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THE DEGRADATIVE EFFECTS OF ACIDS ON THE AMYLOPECTIN COMPONENT OF STARCHES

by

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PREFACE.

I would like to take this opportunity of thanking Dr. C. T. Greenwood for his continued advice and unfailing interest during the period of this research. I also wish to thank Professor E. L. Hirst for the provision of the facilities which made the work possible, and for his subsequent interest.

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C O N T E N T S.

GENERAL INTRODUCTION.

The degradation of high molecular weight materials has been extensively investigated. The first investigations were of the acid-hydrolysis of the naturally occurring high polymers, starch and cellulose. These studies initiated kinetic studies on the degradation of high polymers in general (1). Such studies have provided much information about molecular structure.

In the early experiments, chemical end-group analysis was used to follow the reaction, but there are limitations to the method. For polymers, quantitative results are difficult to obtain, and the results are only easily interpreted if the molecule is unbranched.

Degradation studies are particularly suited to the elucidation of fine structure, since degradation will preferentially occur at any weak anomalous bonds. Recent improvements in methods of molecular weight determination, and of analysis of the degradation products, have been responsible for more precise information being obtained regarding the kinetics of such processes.

The agencies causing polymer degradation may be physical or chemical in nature. (Whilst two types of degradation may occur, the bonds undergoing fission being between monomer units in one case, and between the atoms of the monomer itself in the other, only the former is considered here.) Many physical processes can cause degradation. Thermal degradation gives information on the general mechanisms of the degradation process. Photochemical

degradation, on the other hand, can give more detailed data on the individual steps, so allowing evaluation of rate constants (2) (3). Mechanical forces such as rapid shaking, or rotating, can also set up sufficiently high shearing forces to cause degradation. In the case of ultra-sonic degradation, this shearing force is brought about by high-frequency sound waves. However, experimentally the study of chemical degradation is generally the more simple.

Depolymerisation can be brought about by many chemical reactions oxidation, acid- or alkaline-hydrolysis. Perhaps the most satisfactory of these from the point of view of kinetic analysis is acid-hydrolysis, because the degradation products are stable. This method was studied in this work and is therefore dealt with in outline below.

Much of the previous work carried out on the kinetics of the acid-degradation of polysaccharides which has indicated the presence of weak-linkages, has been on cotton cellulose and its derivatives (4). (At this point it should be noted that alkaline degradation of cotton cellulose has also received much study (5).)

Early studies (6) indicated that hydrolytic breakdown proceeded by a mechanism involving random breaking of bonds which have the same cleavage probability, although this may be complicated in that the end-bonds may be more susceptible to attack than the others. The first experimental evidence that all the inter-unit links in cellulose might not be identical was obtained by Schulz, Husemann and Lohmann (7), studying the hydrolysis of a fraction of nitrated

Egyptian cotton, chain length 3,000. It was found that the molecular weight distribution widened at first, as expected, but then narrowed before widening again. Furthermore, fractionation of the reaction products yielded a large proportion of material with chain-length about 500. These results were taken to indicate the presence of a number of weak-bonds, arranged evenly throughout the chain about 500 units apart. A similar effect has been reported for the parent cellulose by Schulz (8), and a number of authors have interpreted results of degradation in the same way (9).

In the word described in this thesis, the hydrolytic action of acid on starch, and its component amylopectin, has been studied, using conditions mild enough to allow the rate of hydrolysis of any anomalous linkages to be distinguished and measured. (An outline of the chemistry of these polysaccharides is given in the following section as are the methods used to characterise their purity and determine their molecular weights.) The starch granule itself was also subjected to acid attack, since this may yield information about yet another facet of starch chemistry - the fine structure of the granule. This in turn led to improving methods of fractionation, and the examination of the fractions. Finally acid attack on the free, and methylated amylopectin, was studied.

GENERAL CONCEPTS IN STARCH CHEMISTRY.

In land plants, the most abundant and widely distributed reserve food polysaccharide is starch. It occurs as discrete granules, which form a basis for identification, as their characteristics vary from one source to another (10). The chemical and physical properties of the starch also vary with its botanical source. Because of the widespread occurrence and importance of starch, its structure and properties have been extensively investigated. This has resulted in the main structural outlines of the polysaccharide being established. Starch is composed, in the main, of chains of D-glucopyranose units joined by α-1:4-D glucosidic linkages.

The structural heterogeneity of starches has been recognised for some time, but only since the advent of improved techniques for isolation and purification, have the characteristics of the fractions been satisfactorily established. It is now recognised that starch consists of at least two chemically distinct components; (1) amylose, an essentially linear molecule giving an intense blue colour with iodine and (2) amylopectin, a highly branched molecule, whose inter-chain linkages are essentially α-1:6, and which gives a reddish brown stain with iodine (11). (This terminology for the components will be used here, as recommended by the International Union of Pure and Applied Chemistry (12), although A- and B-fractions have been used for amylose and amylopectin respectively by Schoch (13).) This recognition is in no small way due to the first quantitative fractionation of starch, carried out by Schoch (14). (For reviews

see Schoch (15) and Greenwood (16).)

The main differences between amylose and amylopectin may be summarised as follows:-

Amylose	Amylopectin
An <u>essentially linear</u> molecule.	A branched molecule.
Molecular weight values range to ca. 10^6.	Molecular weights range to values very much greater than 10^6.
Forms complexes with iodine and polar molecules.	Does not form complexes with iodine or polar molecules to any appreciable extent.
Retrogrades rapidly from aqueous solutions.	Does not retrograde or only slowly.
Crystalline or fibre X-ray patterns.	Poor or Amorphous X-ray patterns.*
β-amylase hydrolyses to <u>ca</u>. 70%.	β-amylase hydrolyses to 55 $\pm$ 5%.

* However, amylopectin does exhibit crystallinity in the granule, since waxy maize starch and other waxy starches give diffraction patterns.

<u>Amylose</u>.

The amylose fraction causes the instability of starch solutions. Retrogradation is thought to be due to the alignment of molecular chains to form crystallites, which can themselves grow to form visible aggregates. Early methylation studies on amylose (17)(18) indicated a unit chain of 200 - 350 D-glucose residues, and from the fact that the osmotically determined molecular weight of both maize and potato amylose agreed with this chain length Meyer (17) suggested the molecule was linear. This linearity means that

degradative effects may be considerable, and that contamination with only a small amount of amylopectin will have a large effect on the apparent chain length obtained from any measurement of the amount of non-reducing end-group. Periodate oxidation, which is quicker, less tedious and requires much less material than methylation, has also confirmed the essentially linear character (19)(20)(21).

Recent work indicates that amylose consists of a long chain of several thousand glucose residues, linked α-1:4, the degree of polymerisation varying with the source (20)(22). It has also been shown that, due to its ease of degradation, the degree of polymerisa-:tion may vary with the method of extraction (23).

The concept of a completely linear molecule has had to be reviewed in the light of recent β-amylolysis studies. Using highly purified β-amylase it was found that amylose was only hydrolysed to 70% (24)(25). Several explanations have been put forward, none of which solves the problem completely. It has been suggested that a small proportion of β-glucosidic linkages are present (24)(25), or that the molecule is slightly branched (26). Clearly more work is required on this subject before it can be fully resolved.

Amylopectin.

Methylation and hydrolysis of the methylated derivative of potato amylopectin indicated a unit chain length of about 27 D-glucose residues. Since the molecule is non-reducing, and has a molecular weight corresponding to many times this value, Meyer suggested a highly branched structure (27). These results and

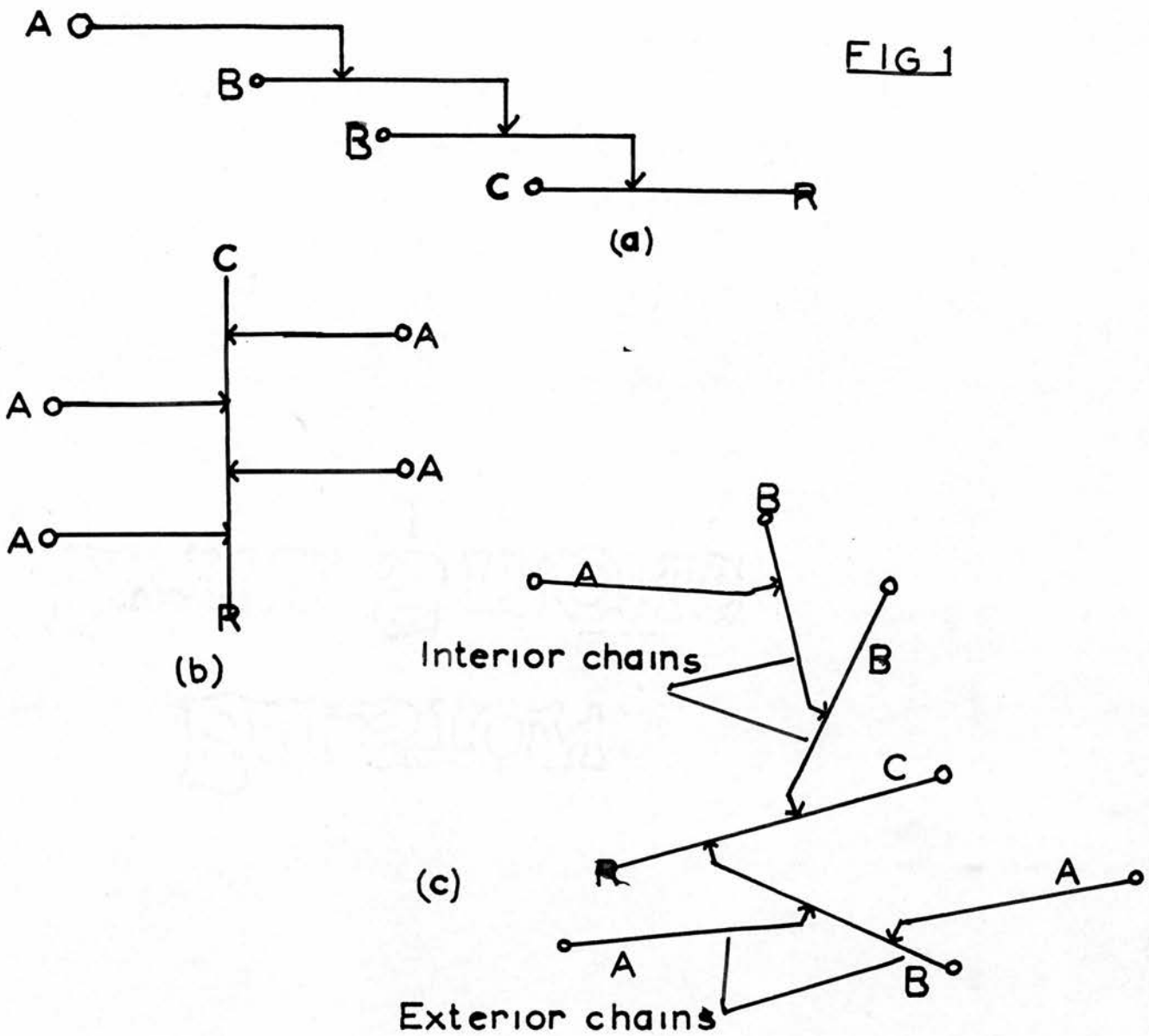
conclusions have been supported by periodate oxidation studies. The chain length and molecular weight have been found to vary with the source of amylopectin (20)(21)(28).

Amylopectin then is composed of several hundred unit-chains, each one containing 20 - 25 α-1:4-linked glucose residues. Each unit chain is linked to an adjacent one through linkages which are predominantly α-1:6 (29).

Various branched structures have been advanced for amylopectin. Since the non-reducing end-group value showed little change for large changes in molecular weight, a laminated structure was proposed by Haworth, Hirst and Isherwood (30), Fig. 1.(a).p.7a This structure, it has been pointed out (31), was the simplest consistent with the data from methylation, but was not intended to be a complete representation. By comparing the viscosities of starch derivatives with those of cellulose having the same molecular weight, Staudinger (32) suggested a 'herring-bone' type of structure, Fig. 1.(b). Meyer (33), on the other hand, proposed a ramified structure to explain enzymic degradation results, Fig. 1.(c).

It was shown by Myrback and Sillen (34), that all these structures contained different arrangements of the same basic linear chains, and Peat (35) suggested that these should be termed A-, B- and C-chains. A-chains are side chains linked solely through their reducing group to the rest of the molecule; B-chains have the A-chains attached to them, and in turn are themselves linked through their reducing group to another group; and the C-chain carries the

FIG 1



o Terminal non-reducing end-group

R Reducing end-group

o→ A chain of 20–25 α-1 : 4-linked glucose units

↓ α-1 : 6-D. linkages

A B and C are types of chains

single reducing end-group. It will be seen then that the three structures differ only in the ratio of A- to B-chains. In (a) there is only one A-chain, in (b) no B-chains, and in (c) approximately equal numbers of A-and B-chains. In addition, that part of a chain between a branch point and the terminal non-reducing group is called the exterior chain, the rest of the chain being referred to as the interior chain. See Fig. 1. By the successive use of a hydrolytic enzyme (β -amylase), and a debranching enzyme (R-enzyme), on waxy maize starch, Peat et al. (35) have concluded that multiple branching is an intrinsic part of the amylopectin molecule.

