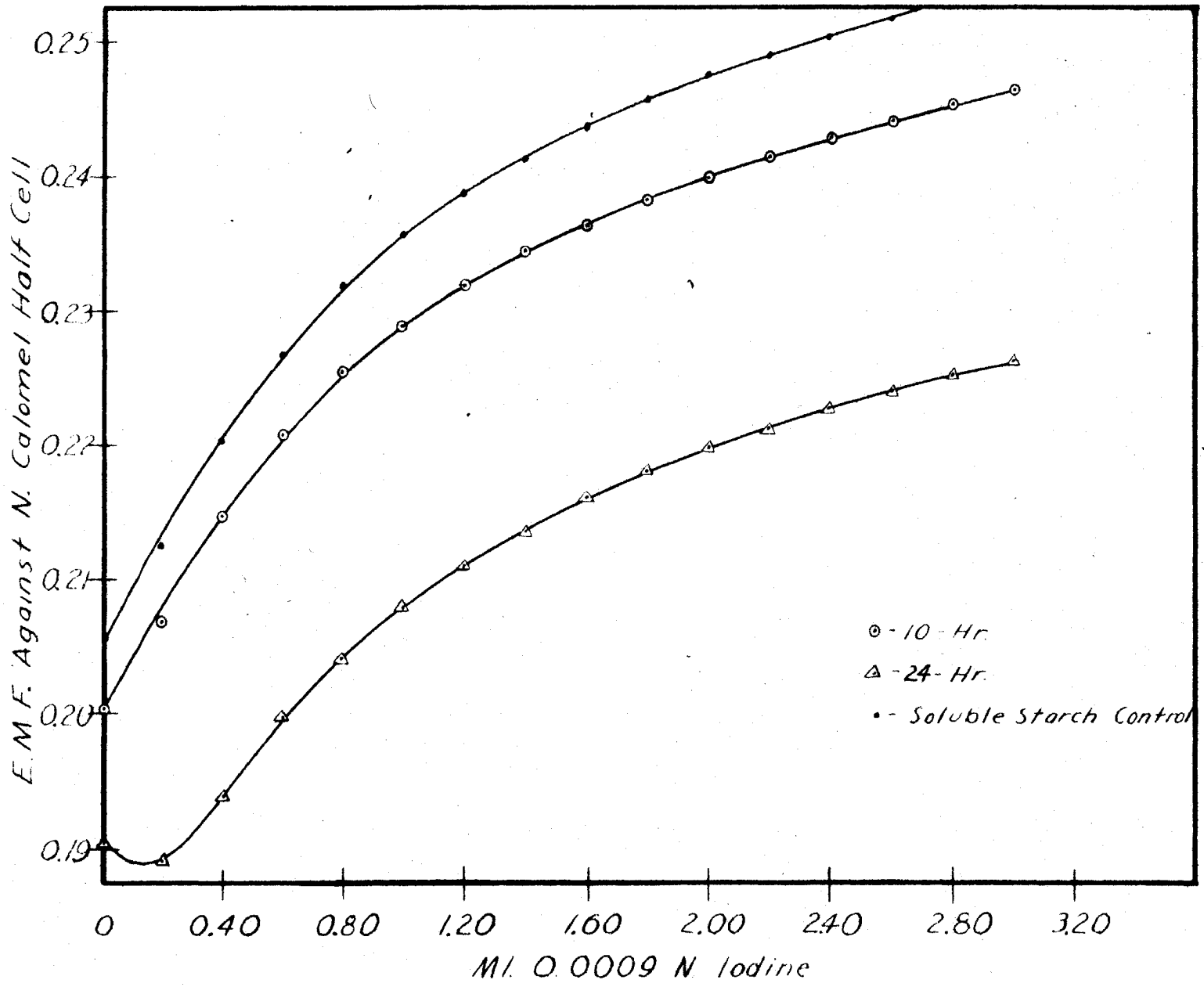

Fig. 6. Iodine Titration of Digest C

could be made on the basis of the change in potentiometric level of the curves from that of the original starch catalyst. However, it must be borne in mind that the curves of Digests A and B represent the titration of 25 ml., or the entire digests, whereas the curves of Digest C represent the titration of 5-ml. aliquots of a 30-ml. digest. A further consideration is the time and velocity of the digestion as influenced by the difference in temperature. Digests A and B were run for 30 minutes at 38°, and Digest C was run at room temperature for several hours.

