here studied may form some glucose, (2) that in such conditions as obtain in the usual determinations of diastatic power the yield of maltose so far predominates as to justify the custom of calculating the reducing powers of the digestion products as if due to maltose alone.

Detailed Data of the Principal Experiments of the Series of 1914–5.

In the above tables, the data are recorded under abbreviated captions, the significance of which are as follows:

(1) is the weight of dry starch used. (2) is the weight of insoluble matter filtered out. (3) is the percentage of the dry starch consisting of such insoluble matter. (4) is the weight of soluble carbohydrates in the total solution; *i. e.*, total solids less activation salts, less enzyme. (5) is the sum of (2) and (4). (6) is the difference between (5) and (1). (7) is the percentage increase in weight over the weight of dry starch used. (8) is the specific rotatory power for the soluble carbohydrates in the total solution ("T"). (9) is the reducing power expressed in terms of maltose, and (10) in terms of glucose for the whole solution. (11) is the amount of alcohol used for the extraction. (12) is the weight of carbohydrates in the extract solution ("X") and (13) is the weight equivalent to the total solution; *i. e.*, (12) $\times$ 1.25, since the extraction was made on four-fifths of the original material. (14) is the specific rotatory power for the carbohydrates in the extract solution. (15) and (16) are the reducing power of the extract solution expressed in terms of maltose and glucose, respectively. (17) is the weight of carbohydrate in the residue solution ("R"), *i. e.*, total solids less four-fifths the weight of added activator. (18) is the portion of soluble carbohydrates in the original total solution corresponding to the weight in the residue solution. (19) is the specific rotatory power of the carbohydrates in the residue solution and (20) and (21) their reducing power expressed in terms of maltose and glucose, respectively. (22) is the sum of (13) and (18) and when subtracted from (4) gives the loss of material (23) due to the process.

LABORATORY OF FOOD CHEMISTRY.

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY OF COLUMBIA UNIVERSITY, NO. 274.]

EXPERIMENTS UPON STARCH AS SUBSTRATE FOR ENZYME ACTION.

BY H. C. SHERMAN AND J. C. BAKER.

Received June 24, 1916.

The forms in which starch has been used as substrate for enzyme action may be grouped as: (1) Natural starch dispersed in water under different conditions of time, temperature, and pressure but without "chemical" treatment; (2) starch which has been subjected to the action of acid (Lintner)[1] or to other chemical treatment (Wolff and Fernbach)[2] to render it "soluble;" (3) fractions of the starch substance which have been separated from the remainder by sedimentation (Tanret),[3] or pre-

[1] *J. prakt. Chem.*, [2] **34**, 378 (1886); see also Ford, *J. Soc. Chem. Ind.*, **23**, 414 (1904).

[2] *Compt. rend.*, 140, 1403 (1905); 143, 363, 380 (1906).

[3] *Bull. soc. chim.*, [4] **17**, 83 (1915).

cipitation (Maquenne and Roux,[1] Fernbach,[2] Malfitano and Moschkoff,[3] Gruzewska[4]).

In order to ensure uniformity of substrate in successive experiments and to avoid difficulties due to imperfect solution or unequal dispersion on the one hand, or on the other hand the use of substrate representing only a part of the original starch and perhaps contaminated by the reagent or solvent used in its preparation, the consensus of opinion of workers in this field has favored the use of "soluble" starch, prepared by Lintner's method of simply soaking the starch grains in cold hydrochloric acid and subsequently removing the latter by washing with cold water. Such "soluble" starch has been used in nearly all the studies of amylases carried on in this laboratory and has always been found a satisfactory substrate except for certain peculiarities encountered in the case of purified malt amylase.

When soluble starch is acted upon by pancreatic amylase either in purified or commercial form under the conditions used for measuring the amylolytic activities of enzyme preparations, the weight of maltose produced is regularly about half of the weight of starch which disappears. In other words the purified pancreatic amylase having many times the power of commercial preparations shows nevertheless the same relation between amyloclastic and saccharogenic powers.[5] Similarly in experiments upon the amylase of *Aspergillus oryzae*, the amyloclastic and saccharogenic activities were found to increase in about the same proportion as the enzyme was concentrated by purification.[6] But when purified malt amylase preparations were compared with malt extracts no such constancy in the ratio of amyloclastic and saccharogenic powers was found. In this case not only had the purification process altered the ratio of amyloclastic and saccharogenic activities, but evidently also the generally accepted method for measuring amyloclastic action (that of Wohlgemuth) yields misleading results when applied to purified malt amylase.

The present investigation had its origin in an attempt to prepare a substrate sufficiently homogeneous to yield reliable results when used as in the Wohlgemuth method, and developed from this into a study of the differing behavior toward enzymes of the two chief constituents of starch. It was thought that the discrepancy noted in the measurements of amyloclastic action of malt amylase might be due to an unequal susceptibility of the two main constituents of starch[7] to the action of the enzyme, in which

[1] *Compt. rend.*, 140, 1303 (1905); *Ann. chim. phys.*, [8] **9**, 179 (1906).

[2] *Proc. 8th Intern. Congr. Appl. Chem.*, **13**, 131 (1912).

[3] *Compt. rend.*, 150, 710 (1910); **151**, 817 (1910).

[4] *Ibid.*, 146, 540 (1908); **152**, 785 (1911).

[5] Sherman and Schlesinger, THIS JOURNAL, **35**, 1784 (1913).

[6] Sherman and Tanberg, *Ibid.*, **38**, 1638 (1916).

[7] Meyer (Untersuchungen über die Stärkekörner) considered starch to be chiefly

case an approximate separation of the substances might yield a more satisfactory substrate. At the same time it seemed very desirable that the material to be used as substrate in our experiments should not be subjected to treatment with strong reagents nor to solution and reprecipitation, which might involve danger of change in its behavior toward enzymes.

Experiments were, therefore, made to test the feasibility of extracting the more soluble component ("β-amylose," "amylose") while leaving behind the less soluble ("α-amylose," "amylopectin") by carefully regulated treatment of purified potato starch with distilled water. By ultrafiltration or by simple sedimentation we did not obtain satisfactory separations but by use of the centrifuge much better results were obtained.

Separation of "Soluble" and "Insoluble" Components of Starch by means of Centrifugal Force.

