CXCVII. AN INVESTIGATION OF THE RATES OF DIGESTION OF STARCHES AND GLYCOGEN AND THE BEARING ON THE CHEMICAL CONSTITUTION.

I. ACTION OF AMYLASES ON STARCHES AND GLYCOGEN.

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STARCHES from different sources show very different rates of hydrolysis by amylases. This is presumably due to differences in the composition of the starch granules. Dietetically, however, starches are usually considered of equivalent food value, provided that they all undergo complete digestion. Having found that potato and rice starches are hydrolysed at very different rates, as judged by the achromic point, and being unable to find in dietetical and chemical literature information concerning this, we decided to investigate the rates of digestion and of the formation of the intermediate hydrolysis products of the starches which are incorporated in our diet. Glycogen was later included in the investigation. In this way it was hoped to gain information concerning the differences in chemical constitution of the different starches and their relative digestibilities.

Concerning the chemical structure of starch little need be said here. The work of Samec [1927], Ling & Nanji [1923], Schardinger [1909; 1911], Pringsheim & Wolfsohn [1924] all supports Maquenne & Roux's [1906] theory that the starch granule consists of two constituents (termed by these workers amylopectin and amylose). The work of Karrer & Nägeli [1921, 1, 2] and of Haworth [1931; 1932; 1935], Hirst *et al.* [1932], on the other hand, supports the now more generally adopted view that starch is homogeneous in structure. Amylose and amylopectin are reported to be formed by successive depolymerization and repolymerization of natural starch and their distinctive characteristics to arise from differences in physical character or from admixture with other substances.

Most workers consider that the unrestricted action of maltase-free amylases on starch leads to the formation of one sugar only, namely maltose, and that by the prolonged action of amylase there is probably at least 80% conversion into α -glucosidic maltose. Certain early workers, however, namely Lintner & Düll [1892; 1893] considered that *isomaltose* is formed in addition to maltose. *iso*-Maltose, as prepared by these workers by the action of malt diastase on potato starch, has a reducing power of 80% of that of maltose, $[\alpha]_D$ 140°, and forms an osazone of m.p. 151° which crystallizes in spherical groups of very fine light yellow needles and is very much less soluble in water and alcohol than maltosazone. Syniewski [1902] confirmed the above work and claimed to have isolated pure *isomaltose* (termed by this worker "dextrinose"). Brown & Morris [1895] showed that Lintner's *isomaltosazone* is not a separate chemical entity but is only maltosazone altered in crystalline form and m.p. by admixture with an amorphous osazone of a simple dextrin. Ling & Baker [1895] are also of this opinion. Pottevin [1899] interpreted the amylolytic hydrolysis of starch

as due to partial hydrolysis of the starch granule by means of an enzyme "amylase", into a series of dextrins, ranging from amylo-dextrins, giving a blue colour with iodine and exhibiting slight Fehling's reducing properties, to achroodextrins or simple dextrins, giving no colour with iodine and having high Fehling's reducing properties. A second enzyme "dextrinase" then converts these dextrins into maltose. According to Chrzyszcz [1911], an amylase contains separate depolymerizing, dextrinizing and saccharifying enzymes, which view was later supported by Pringsheim [1924], but this assumption does not seem to be essential. Nishimura [1928] regards amylases as being composed of one enzyme only, which saccharifies and depolymerizes, the latter notably in the presence of a co-enzyme. Olsson [1923] showed that the saccharifying enzyme is different from the liquefying enzyme because the addition of certain poisons reduced the liquefying action without affecting the saccharifying power. Kendall & Sherman [1910] found that the liquefying action is predominant with pancreatic amylase and the saccharifying action with malt amylase. These two enzyme components have now been successfully separated by Van Klinkenberg [1934]. Karrer [1921] considered diastatic action to consist of two phases, depolymerization and hydration, the latter phase involving the hydrolysis of maltose anhydride to maltose. The saccharification of depolymerized amylose and amylopectin does not proceed at the same rate. Amylose smoothly and quickly gives rise to the theoretical amount of maltose, whereas the saccharification of amylopectin is slower and usually ceases when about 78 % of the theoretical yield of maltose is produced. The remaining residual dextrin can, according to Pringsheim & Beiser [1924] be converted into maltose if a co-enzyme (present in yeast) is added. Kuhn [1924] divided amylases into two groups, the α - and β -amylases according to the point of attack of the starch molecule. α -Amylases produce α -glucosidic maltose as the end-product of starch degradation and β -amylases produce β -glucosidic maltose initially. Amylases from animal sources and takadiastase are α -amylases and vegetable amylases, with the exception of takadiastase, are β -amylases. Kuhn concludes that in view of the behaviour of the starch granule towards plant and animal amylases, the starch molecule consists of alternating α - and β -glucosidic linkages. If both α - and β -glucosidic groupings occur in the starch molecule it should be possible to obtain glucose from starch if both α - and β -amylases are made to act on starch paste. Pringsheim & Liebowitz [1925] showed this to be true. Ohlsson [1930] showed that his "dextrinogenamylase" from malt was identical with Kuhn's α -amylase and his "saccharogenamylase" with Kuhn's β -amylase. As judged from the relative permanence of the starch-iodine reaction, Ohlsson concluded that the former enzyme breaks down starch into progressively smaller molecules until maltose is produced, whereas the latter enzyme detaches maltose in successive stages. Van Klinkenberg [1932], in opposition to Kuhn, regards the α - and β -glucosidic linkages as occurring not in the same molecule but in different molecules, in α - and β -starch, respectively.