The low potential level of the 24-hour curve for Digest C indicates long chain-length. The steep slope shows a wide range of chain-lengths. About 0.5 ml. of the iodine solution was required for the complex formation. Consequently, if 0.39 mg. of synthetic polysaccharide was formed in the aliquot taken, about 0.66 ml. of the iodine solution would be required for complex formation in case the entire amount were amylose. This observation seems to indicate that the greater part of the polysaccharide was of the straight-chain, or amylose, type[1].

It has been found by Hanes (14,15) and by Cori and coworkers (12) that purification of an enzyme extract can be

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[1]Such a conclusion is not inconsistent with the interpretation given for Digest B in view of the fact that the velocity of the reaction at 38° would permit equilibrium to be reached in a relatively short time; Digest C probably had not attained equilibrium. As will be pointed out with regard to Digest D, it is possible that amylopectin is formed from amylose as a "delayed reaction". In this event it is conceivable that Digest B had progressed to the formation of amylopectin, whereas Digest C had not passed the amylose stage.

Table 17

Iodine Titration of Digest C

Ml. 0.0009 N I_2	E. M. F.		
	Initial	10-hr.	24-hr.
0	0.2057	0.2002	0.1903
0.20	0.2124	0.2069	0.1891
0.40	0.2202	0.2147	0.1939
0.60	0.2267	0.2207	0.1996
0.80	0.2319	0.2253	0.2041
1.00	0.2356	0.2288	0.2078
1.20	0.2387	0.2319	0.2110
1.40	0.2414	0.2344	0.2135
1.60	0.2437	0.2363	0.2160
1.80	0.2457	0.2382	0.2179
2.00	0.2475	0.2399	0.2197
2.20	0.2490	0.2414	0.2212
2.40	0.2504	0.2427	0.2226
2.60	0.2517	0.2440	0.2240
2.80	0.2528	0.2452	0.2251
3.00	0.2536	0.2463	0.2262

brought about by dialysis. A specific advantage of dialysis is that the process removes excess inorganic phosphate. For a fourth experiment in this series an aqueous extract was dialyzed for 23 hours in the refrigerator against 8 l. of 0.2 M KCl. The slight dialysis precipitate was removed by centrifugation. The precipitate formed between 0.29 and 0.39 ammonium sulfate saturation was suspended in 0.1 M citrate buffer and made up to 25 ml. This precipitate represented the enzyme present in 750 ml. of original extract.

The digest (Digest D) contained 25 ml. of enzyme suspension, 0.2 ml. starch, 1 ml. 0.1 M K_2 glucose-1-phosphate,

and 3.8 ml. of 0.1 M citrate buffer, making a total of 30 ml. Digestion was at room temperature. Aliquots were removed as follows: "0" time (actually 1.5 minutes after start of digestion)--5 ml. for free-P, 5 ml. for iodine titration; 12 hours--same as for "0" time; 24 hours--4 ml. for determination of free-P and of free- + ester-P, and 5 ml. for iodine titration. Data for the iodine titration of Digest D are presented in table 18.

Fig. 7 was constructed from the data of table 18. The curve for the starch catalyst added to the digest was included for reference, as was also a 0.05 N KI titration curve. From the phosphorus analyses it was calculated that 14.3 mg. of polysaccharide was formed in this digestion. For the 5-ml. aliquots the following amounts of polysaccharide were synthesized during the time intervals indicated:

1.5 min.	- 12 hrs.....	1.34 mg.
12 hrs.	- 24 hrs.....	0.72 mg.
1.5 min.	- 24 hrs.....	2.06 mg.
0	- 1.5 min.....	0.32 mg.(calcd.)
0	- 12 hrs.....	1.66 mg.(calcd.)
0	- 24 hrs.....	2.38 mg.(calcd.)