When thin starch paste is submitted to centrifugal force it is separated into a lighter, clearer and more limpid layer containing the more soluble constituent (β-amylose of Meyer, amylose of Maquenne and Roux) and a heavier, opalescent, very viscous layer containing the less soluble component (α-amylose of Meyer, amylopectin of Maquenne and Roux, amylophosphate of Tanret). For the purpose of making such separations we have employed three different types of centrifuge, but in all of the experiments here described the Size 1, Type A, 8-tube centrifuge of the International Instrument Company has been used. In these experiments 50 cc. of the starch paste are placed in each of the 8 tubes of the centrifuge and rotated for 30 min. at about 2400 revolutions per min. ("relative force 1024 times gravity"). At the end of such treatment the liquid in each tube is found to have separated into two distinct layers, the heavier opalescent layer usually occupying from one-third to four-fifths of the total volume.

It was thus found possible to carry out a series of experiments, heating the starch with water under different conditions, separating the two por-

composed of two substances to which he gave the names α-amylose and β-amylose. Maquenne and Roux (*Loc. cit.*) call about four-fifths of the starch substance "amylose" and about one-fifth "amylopectin." Gruzewska (*Compt. rend.*, 146, 540; 152, 785) by solution in alkali and precipitation with alcohol estimated the "amylopectin" to be 40 to 45% of the starch. Samec (*Koll. Beihefte*, 6, 32) by a modification of Gruzewska's method found 56% of "amylopectin" and only 44% of "amylose," the latter being practically phosphorus-free while the former contained phosphorus as an essential constituent. Tanret (*Bull. soc. chim.*, [4] 17, 83) by successive treatment of starches with hot water, sedimentation and decantation, finds from 70 to 80% of "amylopectin" or "amylophosphate" and 20 to 30% of "amylose." For the material present in larger quantity the term α-amylose not only has priority but is much more appropriate than the term amylopectin. We have, therefore, given preference to Meyer's terminology in this paper, even though some of Meyer's views as to the chemical nature and relationships of the α- and β-amyloses may not be tenable.

tions, observing their properties, and using them as substrates for the study of diastatic hydrolysis.

Experiments upon Commercial Potato Starch.—In the earlier of our experiments a good grade of commercial potato starch was used. The starch was handled in air-dry condition and allowance was made for the moisture which it contained when weighing out portions for experiment. All statements of weight or concentration of starch refer, therefore, to the water-free substance. Only one of these earlier experiments will be described here.

In this case starch was gelatinized in 100 times its weight of water at 85°, and the temperature maintained by means of a thermostat while the liquid was stirred continuously. Portions of the liquid were withdrawn and centrifuged at intervals. The centrifugates were studied with reference to volume, viscosity, concentration, and behavior under the action of pancreatic and malt amylases. In the enzyme experiments the time required for the digestion of starch to disappearance of blue iodine reaction, and to disappearance of red reaction with iodine, was observed, and the maltose which had been formed at the time of disappearance of blue iodine reaction was determined. The iodine tests were applied under the conditions prescribed by the Wohlgemuth method and the "disappearance of blue" was considered to be that point at which the color obtained with iodine changed from red-violet (RV) to violet-red (VR), the colors being always determined by comparison with the Milton-Bradley Standard Color Chart as given in Mullikin's *Identification of Pure Organic Compounds*. The data obtained are shown in Table I.

TABLE I.—PROPERTIES OF UNFILTERED CENTRIFUGATES FROM 1% POTATO STARCH PASTES PREPARED AT 85°.

Time of heating	1 hour	2 hours	4 hours	6 hours	8 hours
Volume of lower layer	60%	66%	65%	61%	47%
Volume of centrifugate	40%	34%	35%	39%	53%
Viscosity of centrifugate[1]	1.445	1.572	2.130	2.335	3.06
Concentration of starch in centrifugate	0.384%	0.444%	0.572%	0.716%	0.816%
Digestion with pancreatin:					
To disapp. of blue react. (Mins.)	10.0	11.5	14.5	18.5	20.0
To disapp. of red react. (Mins.)	20.0	24.0	32.0	42.0	46.0
Maltose at end of blue in percentage of starch	(60.5)	48.5	46.2	48.8	44.7
Digestion with malt amylase:					
To disapp. of blue react. (Mins.)	18.0	19.0	20.0	24.0	26.0
To disapp. of red react. (Mins.)	24.0	28.0	40.0	55.0	65.0
Maltose at end of blue in percentage of starch	83.2	81.2	75.0	69.2	68.0

[1] Viscosities are here expressed as time of flow of solution divided by time of flow of water, both at 30°, Ostwald viscosimeters being used.

When starch pastes are separated by centrifugal force as above described, the lower (heavier) layer is found to consist largely of greatly swollen grains. The swelling of the grains increases with duration of heating in water and appears to be due to absorption of water by the "insoluble" constituent of the starch grain, the "soluble" constituent going into solution.

From the data given in Table I for volumes of lower layers and centrifugates from pastes prepared by heating for five different lengths of time at each of three temperatures, it would appear that in this particular series of experiments the swelling of the grains and liberation of the soluble component was the chief effect of heating the starch in 100 times its weight of water up to about 2 hours at 85°, and that the chief result of further heating at 85° was dispersion (or hydration) of the insoluble component into forms (or derivatives) not removable from the centrifugate by the force here employed (about 1000 times gravity for 30 minutes). Microscopic examination showed that the upper layer did contain some swollen grains. In subsequent experiments these were removed by filtering through paper covered with a thin layer of absorbent cotton. Observations upon the iodine reactions and phosphorus content of the two layers obtained in this and several subsequent experiments also bear upon this phase of the problem. The results of the action of amylases upon the centrifugates obtained after different periods of heating in water at 85°, also throw light upon the nature of the starchy material contained in this layer, and upon the differing ratios of amyloclastic to saccharogenic powers as between pancreatic and malt amylases. Thus it will be seen from the data given in Table I that the pancreatic amylase acted in much the same way upon the different substrates. The time required for the digestion of the material to a point where iodine no longer gave a blue or violet reaction ("disappearance of blue") was approximately proportional to the amount of starch material present, the time required for "disappearance of red" was approximately twice that for "disappearance of blue" in all cases, and the maltose found at the time when the blue reaction had just disappeared was always about half the weight of the original substrate, as had been found by previous workers in this laboratory in experiments in which the enzyme acted upon soluble starch prepared by the Lintner process. On the other hand, it is apparent that the malt amylase acted differently upon the substrates obtained after different lengths of heating. The time required for disappearance of the red reaction with iodine increased more than did the concentration of the substrate in the successive portions; and the relative yield of maltose at the time of disappearance of the blue reaction is greatest for the substrate first obtained and decreases progressively for the substrates prepared by longer heating.