Little work has been done on the rates of digestion of different starches and the results that are quoted are very discrepant. The chief reason for this discrepancy is that two different methods are used for determining the amount of digestion that has occurred. These two methods, the achromic point and a sugar determination, are actually estimating different things. The achromic point determines when all the starch has been broken down at least as far as achroodextrin, although this does not necessarily mean that there is any sugar present. A sugar determination, on the other hand, estimates the maltose present and also any reducing dextrins, but there may at the same time be a considerable

amount of unchanged polysaccharide left. In order to get an accurate conception of the amount of starch degradation that has taken place at any time it is necessary to determine at least four factors; the achromic point, the unchanged polysaccharide, the total reducing power and the reducing power due to maltose alone. O'Sullivan [1904] found that a much lower percentage of maltose was formed by the action of malt amylase on potato starch than on cereal starches. Stone [1904] found that potato starch was more readily digested by pancreatin than were cereal starches. Ford [1904] showed that when rice, barley, maize, wheat and potato starches were purified by the same method, they were digested equally by malt extracts. Nagao [1911] found that wheat and rye starches were hydrolysed at equal rates by pancreatic amylase and that oat and barley starches were digested at approximately the same rate, but that the rate of hydrolysis of the latter two was considerably higher than that of wheat and rye. Sherman *et al.* [1919] showed that, when similarly purified by washing with dilute alkali to extract any gluten, wheat, maize and rice starches were hydrolysed at equal rates. Potato starch generally showed a rate of hydrolysis equal to or slightly greater than the cereal starches. These experiments were performed with both pancreatic and salivary amylases. The percentage hydrolysis was determined in each case at the end of 30 min. by estimating the reducing sugar by Fehling's method.

Concerning the breakdown of glycogen, Musculus & Mering [1879] showed that glycogen gave the same hydrolysis products as starch. Cremer [1894] found that the amylolytic hydrolysis of glycogen was much slower than starch. Tebb [1897] found that when glycogen was digested by ptyalin, amylopsin and malt diastase, both maltose and isomaltose were formed. Osborne & Zobel [1903] found that both pancreatic and malt amylases formed isomaltose alone as the end-product, whereas both takadiastase and salivary amylase produced glucose in addition to isomaltose. Horton [1913] showed that the hydrolysis of glycogen by pancreatic amylase proceeded much faster in the early stages, but the subsequent breakdown of dextrin was very slow and there was seldom a quantitative conversion into maltose. Pringsheim's view that amylopectin and glycogen are identical was further substantiated by the work of Pringsheim & Beiser [1924] who showed that a co-enzyme was necessary for the complete hydrolysis of glycogen, just as in the case of amylopectin. Barbour [1929] showed that with salivary amylase the chief end-product was isomaltose, and there was also occasionally glucose present, but never any maltose. With pancreatic amylase, both isomaltose and glucose were formed. He found that there was a serial degradation of glycogen with pancreatic amylase, the increased reducing power corresponding with disappearance of glycogen, whereas with salivary amylase there was no such correspondence.