From these curves it can safely be concluded that some amylose was present in the 1.5-minute and the 12-hour aliquots (Curves 1 and 2) as shown by the straight-line portions of these curves. Curve 3, representing 24-hour digestion, is very different in type from Curves 1 and 2 and resembles closely an amylopectin curve. Calculations and comparisons with Bates' analyses indicated that the proportion

Iodine Titration of Digest D

Ml. 0.0009 N I_2	E. M. F.				
	Initial:	1.5 min.:	12-hr.:	24-hr.:	0.05 N KI
0	0.2046	0.1936	0.1931	0.1925	0.2284
0.20				0.1972	
0.24	0.2107	0.1947	0.1943		
0.40				0.2010	
0.48	0.2173	0.1983	0.1970		
0.60				0.2052	
0.72	0.2224	0.2016	0.2015		
0.80				0.2089	
0.96	0.2259	0.2052	0.2065		
1.00				0.2121	0.2453
1.20	0.2290	0.2082	0.2104	0.2149	
1.40				0.2171	
1.44	0.2315	0.2111	0.2138		
1.60				0.2191	
1.68	0.2333	0.2130	0.2169		
1.80				0.2210	
1.92	0.2351	0.2152	0.2191		
2.00				0.2225	0.2527
2.16	0.2378	0.2170	0.2213		
2.20				0.2238	
2.40	0.2390	0.2187	0.2229	0.2252	
2.60				0.2265	
2.64	0.2401	0.2202	0.2246		
2.80				0.2276	
2.88	0.2410	0.2216	0.2261		
3.00				0.2287	0.2606
3.12	0.2419	0.2228	0.2273		
3.36	0.2428	0.2240	0.2286		
3.60	0.2435		0.2297		

of amylose represented by Curves 1, 2, and 3 became progressively less as the digestion proceeded. From the data it is difficult to avoid the conclusion that on the basis of the