All of the results are consistent and together they indicate plainly that the continued heating results in dispersion of more and more of the less soluble component of the starch (α-amylose, amylopectin) into the solution of the more soluble component (β-amylose, amylose).[1]

With pancreatic amylase the digestion of the two components proceeds proportionately, so that the ratio of apparent amyloclastic and saccharogenic activity is not materially disturbed by the difference in nature of the substrate; but with malt the two components are hydrolyzed quite differently, so that with increasing proportion of the less soluble component (α-amylose, amylopectin) in the substrate the ratio of times required for disappearance of the blue and red reactions and the ratio of amyloclastic and saccharogenic action are both altered.

Experiments upon Potato Starch Prepared in the Laboratory.—For the main series of experiments, starch was prepared in the laboratory from mature August potatoes, with care to prevent contamination by organisms, dust, fumes, or water containing electrolytes. Pastes made by heating this starch in pure water could not be separated by centrifuging as described above. Experiments were then made to test the effect of adding small amounts of known electrolytes to the starch paste before centrifuging, and it was found that the addition of a very small amount of hydrochloric acid or sodium chloride sufficed to ensure a good separation. Since sodium chloride (at least in small amounts) is known to have no injurious effect upon amyloclastic enzymes its use was decided upon, and in all subsequent centrifugal separations of 1% starch pastes enough sodium chloride was added to bring the concentration of salt in the final mixture to 0.001 *M*. When 2% starch pastes were to be separated a 0.002 *M* concentration of salt was employed.

The effect of varying the time and temperature of heating was studied in repeated experiments, in all of which the paste resulting from dispersion of the starch was separated by the centrifuge in the manner already described, the centrifugate filtered through filter paper covered with a thin layer of cotton, and the concentration of starch material in the filtered centrifugate was determined by evaporating and drying to constant weight at 80° *in vacuo*. These experiments showed that the bulk of the readily soluble material was extracted from the starch grains very quickly by water at 70 to 80°. When one part of starch suspended in 13 parts of cold water was poured into 87 parts of water heated sufficiently to give the entire mixture the desired temperature and containing sufficient salt to give the desired final concentration, thoroughly stirred for at least one minute until the viscosity no longer seemed to increase, and the paste

[1] There remains the possibility that by heating with water the less soluble component of the starch grain may be slowly changed into the more soluble—α-amylose into β-amylose as suggested by Meyer.

then centrifuged as promptly as practicable (within 10 minutes of the first exposure of the starch to the hot water) the concentration of amylose in the centrifugate was about the same as when the heating was continued for one hour. Prolonged heating (up to 30 hours) caused a very slow gradual increase in the amount of material going into the filtered centrifugate. Thus the concentration after heating at 80° for ten minutes to one hour averaged 0.20%; after 19 hours, 0.30%; after 30 hours, 0.35%.

In several cases the lower layer from the centrifugal separation was again treated with hot water and centrifuged a second and a third time, and the material thus extracted was compared as to amount and iodine reaction with the residual material and with the material of the first centrifugate. In this way it was found that the material brought into solution by repeated treatment with hot water, and, therefore, presumably the material which comes slowly into solution on prolonged heating with large excess of water, is different from that contained in the first centrifugate (original upper layer), and apparently consists of a mixture of the β-amylose which constitutes the chief solid of the upper layer and the α-amylose which is the chief solid of the lower layer or residual gel.

To develop a given *intensity of blue color with iodine* requires from 13 to 18 times as much of the twice washed solids of the residual gel (crude α-amylose) as is required of the solids of the original centrifugate (crude β-amylose); or, in other words, the solid matter of the β-amylose fraction had 13 to 18 times the blue-forming property[1] of the solids of the α-amylose fraction, and the solids of the washings (second and third centrifugates) were of intermediate character.

No attempt was made to investigate the exact significance of these differences, which we recognize to depend to a considerable extent upon the amount of iodine arbitrarily chosen by Wohlgemuth for making such tests, because for the purposes of this investigation it was desired that the material going into the substrate should be as nearly homogeneous and as little changed from the form in which it exists in the natural starch as is possible. For these reasons it was decided to use in the separation of β-amylose only a short exposure of the starch to hot water as decribed above, centrifuging within 10 minutes and determining the solids in the centrifugate after filtering as already explained. Experiments at different temperatures indicated that the best results were obtained in heating at 75° in the preparation of 2%, or 80° in the preparation of 1% pastes.

The viscosities as influenced by heating and by treatment with *acid or alkali* also furnish evidence of a pronounced difference between the

[1] It is hardly necessary to say that in speaking of this as a property which helps to differentiate the two fractions, we do not mean to convey the impression of a definite chemical reaction in either case.

substances contained in the two layers separated by the centrifuge, the "β-amylose" of our fractionation resembling the "amylose" of Gruzewska and of Samec in this respect and differing greatly from the α-amylose fraction ("amylopectin").

Thus in an experiment in which the filtered β-amylose layer as obtained had a concentration of 0.4% and a viscosity of 1.13 the α-amylose layer when diluted to the same concentration showed a viscosity of 1.18. On heating the β-amylose solution its viscosity was lowered to 1.03 after 5 minutes' and 1.02 after 20 minutes' boiling. Boiling the α-amylose solution of the same concentration for 5 minutes raised its viscosity to 1.38, and heating for 1 hour in the autoclave at 125° increased it to 1.81; further heating in the autoclave caused decrease of viscosity—to 1.13 at the end of 2 hours' and 1.12 at the end of $3^1/_2$ hours' heating at the same temperature.[1]

The addition of sufficient hydrochloric acid to give a concentration of 0.02 *N* did not change the viscosity of a β-amylose solution, and sodium hydroxide in equivalent concentration increased the viscosity only slightly. This is in accordance with the results of Samec[2] who finds that the viscosity of a starch solution (chiefly α-amylose) is descreased by hydrochloric acid and increased by sodium hydroxide to a very marked extent while the viscosity of a solution of "amylose" (β-amylose) prepared by his modification of Gruzewska's method was not appreciably affected.