In view of these rather discordant results and conclusions the present investigation was carried out using potato, maize, wheat and rice starches (all of these having been obtained from B.D.H.) and glycogen (Kahlbaum).

The ash and P contents of all the samples were determined; as a further test of purity nitrogen analyses were performed but the results were negative in all cases. The values obtained are given in the following table, all the estimations being performed on the dried material.

Polysaccharide	% Ash	% P
Maize starch	0.084	0.055
Wheat starch	0.185	0.142
Potato starch	0.285	0.119
Rice starch	0.284	0.068
Glycogen	1.100	0.298

The ash in each case gave a positive iron reaction. The P was determined by Neumann's method using 0.5–2.0 g. of dried material. The results are of the same order as those obtained by Samec & Haerdtl [1920] and the higher value obtained for glycogen substantiates the generally adopted view that glycogen has a higher electrolyte content than starches.

Achromic point.

The achromic points were determined for each polysaccharide, using a 1% solution and hydrolysing it in turn with saliva, takadiastase, malt diastase and a solution of "Holadin" (a pancreatic preparation particularly rich in amylopsin) at the appropriate optimum pH. Whichever amylase was used, the achromic points were always in the same order, this point being reached very much more quickly with potato starch and glycogen than with rice, wheat and maize starches, these latter three, however, all giving the achromic point at approximately the same time.

Amylase	Starch				Glycogen
	Potato sec.	Maize hours	Rice hours	Wheat hours	
Salivary	20	1.5	1.5	1.5	Few sec.
Pancreatic	5	2.25	2.25	2.25	Immediate
Takadiastase	20	4	4	4	Few sec.
Malt	240	3	3	3	2 min.

As will be seen from the following results, the achromic point is a very unreliable index of the amount of digestion that has taken place.

According to Van Klinkenberg [1934] iodine-colouring atomic groups are present in α -starch only and the iodine reaction therefore only disappears by the action of α -amylase.

Rate of degradation of the different starches and of glycogen.

The course of the enzymic hydrolysis of glycogen and of each of the starches was followed using pancreatic amylase ("Holadin"). The salivary and malt digestions of wheat starch and glycogen were also followed.

The maltase activity of all the enzyme preparations was tested by incubating 2% maltose solutions with relatively large amounts of the enzyme and testing for glucose by an osazone test after 3 days. Except with the malt preparation, glucose was produced in every case. On diminishing the concentration of enzyme, however, no glucose was formed even with concentrations of enzyme ten times as great as those used in the subsequent hydrolyses. It is concluded that these enzyme preparations contain only traces of maltase which are ineffective in the low concentrations in which they were employed and would therefore be overlooked in most cases.

The following estimations were performed:

- (1) estimation of unchanged polysaccharide;
- (2) estimation of total reducing power using the Wood-Ost method;
- (3) estimation of reducing power due to sugar alone using the same method.

In the later experiments Hanes's [1929] method of sugar estimation was substituted for that of Wood-Ost. From these data it is possible to calculate the amount of polysaccharide that has been broken down, the amounts of reducing dextrin and maltose formed, and, by deduction, the amount of non-reducing dextrin.

These three estimations were performed on samples of the digest immediately after mixing and 15, 30, 60 or 90, 120, 180, 330 min., 24 or 72 hours after the beginning of the experiment.

An osazone test was also performed on samples from each digest, after alcohol precipitation of any residual starch and dextrans followed by evaporation of the alcohol from the filtrate. In no case was a typical maltosazone observed. Most frequently the osazone consisted of relatively large rosettes of very fine needles but very often these needles were tufted at their free extremities. It seems impossible to place any reliance on the appearance of the osazone; either the crystalline form typical of maltosazone had become modified by admixture with another substance (perhaps dextrans which had not been completely removed by alcohol precipitation) or else maltose is not the sole product of hydrolysis.

Different methods were employed for the estimation of starch and glycogen.

The starch was estimated by a modification of the von Fellenberg method [1916]. This method depends on the principle that starch dissolves in concentrated CaCl_2 solution, from which it can be precipitated by iodine. Protein, which might be present in small amounts, is insoluble in CaCl_2 solutions and by dextrans are not precipitated from CaCl_2 solutions by iodine.