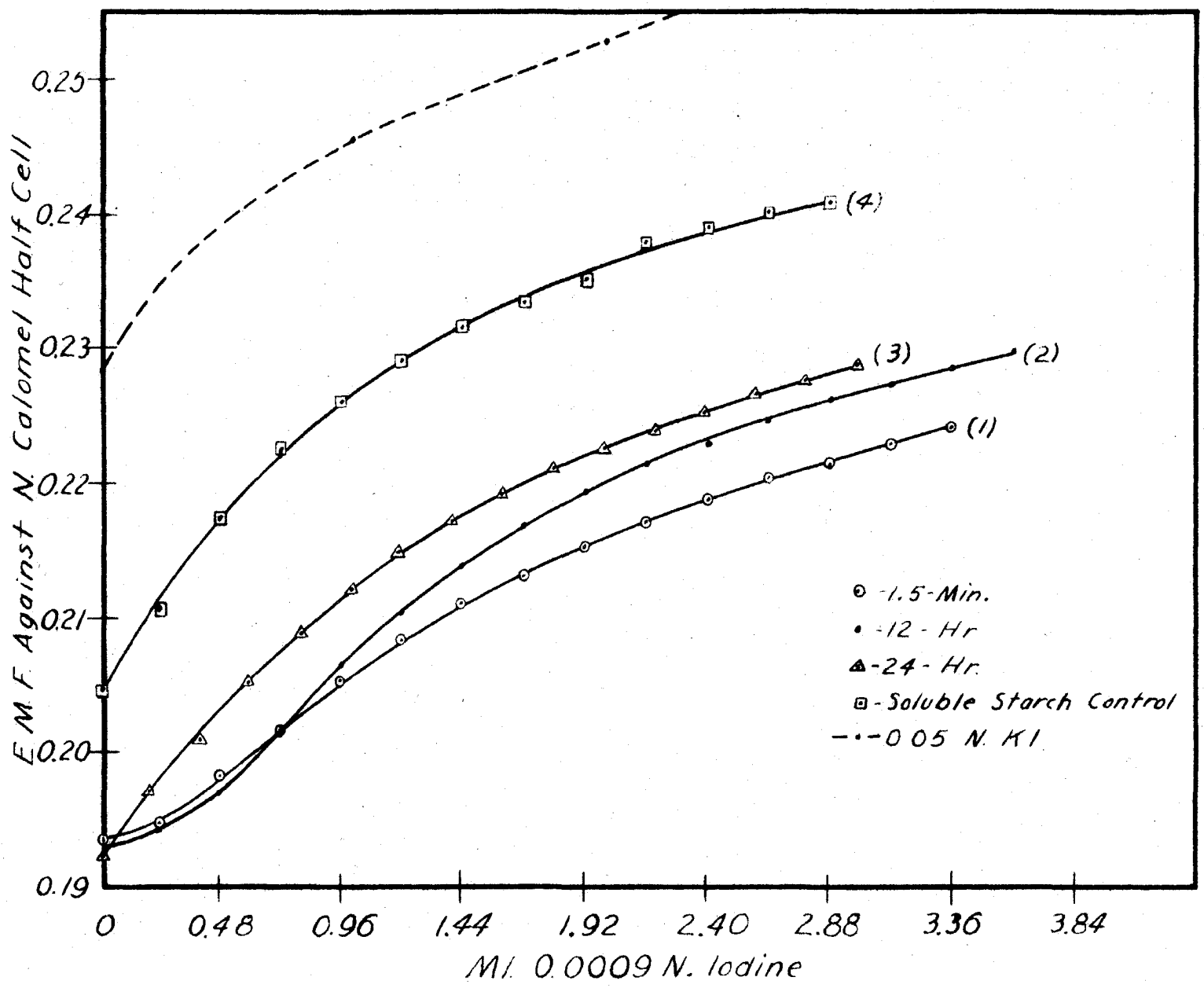

Fig. 7. Iodine Titration of Digest D

total polysaccharide synthesized the proportion of amylopectin increased during digestion. Previous digests (A, B, and C) have all indicated the formation of some amylose, However, in Digest D the obvious change in the character of the starch being synthesized was especially noticeable during the 24-hour period. To hypothesize that branching had occurred after the formation of the straight-chain amylose during the synthesis of an additional 0.72 mg. of starch (per 5 ml.), or after two-thirds of the synthesis had been completed, would not be consistent with the findings of Meyer (66), who showed that only 8 to 9 glucose units separated the branch-points of amylopectin. However, the 24-hr. curve shows either amylopectin or very short chain amylose (of 50 glucose units or less) because of the high level of the potential. On the basis of the calculations of starch formation and the amount of iodine taken up by comparable amounts of typical amyloses (as shown by Bates et al.[1]) if any amylose was present at all, it constituted a very small proportion of the total amount of the starch synthesized. The remainder, then, was assumed to be amylopectin in nature.

Another interpretation is conceivable. Since it has been found that the flat portion of amylose curves may represent either the presence of relatively few long straight-chain molecules or the presence of many relatively

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[1]See page 77.

short straight-chain molecules, it is entirely possible that many of these unbranched portions were built up during the first 12 hours of synthesis (by 1,4-phosphorylase) and that during the subsequent time the 1,6-phosphorylase came into action and branched the amylose chain, forming amylopectin.

It has been shown by Bates and co-workers that the existence of starch components with degrees of branching intermediate between those of amylose and amylopectin is very unlikely on the basis of the marked discontinuity in the ability of all the possible molecular species of starch to react with iodine. The 24-hr. curve is amylopectin in character, as has been noted, whereas the 1.5-min. and the 12-hr. curves have amylose characteristics. An explanation alternative to that of "delayed branching" might be that the reaction of the digest had reversed and that glucose-1-phosphate was being formed, with the possibility that only the original soluble starch was left to give its original curve. The unlikeliness of such a proposal is evident in view of Hanes' findings (15,44) with regard to the equilibrium ratio of the phosphorylase reaction. At a pH of 6.0, at which Digest D was run, the ratio of free-P to free- + ester-P at equilibrium is about 0.86. This ratio was not attained at the end of the digestion. Therefore, a reversal of the reaction appears out of the question.