The two fractions into which starch is divided by the process above described and which, although not quantitatively free from each other, are for convenience referred to simply as α- and β-amylose, also differ from each other in their behavior as regards *retrogradation*. We have had no evidence of retrogradation in any of our α-amylose solutions, while the β-amylose solutions show the phenomenon to a pronounced degree, the more so the lower the temperature or the higher the concentration.

In an experiment to test this property β-amylose solutions of 0.6 and of 0.2% were allowed to stand in the refrigerator for three weeks and were then filtered. The filtrates contained only 0.04 and 0.037%, respectively. The completeness of this retrogradation is a further indication of the identity of this material with Gruzewska's amylose.[3] It seems not improbable that in ordinary starch pastes the α-amylose may act as a protective colloid to the β-amylose. In all of the cases observed by us the retrogradation of the β-amylose has occurred more readily in the absence than in the presence of amylase, the enzymes being employed in purified form and low concentration. The suggestion that the amylase may exert a

[1] For discussion of effect of heat treatment upon viscosities of starch solutions see Harrison, *J. Soc. Dyers and Colorists*, April, 1911.

[2] Samec, *Koll. Beihefte*, 4, 132; 6, 32.

[3] *Compt. rend.*, 152, 785 (1911).

coagulating influence upon the substrate seems superfluous, at least as regards enzymes of the degree of purity used in these experiments. The phenomena observed by Maquenne and Roux and attributed by them to an amylocoagulase may possibly have been due to the digestion of α-amylose and consequent removal of its protective effect upon the β-amylose.

In order to avoid retrogradation in our digestion experiments the solutions were autoclaved, allowed to cool to 40° and then promptly mixed with the enzyme solution, the activating salts being added at the same time (not mixed with the substrate in advance). Omission of such precautions is likely to result in cloudy digestion mixtures and delayed iodine end points.

The phosphorus content of the two fractions separated by the centrifuge was studied as a further indication of the relative amounts of α- and β-amyloses and the extent to which they are differentiated by the procedure here used.

According to Samec, the phosphorus of starch is all contained in the α-amylose ("amylopectin") portion. If, then, the centrifuge separates the α- and β-amylose, the phosphorus should be found in the lower layer; but since in Northrop and Nelson's experiments[1] about one-tenth of the phosphorus escaped recovery it is possible that the α- and β-amyloses might be separated by the centrifuge and still a fraction up to 10% of the original phosphorus might be found in the upper layer or diffused throughout the dispersion medium. In several experiments in which we have determined the phosphorus, the centrifugates (upper layers) constituting about 70% of the total volume have never contained more than about 2% of the total found. Our results are thus in harmony with the findings of Northrop and Nelson and with the view that α- and β-amylose ("amylopectin" and "amylose") are separable by heating with water and centrifuging as here described.

The amounts of phosphorus found in the β-amylose fraction are but little, if any, above the limits of experimental error, and for most purposes might be regarded as negligible, but since the concentration of solids in this layer is only about 0.20% the percentage of phosphorus in the solids is about one-tenth as high for this fraction as for the whole starch, so that it seemed desirable to ascertain if possible whether the phosphorus of the β-amylose layer were present merely as diffusible phosphate or in the form of α-amylose or other non-diffusible material.

The centrifugates (β-amylose layers) from several experiments were saved and united, a portion of the solution reserved and the remainder dialyzed in a collodion bag against 5 to 8 times its volume of redistilled water for 8 days, the water being changed every day. A portion of the

[1] THIS JOURNAL, 38, 472 (1916).

centrifuge residue (α-amylose layer) was diluted to a similar concentration and dialyzed under the same conditions. With the kind assistance of Dr. I. Greenwald of the Harriman Research Laboratory, the phosphorus content of these solutions was determined nephelometrically by precipitating as strychnine phosphomolybdate according to the method of Pouget and Chouchak.[1] The β-amylose layer showed before dialysis 0.00002% and after dialysis 0.00001% of phosphorus; the α-amylose layer after dilution to corresponding concentration of solids showed 0.00195% before, and 0.00180% after dialysis. Thus the eight-day dialysis had removed half of the phosphorus of the β-amylose layer and somewhat less than one-tenth of that of the α-amylose layer. Calculating the phosphorus in percentage of the starch solids present it is found before dialysis to constitute 0.068% of the α-amylose fraction and 0.010% of the β-amylose fraction, or about one-seventh as much in the latter case as in the former. If the phosphorus were assumed to be present only in the form of α-amylose it would follow that the amylose of the upper layer from the centrifuge is about one-seventh α-amylose and six-sevenths β-amylose; but since at least half of the phosphorus of this fraction is dialyzable it appears probable that well over nine-tenths of the starch material in the upper layer from the centrifuge tube is β-amylose (using this term to designate the phosphorus-free fraction of starch which gives a pure blue reaction with iodine). From the comparison of iodine color reactions and solubilities it also appears probable that the starchy material of the lower layer after separation and two washings in the centrifuge is at least nine-tenths α-amylose.

Thus it appears that by the method of regulated dispersion and centrifugal separation here described it is feasible to divide potato starch into two fractions, one of which is more than nine-tenths α-amylose and the other more than nine-tenths β-amylose. If amylases behave differently toward α- and β-amylose it should be possible to study their differences in behavior by experiments in which these two fractions are used side by side as substrates for the action of the enzyme. The data recorded below are typical of those which we have obtained in several series of comparitive experiments of this sort.

Experiments with Enzymes.

In the experiments with enzymes recorded in this paper we have used each enzyme in the presence of its optimum concentration of salt and phosphate as determined in previous work in this laboratory.

Except as different enzymes with their appropriate activators were used, the only variable factor was the nature of the substrate. All digestions were conducted at 40° with all the precautions as to purity of reagents and water used, and accurate control of time and temperature,

[1] *Bull. soc. chim.*, [4] 5, 104 (1909); 9, 649 (1911).

which have been described in previous papers.[1] The enzyme hydrolysis of four potato starch substrates—α- and β-amyloses, Lintner soluble starch, and whole starch autoclaved—has been studied at two concentrations of substrate, 0.16% and 0.5%. For the sake of brevity only the latter will be described.