The digestion mixture in the case of the pancreatic digestion consisted of 225 ml. of 1 % starch solution in 0.5 % NaCl , 70 ml. of borate¹ buffer of pH 6.8 and 5 ml. of 0.1 % solution of holadin in distilled water. This mixture was left in a thermostat at 37° and samples were removed at the time intervals stated above.

The total reducing power was determined by the Wood-Ost method, introducing 5 ml. of the digest into 20 ml. of the Wood-Ost solution. This was performed in duplicate.

In order to determine the reducing power due to maltose alone, 10 ml. of the digest were removed, boiled to inactivate the enzyme, cooled and made up to 50 ml. in a graduated flask with absolute alcohol. This was left overnight, filtered and 20 ml. portions of the filtrate used for the Wood-Ost determination. In many cases a further estimation was carried out. A portion of the alcohol filtrate, which should contain maltose alone, was heated on a water-bath to drive off all the alcohol and the reducing power determined after acid hydrolysis. This should indicate whether maltose is the sole sugar formed.

Furthermore, in a few cases, polarimetric determinations were carried out on the alcohol filtrates, after the alcohol had been driven off and the subsequent solution considerably concentrated and the turbidity removed, if necessary, by the addition of a small amount of basic lead acetate.

At the end of each experiment the digest was tested for lactic acid by Uffelmann's reagent. This test was in all cases negative.

The digestion mixture for the glycogen hydrolysis was the same as for the starches. The total reducing power and the reducing power due to sugar were determined in exactly the same way as described for starch. In the determination of the residual glycogen, the observations of Good *et al.* [1933], that the optimum concentration of alcohol for the precipitation of glycogen is 50 % and of Osterberg [1930] that this precipitation is assisted by the use of Na_2SO_4 , which carries down mechanically the precipitated glycogen with the precipitate of Na_2SO_4 , were made use of. For the glycogen determinations 3 ml. samples of the digest were employed and the glucose, after acid hydrolysis was estimated by Hanes's method.

¹ Phosphate buffers cannot be substituted for borate buffers, since it was found that boiling the starch and phosphate mixture with CaCl_2 led to the complete disappearance of the starch.

The results are represented diagrammatically (Fig. 1) and in addition three graphs are given plotting respectively the percentage disappearance of polysaccharide, the percentage formation of reducing dextrin and maltose, and the

Fig. 1. Representing the formation of the products of digestion of starches and glycogen.

Fig. 2. Digestion of starches and glycogen by pancreatic amylase.

percentage formation of maltose against time. (The time axis is shortened for economy of space.)

Nature of sugar formed on hydrolysis.

As has already been stated most observers agree that maltose is the sole sugar formed on hydrolysis of starch or glycogen by amylases. The present results are not entirely in agreement with this. The same sugar was formed from all the starches and from glycogen but as judged from the appearance of the osazone, the amount of glucose formed on acid hydrolysis and the optical rotation of the sugar, this sugar could not be identified with maltose. The appearance of the osazone has already been described. On acid hydrolysis of the digest, after precipitation of the unchanged polysaccharide and dextrins with alcohol, 120–140% yield of glucose was obtained (assuming that the sugar before

hydrolysis was maltose). This means that the reducing power of the sugar before acid hydrolysis was only 70–85 % that of maltose. In no case, as judged by the osazone was any glucose present, but if any were present this would mean that the other sugar would have a reducing power even further removed from that of maltose than the value given above.

In a few cases only was the optical rotation determined, but when this was done it was invariably found that there was lack of agreement between the optical rotation and the reducing power (assuming the sugar to be maltose only). The optical rotation was in each case 124.5 % that of maltose. This value is very similar to that given for α -maltose, but this possibility is excluded because no mutarotation was observed after treatment with alkali. The constancy of this figure obtained both from potato and wheat starches at different stages of hydrolysis indicates that only one sugar could be present. If a mixture of sugars were present, it is extremely unlikely that they would be present in exactly the same proportions under different conditions. These results might be explained by the presence of reducing dextrans, but since the final concentration of alcohol before filtration of the unchanged polysaccharide was 80 % this seems improbable.