It has already been noted that <u>in vivo</u> waxy maize

phosphorylase must contain both the 1,4- and the 1,6-component. Meyer's observations (7) on the possibility that 1,6-phosphorylase is often lost in the process of extraction of the enzyme has been mentioned. One primary purpose of the present preliminary experiments with the potentiometric iodine titration method was to ascertain whether both components of the enzyme system were present in the waxy maize extracts.[1]

From the evidence presented in the experiments described and from the general observations of numerous investigators in the field of starch chemistry, the explanation offered by the possiblity of "delayed branching" seems more plausible than any other at hand. Acceptance of this theory would imply the presence of both 1,4- and 1,6-phosphorylase in the extract.

Inasmuch as work on the standardization of the application of the potentiometric iodine titration to starch analyses is still in progress, an interpretation of the data presented in the preceding pages is subject to revision. However, it is apparent that the method has unlimited possibilities as a tool in phosphorylase study.

Bates and co-workers (64) consider the procedure well standardized for the analysis of amylose but are yet uncertain as to the exact behavior of amylopectin in the titration. Further investigation is now being carried on

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[1]Meyer's method of approach to the subject would require more polysaccharide than was available for study.

in their laboratories.

The results obtained in the present experiments indicate that this potentiometric titration may eventually be used successfully for the complete characterization of synthetic polysaccharides and thus for the definite identification of phosphorylases as of the 1,4- or the 1,4- and 1,6-type. However, realization of this application to the fullest extent waits upon the standardization of the method with regard to amylopectin.

SUMMARY

1. A method of phosphorus determination adaptable to the phosphorylase enzyme system of waxy maize was developed after a critical study of the methods reported in the literature. This problem involved the construction of a photoelectric colorimeter, the selection of a suitable filter combination, the calibration of the instrument, and the standardization of the method of phosphorus determination.

2. Preliminary studies were made to obtain an estimate of the role of phosphorylase in the development of the waxy maize, to show the trend of the equilibrium reaction of starch + inorganic-P $\rightleftharpoons$ glucose-1-phosphate in the presence of phosphorylase, and to form a basis for further experiments in purification and concentration of the enzyme.

3. Phosphorylase studies involved the necessity of the preparation of glucose-1-phosphate (the Cori-ester) in quantities suitable for use as substrate for enzyme action. The chemical synthetic (rather than the enzymic) method of preparation of glucose-1-phosphate was used, and several adaptations of the previously reported methods are described.

4. The Green and Stumpf method of expressing the activity of the enzyme in terms of phosphorylase units was studied and adapted to this work.

5. Waxy maize collected from the field during the entire time of growth from 16 days after pollination to maturity was tested by precipitation of the enzyme from extracts and determination of the phosphorylase units present. Data for the 1942 corn showed a considerable activity of phosphorylase in the earliest corn tested (16 days after pollination). Phosphorylase precipitates were obtained from corn at the later stages of growth; these precipitates apparently exhibited fairly constant (or possibly decreasing) activity up to maturity.

6. The techniques reported in the literature for the purification and concentration of the enzyme were tested to determine the method which would be most adaptable to the preparation of phosphorylase concentrates from waxy maize. Fractional precipitation with ammonium sulfate at concentrations between 28 and 34% of saturation yielded the most active precipitates.

7. The potentiometric iodine titration method of Bates, French, and Rundle was used in an attempt to characterize the polysaccharide synthesized by the action of waxy maize phosphorylase on glucose-1-phosphate. Results of the experiments seemed to indicate that this product contained amylopectin and, therefore, that the waxy maize phosphorylase had both 1,4- and 1,6-linkage ability.

ACKNOWLEDGMENTS

The author wishes to express appreciation for the advice and helpful criticism of Prof. Nellie M. Naylor.

Prof. R. M. Hixon and Prof. I. B. Johns contributed valuable suggestions and encouragement throughout this investigation.

The author is indebted to Dr. G. F. Sprague, of the Agronomy Department, for furnishing the waxy maize for these experiments, and to the Botany Department for the use of the refrigerated storage room.

The photoelectric colorimeter was designed by Donald Howe, and its construction was under his supervision.

F. Leslie Bates made possible the work on the potentiometric iodine titration. His apparatus for this work was put at the disposal of the author, and his constant advice was invaluable in the interpretation of the results.

Acknowledgment is also made for grants from the Iowa Agricultural Experiment Station.

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