Preparation of Substrates.—The α-amylose substrate was prepared by centrifuging a 1% starch paste made at 85° in a concentration of 0.001 *M* sodium chloride as previously described. The supernatant β-amylose layer was decanted off and an equal volume of hot 0.001 *M* sodium chloride stirred into the α-amylose layer and this again centrifuged. This washing was repeated and the residue then diluted to the original volume and this solution (about 0.8%) heated in autoclave at 125° for 2½ hours. The exact concentration was then determined by evaporating a portion *in vacuo* at 80°. When about to start an experiment, this solution was sufficiently diluted with water so that when mixed with the activator and enzyme solutions the desired final concentration of 0.5% substrate would be obtained.

The β-amylose substrate was prepared by centrifuging a 2% starch paste prepared at 75° containing sodium chloride in 0.002 *M* concentration. The supernatant liquid from the centrifuge tube was filtered through paper covered with a thin layer of absorbent cotton. About 170 cc. of 0.4% filtered β-amylose solution were obtained from 400 cc. of 2% starch paste. Two such filtrates were usually united. A portion of this solution was evaporated *in vacuo* at 80° to determine the exact concentration of β-amylose and a larger measured volume (sufficient for the experiment contemplated) was concentrated by evaporation to about 0.8%, autoclaved in the same manner as the α-amylose solution and finally brought exactly to the desired concentration.

"Autoclaved starch" was prepared by heating an 0.8% starch paste in the autoclave as described under α-amylose, and then bringing to the proper concentration.

"Soluble starch" (Lintner) was "dissolved" by boiling with water for 10 minutes and was not heated in the autoclave.

The initial reducing power of each substrate was determined and this value subtracted from that found in the digestion experiments.

Each digestion experiment was started by measuring the enzyme solution into a flask and pouring into it the proper volumes of substrate solution and of the solution of activating salts so that the concentration of substrate in the final digestion mixture was exactly 0.5%. The substrate and activator solutions were measured at 40° and this temperature was maintained throughout the experiment, portions being withdrawn by

[1] THIS JOURNAL, 32, 1073, 1087; 33, 1195; 35, 1617; 37, 623.

pipet from time to time without removing the main solution from the thermostat.

The four experiments of a set were started at intervals of one minute so that portions of each might be withdrawn in succession after exactly equal digestion periods. Each measured portion as withdrawn was at once delivered into a flask containing mixed Fehling solution, and the reducing sugars determined at once, following the procedure described by Sherman, Kendall and Clark[1] and calculating the result as maltose by means of Defren's table.[2] In order to simplify the tabular statement and comparison of the results, the weights of reduced copper and the corresponding weights of maltose are omitted and only the maltose as calculated in terms of the amount theoretically obtainable from the substrate and the corresponding velocity constant are printed. Since it has been shown[3] that even purified amylases may form appreciable amounts of glucose as well as maltose, it is possible that the figure for maltose calculated from the reduction of Fehling solution may, in prolonged digestions or under special conditions of enzyme or substrate, exceed the theoretical yield.

Action of Pancreatic Amylase.—The four substrates above described were quantitatively compared as regards their digestion by pancreatic amylase, first in the form of a high grade commercial pancreatin (Pancreatin 6) and then in the form of a highly purified laboratory preparation (Preparation 59). The results are shown in Tables II and III, respectively.

TABLE II.—HYDROLYSIS OF DIFFERENT SUBSTRATES BY COMMERCIAL PANCREATIC AMYLASE (PANCREATIN 6).

	Soluble starch.		Autoclaved starch.		α-Amylose substrate.		β-Amylose substrate.	
Time.	Maltose in terms of theory. %	k* × 10⁵.	Maltose in terms of theory. %	k × 10⁵.	Maltose in terms of theory. %	k × 10⁵.	Maltose in terms of theory. %	k × 10⁵.
30 min.....	4.3	63	4.3	63	4.6	67	5.2	77
60 min.....	8.4	63	8.5	64	7.7	58	10.7	81
90 min.....	12.5	64	13.0	67	11.2	57	15.2	79
120 min.....	16.2	64	17.5	69	14.9	58	20.4	82
150 min.....	20.0	64	21.0	68	18.4	58	25.6	85
180 min.....	23.0	63	24.4	69	21.2	57	31.3	91
210 min.....	26.7	64	28.1	68	24.4	57	35.6	91
240 min.....	29.5	63	31.1	67	26.9	56	39.9	92
6 hours.....	38.6	58	40.2	62	35.6	53	52.4	91
18 hours.....	56.2	33	58.0	34	52.8	30	75.2	56
24 hours.....	59.3	26	61.2	28	56.9	25	77.5	45

[1] THIS JOURNAL, 32, 1072 (1910).

[2] *Ibid.*, 18, 749.

[3] Sherman and Punnett, *Ibid.*, 38, 1877 (1916).

* From the formula $(1/t \log a/a - x = k)$ in which t = time in minutes; a = the amount of maltose theoretically obtainable (starch × 1.05) and x = maltose found at time t.

TABLE III.—HYDROLYSIS OF DIFFERENT SUBSTRATES BY PURIFIED PANCREATIC AMYLASE (PREPARATION 59).

	Soluble starch.		α-Amylose substrate.		β-Amylose substrate.	
Time.	Maltose in terms of theory. %	$k \times 10^5$.	Maltose in terms of theory. %	$k \times 10^5$.	Maltose in terms of theory. %	$k \times 10^5$.
30 min.....	4.4	65	4.2	62	6.1	91
60 min.....	10.8	83	7.5	56	11.3	87
90 min.....	13.1	67	11.4	58	16.8	86
120 min.....	17.0	67	14.9	58	22.6	93
150 min.....	21.9	71	18.0	57	28.2	96
180 min.....	25.7	72	21.0	57	33.7	99
210 min.....	28.8	70	24.0	57	38.7	101
240 min.....	31.5	68	26.5	56	43.2	102
6 hours.....	39.0	59	34.8	51	55.0	96
24 hours.....	59.4	27	55.1	24	84.5	56

It will be seen that under the action of pancreatic amylase, whether in commercial or purified form, the yield of reducing sugar is higher for the β-amylose than for the α-amylose fraction at each of the stages at which the digestion mixture was tested, the difference becoming greater as the digestion proceeds. The amount of enzyme here used was small in order that the progress of the reaction might be slow enough to be followed experimentally through the earlier stages. Under these conditions, at the end of 24 hours, when the last of the digestion mixture was withdrawn for testing, the reaction was still in progress and the yield of maltose had reached in the two series of experiments, respectively, only 56.9 and 55.1% of theoretical in the α-amylose, and 77.5 and 84.5% in the β-amylose fraction.