The appearance of the osazone and the reducing power corresponded fairly closely with those of *isomaltose* as described by Lintner & Düll. The specific rotatory power, however, was different. The figure given for *isomaltose* is $[\alpha]_D = 140^\circ$, which is only very slightly different from that of maltose.

DISCUSSION OF RESULTS.

There are at least four different criteria of the rate of degradation of the polysaccharides:

- (1) rate of disappearance of polysaccharide;
- (2) rate of formation of maltose and reducing dextrin;
- (3) rate of formation of maltose;
- (4) achromic point.

The following table represents the order of these events, the polysaccharides being arranged in order of decreased speed of hydrolysis by pancreatic amylase as indicated by the different criteria.

Achromic point	Rate of disappearance of polysaccharide	Rate of formation of reducing dextrin and maltose	Rate of formation of maltose
Glycogen	Potato, maize	Wheat, potato	Wheat, rice
Potato	Wheat, rice	Maize, rice	Potato
Maize, wheat, rice	Glycogen	Glycogen	Maize Glycogen

It is difficult to reconcile the very quick achromic point of glycogen with its slow rate of breakdown and formation of dextrin and maltose. It must be the figures for the achromic point which are at fault, since it was found very difficult to determine this value when very weak glycogen solutions were used, the colour being only with difficulty distinguishable from that of a weak iodine solution.

It is interesting to note that in the case of glycogen, the rate of formation of maltose reached a steady state after 2 hours, although the formation of reducing dextrin and the disappearance of glycogen were still occurring. (The decrease in the 24 hours value for maltose seen in the results for wheat and maize starches is presumably due to fermentation.)

In the case of the salivary digestion of glycogen and wheat starch, although the salivary amylase was obviously relatively very much stronger than the

pancreatic amylase, the results are in the same order, the figures for glycogen being in every case smaller than those for wheat starch.

In the case of the digestion of glycogen and wheat starch with malt there is a slightly greater breakdown of glycogen than with pancreatic and salivary amylases. Malt contains chiefly β -amylase, but this does not warrant the assumption that glycogen contains a preponderance of β -linkages. The breakdown of glycogen by pancreatic, salivary and malt amylases is essentially similar in character.

These differences in the hydrolysis rates of the different starches must be due to differences in the chemical constitution of the starch granules, since they cannot be attributed to differences in the enzyme systems.

The rate of hydrolysis of all the starches is very much quicker than that of glycogen.

Factors which could influence the rate of breakdown.

(1) *Molecular dimensions of the polysaccharide.* The smaller the molecular size, the quicker one would expect the breakdown to be. But the size of the glycogen molecule is (according to Haworth) approximately half that of the starch molecule.

(2) *Proportion of amylopectin to amylose.* According to most modern workers the starch granule is homogeneous. But supposing that two constituents are present, the advocates of this theory agree with the original view of Maquenne & Roux [1906] that the viscosity of a starch solution is produced by the amylopectin. According to this view, potato starch, which gives the most viscous solution, should show the lowest rate of hydrolysis, since amylopectin is considered to be hydrolysed more slowly than amylose. This, however, is not the case, potato starch showing, if anything, a higher rate of hydrolysis in the initial stages than the other starches. The lower rate of hydrolysis of glycogen, however, could be accounted for if, as claimed by Pringsheim, glycogen consists of amylopectin.

(3) *Inorganic content of polysaccharide.* According to Samec & Haerdtl [1920] a low rate of hydrolysis can be correlated with a high P content. Potato and wheat starches have much higher P contents than the other starches, but their rates of hydrolysis are no lower. The lower rate of hydrolysis of glycogen, however, could be explained according to this theory by its high P content.

(4) *Method of breakdown.* As has already been mentioned Ohlsson considers that α -amylases break down starch into smaller and smaller molecules until maltose is finally produced whereas β -amylases detach maltose successively. In the salivary and pancreatic digestions of glycogen and the various starches employed in this investigation, an early appearance of maltose was invariably observed. When malt, which contains β -amylase in addition to α -amylase, was used as the hydrolysing agent, no increased formation of maltose was observed. These results therefore do not substantiate the findings of Ohlsson. Moreover, Ohlsson's work was based on the relative permanence of the starch-iodine reaction, and since it is not known with certainty that this colour is a function of the molecular dimensions, his conclusions seem rather unreliable.