Of the three postulated structures, the one proposed by Staudinger, along with a modification of Hess and Lung (36), has been disproved. Both structures would give rise to greater yields of maltose, and a limit dextrin of lower molecular weight than was actually found on β -amylolysis (33)(37). Further, acid-degradation experiments carried out by Hirst and Young (38)(39)(29) (see later) gave a good yield of a homogeneous high molecular weight product, whereas this structure would give rise to low molecular weight heterogeneous products. The true structure probably lies between (a) and (c).

Glycogen (40) - the food reserve polysaccharide of animals - has a highly branched structure very similar to amylopectin, and it is now accepted that the structure is similar to the tree-like one proposed by Meyer for amylopectin, Fig. 1(c). The average

chain-length of amylopectin is approximately twice that of glycogen, 20 - 25 as against 12 (41)(42)(43). The iodine binding power of glycogen is much lower than that of amylopectin, and serves as a means of characterising the two structural types (28).

Profound differences in physical behaviour exist between the two polysaccharides. Viscosity measurements indicate that amylopectins are more asymmetric than glycogens (30b), as do the majority of measurements on molecular size. Staudinger and Husemann (44) suggested the glycogen molecule was spherical. The viscometric data, however, together with ultracentrifuge measurements (45)(46), show that glycogens have a molecular asymmetry, which varies according to the source. It has been found that the limiting viscosity number of rabbit-liver glycogen is ten times lower than that of rubber-seed amylopectin (47). Hence amylopectin must have an appreciably greater degree of molecular asymmetry than glycogen, the steric arrangement of the multiple branched chains being different. The fact that amylopectin is less compact than glycogen has been shown by its greater susceptibility to enzymic attack. Any investigations resulting in further information regarding the nature of the fundamental differences between the two polysaccharides are therefore most important.

A limit has been reached, however, in the study of polysaccharides beyond which it is impossible to proceed with purely chemical methods. Many problems still remain, mainly connected with the fundamental details of fine structure, size and shape of the polysaccharides and their components. Further progress is now dependent upon full use

being made of physical and enzymic studies to supplement the earlier results from purely chemical methods of study.

SECTION I.

EXPERIMENTAL METHODS.

INTRODUCTION.

In the physico-chemical study of any starch, the first essential requirement is an effective method of fractionation. But before studying the size, shape, or fine structure of the resultant components, it is necessary to characterise them. Therefore two lines of study are required. These entail measurements of (a) the purity of the products, and (b) their size, shape and fine structure. In this work (a) has been obtained from measurements of iodine uptake, extent of enzymic degradation, and chain length by periodate oxidation, whilst (b) has been obtained from viscosity and sedimentation measurements.

1. MEASUREMENT OF IODINE UPTAKE.

Introduction.

After Schoch carried out the first quantitative fractionation of starch (14), using selective precipitation of amylose from solution by organic, polar molecules, methods of characterising the fractionation products based on their differing affinities for iodine were developed. These included colorometric (18)(48) and potentiometric titration (49) techniques. The former, while suitable for comparative work, are often not absolute, and are not very accurate. The potentiometric titration technique has now developed into a standard analytical method for the determination of the purity of fractionation products and the amylose/amylopectin ratio of whole starches.

The interaction of amylose and iodine to give a characteristic blue colour has been known for some time, but only since the use of this technique has it been possible to determine the mode of interaction. It appears that a definite inclusion complex is formed and not a solid solution (4), and further that the interaction is of a dipolar nature because of the great difference in the molar extinction coefficient of iodine in starch and in non-polar solvents (50). Evidence that a helical configuration existed in the complex has been obtained from optical studies on crystalline amylose, both before and after staining with iodine (51), and X-ray studies of butanol/amylose and iodine/amylose complexes (51)(52). The latter studies also indicated that each turn of the helix contained six D-glucose units, and that there was one

molecule of iodine per turn. Gilbert and Marriott (53) were able to show from potentiometric studies that the constitution of the complex is ($3\ I_2 . 2\ I^{\ominus}$) or ($I_8^{\ominus\ominus}$) and this resonating ion is thought to be responsible for the blue colour.

Amylopectin, in contrast to amylose, gives a red colouration with iodine, and under the same conditions binds much less iodine (49). Mikus, Hixon and Rundle (54) consider this low iodine binding power cannot be explained by hydrogen bonding, as has been suggested by Whistler and Hilbert (55), but rather that the large number of branch points prevent helix formation and hence complexing. The amount of helix formation and hence the amount of iodine uptake must depend upon the degree of branching.

The most satisfactory method for accurate work at the low free iodine concentrations required is the differential one of Gilbert and Marriott (53). It should be pointed out that this method has the disadvantage of a logarithmic decrease in the sensitivity during the increase in free iodine concentration occurring in the course of a titration.

This method does not require reagent blank titrations since the starch- and blank-solutions form two opposing half-cells connected by a salt bridge, allowing the equilibrium free-iodine concentration to be determined directly. Mould (56) pointed out that the applicability of this method depended upon obtaining a null potential indicator which was sensitive enough, yet gave good zero stability. Such an electrometer was described by Anderson and Greenwood (57).

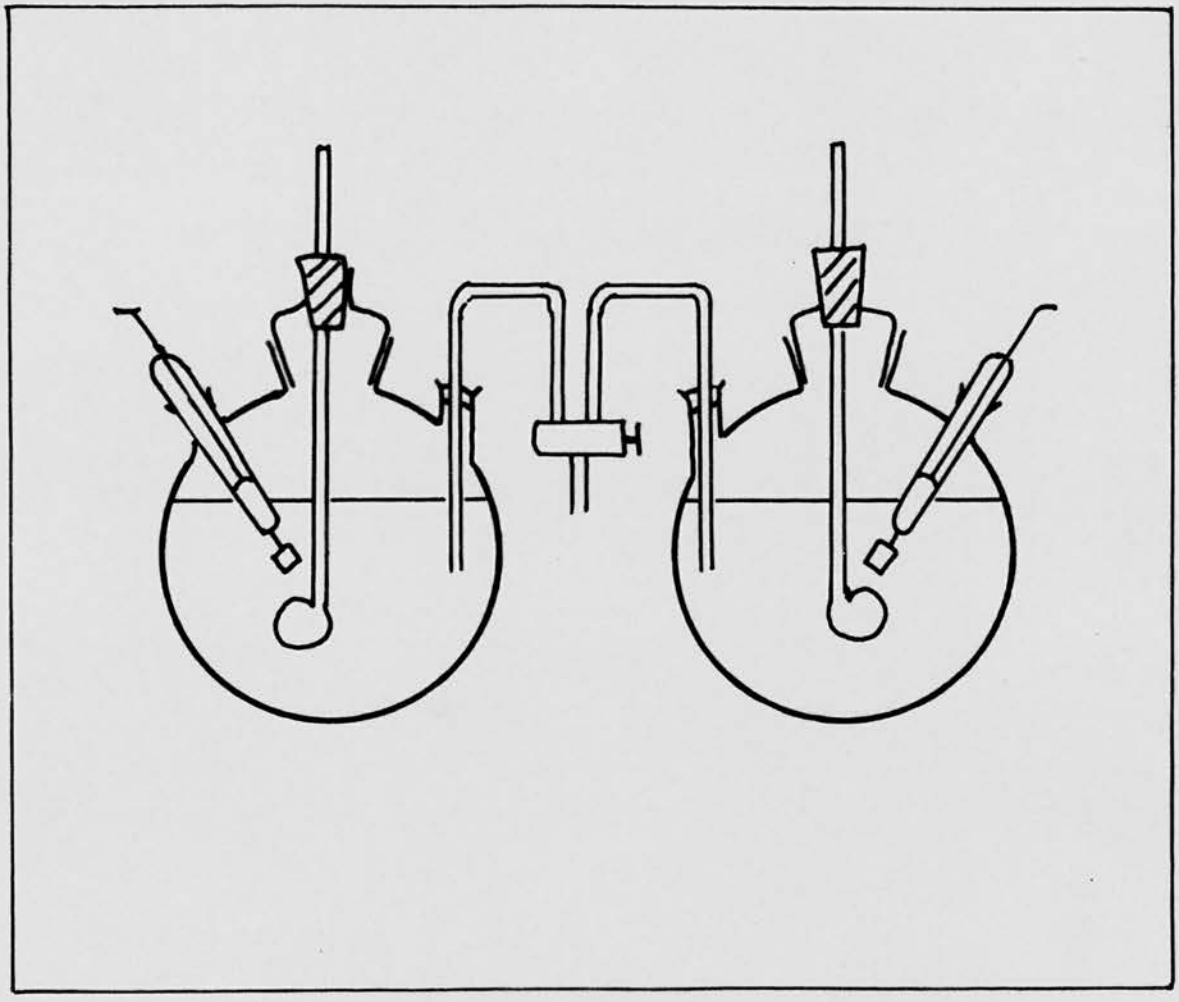
The stability and lack of external electrical interference was obtained by the use of a matched pair of valves as two resistors in a Wheatstone bridge arrangement. The sensitivity was further increased, without loss of stability or increase in interference, by using a further pair of matched valves as a cathode coupled amplifier. These pairs were obtained from experiments involving a number of valves until the best pairs were obtained.

METHOD.

The experiments were carried out in two 1 l. pyrex flasks with connecting salt bridge as shown in Figure 2. p. 14a The electrolyte solution was prepared by diluting 200 ml. 0.1M potassium iodide and 15 ml. M/15 phosphate buffer pH 5.85 to 2 l. 800 ml. of this solution were placed in each half-cell and allowed to reach temperature equilibrium. During this time and throughout the experiment the solutions were stirred continuously. The stirrer passed through a quickfit gland, and since the electrodes were fitted with a quickfit joint the whole apparatus could be sealed. The neutralised sample and blank solutions were then added to their respective half-cells, and the flasks rinsed with electrolyte solution. Provided that sufficient time had been allowed for temperature equilibrium to be reached, and care taken in making up solutions, there was no significant off-balance potential before the commencement of the titration. This rendered the depolarisation procedure of Gilbert, Greenwood and Hybart unnecessary (13).

A given volume of 0.01M iodine was added to the sample cell

FIG 2



Cell Arrangement

Iodine Titration Apparatus

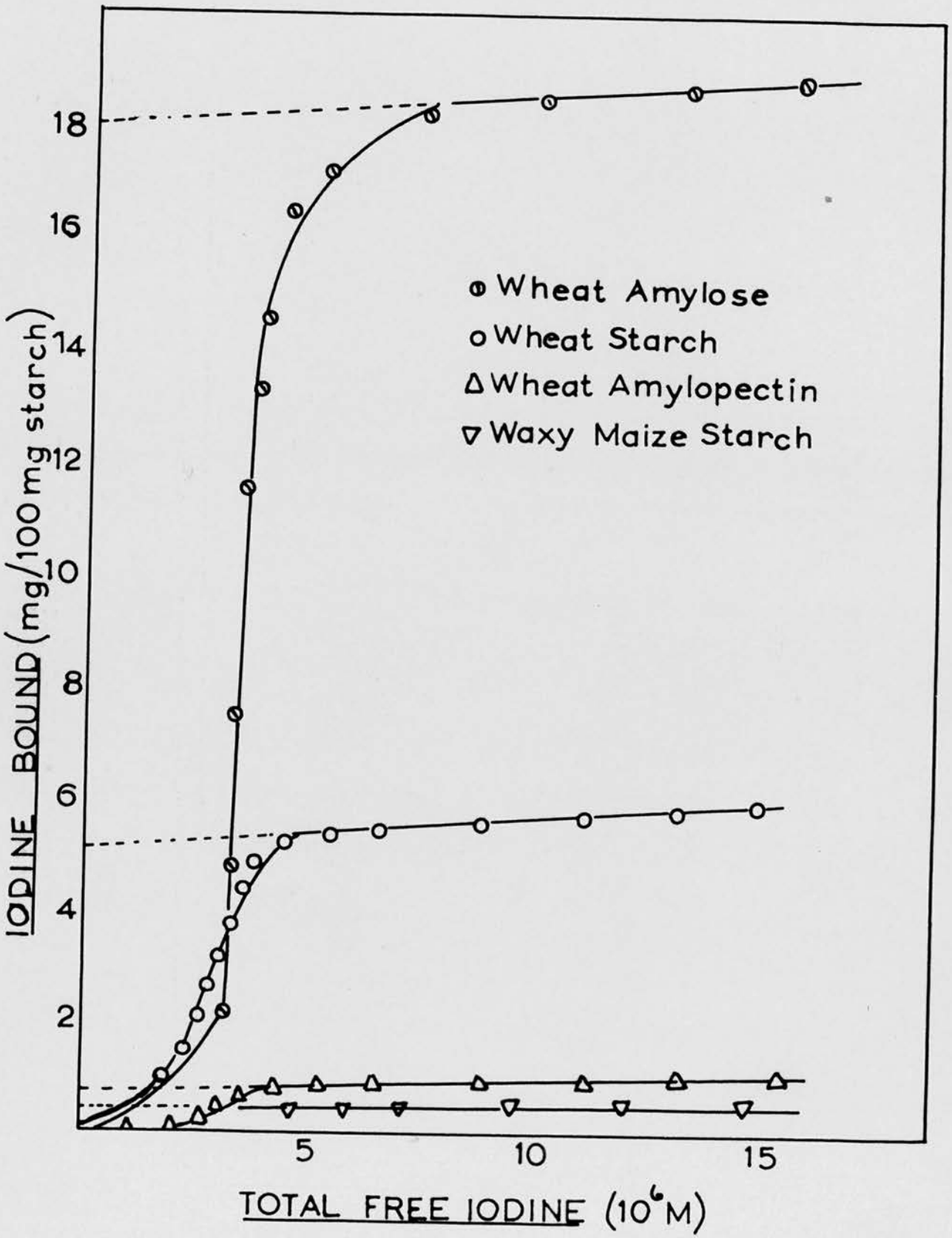
through a fourth neck from an Agla micrometer syringe. The same iodine solution was then added to the blank until there was the same free iodine concentration in both, as shown by no deflection of the galvanometer. The difference between the two volumes gave the iodine bound by the sample, and by plotting the free iodine concentration against mg. of iodine bound per 100 mg. of poly-:saccharide the titration curves were obtained. It is convenient to mention here that the high insulation required for the two-way switch was obtained by using a block of wax with pools of mercury, these being connected by a tilting copper wire framework.