Since each of the successive reactions by which maltose is formed is an hydrolysis taking place in water solution, and, therefore, essentially a unimolecular reaction, and it is the catalytic effect of the enzyme which causes these reactions to proceed at a measurable rate, it follows that the speed of formation of maltose may be expressed in terms of k of the unimolecular reaction formula ($1/t \log a/a - x = k$) and that (under a given set of conditions as in either one of the two sets of experiments here described) the magnitude of k at any given stage in the experiment may be regarded as an indication of the efficiency with which the formation of maltose is catalyzed at that stage. Constancy of k from the beginning of the experiment until a practically theoretical yield of maltose is obtained would indicate that the group of hydrolyses which produce maltose (that of starch and of each of the resultant dextrins) is equally catalyzed by the enzyme throughout; whereas if the digestion yields at any stage a dextrin which is less readily hydrolyzed by the enzyme, the value of k must diminish. A fall in the value of k may, therefore, serve as an indication of the formation of a "resistant dextrin." When equal amounts of the same enzyme act on equal amounts of different sub-

strates under identical conditions, a comparison of the values for k may indicate an earlier or a larger production of "resistant dextrin" from one substrate than from the other.

The data in Table II show in all cases a decided diminution of the velocity of reaction (expressed as value of k) between the 6th and the 18th hour. Comparing the yields of maltose from the different substrates at these periods it is plain that the decrease in value of k began at a lower yield of maltose in the α-amylose than in the β-amylose fraction, indicating that the former yields more of the relatively resistant dextrin than the latter, or that its dextrin is more resistant than the corresponding dextrin from β-amylose. Lintner soluble starch and autoclaved whole starch gives intermediate results, but approach the behavior of α-amylose more closely than that of β-amylose as is consistent with the other evidence that they consist much more largely of the former than of the latter.

Similar comparison of the data obtained in experiments with other amylases described below will show that the stage of digestion at which the value of k begins to diminish depends upon the enzyme as well as the substrate but that in all cases the indications of "resistant dextrin" are much more pronounced in the α-amylose than in the β-amylose fraction.

There are, of course, many causes which may contribute to the slowing of the reaction as the hydrolysis proceeds, such as the destructive action of water upon the enzyme, inactivation of enzyme by combination with hydrolytic products, etc. Without entering into discussion of such factors here, it may be pointed out that the arrangement of these experiments was such that any distinctly different behaviors of k in experiments recorded *in any one table* (such as discussed in the above paragraph referring to Table II) may be interpreted as due to some difference in the substrate or its hydrolytic products. The general relations noted from the data of Table II will be seen to hold true for those of Table III also.

Action of Malt Amylase.—Parallel tests upon the α- and β-amylose substrates at 0.5% concentration were made with a freshly prepared malt amylase preparation of a diastatic power of 957[1] prepared essentially as described in previous papers from this laboratory, but precipitated at a concentration of alcohol slightly higher than usual, *viz.*, 65–70%; and also with a preparation purified in the usual way and in which the final active precipitate was obtained at a somewhat lower concentration of alcohol, *i. e.*, Preparation 151, precipitated at 57.5 to 65% alcohol, diastatic power 1240.[1] Except for the kind and amount of enzyme used, the experiments were in all respects similar to those with pancreatic amylase just described. The results with these two malt amylase preparations are given in Tables IV and V, respectively.

[1] Expressed on the scale used since 1910 (Sherman, Kendall and Clark, THIS JOURNAL, 32, 1073.

TABLE IV.—HYDROLYSIS OF α- AND β-FRACTIONS OF STARCH BY MALT AMYLASE PRECIPITATED AT 65–70% ALCOHOL.

	α-Amylose substrate.		β-Amylose substrate.	
Time.	Maltose in terms of theory. %.	$k \times 10^5$.	Maltose in terms of theory. %.	$k \times 10^5$.
30 min	10.2	156	5.6	84
60 min	19.2	154	10.8	82
90 min	26.4	148	15.5	81
120 min	32.2	141	22.1	90
150 min	36.7	131	27.4	93
180 min	40.3	124	32.5	95
210 min	43.2	117	36.4	94
240 min	45.8	110	39.8	92
6 hours	50.4	84	53.2	91
24 hours	58.1	26	82.2	52

TABLE V.—HYDROLYSIS OF DIFFERENT SUBSTRATES BY MALT AMYLASE PREPARATION 151, POWER 1240.

	Soluble starch.		Autoclaved starch.		α-Amylose substrate.		β-Amylose substrate.	
Time.	Maltose in terms of theory. %.	$k \times 10^5$.	Maltose in terms of theory. %.	$k \times 10^5$.	Maltose in terms of theory. %.	$k \times 10^5$.	Maltose in terms of theory. %.	$k \times 10^5$.
30 min	29.2	499	26.6	448	28.3	482	19.8	321
60 min	48.4	477	44.0	418	46.8	462	38.8	355
90 min	58.5	424	54.9	384	53.6	401	56.3	399
120 min	62.2	352	59.8	330	57.9	313	70.0	436
150 min	64.3	298	63.6	292	60.3	267	81.8	493
180 min	65.2	255	65.8	258	61.6	231	91.3	590
210 min	66.5	226	67.7	234	62.4	202	97.6	766
240 min	68.2	208	69.5	216	62.7	179	98.7	785
270 min	68.7	188	70.4	198	63.1	162	99.0	748

Comparison of the data of Tables IV and V shows that the purified malt amylase preparations exert in the earlier stages of the digestions a greater saccharifying action upon the α-amylose than upon the β-amylose substrate, while in the later stages the β-amylose shows the greater yield of maltose.