In order to ascertain which of the above-mentioned methods of breakdown was involved or which method was predominant, the following ratio was calculated for each digestion mixture throughout each hydrolysis:

$$\frac{\text{Polysaccharide destroyed in mg./100 ml.}}{\text{Maltose formed in mg./100 ml.}}$$

If this ratio remains constant throughout a hydrolysis it would indicate that maltose was liberated successively. If, on the other hand, the ratio decreases as digestion proceeds it would mean that maltose is formed only, or principally, in the final breakdown of the simple dextrins. These ratios, calculated from determinations made at 15 min. from the beginning of each experiment and at the subsequent intervals already mentioned, are tabulated below. The values cited represent values for this ratio obtained at the beginning, middle and end, respectively, of each experiment:

Substrate	Pancreatic amylase			Malt diastase			Salivary amylase		
Glycogen	2.16	2.46	3.15	1.24	1.40	1.50	2.52	1.98	1.39
Potato	4.00	3.52	2.78	—	—	—	—	—	—
Wheat	1.87	1.83	1.86	1.27	1.13	1.09	1.39	—	1.04
Rice	2.60	2.56	1.89	—	—	—	—	—	—
Maize	4.38	2.89	2.70	—	—	—	—	—	—

The great variability of this ratio probably indicates that both methods of breakdown are occurring simultaneously. It is difficult to accept the deduction that the hydrolysis of wheat starch by pancreatic amylase is the one instance amongst all those cited in which the former method of breakdown alone occurs. An increase in this ratio as hydrolysis proceeds, observed with glycogen in two out of the three cases, seems very difficult to explain, especially as there was no glycogen resynthesis.

In the case of glycogen, the formation of maltose from dextrin appears to be very slow, which indicates that the hydrolysis is retarded at this stage, suggesting a reduced saccharification process. But since the same enzymes were used in the starch and glycogen digestions, this cannot be accounted for by the enzyme. Perhaps, as suggested by Pringsheim, a co-enzyme is necessary for the complete hydrolysis of glycogen.

(5) *Nature of linkages in polysaccharide.* As discussed in the previous section, the slow rate of breakdown of glycogen cannot be explained by assuming a preponderance of β -linkages in the glycogen molecule.

According to Van Klinkenberg the iodine reaction is characteristic of α -starch and only disappears by the action of α -amylases. The very rapid attainment of the achromic point observed with glycogen appears at first sight to substantiate Van Klinkenberg's view that glycogen is α -starch. But this does not explain the slow breakdown of glycogen in comparison with the starches. If glycogen consisted entirely of α -starch, a very quick breakdown by α -amylases would be expected.

It is thus seen that the theories of starch and glycogen structure so far enunciated do not explain satisfactorily the results of the amylolytic hydrolysis of these polysaccharides. There must be some difference in structure between the starch and glycogen molecules to account for the much lower rate of hydrolysis of the smaller glycogen molecule. There are also probably slight differences in structure between the different starches. It is also difficult to accept the identity of the starch and glycogen molecules as advocated by Haworth.

SUMMARY.

1. The course of digestion of potato, wheat, maize and rice starches and of glycogen by pancreatic amylase and in addition of wheat starch and glycogen by salivary amylase and malt diastase have been followed quantitatively.

2. The estimations included determinations of the achromic point, the residual polysaccharide, the total reducing power and the reducing power after the precipitation of the dextrans. Polarimetric observations were also made in a few cases.

3. The relative rates of formation of the various hydrolysis products were different with the different starches. The rate of hydrolysis of glycogen was in every case very much lower than that of the starches.

4. The same end-product was formed from all the starches and from glycogen.

5. The properties of the reducing sugar formed as end-product did not correspond with those of maltose, or, in all respects, with those of isomaltose.

6. Glucose was not formed in any of the above digestions, or in the case of pancreatic amylase over the range pH 5.0–7.5. When, however, a very large amount of enzyme was employed, some glucose was produced.

7. The bearing of these results on the chemical constitution of the starch and glycogen molecules and on the method of breakdown is discussed.

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