The samples, all of which had been exhaustively defatted, then dried for several hours in vacuo at 70°C, were weighed from a tared weighing stick into a 50 ml. B14 conical flask. They were dissolved by the addition of 10 ml. 0.2M potassium hydroxide. In most cases heating in a water bath at 95°C for a few moments was necessary, and in some shaking overnight was required in addition. It has been shown (12) that for whole starch, ageing at room temperature has no effect on the iodine affinity, and that the effect of heating in 0.2 and 1M potassium hydroxide for 30 minutes is negligible. Prior to addition to the half-cell the samples were adjusted to pH 5.85 with a known amount of 0.4M phosphoric acid. The blank solutions were prepared in the same way but contained no polysaccharide. Weights found to give satisfactory curves were amylose 5 mgs., starch 10 mgs. and amylopectin 30 mgs.

The method measures the "iodine affinity" of a starch sample. At the free-iodine concentration required to saturate the amylose,

however, (2×10^{-6}M), amylopectin also binds a measurable amount (59)(57) and in order to avoid including this, the linear portion of the curve was extrapolated to zero free iodine concentration. From this "iodine affinity" value the percentage of amylose in any sample can be calculated, if the value for pure amylose is known (49). Although Higginbotham (60) suggested that all amyloses have the same maximum iodine binding power this is improbable, the value probably depending on the botanical source, fractionation procedure and the potentiometric titration conditions. Schoch (61), for instance , found the iodine affinity of several amyloses varied between 18.5 and 20.0%. Thus as suggested by Schoch (13), unless a rigorous fractionation has been carried out leading to a pure amylose component, only the iodine affinity should be given. If, however, the maximum iodine binding power of the pure amylose is obtained, then it is possible to calculate the percentage of amylose present. Typical curves obtained are as shown in Fig. 3, p. 16a.

FIG 3


IODINE BOUND (mg/100 mg starch)
18
16
14
12
10
8
6
4
2
5
10
15
TOTAL FREE IODINE (10⁶M)
Wheat Amylose
Wheat Starch
Wheat Amylopectin
Waxy Maize Starch

2. ENZYMIC DEGRADATION.

Introduction.

Purified hydrolytic enzymes, or polysaccharases, have become increasingly useful in structural analysis of polysaccharides. With the availability of pure substrates of known molecular structure, it has been possible to work out the action pattern of many enzymes. In turn this has made it possible to use these enzymes for the investigation of fine structure.

Four main groups of hydrolytic enzymes attack starch (62).

(1) α-amylases which catalyse a random hydrolysis of α-1:4 linkages. The products are maltose and α-dextrins consisting of 6 - 10 glucose residues. The α-dextrins from amylose are linear, those from amylopectin and glycogen branched, since α-amylase cannot hydrolyse α-1:6 glucosidic inter-chain linkages.

(2) Glucose producing enzymes which catalyse a stepwise hydrolysis of every linkage in a chain of α-1:4 linked glucose residues, yielding glucose as the primary product. It has been found that they can either attack or bypass α-1:6 linkages, thereby being able to attack interior chains. (see Fig. 1. p7a).

(3) Debranching enzymes.

These catalyse the hydrolysis of α-1:6 inter-chain linkages. Five such enzymes have been reported to date.

(4) β-amylases.

β-amylases catalyse the stepwise hydrolysis of alternate

α-1:4 glucosidic linkages, β-maltose being produced. The reaction, which involves a Walden inversion, begins at the non-reducing end-group of the substrate and is stopped by any linkages other than α-1:4. Thus linear molecules are completely degraded, while in branched α-1:4 glucosans such as amylopectin and glycogen only exterior chains are attacked, yielding maltose and a β-dextrin of high molecular weight. The exterior chains of the dextrin consist of only two or three glucose residues (63)(64). β-amylases occur only in higher plants, examples being those from soya bean, barley, sweet potato and wheat, and the action pattern does not appear to depend upon the source.

In this work, β-amylase has been used to detect the presence of anomalous linkages in amylose, and to differentiate between amylose and amylopectin structures.

Meyer's view (17) that amylose was a linear molecule was supported by the fact that it was completely hydrolysed by β-amylase. Later workers, however, reported values ranging from 70 - 100% conversion (62). Peat et al. (24) explained the incomplete hydrolysis of amylose by the presence of 'Z-enzyme'. According to these workers <u>pure</u> soya-bean β-amylase hydrolyses pure potato amylose only to 70% conversion into maltose. Control experiments led to the conclusions that neither inhibition of enzyme action, nor "ageing" of the substrate, was responsible for the low limit. (Meyer (65) had previously suggested that "ageing" caused the low limits by amylose molecules coalescing to make them unavailable to enzymic attack. This resistance was shown to be overcome by a brief treatment with hot, dilute alkali.) Since <u>impure</u> soya-bean β-amylase hydrolysed the amylase completely, Peat et al. suggested that the incomplete

hydrolysis was due to the presence of structural anomalies which were resistant to pure β-amylase. These were either hydrolysed or bypassed by a second enzyme - Z-enzyme - present in impure β-amylase, thus allowing the limit to increase. They concluded that Z-enzyme is a true debranching enzyme, but that the barrier to complete β-amylolysis is not α-1:6 linkages. Since it hydrolyses certain carbohydrates containing β-linkages, e.g. β-1:3 linkages in laminarin (25) Peat and co-workers concluded it was a β-glucosidase and that the barrier is a β-glucosidic linkage. On this basis, they suggested that the Z-labile linkage joins single glucose residues to the main chain. Baum, Gilbert and Scott (66) have suggested that the anomalous linkages are introduced by oxidation during fractionation, but this has not been substantiated by later workers (67). Hopkins and Bird (68) suggested that Z-enzyme is simply a weak α-amylase. The enzymic work of Peat et al. (25)(69) is against this, as is the constancy of the molecular weight during β-amylolysis (70), which indicates that random α-amylolysis is not occurring.

Thus it will be seen that neither the nature of any anomalous linkages in amylose is yet known, nor is the specificity of Z-enzyme.

Two enzyme preparations were used in the experiments carried out in later sections. The first was a commercial preparation purchased from the Wallerstein laboratories (New York), and kindly provided by Dr. D. J. Manners. It was found to be free from maltase and α-amylase, and had an activity of 100 units per mg. as determined

by the method of Hobson, Whelan and Peat (71) (i.e. the number of mgms. of maltose liberated by 1 mg. (or 1 ml.) of enzyme in 30 minutes from soluble starch solution). It does however contain "Z-enzyme". The second, soya-bean β -amylase, was prepared by the method of Peat, Pirt and Whelan (24). The contaminating Z-enzyme is inactivated by heating at 60°C for 30 minutes at pH 4.6 The activity was about 20,000 units/ml. as determined by Hobson, Whelan and Peat's method (71).

Method:-

Digests were prepared either from the butan-1-ol amylose complex or from dried amylose.

Digest Conditions.

The well-centrifuged butan-1-ol complex (20 - 40 mgs.) was dissolved in water, and incubated at 35°C with buffer at pH 4.6 (10 ml.), barley β -amylase solution (20 mgs., 4 ml.), after dilution to 50 ml. in a standard flask. The digests were incubated at pH 4.6 as Z-enzyme is acid-labile, being deactivated at pH 3.6 (25). Dried amylose samples were moistened with ethanol before dissolving in 0.2N-potassium hydroxide with shaking, and slight warming if necessary. The solution was then neutralised with N-hydrochloric acid, using phenolphthalein as indicator. Reducing sugars were determined by the alkaline ferricyanide method (see Viscometry section), which had been calibrated against maltose and glucose.

In the case of the soya-bean β -amylase, digests were made up as above, with 0.1 ml. pure soya-bean β -amylase instead of the

Barley β -amylase.

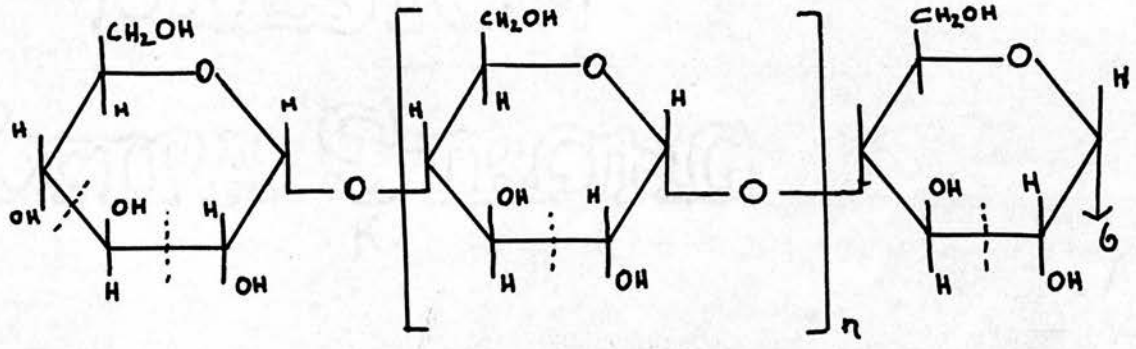
Incubations were normally allowed to proceed overnight, although it was found that the reactions were complete in several hours.

3. PERIODATE OXIDATION.

Introduction.

Malaprade (72) showed that periodate ion caused selective oxidative fission of α-glycols, the rate being greater for cis than trans-α-glycols. In 1936 the reaction was applied to the study of sugar ring forms by Jackson and Hudson (73), who then extended it to starch and cellulose (74).

Periodate will attack 1, 2, 3-triol groupings with the liberation of formic acid. Hence in the case of starch,



(n + 2) molecules of periodate willbe consumed in oxidising the $C_2 - C_3$ bonds in each ring, and liberating one mole of formic acid from the non-reducing terminal D-glucose unit. Thus estimation of the formic acid released per anhydroglucose unit will enable the average chain length to be found.

In an unfractionated starch, the amounts of formic acid liberated from the single non-reducing end-group in the amylopectin component, and from the non-reducing and reducing end-groups in the amylose, are so small that they may be neglected to the first approximation.

The advantages of this method over the classical methylation method are that it is quicker, less tedious, and can be carried out on ten times less material, which often may be of prime importance. It also eliminates the possibility of any degradation which might occur during methylation.

In general, periodate studies have supported the earlier results from methylation studies. It can also give additional structural evidence. It can theoretically indicate the presence or absence of inter-chain linkages involving C_2 or C_3 (75), since such units would not be attacked, and D-glucose would be found in the hydrolysate of the oxidised starch (76 - 82). The fact that the average chain length of the amylopectin component is the same when determined by both methods (83), further indicates that no glucose units are present which are linked solely through C_1 and C_6.

However, one of the difficulties in the periodate method is the danger of over-oxidation before the theoretical stage is reached. In attempts to minimise this, Brown, Halsall, Hirst and Jones (93) used the sparingly soluble potassium salt at room temperature, and in addition kept the formic acid concentration low. Anderson, Greenwood and Hirst (79) also examined the conditions nec-:essary for avoiding this, and for the correct estimation of the liberated formic acid. Their method has been applied to the unfractionated cereal starches. In the case of potato amylopectin, the method of Potter and Hassid (20) was used. These authors suggested the use of sodium meta-periodate at 2°C. The methods will be described separately.

EXPERIMENTAL.

Oxidation with Potassium meta-periodate at 16°C. (79)

Wheat and oat starches were treated by this method. Samples (250 - 400 mg.) were suspended in 0.56M-potassium chloride (60 ml.) to which was added 0.2M sodium meta-periodate. The flasks were then shaken in the dark in a constant temperature room at 16°C. After various times 10 ml. samples were withdrawn by pipette, 1 ml. ethylene glycol added, and the mixture shaken in the dark for at least 10 minutes to destroy excess periodate. The suspension was then titrated potentiometrically with carbonate-free 0.01M-sodium hydroxide to pH 6.25, when quantitative estimation of the formic acid is complete (79). Carbonate-free nitrogen was bubbled through the suspension before and during the titration. This method was found to require no correction for the acidity of the samples or reagents.