The notable feature of the action of malt amylase upon the β-amylose substrate is the striking way in which the speed of formation of maltose is maintained as compared either with the action of this enzyme upon other substrates or the action of other amylases upon this substrate. In the experiment with a small amount of enzyme (Table IV) the values of k show no decrease until the formation of maltose is nearly complete, and with a larger amount of enzyme (Table V) the values of k increase, the time curve being substantially linear, up to a nearly theoretical yield of maltose. This may be expressed as a maintenance throughout the process of the linear relationship usually noted in the earlier stages of such

hydrolytic enzyme actions. The explanation is probably to be found in accumulation of hydrolyzable products and the efficiency with which the enzyme catalyzes the later of the series of consecutive hydrolyses by which maltose is produced. Simultaneous effective catalysis of the successive steps in the process would naturally tend to increase the value of k as computed from the simple unimolecular reaction formula and this tendency will be further augmented by the fact that the velocity of the reaction during the later stages is compared with that shown at the beginning of the process where in the case of this enzyme the β-amylose is hydrolyzed less readily than are the other substrates. With the small amount of enzyme used in the experiments of Table IV this tendency toward a rise in the value of k suffices merely to balance the factors which tend toward a loss or inactivation of the enzyme; whereas with the larger concentration of enzyme in the experiments of Table V the latter factors are not appreciable and the rise in value of k becomes very pronounced. Thus the detailed study of the course of the hydrolysis under the influence of a precipitated malt amylase of this character brings out a new difference in behavior between the α- and β-fractions of the starch.

In all cases the saccharification of the substrate had proceeded to a much higher yield of maltose from β-amylose than from α-amylose when the concentration of enzyme was such as would be used in determinations of diastatic power and the experiments were sufficiently prolonged to produce maltose up to or beyond half the theoretical yield.

When the amount of enzyme used is much larger the same result may be obtained in a short time as shown in Table VI.

TABLE VI.—HYDROLYSIS OF α- AND β-SUBSTRATES WITH A RELATIVELY LARGE AMOUNT OF PURIFIED MALT AMYLASE (PREPARATION 153).

	Reducing sugar as percentage of theoretical yield of maltose.	
Time.	From α-amylose substrate.	From β-amylose substrate.
20 min.....	54.5	72.4
40 min.....	67.1	100.0
60 min.....	68.7	100.2
154 min.....	71.4	100.8

Evidently even a relatively large amount of enzyme does not readily hydrolyze the material which remains in the α-amylose fraction after two-thirds of the theoretical amount of maltose has been produced, while in the β-amylose fraction under the influence of the same amount of enzyme the hydrolysis runs rapidly to a theoretical yield of maltose. It thus appears that the "resistant dextrin" emphasized by many previous investigators as a product of the action of malt amylase upon starch is derived only from the α-amylose and not from the β-amylose fraction. After the theoretical yield of maltose from β-amylose has been obtained, the increase in reducing power is exceedingly slow, indicating that even

this amount of purified malt amylase has only a slight hydrolytic action upon maltose.

The rapid digestion of β-amylose in this experiment explains the statement of Maquenne and Roux[1] to the effect that "amylose" (β-amylose) is immediately hydrolyzed to the theoretical amount of maltose by malt, since in their experiments very liberal amounts of malt extract were used.

Action of the Amylase of Aspergillus Oryzae.—Experiments upon Lintner soluble starch, autoclaved whole starch, and α- and β-amylose substrates all at 0.5% concentration have been carried out as described above with the amylase of *Aspergillus oryzae* prepared in this laboratory by Dr. A. P. Tanberg from takadiastase by the purification method described elsewhere.[2] The preparation here used was No. 22*b* having a diastatic power of 502 as expressed on the scale in use in this laboratory. This corresponds to a Lintner value of about 750. The Wohlgemuth value of this preparation was about 1,000,000.

The results obtained with this amylase preparation are shown in Table VII.

TABLE VII.—HYDROLYSIS OF DIFFERENT SUBSTRATES BY AMYLASE OF ASPERGILLUS ORYZAE (PREPARATION 22*b*).

	Soluble starch.		Autoclaved starch.		α-Amylose substrate.		β-Amylose substrate.	
Time.	Maltose in terms of theory. %	$k \times 10^5$.	Maltose in terms of theory. %	$k \times 10^5$.	Maltose in terms of theory. %	$k \times 10^5$.	Maltose in terms of theory. %	$k \times 10^5$.
30 min.....	10.5	161	11.9	183	12.2	188	14.3	224
60 min.....	19.8	159	23.0	189	23.4	193	28.5	243
90 min.....	29.8	171	32.7	191	33.3	195	41.3	257
120 min.....	35.1	156	39.0	179	39.5	182	51.4	261
150 min.....	40.0	148	45.7	176	43.4	165	58.9	258
180 min.....	43.8	139	49.7	167	47.5	155	64.1	247
210 min.....	46.7	130	53.6	159	50.4	145	67.7	234
240 min.....	48.8	120	55.6	147	53.2	138	70.0	218
360 min.....	53.5	92	63.4	121	59.4	108	75.0	168
24 hours.....	62.8	29	75.4	42	..	...	..	...

From the data given in Table VII together with the results of two other series not reproduced here, it may be concluded that the purified amylase of *Aspergillus oryzae* exerts essentially the same saccharogenic action upon Lintner soluble starch, autoclaved whole starch and the α-amylose substrate, while upon the β-amylose substrate it shows a greater saccharogenic action at whatever stage in the digestion the results be compared. The speed of hydrolysis was not so well sustained in this case as with the other amylases; even in the case of the β-amylose substrate there is here a falling off of the rate of saccharogenic action before the reducing

[1] *Compt. rend.*, 140, 1303 (1905).

[2] Sherman and Tanberg, THIS JOURNAL, 38, 1638 (1916).

sugar reaches the equivalent of 70% of a theoretical yield of maltose, notwithstanding the fact that this amylase is more active in hydrolyzing maltose to glucose than is either pancreatic or malt amylase. This property is doubtless related to the high ratio of amyloclastic to saccharogenic activity of this enzyme which will be considered in the next section.