The time necessary for the uptake of periodate to reach the theoretical value of 1.03 - 1.05 moles of periodate per anhydroglucose unit was obtained by Fleury and Langes (84) indirect method for determining the residual periodate concentration.

A number of individual mixtures, each containing about 50 mgs. of starch were set up, and analysed at the required times as follows. Excess sodium bicarbonate was added to buffer the solution, then the reduction of IO_4^- to IO_3^- was brought about by the addition of excess 0.1M-sodium arsenite together with potassium iodide, the last acting as a catalyst. After shaking in the dark for 15 minutes, the excess

arsenite was back-titrated with 0.05M-iodine.

Oxidation with Sodium meta-periodate at 2°C. (20)

Potato amylopectin (200 mgs.) in 3% sodium chloride (5 ml.) and 0.37M-sodium meta-periodate (5 ml.) was shaken in the dark for 25 hours at 2°C. 1 ml. of ethylene glycol was then added and, after standing for one hour at room temperature, the solutions were titrated with 0.01M-sodium hydroxide. A mixed indicator, methyl-red (0.04%, 10 vols.) and methylene-blue (0.04%, 1 vol.), was used by titration to a green end-point (pH 6 - 6.5). In this method a reagent blank was necessary.

4. VISCOMETRY.

Introduction.

The measurement of the viscosity of a polymer solution does not give an absolute value for the molecular weight of the polymer, as the method requires calibration. For this reason, notwithstanding the ease and accuracy with which they can be carried out, viscosity measurements are normally only used for the comparison of the molecular weights of a series of samples.

In this work viscosities were measured in a modified Ubbelohde viscometer (85)(86) in a bath controlled to 225 $\pm$ 0.001. In a capillary viscometer of this type, the viscosity (η) is given by

$$\eta = K\,dt - \frac{B\,d}{t} \qquad (12)$$

where t is the flow time of a fixed volume,
K and B are constants,
and d is the density of the liquid.

B is the kinetic energy factor which may be determined by measuring t with different liquids of known viscosity and density. The value of B may be reduced by increasing the flow time well over 100 seconds and, for the viscometer used in this work, B was negligible.

Hence $\eta = K\,dt$ solution viscosity

and $\eta_o = K\,d_o t_o$ solvent viscosity.

Staudinger defined the specific viscosity (η_{sp}) as

$$\eta_{sp} = \eta/\eta_o - 1.$$

Hence $$\eta_{sp} = \frac{K\,dt}{K\,d_o t_o} - 1$$

$$= \frac{K\,dt - K\,d_o t_o}{K\,d_o t_o} .$$

Now for dilute solutions the approximation $d = d_o$ is valid,

$$\therefore \quad \eta_{sp} = \frac{t - t_o}{t_o}$$

η_{sp} and the concentration (c) of a polymer solution are related by the series

$$\eta_{sp} = Ac + Bc^2 + Cc^3 + \ldots \quad \ldots$$

and for dilute solutions ($c < 1$ gm./100 ml.) terms above the square are negligible, leaving

$$\eta_{sp} = Ac + Bc^2$$

or

$$\eta_{sp}/c = A + Bc.$$

This quantity η_{sp}/c is known as the viscosity number and by extrapolation of the graph of η_{sp}/c versus c to zero concentration, the limiting viscosity number $[\eta]$ is obtained. This latter is characteristic of the polymer solution and from it the molecular weight of the polymer can be obtained.

Staudinger proposed the empirical relationship (87)

$$[\eta] = K\,M$$

where K is a constant, M is the molecular weight of the polymer.

This has been modified by Mark (88) and Houwink (89) to

$$[\eta] = K M^a$$

K and a must be evaluated for each polymer-solvent system, using fractions of known molecular weight.

The viscosity average molecular weight for a specific value of (a) is

$$\bar{M}_v = \left[\frac{\sum n_i m_i^{1+a}}{\sum n_i m_i} \right]^{1/a}$$

where n_i is the number of gram moles of molecular weight m_i the summation being taken over all values of i.

If we compare this expression with those for (90)(91)

(1) Number average molecular weight $\bar{M}_n = \frac{\sum n_i m_i}{\sum n_i}$

and (2) Weight average molecular weight $\bar{M}_w = \frac{\sum n_i m_i^2}{\sum n_i m_i}$

then we see that if 'a' has the value unity, the viscosity average is identical with the weight average. Since 'a' is normally between 0.5 and 1.5, molecular weights of samples for calibration purposes should be obtained using methods which give weight average values.

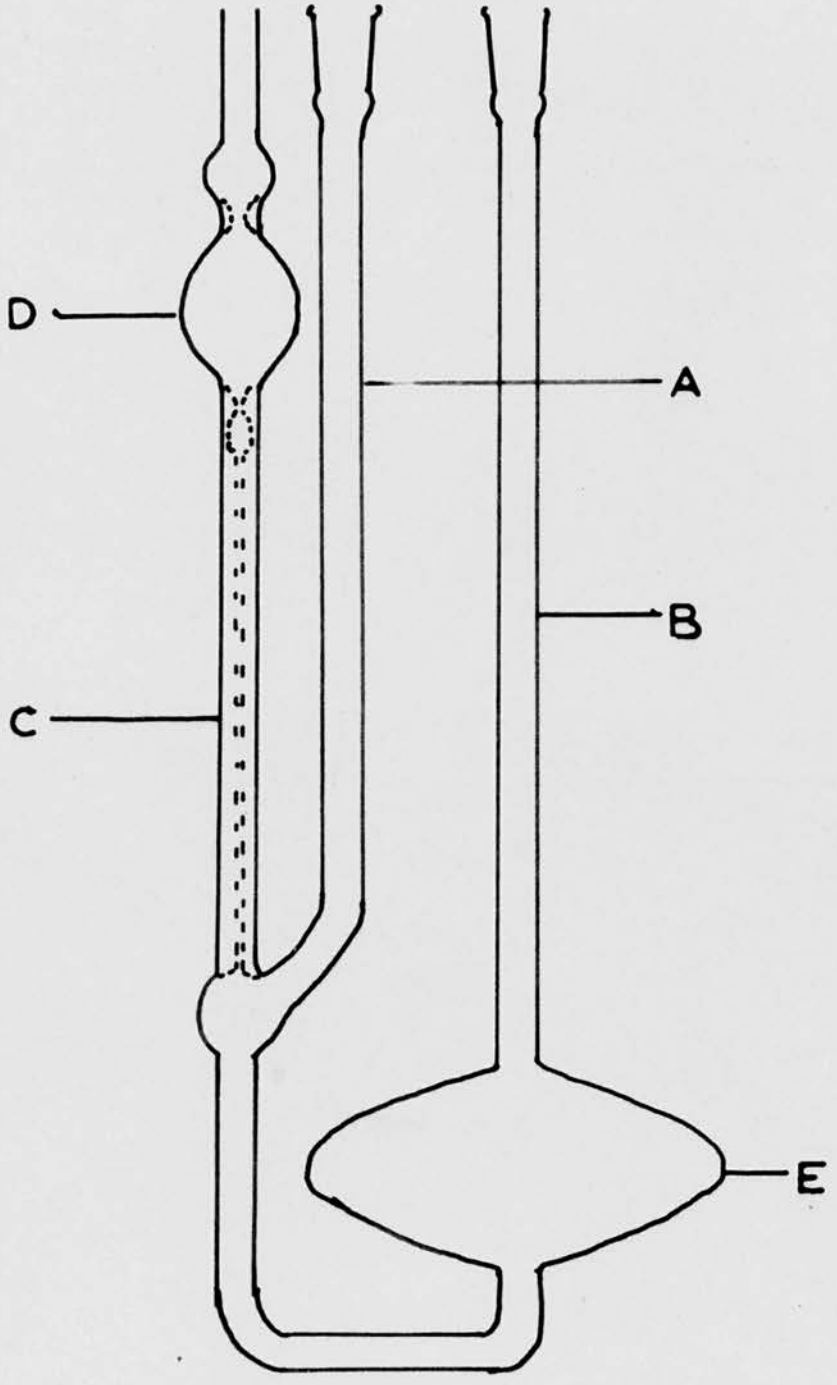
Experimental.

The solvent used for all the viscosity measurements was M-potassium hydroxide. Both solution and solvent were carefully filtered through G3 and G4 sintered-glass filters before adding to

the viscometer, since the fine capillaries were very easily blocked. The viscometer (see Fig. 4, p.29a) required a minimum of 10 mls. of solution. This was introduced into tube A, which was then closed with a ground glass stopper (B.10) and pressure exerted on B, forcing liquid up tube (C) to fill bulb (D). When the pressure was released the liquid level fell and time taken for it to pass between two marks, one above and one below (D) noted. The average of at least two concordant determinations was taken, using a stopwatch reading to 0.05 second.

This type of viscometer had the advantage of allowing dilutions to be made in situ. Solvent equilibrated with the temperature of the bath was used for dilution so cutting out the time required for temperature equilibrium to be reached after each dilution. The required amount was pipetted down tube (A) and mixed in bulb (E). Concentrations of the solutions were determined by two methods. The amylopectin fractions, which had been freeze-dried, were weighed out directly and dissolved in a given volume of solvent. The amylose fractions were dissolved directly from the well centrifuged butan-1-ol complex. The concentration was then determined, in triplicate, by acid hydrolysis of an aliquot and estimation of the liberated glucose by the alkaline ferricyanide method (92)(93) as follows. The aliquots were transferred by pipette to 6" x 1" Pyrex tubes fitted with a ground glass stopper and a mark at 10 ml. After neutralising, 1 ml. of 3N sulphuric acid was added and the sample hydrolysed for two hours on a boiling water bath. The tubes were then cooled, one drop of bromo-cresol green added, the solution

FIG 4



Modified Ubbelohde Viscometer

neutralised, and then made up to the mark. This was then oxidised by heating with alkaline ferricyanide, and the ferrocyanide produced determined by a slight modification of Hassid's method (94). The cooled oxidised solution was acidified with 5 ml. 5-N sulphuric acid and titrated with 0.02N ceric sulphate, using two drops of 0.4% xylene cyanol F.F. as indicator. The colour change at the end point is "sage-green" to "whisky yellow"; mechanical agitation aided the end-point. Calibration was carried out with glucose solutions subjected to the same treatment with acid as that used to hydrolyse the starch components.

5. ULTRACENTRIFUGATION.

Introduction.

By the use of dynamic or equilibrium measurements in the ultracentrifuge it is possible to obtain the molecular weight of a polymer. Information can also be obtained regarding its dispersity and the shape of the molecules in solution.

A solid particle suspended in a liquid tends to migrate through the liquid due to the gravitational force field. As the size of the particles diminishes, so does the rate of sedimentation, while diffusion increases. To produce measurable sedimentation in a measurable time, and without excessive diffusion, use must be made of higher fields of force than gravity alone. Such force-fields can be obtained by rotation of the solution, that is exposing it to the influence of a centrifugal field.

Svedberg and co-workers (95)(96) initiated studies into the use of high centrifugal fields for quantitative measurements. The publication by Svedberg and Pedersen in 1940 of "The Ultracentrifuge" (97) forms the standard reference work for all phases of ultracentr-:fugation. A more recent review has been published by Pickels (98).

Several difficulties arose in developing the method to produce measurable sedimentation conditions. To obtain convection-free sedimentation, an essential requirement is that the containing vessel or cell must be sector-shaped.

This ensures that any particles close to the walls continue to pursue a course parallel to them during sedimentation, thus preventing any sedimentation from or to the walls, which would produce convective

disturbances. The cell fits into a rotor, and rotors have been developed which are capable of routine operation at 60,000 r.p.m. The work described here was carried out on a Spinco Model E, electrically-driven ultracentrifuge (Spinco Division, Beckman Instruments Corporation, Belmont, California), capable of speeds of up to 60,000 r.p.m. (Fig. 5, p.32a)

The type of measurement used in this work was of sedimentation velocity using high speeds of centrifugation. From such measurements the sedimentation constant of the solute is obtained. This is usually expressed in S or Svedberg units.

Svedberg (97) showed that the molecular weight (M) of a polymer could be calculated from the following expression,

$$M = \frac{R\,T\,S}{D(1 - V\rho)},$$

where S is the sedimentation constant, D is the diffusion constant, V is the partial specific volume of the solute, ρ is the density of the solution, and R and T the gas constant and absolute temperature respectively. The diffusion constant can usually be measured in a separate experiment, for example, in an Antweiler microelectrophoresis and diffusion apparatus (99). The partial specific volume may be obtained by the use of a pyknometer (100).

In a dilute polydisperse polymer solution, separation occurs in the ultracentrifuge because of variation in the sedimentation constants of molecules of different sizes. If the variation is large, and diffusity not too great, then a sharp boundary forms for

FIG 5

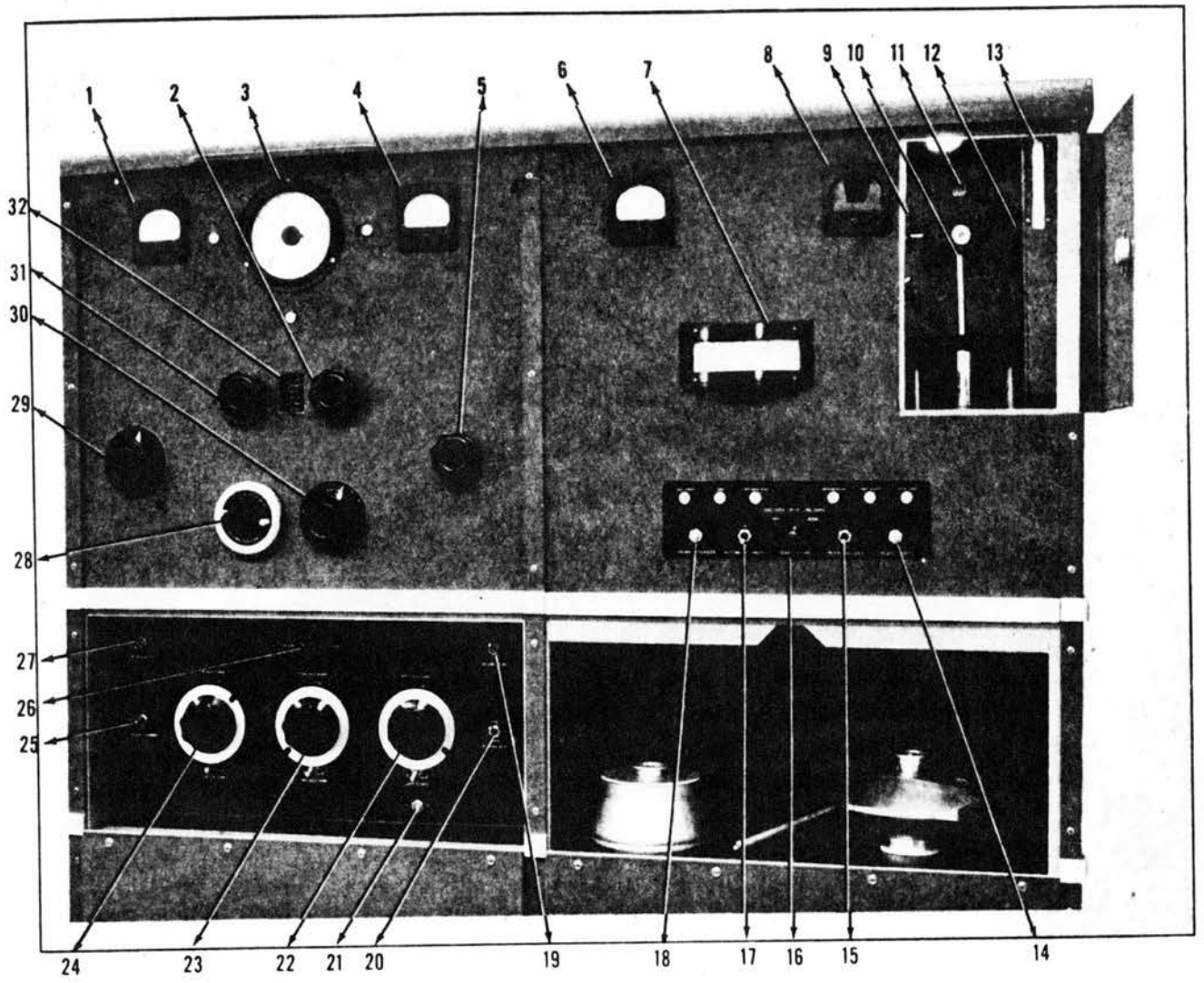


Fig. 5. INSTRUMENTS AND CONTROLS OF MODEL E ULTRACENTRIFUGE

1. Drive Motor Voltmeter
2. Individual Speed Selector Knob
3. Tachometer (Rotor Speed in 1,000th RPM)
4. Drive Motor Ammeter
5. Drive Motor Voltage Control Knob
6. Vacuum Indicator
7. Thermocouple Meter
8. Viewing Screen
9. Schlieren-Diaphragm-Angle Adjustment Knob
10. Reference Thermocouple Thermometer
11. Schlieren-Diaphragm-Angle Indicator
12. Photographic Plate Position Indicator
13. Slot Receiving Photographic Plateholder
14. Plate Shift Push Button
15. Plateholder Travel-Direction Switch
16. Thermocouple Selector Switch
17. Automatic Photo Switch
18. Viewing Screen Push Button
19. Vacuum Gauge Switch
20. Braking Rate Switch
21. Lightsource Intensity Switch
22. Lightsource Water Valve and Lightsource Switch
23. Diffusion Pump and Drive Motor Cooling Water Valve and Diffusion Pump Switch
24. Vacuum Chamber Air Valve and Vacuum Pump Switch
25. Vacuum Chamber Hoist Switch
26. Vacuum Gauge Adjustment Knob
27. Refrigeration Switch
28. Exposure Time Adjustment Dial
29. Time Switch
30. Exposure Interval Selector Knob
31. Speed Range Selector Knob
32. Speed Setting Indicator

each species enabling the amount of each to be estimated. If there is a small difference in molecular size, then diffusion causes an overlapping of the boundaries. Spreading of the sedimenting layer has been used by Gralen (101) to estimate polydispersity.

Description of Instrument.

The rotor spins within a heavy steel chamber, which can be evacuated to pressures below 1 μ Hg. by means of a water-cooled oil diffusion pump and a mechanical backing vacuum pump. (For added protection, in the event of rotor breakage or escape, there is a 2" inner layer of armour steel.) The drive shaft, consisting of 1/10" piano wire, is flexibly supported, serving to make the rotor self-balancing so that precision weighing of the cell and counterpoise is not required, and leakages from the rotor cell cause no harm.

The rotors were of duralumin and contained two symmetrically placed holes, one accommodating the solution cell, the other the counter-poise. The cell consists of a housing into which is placed the centrepiece, which has a sector-shaped slot. Quartz discs, each held in a sector-cup, close off the ends of the slot by making a seal with the faces of the centrepiece. A screw ring engaged by a special torque ensures even tightening of the cell assembly every time. The cavity so formed is filled with the sample through a small hole in the centrepiece and cell by means of a syringe. The hole is then sealed with a plastic washer and screw plug. Cells with both 12 mm. and 30 mm. length centrepieces, were used, and for the alkaline solutions Kel-F centrepieces (polymono-chlorotrifluorethylene) were necessary.

Temperature gradients in the fluid sample, giving rise to convective disturbances, must also be avoided. The rotor temperature rise was found to be 0.6°C/hour at 60,000 r.p.m., and correspondingly less at lower speeds. Even this can be virtually eliminated by using the refrigerating system to keep the average temperature of the surroundings about 10 - 15° below that of the rotor. Since runs were normally completed within 30 minutes, and speeds of less than 60,000 used, this was not used.

Optics.

In this instrument the refractive-index gradient in the ultracentrifuge cell is measured directly by means of a Schlieren optical system (102)(103). The boundary, which is associated with the maximum rate of change of concentration, causes the maximum deviation of light, since the refractive index is directly proportional to the solute concentration. In essence the system converts a vertical deviation of light at the boundary into a horizontal displacement of a light point on a photographic plate, without alteration of the point's vertical height which itself corresponds to a certain level in the boundary. These displacements corresponding to positions within the boundary result in the light-points forming a smooth continuous curve on the plate. The deviation of light is thus obtained as a function of cell-height (distance from axis of rotation).

The source of illumination is a water-cooled mercury arc lamp. A collimating lens ensures parallel light passes through the cell.

The light leaving the rotor chamber is directed, by means of a condensing lens, upon a full surface mirror inclined at 45°, which deflects the vertical beam in a horizontal direction. The beam then passes through a Schlieren diaphragm, which has an inclined bar or wire, after which it is focussed on the photographic screen. By the use of a partially reflecting mirror, it is possible to view the Schlieren diagram and photographs were taken manually.

Experimental Techniques.

Studies on starch components have been carried out on derivatives dissolved in organic solvents (104), but the formation of such derivatives may lead to degradation (16). The study of aqueous solutions is complicated by the fact that both amylose and amylopectin tend to aggregate in such media. Dilute alkali was found to be a suitable solvent, preventing aggregation and yielding solutions which were stable for the time necessary for measurements to be made.

In the filamentous molecules studied here, interaction occurs between molecules in concentrated solutions (e.g. > 0.4g./100ml. for amylopectin). Free sedimentation is only achieved at low concentrations and the sedimentation and diffusion constants are dependent upon concentration. Hence, measurements were made at several different low concentrations, and the results extrapolated to infinite dilution (101). Amylopectin (ca. 25 mgs.) was dissolved in 0.2M-potassium hydroxide (5 ml.), giving an initial concentration of 0.5 gm./100 ml. Solutions of decreasing concentration were then prepared from this by dilution. Due to the very large concentration dependence of

amylopectin (see later), an Agla micrometer syringe was used to give accurate dilutions. Each solution was spun and the movement of the boundary photographed. It was found by experiment that the optimum speed for undegraded amylopectin was 15,000 r.p.m., although some degraded samples required higher speeds. The rates of sedimentation were obtained by measurement of the position of the boundary to 0.01mm. directly from the photographic plate. This was carried out using a two-dimensional travelling microscope. The sedimentation constants were evaluated from the equation -

$$S = \frac{2.303 \, d \log_{10} x}{dt} / w^2$$

where x is the distance in cms. from the centre of rotation,

t is the time in seconds from the start of acceleration, and

w is the angular velocity in radians.

The plot of $\log_{10} x$ against t was linear, and allowed S to be calculated. The time before sedimentation started could be obtained by noting where these lines cut the time axis at the value of $\log_{10} x$ for the meniscus.

The relative temperature of the rotor was measured before and after the completion of a run, by means of a thermocouple inserted in the base. The temperature at any time during the run was obtained by linear interpolation. Sedimentation constants were reduced to values which theoretically would be obtained if the medium had the viscosity and density of water at 20°C (97). Thus results are given as S_{20}.

SECTION II.

STUDIES ON CEREAL STARCHES.

INTRODUCTION.

The starches studied in this Section were isolated from oats (var. Milford) and wheat (var. Victor II). This section deals with their isolation from the grain, and purification. Fractionation methods are then evaluated, and sub-fractions of the amyloses examined.

II.(1). ISOLATION AND PURIFICATION OF STARCH SAMPLES.

The procedure for the isolation of the starch from oats and wheat was identical. The whole grain was lightly milled, then immediately immersed in boiling aqueous ethanol in order to deactivate enzymes and extract fats. The defatted flour was then shaken vigorously with distilled water (1 l./200 g.) and toluene (10 ml./litre) for 24 hours, and filtered through a double layer of muslin. The procedure was repeated until yields became small, when the extracts were combined and centrifuged to yield an impure starch deposit.

These products were then purified from contaminating protein using a modified Swag technique (105)(106). The impure starch was suspended in M-sodium chloride to give a suspension of sp.gr. 1.07, and toluene ($\frac{1}{8}$ vol.) was added.

After shaking for several hours the mixture was centrifuged, first for five minutes at 750 r.p.m. (M.S.E. "Major" centrifuge), followed by an increase in speed to 1,200 r.p.m. over the next five minutes. This procedure yielded two layers; the upper proteinaceous layer was discarded, while the lower one, consisting mainly of starch, was resuspended, shaken and centrifuged. This was repeated until the protein content remained constant. Salts were then removed by decantation, and the purified starches exhaustively defatted by placing in a thimble, and extracting in a Soxhlet apparatus for 24 hours, with 80% aqueous methanol in the reservoir flask. The starches were then stored at $0^{\circ}C$ under aqueous methanol.

Results

1. Determination of % protein.

The percentage protein was obtained from %N x 6.25, the percentage nitrogen being determined by the semi-micro Kjeldahl method (107).

	% Protein	
Starch	Impure	Pure
Oat	4.9	0.24
Wheat	6.5	0.33

2. Determination of % Amylose.

For the method of dissolving the sample and titration conditions see Section I.

Wheat Starch

a	b	c (x-axis)	d	e	f (y-axis)
5	4.05	4.05	0.95	0.95	0.14
5	2.30	6.35	2.70	3.65	0.53
5	1.70	8.05	3.30	6.95	1.00
5	1.80	9.85	3.20	10.15	1.46
5	1.20	11.05	3.80	13.95	2.01
5	0.85	11.90	4.15	18.10	2.60
5	1.05	12.95	3.95	22.05	3.17
5	1.05	14.00	3.95	26.00	3.74
5	1.00	15.00	4.00	30.00	4.32
5	1.00	16.00	4.00	34.00	4.89
5	2.60	18.60	2.40	36.40	5.24
5	3.95	22.55	1.05	37.45	5.39
5	4.30	26.85	0.70	38.15	5.49
10	9.30	36.15	0.70	38.85	5.59
10	9.25	44.40	0.75	39.60	5.70

Graph - See Fig. 6, p. 40a

Key

(a) = additions iodine in Agla units (50 units = 1 ml.) to starch half-cell.

(b) = additions iodine in Agla units to blank half-cell.

(c) = Σb ; (d) = a - b ; (e) = Σd.

(f) = e x 0.1439, the factor which converts iodine uptake in Agla units of 0.00942 N iodine per 16.63 mgs. starch into mg. of iodine bound per 100 mg. starch.