Relation of Amyloclastic to Saccharogenic Action.—In many of the experiments comparing the different substrates the time of disappearance of the blue or violet reaction with iodine was noted and the amount of maltose which had been formed at this time was found either by direct determination or by interpolation from the determinations of reducing power made at known intervals in the course of the same experiment. While no great accuracy is to be expected of such attempts to determine the stage of digestion at which the starch disappears, as judged by the iodine test, yet they permit comparisons of the amyloclastic and saccharogenic activities which at least are not open to such gross discrepancies as may arise when comparisons are made between saccharogenic powers as ordinarily determined and amyloclastic powers determined by the Wohlgemuth method.[1]

In the case of pancreatic amylase the present experiments show with each of the four substrates a maltose production from 35 to 50% of the theoretical at the point of disappearance of the blue or violet color reaction with iodine. Allowing for the difference in experimental conditions this may be considered as a confirmation for all four substrates of the ratio of about 2 : 1 previously established for the action of pancreatic amylase upon Lintner soluble starch.

In the experiments with the amylase of *Aspergillus oryzae* the iodine end point occurred at a much earlier stage in the saccharogenic action, usually when from 20 to 30% of the theoretical yield of maltose had been produced. This high ratio of amyloclastic to saccharogenic activity and the relatively early decrease in velocity of maltose formation noted above are both indications that this amylase exerts a more pronounced catalytic effect upon the earlier than upon the later of the hydrolyses involved in the transformation of starch through dextrins into maltose.

Malt amylase, on the other hand, is, as shown by the data of Tables IV and V, an active catalyst of sugar production even in the relatively advanced stages of the process; and apparently it does not correspondingly catalyze the complete disruption of the material to which the iodine test is due since the iodine end point is found only at a much more advanced stage of sugar production with this than with either of the other amylases. When acting upon Lintner soluble starch, autoclaved whole starch, or the α-amylose substrate the malt amylase preparations caused disap-

[1] For a discussion of these discrepancies see Sherman and Schlesinger, THIS JOURNAL, 35, 1784–90.

pearance of the iodine test only when from 65 to 80% of the theoretical yield of maltose had been produced; and when acting upon the β-amylose substrate, only when the maltose production had reached 85 to 95%. Thus the "delayed iodine end point" remarked in previous papers as characteristic of the hydrolyses induced by purified malt amylase, is found to be a noteworthy feature of its action upon both α- and β-amylose.

Summary.

Dispersions of commercial potato starch in water or of purified potato starch in water containing a small amount of electrolyte (sodium chloride) have been separated by centrifugal force into a heavier, very viscous, opalescent layer containing the more abundant, less soluble component of the starch (Meyer's α-amylose, Maquenne's amylopectin), and a lighter, limpid solution containing the less abundant, more soluble component (Meyer's β-amylose, Maquenne's amylose).

In designating these two chief components (or derivatives) of starch the terminology of Meyer is preferred to that of Maquenne, not only on grounds of priority but also as being much more appropriate in view of our present knowledge of the relative abundance of the two components, their properties as compared with the typical properties of starch, and their behavior toward the amylases.

The centrifugal method here described does not completely separate either component from the other but affords a means of approximate separation in which the danger of contamination, denaturization, or retrogradation is minimized and which is well adapted to the study of the effects of the different amylases.

Pancreatic amylase both in commercial and in highly purified form produced reducing sugar more rapidly from β-amylose than from α-amylose, autoclaved starch, or Lintner soluble starch, the last three giving very similar results when used as substrate for this enzyme. Not only does the β-amylose substrate show larger yield of maltose at each of the various time intervals tested, but the initial speed of hydrolysis is better maintained with this substrate than with either of the others.

Purified malt amylase shows in the earlier stages of its action a somewhat greater yield of maltose from α- than from β-amylose. As the digestion proceeds, the saccharogenic action of this enzyme upon α-amylose becomes slower while its action upon β-amylose is well sustained so that in cases in which the hydrolysis proceeds to the production of more than half the theoretical yield of maltose, the final result shows a greater saccharogenic action upon β- than upon α-amylose. The results obtained upon autoclaved starch and Lintner soluble starch are very similar to those found with α-amylose.

The amylase of *Aspergillus oryzae* digests Lintner soluble starch, autoclaved whole starch and α-amylose at about equal rates, and β-amylose

at a somewhat higher rate. Its action upon the β-amylose substrate is, however, not so well sustained as that of pancreatic or malt amylase. This relatively early falling off in the speed of sugar formation together with the high ratio of amyloclastic to saccharogenic power indicate that this amylase is a more active catalyst of the earlier than of the later stages of the hydrolysis.

Contrasting the action of comparable amounts of the three different amylases it appears that they catalyze the successive stages of the hydrolysis of β-amylose and its products at relatively different velocities. The time curve for pancreatic amylase is practically logarithmic up to the production of about three-fourths the theoretical amount of maltose while beyond this point the reaction proceeds at a lower velocity. Compared with this result, the catalytic effect of the amylase of *Aspergillus oryzae* is more pronounced in the earlier and less pronounced in the later stages, while purified malt amylase is relatively less efficient in the earlier stages but catalyzes the later stages more efficiently.

In the digestion of α-amylose all of the amylases showed more pronounced catalytic effect upon the earlier than upon the later stages of the digestion. Starch pastes made at low temperatures (65–80°), autoclaved starch, and Lintner soluble starch all resemble the α-amylose rather than the β-amylose substrate in their behavior toward all three amylases, doubtless because α-amylose is the chief component of all these forms of starch.

The separation of starch into its α- and β-fractions made possible a more satisfactory study of the course of the amylase hydrolyses because of the greater homogeneity of the new substrates. All the data pertaining to the earlier stages of these hydrolyses indicate that Lintner soluble starch is well adapted to its purpose as substrate for testing the activities of the different amylases and that its use leads to conservative estimates of the diastatic powers of purified preparations.

Tested upon any of the four substrates here studied, the three amylases show distinctly different ratios of amyloclastic to saccharogenic powers.

LABORATORY OF FOOD CHEMISTRY.
NEW YORK, N. Y.

CORRECTIONS.

Page 1366, line 7 from bottom: after the words "to 170°" insert "with aniline."

In the article by C. E. Hudson in the August number of THIS JOURNAL (p. 1572, line 22) the specific rotation of trehalose octacetate in chloroform should be "+162°" instead of "—162°."

On page 1569, line 11 from the bottom, "melibiose" should be "gentiobiose."