From Graph, Iodine Affinity (I.A.) = 5.0.

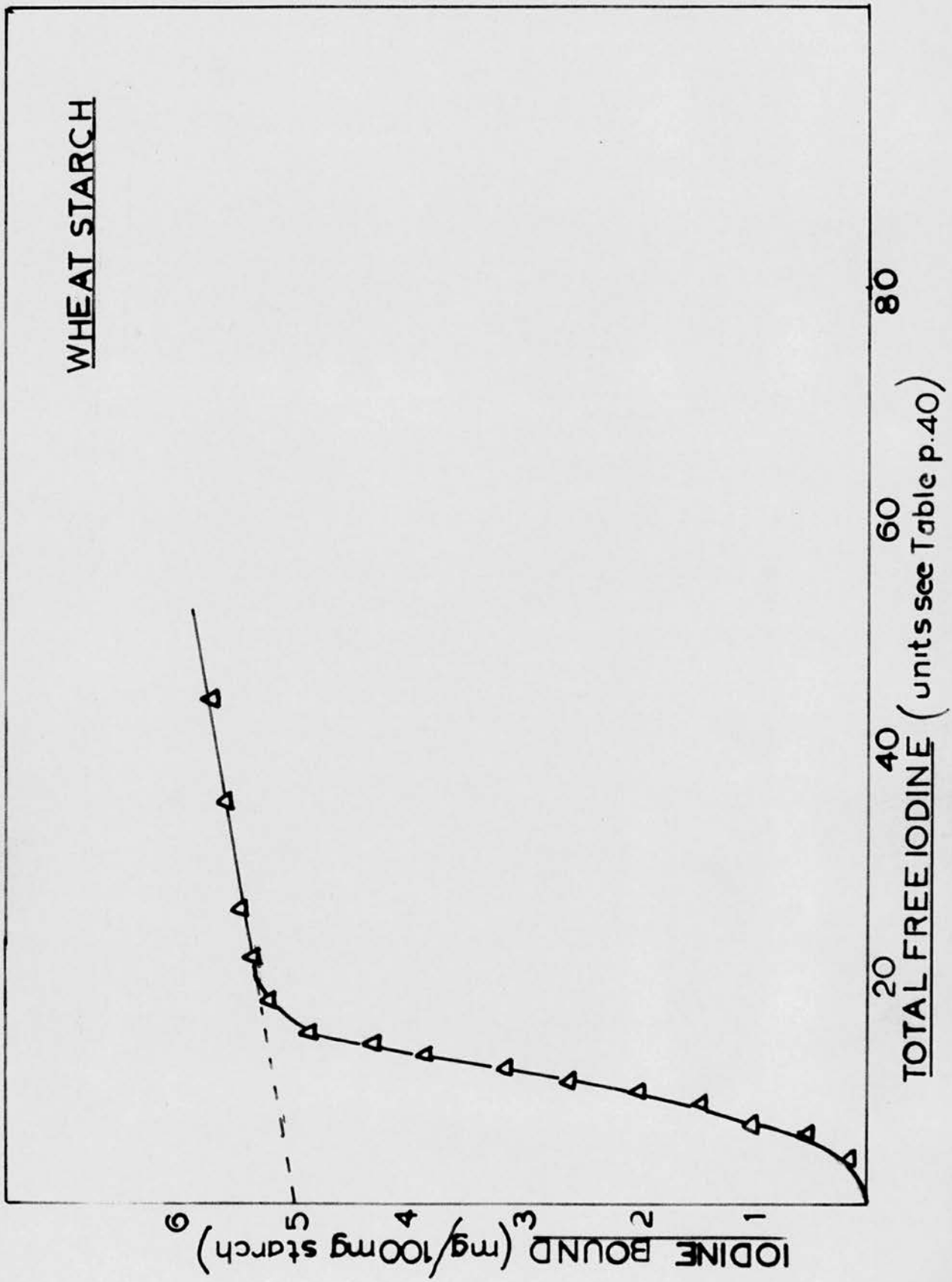
Under these conditions, pure wheat and oat amyloses bind 19.0% of their own weight of iodine (see later).

$$\therefore \quad \% \text{ Amylose} = \frac{5.0}{19.0} \times \frac{100}{1} = \underline{26.3\%}$$

Similarly for oat starch, I.A. = 5.13.

$$\therefore \quad \% \text{ Amylose} = \underline{27.0\%}$$

FIG 6


WHEAT STARCH
IODINE BOUND (mg/100mg starch)
1
2
3
4
5
6
20
40
60
80
TOTAL FREE IODINE (units see Table p.40)

3. Determination of Periodate Uptake - average value of ($\bar{R}$) and calculated chain length for Amylopectin (Section I).

$$\bar{R} = \frac{1000 \times x}{y \times z \times 162}$$

where x = gms. starch sample,

y = molarity of NaOH,

z = titre of yM-NaOH.

Starch	Average Value ($\bar{R}$)[a]	Amylose Content[b] (%)	Calc. C.L. for amylopectin.
Oat	26.8	27.0	19.6
Wheat	25.6	26.3	18.9

(a) Ratio of terminal to non-terminal groups.

(b) From potentiometric iodine titration curves.

DISCUSSION.

It is essential that the starches should be freed from fatty acids and protein before structural studies are undertaken, for both interfere in fractionation (14), interaction with iodine (28), and oxidation with periodate (79). On the other hand, care must be taken that the methods adopted do not cause degradation if studies of fine structure and molecular size are to be made.

Since typical fat solvents (e.g. carbon tetrachloride, ether) were unable to remove lipid material from certain starches (108), it was at one time thought that this material was present as an ester. That it was, in fact, simply adsorbed on the starch as an impurity was shown by its removal with water-miscible fat solvents with sufficient hydrophilic loading (e.g. lower aliphatic alcohols, aqueous dioxane) (109)(110).

For both starches studied here, the purely physical methods of purification used did not reduce contaminating protein below about 0.3%; a value about ten times that for potato starch. Johnston's method (111), which involved extraction with 1% ammonium oxalate was also unsatisfactory, a similar percentage of protein remaining. In fact, tightly-bound protein appears to be a characteristic of these laboratory-prepared granular starches, and it may well be incorporated in the granule. Although its significance with regard to granular structure is not known, it may well hinder the swelling properties of the granule and so influence its dispersive properties. This may in turn influence fractionation since this requires complete dissolution of the starch.

II.(2). FRACTIONATION OF PURIFIED STARCH SAMPLES.

Introduction.

Maquenne and Roux (112) in 1905, using retrogradation from starch sols as a fractionation procedure, were the first to attempt to isolate and study the components of starch. Progress was slow, however, and there was a great deal of confusion until improved techniques were developed for isolation and purification of starches, and for obtaining and characterising their components. Many of the difficulties arose through the use of samples degraded by the drastic conditions of isolation or fractionation. For example, the use of a Waring Blendor to disintegrate swollen granules prior to fractionation causes mechanical degradation of the amylopectin component (61). In the preparation of waxy-maize starch it has been shown that shortening the period of contact with sulphur dioxide - water gave an equally pure starch but with a considerably higher viscosity (113).

Methods which have been suggested for fractionation include electrophoresis, selective retrogradation, selective adsorption, aqueous leaching, and selective precipitation.

Electrophoresis depends upon the polarity imparted to one component when a dilute starch sol is subjected to electromigration. The effect is due to non-carbohydrate material present e.g. organic phosphate, fatty acids. Since this method presupposes, erroneously, that any such material is linked only to one component, separations are slow and incomplete. Furthermore the electrolysis may cause

localised acidity or alkalinity leading to degradation.

Selective Retrogradation was used by Maquenne and Roux as mentioned above. They found that starch pastes, or autoclaved solutions, on standing at room temperature formed a precipitate. This method has been reported by other workers, but retrogradation of the amylose necessarily co-precipitates a large amount of amylopectin.

Selective Adsorption has not been used to effect a complete fractionation, but rather to remove traces of amylose from the amylopectin fraction. Tanret (114) found that by dissolving the amylopectin in water, and treating with cotton wool, that the cotton wool selectively adsorbed any amylose. Pacsu's work (115) seemed to support this. It has been shown, however, that fatty acids were being removed from the cotton wool, and suppressing the formation of the iodine complex by which the amylose is detected (60). Similarly, Meyer and Gibbons (116) reported the amylose could be removed as an insoluble complex with stearic acid. Again this was shown to be due to retention of stearic acid (58).

Aqueous Leaching of the whole granule has often been used to fractionate starch (117 - 120). Reports as to the efficiency of this method are at variance, but since information can be obtained about granular structure, it was examined here.

The most successful method of fractionation is based on the Selective Precipitation of amylose as an insoluble complex from an aqueous dispersion of the granules. Successful fractionation

requires the complete dissolution of the granule without either hydrolytic degradation or retrogradation. Alsberg in 1926 (121) noticed the formation of crystals when starch sols were treated with alcohol, but it was Schoch (14) who indicated its applicability as a mode of fractionation. The original precipitant used by Schoch was butan-1-ol, but with the development of the potentiometric iodine titration method for estimating the purity of the fractions, the efficiency of various reagents could be compared.

Whistler and Hilbert (55) suggested any water-soluble compound with either donor or acceptor groups capable of hydrogen bonding were suitable. They studied the nitro-paraffins, but the lowered iodine-binding powers of the amyloses (14.2 to 18.2%) indicate they were contaminated with amylopectin.

It was found that thymol and cyclohexanol were suitable complexing agents for potato starch amylose (122)(123). Higginbotham and Morrison (60) found butan-1-ol and pyridine gave similar results, while iso pentyl alcohol was less efficient. Cowie and Greenwood (124) found that for potato starch, pyridine was inefficient while pentan-1-ol was comparable to thymol.

A general method for obtaining both pure amylose and amylopectin appears to be that involving the use of thymol for the first fractionation, after which the amylose is recrystallised as the butan-1-ol complex (78).

The possibility of inadvertent degradation may occur during fractionation. Schoch's original method used autoclaving as a

means of dispersing the granules, but this is likely to lead to degradation (61)(60). The amylose component is more seriously affected than the amylopectin, and the only conclusive test for degradation is to measure the molecular size. For this purpose, viscosity measurements are the most convenient, since measurements of changes in observed reducing power are not sensitive enough. The degradation of amylose at elevated temperatures has been studied by Bottle, Gilbert, Greenwood and Saad (23), and they suggest dissolution should be accomplished by heating under reflux in an oxygen-free atmosphere.

In some cases (for example, pea starches), the usual method of refluxing - or even autoclaving - does not give complete dispersal. For these pre-treatment with sodium hydroxide (125)(60) or liquid ammonia (126)(127) to cause gelatinisation has been suggested.

EXPERIMENTAL.

(a) Aqueous leaching at 70°C.

A suspension of starch (2% in water) was stirred at 70°C in a water bath for 1 hour under nitrogen. Gelatinisation occurred within 10 minutes. After cooling to 50°C the mixture was centrifuged for 15 minutes at 17,000 r.p.m. (M.S.E. Major centrifuge). After filtration through a sintered glass filter (G.3), excess butan-1-ol was added. Amylose complex formation occurred immediately, but the mixture was allowed to stand for 24 hours at room temperature to ensure complete precipitation. After centrifugation, the precipitate was stored under butan-1-ol-saturated water. Where dried samples were required (for example, for measurement of iodine uptake), they were obtained by dehydrating a portion of the complex by stirring several times with butan-1-ol, and then drying *in vacuo* at 80°C.

(b) Aqueous leaching at 98°C.

A suspension of starch (0.5% in water) was deaerated at room temperature by means of a stream of oxygen-free nitrogen for 30 mins. The suspension was then placed in a boiling water bath, and stirred for a further 10 minutes under nitrogen. The resultant gelatinised mixture was cooled to 30°C under nitrogen then centrifuged down (2,500 r.p.m., M.S.E. 20 mins.). The supernatant was filtered through a sintered glass filter (G.3) to remove any solid particles, and saturated with butan-1-ol. There was no immediate precipitate in this case, but it occurred on standing overnight at room temperature. This was then centrifuged and stored as before. The supernatant

liquor on freeze-drying yielded a small amount of material. The gelatinised sediment was then washed with distilled water (6 times), and redispersed in water under nitrogen, in a boiling water bath, for a further 10 minutes. After isolation as before, the solid obtained was washed with distilled water, and freeze-dried to yield the "amylopectin" fraction. The supernatant was saturated with butanol but no precipitation occurred after this, and freeze-drying yielded no product.

(c) Selective Precipitation.

(i) Without pre-treatment of the granule.

Preliminary experiments with Mr. J. M. G. Cowie had shown that thymol was a more suitable initial precipitant than cyclohexanol, butan-1-ol, pentanol or pyridine. Accordingly, this was used for oat and also for wheat.

The fractionations were carried out in a three-necked Quickfit flask. One neck held a reflux condenser, the second a stirring gland and the third was used for the nitrogen inlet. Starch (0.5%), defatted and free from protein, was dispersed in boiling water with vigorous stirring. In order to prevent oxidative degradation at this temperature (23), nitrogen was bubbled through continuously.

To ensure the nitrogen was free from oxygen, it was first bubbled through a series of wash-bottles containing alkaline pyrogallol (128). This is prepared by mixing 3 volumes concentrated potassium hydroxide (500 gm./500 ml. water) with 1 volume pyrogallol (30 gm./100 ml. water).

After boiling for 1 hour, the temperature was allowed to fall to 60°C, powdered thymol added (1 gm./litre), and stirring continued for a further 30 minutes. The mixture was then left for 3 days at room temperature, and the amylose-complex which precipitated was removed on the Sharples supercentrifuge. The supernatant liquor containing the amylopectin component was freeze-dried, refluxed with methanol three times, then redissolved in water and freeze-dried. This procedure was modified to yield a more soluble product; the thymol was extracted by shaking twice with ether, the ether allowed to evaporate, and the liquor freeze-dried.

The amylose-thymol complex was directly dispersed in boiling water under nitrogen, and the amylose reprecipitated by saturating the solution with butan-1-ol, stirring at 95°C for 1/2 hr., and then allowing the solution to cool slowly under nitrogen. This recrystallisation was normally repeated a further twice, all three butan-1-ol complexes being removed on the centrifuge.

The effect of carrying out the fractionation in a buffered solution was also examined. The procedure was the same with phosphate buffer (pH 6.47, 2 ml./100 ml. dispersion) being added prior to the addition of the starch.

(ii) With potassium hydroxide pre-treatment of the granules.

Oat starch (2 gm.) was suspended in 90 ml. water, cooled to 3°C, 10M-potassium hydroxide (10 ml.) at 3°C was added slowly and the mixture stirred for 10 minutes. Cold water (100 ml.) was added and the mixture stirred for a further 5 minutes. After

neutralising with glacial acetic acid (pH 5.8 - 6.1, pH-meter), the solution was boiled for 1 hour under reflux, with stirring and under a nitrogen atmosphere. On addition of amyl-alcohol (80 ml./litre) the solution became turbid. This was allowed to cool over 1½ hours, and kept at 3°C for 4 days. The amylose-complex was removed on the Sharples supercentrifuge. A further volume of amyl-alcohol was added to the supernatant, and after keeping at 3°C for 3 days, any precipitate was centrifuged off. The amylopectin was then precipitated with an equal volume of methanol, then redissolved and reprecipitated a further twice to remove salts and finally freeze-dried.

Wheat starch was similarly fractionated, but after the first methanol precipitation the amylopectin was dialysed for 5 days before freeze-drying.

CHARACTERISATION OF STARCH PRODUCTS.

(a) Amylopectin

From potentiometric measurement of the iodine bound, the iodine affinity of the product, and hence the amount of contaminating amylose present, could be calculated.

(b) Amylose.

The purity was determined as for the amylopectin above. A measure of the molecular-weight was obtained from the limiting viscosity number $[\eta]$ in 1M-potassium hydroxide. Values for the average degree of polymerisation ($\overline{D.P.}$) were calculated using the relationship $\overline{D.P.} = 7.4 \times [\eta]$. This was obtained for potato amylose (129), and although it may not be extremely accurate when applied to these cereal amyloses, the calculated values are likely to be of the correct order of magnitude. The β-amylolysis conversion limits were also determined.

The salient features of the results of various fractionation experiments are summarised in Tables 1 and 2 below. The viscosity results are shown in Figure 7, p. 53a

TABLE 1. Properties of the components of oat starch.

Expt.	Method of Fractionation	Amylopectin Purity (%)[a]	Amylopectin % of total amylose retained[b]
F 1	70° Aqueous leach	82	67
F 2	98° Aqueous leach	94	23
F 3	Thymol/Bu^{n}OH dispersion without buffer.	92	31
F 4	Thymol/Bu^{n}OH dispersion with buffer (pH 6.47)	93	27
F 5	KOH pre-treatment	99	<4

	Amylose I.A.[c]	Amylose $[\eta]$ in M-KOH	Amylose D.P.[d]	Conversion limit[e] (i)	Conversion limit[e] (ii)
F 1	19.1	160	1190	95	100
F 2	18.0	340	2500	79	97
F 3	13.7	180	1330	71	86
F 4	14.7	212	1570		
F 5	16.8	263	1950	72	91.

a calc. from (iodine affinity/19.0) x 100.

b % of total amylose in starch retained as impurity in the amylopectin assuming the original starch contains 27% of amylose.

c iodine affinity.

d approximate degree of polymerisation calc. from $\overline{D.P.} = 7.4[\eta]$ (see text)

e β-amylolysis limits for (i) pure β-amylase, and (ii) β-amylase + Z-enzyme, expressed as % conversion into maltose. Accuracy ± 2%.

TABLE 2. Properties of the Components from Wheat Starch.

Expt.	Method of Fractionation	Amylopectin Purity (%)[a]	Amylopectin % of total amylose retained.[b]
F 6	70° Aqueous leach	81	74
F 7	98° Aqueous leach	94	23
F 8	Thymol/Bu^{n}OH dispersion without buffer	96	12
F 9*	Thymol/Bu^{n}OH dispersion without buffer	95	18
F10	Thymol/Bu^{n}OH dispersion with buffer (pH 6.47)	96	12
F11	KOH pre-treatment	96	12

	Amylose I.A.[c]	Amylose $[\eta]$ in M-KOH	Amylose $\overline{D.P.}$[d]	Amylose Conversion limit[e] (i)	Amylose Conversion limit[e] (ii)
F 6	17.8	145	1070	98	-
F 7	15.8	300	2240	66	89
F 8	19.0	260	1920	65	96
F 9*	17.2	133	980	-	-
F10	17.6	280	2060	-	-
F11	18.0	258	1910	-	-

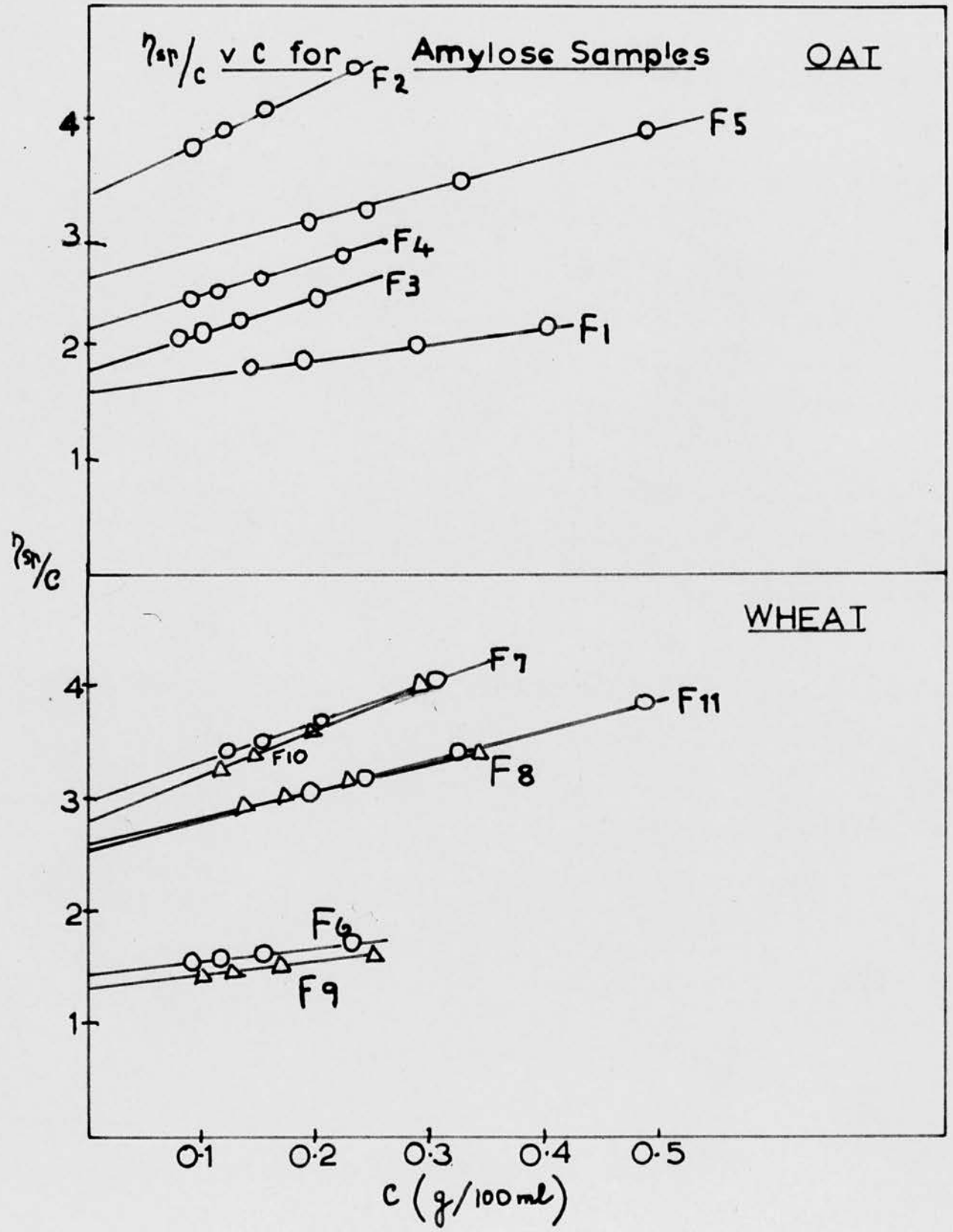
a Calc. from (iodine affinity/19.0) x 100.

b Calc. as for Table I assuming original starch contains 26.3% of amylose.

c, d, and e as for Table I.

* Sample of commercial starch.

FIG 7


ηsp/c v C for Amylose Samples
OAT
F2
F5
F4
F3
F1
ηsp/c
WHEAT
F7
F11
F10
F8
F6
F9
4
3
2
1
0·1
0·2
0·3
0·4
0·5
C (g/100ml)

DISCUSSION.

Satisfactory fractionation of laboratory-prepared cereal starches into their component amylose and amylopectin appears to be more difficult than is the case for potato starch. McWilliam and Percival, who studied barley starch (130), found that they could not obtain amylopectin free from amylose. The conventional methods using thymol, butan-1-ol and pyridine, yielded amylopectin with 5-8% of amylose impurity. Similarly, conventional aqueous dispersive and leaching methods of fractionation were unsuccessful for the fractionation of the starch from sweet corn, Zea mays (131). The two cereal starches prepared in this work proved extremely difficult to disperse prior to a conventional fractionation using precipitants. The resultant dispersions were turbid even after two hours' boiling, and the addition of sodium chloride or phosphate buffer made no improvement. This difficulty may well be due to the residual tightly-bound protein, which cannot be removed by the mild physical conditions used here to prevent degradation of the starch. Difficulty was also found in recrystallising the amyloses from butan-1-ol solutions; in some cases an iodine affinity of 19.0 (the maximum value found for the amyloses from these starches) could not be achieved. This effect was more noticeable in the case of the oat starch. As an example, F3 amylose after three recrystallisations had an iodine affinity of 13.7, which corresponds to a purity of only 72%. An indication of the purification occurring on recrystallisation may be obtained from Table 3.

TABLE 3.

F5 Amylose	No. of precipitations with Bu^nOH.	I.A.	Purity[a] (%)
	2	11.0	57.9
	3	14.9	78.4
	4	16.8	88.0

a - Calc. from (Iodine affinity/19.0) x 100.

The limiting viscosity numbers of the 98°C-leached products (F2 and F7) are higher than the other amylose fractions. This suggests that degradation must have occurred during the dispersions, even in a nitrogen atmosphere. Both amyloses seem to be more susceptible to hydrolysis than potato amylose. The two amyloses F2 and F7 were redispersed in boiling water under nitrogen, and heated on a boiling water bath for an hour. On reprecipitation and measurement of the limiting viscosity numbers of the products the following changes were observed.

Amylose	$[\eta]$ before heating	$[\eta]$ after heating
F2	340	260
F7	300	280

In view of the apparent lability of the amylose components of oat and wheat starch, leaching at 98°C for a short time yields amylose of higher limiting viscosity number than that from a conventional dispersion. Similar results were obtained by Baum,

Gilbert and Wood (120) in studies on wheat starch. The purity of the amylopectin obtained by this method may not be high, for instance in F7 23% of the total amylose was retained, whereas in F8 only 12% was retained. This is in agreement with results of experiments on barley starch (132) and potato starch (129). Schoch (15) in an investigation of aqueous leaching studied leaching at temperatures from 70°C to 90°C and suggested that it was inefficient due to concurrent leaching of the amylopectin as the temperature was increased.

In conclusion this procedure results in the extraction of material which is predominantly amylose of high limiting viscosity number but lowered purity, leaving a residual granular network which is predominantly amylopectin.

Leaching at 70°C gives rise to an amylose which is purer than that obtained at 98°C. On the other hand, it is smaller. This suggests, in agreement with Meyer et al.(65),that subfractionation of the amylose has occurred, short chains being preferentially leached at this temperature, whilst the larger ones are unable to diffuse out until the granular structure is further disrupted. Schoch (15) obtained similar results but suggested that the method was inefficient due to the fact that 50% of the amylose retrograded in situ in the granule. It is more likely that simple subfractionation occurs since it has been shown that for potato starch the granules can be redispersed at 98°C (129). Insolubilised retrograded amylose would not disperse under the conditions used.

The figures obtained from fractionation involving complete dispersion indicate the very appreciable difficulties involved in

fractionating cereal starches to give both components pure and undegraded. In both cases, the addition of phosphate buffer causes little change in the purity of the amylopectin or the $\overline{D.P}$, of the amylose.

Pre-treatment of the starches with alkali (125) appears to be the most satisfactory method of fractionation. In the case of oat starch, a pure amylopectin is obtained, although the amylose is still impure. There does not seem to be any marked improvement over normal fractionation for wheat. It should be noted that there is little evidence of any degradation in either amylose.

In order to compare laboratory-prepared cereal starches with those prepared commercially, a commercial sample of wheat starch was examined. On fractionation, using normal dispersion, an amylose with a low limiting viscosity number was obtained. The decrease was relatively much larger than between laboratory- and commercially-prepared potato starch (124).

	$[\eta]$ Laboratory prepared	$[\eta]$ Commercial
Wheat	260	133
Potato	500	340

This is perhaps related to the more vigorous purification necessary to remove protein in the manufacture of cereal starches. The low value of the limiting viscosity number, indicating that the amylose is degraded, emphasises the fact that it is inadvisable to use industrial samples of unknown origin and treatment for fundamental studies.

UNIFORMITY OF STRUCTURE OF AMYLOSE.

The aqueous leaching and fractionation experiments indicate that there may be two amylose fractions, (1) easily accessible material of relatively low $\overline{D.P.}$ and (2) a fraction of higher $\overline{D.P.}$ requiring disruption of the granule before it can be obtained. To investigate the possibility that these might differ in structure, β-amylolysis experiments were carried out. Pure β-amylase will degrade a linear molecule completely to maltose, while its hydrolytic action stops at any branch point or other anomaly. The concurrent action of β-amylase and Z-enzyme also enabled a calculation to be made of the amylopectin impurity in some fractions, assuming that 56% of the amylopectin was converted into maltose. The values obtained agreed well with those from iodine affinity measurements.

The results of β-amylolysis are summarised in Tables 1 and 2, while Table 4 gives a summary of the results compared with previous values obtained for potato amylose (124).

TABLE 4. Comparison of β-amylolysis limits for various fractions of amylose from laboratory-prepared starches.

Prep. of sample in N_2	Potato†			Oat			Wheat		
	% of total amylose	$[\eta]$	β-limit*	% of total amylose	$[\eta]$	β-limit*	% of total amylose	$[\eta]$	β-limit*
Aq. leaching at 70°	40	240	100	33	160	95	28	145	98
Aq. leaching at 98°	80	370	86	77	340	80	77	300	69
Dispersion of granule	100	440	77	100	180	77	100	260	66

† Results from ref. 129.

* Expressed as % conversion into maltose. Calculated assuming 56% conversion of any amylopectin impurity into maltose.

The almost complete conversion to maltose of the amylose leached at 70°C supports the view that at this temperature limited swelling of the granule and diffusion out of short-chain essentially linear amylose occurs. Extraction at higher temperatures gave amylose which was incompletely hydrolysed, the amount of resistant material increasing with increasing temperature, and consequent swelling and disruption of the granule. Up to the present, the nature of the barrier to β-amylolysis is uncertain, and difficulties in its elucidation have been discussed (67). It is possible that since disruption of the granule is involved, branching in the amylose molecule is not improbable. Kerr and Cleveland (26) have in fact suggested, on the basis of enzymic work, that potato and tapioca amyloses are singly branched and contain 1 - 3 branches per molecule. Potter and Hassid (21)(22) have put forward chemical evidence for branching in a number of amyloses from other plant sources. Studies on the molecular structure of amylose (67) suggest that if branching does occur, then the inter-chain linkage is not of the α-1:3 or α-1:6 type.

For oat amylose, if about 35% is linear, and the whole is hydrolysed to 77% with β-amylase, then the portion containing an anomaly must be hydrolysed to about 65%. For wheat amylose, similar calculations show that the portion containing an anomaly must be hydrolysed to about 50%. These results would suggest that the barrier to β-amylolysis in these amyloses is essentially randomly situated. Thus individual amyloses appear to differ both in $\overline{D.P.}$ and β-amylolysis limits, indicating that variations exist in the

relative proportion and distribution of the barriers.

Although Peat (24), Hopkins (68) and Hassid (133) and their collaborators have suggested that pure β-amylase converts only about 70% of the amylose into maltose, the experiments carried out here on amylose sub-fractions indicate that amyloses obtained by dispersive methods consist of two types of molecules, some being linear and others having a randomly-situated barrier to this enzymic hydrolysis. On this basis, the percentage of linear material (L) in any amylose fraction can be calculated from the expression

$$\frac{(T - L)}{(100 - L)} = \frac{1}{2},$$

where T = the percentage conversion into maltose for the total fraction. Since aqueous leaching is not necessarily quantitative, values of L from calculation and experimental determinations may not agree. The results from such calculations (i.e. potato, *ca.* 50; oat, *ca.* 50; wheat, *ca.* 30) suggest that the amount of linear material may vary from starch to starch.

SECTION III.

ACID TREATMENT OF WHEAT STARCH GRANULES.

INTRODUCTION.

Although a great deal of work has been carried out on starch, little is yet known about the fine structure of the granule and the way in which the amylose and amylopectin are incorporated into it.

As indicated previously, one of the unique characteristics of starch is its almost universal occurrence in the form of granules.

The properties of these granules vary with the source. They show a wide range in size, from an average of 5 microns for rice starch, to 50 microns in the case of sweet potato. The shape also varies from round or elliptical to polygonal (10).

Granules, as laid down in the plant, are surrounded by a thin protein layer, and in some storage organs such as maize-seed endosperm, are held in a larger and heavier, cellulosic cell structure. It appears impossible to remove entirely this protein matrix by purely physical methods, and it is possible that the residual 0.2% protein is incorporated into the granule. The mode of synthesis and deposition of starch in the granule is still uncertain, but it is worthy of note that gradual expansion of the enzyme containing, protein sac during growth may result in the amylopectin component being synthesised in an essentially two-dimensional arrangement (16).

On most granules there may be seen a point of intersection of two, or more, lines or creases. This point of intersection is known as the hilium, which may be the organic centre or nucleus from which the granules develop. The hilium is best located by means of polarised light, under which it appears as the centre of a black cross. The

hilium may occupy a central position in the granule, but is more generally eccentrically placed. Encircling the hilium may be seen a number of layers, due to discontinuities in refractive-index of the deposited material (134). The exact nature of these layers is not yet established, although it has been suggested that they are either growth rings, or have developed as a result of physical strains.

Since successful fractionation depends upon complete solution of the starch, much work has been carried out on the behaviour of the granule in water. Intact granules are insoluble in cold water. As the temperature is increased, swelling takes place in three distinct phases. In the first, swelling is limited and there is no change in birefringence or viscosity. Then at a definite temperature irreversible, rapid, swelling occurs, with a loss of birefringent properties and large increase in viscosity. This gelatinisation temperature is characteristic for different types of starches, and is obviously related to the closeness of molecular packing and degree of association. Only a limited amount of material diffuses out at this stage, but in the final stage, at a higher temperature, there is rapid diffusion of material from some granules, and rupture of others leaving numerous formless 'sacs'. These 'sacs' are not regarded as the granule wall or membrane, but as artefacts produced in the swelling process (16).

The problem of the fine-structure of the granule is complex. Various suggestions regarding the distribution of the components

have been made. Whilst it has been suggested that the amylose is concentrated at the centre of the granule (135)(136), evidence for an even distribution has also been presented (16). On acid hydrolysis of the granule, if the amylopectin was concentrated at the surface, it would be preferentially attacked, whereas if there was an even distribution, both components would be degraded. In work of this type carried out previously, conflicting results had been obtained, and it was for this reason that the present work was undertaken.

Kerr (137) studied the effect of treating maize starch with 0.1 - 0.15N sulphuric acid at 50°C, a heterogeneous reaction medium. After neutralisation, the samples were fractionated by the general method of Schoch (14), using pentasol for the primary precipitation of the amylose, and precipitating the amylopectin with methanol. Molecular weights were determined by osmotic pressure after converting the fractions into their tri-acetates by the method of Mullen and Pacsu (138). Their method involves dispersion of the component in hot aqueous pyridine followed by removal of water as the azeotrope before addition of the acetic anhydride. At this point, the method was slightly modified as suggested by Kerr and Cleveland (139). Fused sodium acetate was added and the mixture refluxed for one hour on each of two successive days. The osmotic pressure of a dilution series of the acetate in chloroform was then measured (139), results being expressed as number average degree of polymerisation. Kerr's data cannot be compared directly as he used 'fluidity grades' -

an arbitrary industrial scale - to express the time of acid treatment. Evaluation of the work is further complicated by the fact that his osmotic pressure data were evaluated using a method (138) which has not yet been substantiated and yields a negative slope of the π/c versus c curve at infinite dilution (140). In addition, the method of acetylation employed prolonged heating, which may cause degradation (16). Kerr's results indicated preferential degradation of the amylopectin, since he found a large initial drop in the degree of polymerisation of this component, while the amylose remained relatively unchanged.

An inherent experimental difficulty found by Kerr was that fractionation of the acid-modified starches was difficult, and that neither dispersion at 100°C, nor autoclaving, was successful. This was shown by the fact that the iodine-binding powers of the amyloses isolated after acid treatment were lower than that from the original untreated starch. Kerr suggested, in fact, that the amylose becomes more firmly associated with the amylopectin, giving a resultant complex which protects the amylose from acid attack.

This preferential attack by acid on the amylopectin in the granule has been confirmed by Ulmann (141) using alumina column chromatography to study the degradation products. The starch was treated with 0.125% hydrochloric acid and then the components and degradation products were separated on an hydrochloric acid treated alumina-absorption column. Ulmann found that the amylopectin was degraded first yielding a dextrin which gave a brown colour with iodine, and that not until most of the amylopectin was degraded was

the amylose affected. Similarly, after passage through different cation exchange resins he was able to show, again with the aid of alumina columns, that the amylopectin was degraded first giving dextrins, then low molecular weight sugars.

Similar experiments carried out by Meyer and Manzi (135) gave different results. In this case changes in molecular weight occurring on acid treatment were measured by the dinitro salicylic acid method (142), which has been shown to be unreliable for absolute molecular weights (16). Maize starch was treated with hydrochloric acid of strengths varying between 0.2 and 1N at 50°C, and potato was treated with from 0.2 to 3N at 45°C. The results indicated that both components were hydrolysed in the same proportions, as the amount of degradation of both was the same. In addition the iodine-binding powers of the acid modified starches were unchanged, the curves being identical. Previous work by Meyer and Bernfeld (143) on the fine structure and swelling of starch granules had led to the postulate that the concentric shells round the hilium were composed of radially arranged spherulites; that is spherical aggregations of needle-shaped crystals. The outer layer of the granule was shown to consist mainly of amylopectin with which high molecular weight amylose co-existed as "mixed crystals" (135), while the inner layers were composed of well crystallised low molecular weight amylose.

The crystallinity of amylopectin was supported by birefringence studies on waxy-maize starch which contains no amylose (143). This showed no distinguishable difference from ordinary maize starch

containing 21% amylose. Further work by Baker and Whelan (144)(136 on starches containing variable amounts of amylose yielded similar results. Their work indicated that the intensity of birefringence bears no obvious relation to the amylose-amylopectin ratio. They were also able to show by microscopic examination of iodine-stained waxy starches, that blue-staining "cores" are present. As a result, they represent the granule as being built up of alternate layers of amylose and amylopectin, the thickness of the former decreasing, and of the latter increasing with distance from the centre of the granule. This model has since been criticised by Badenhuizen (145) on the basis of 'lintnerisation studies'. The starches were subjected to long periods of attack by 7.5% hydrochloric acid, then stained with iodine either with or without removal of the acid, and the granules studied microscopically. For potato starch (25% amylose) the nucleus, located by birefringence, was of the same purple colour as the surrounding layers, suggesting that there was a regular distribution rather than a gradual shift in chemical composition. Similar results were obtained with corn starch. Waxy corn starch (< 1% amylose) on the other hand, had a blue 'core' suggesting the amylose was concentrated at the centre of the granule. An intermediate type was found in the starch from Granadilla or passion fruit, first isolated by Allie and Joubert (146). They showed by potentiometric titration that there was less than 1% amylopectin present, although Badenhuizen, by a rough colorometric method calculated it to be 6.5%. After five weeks lintnerisation, a blue nucleus was observed on staining. However, this was

surrounded by one or two blue layers, the inner one being darker than the outer. It would appear, therefore, that Badenhuizen's results are rather inconclusive.

The position then is far from clear and the work reported here was undertaken in an attempt to clarify it. For comparison with Meyer and Menzi's results the conditions used were 0.2N-hydrochloric acid at 45°C.

EXPERIMENTAL METHODS.

Wheat starch (15 g.) was washed free from methanol and stirred slowly (20 rev./min.) in 0.2M-hydrochloric acid (750 ml.) at 45°C under nitrogen, in a water bath at 45°C. Samples (150 ml.) were removed at different times. The starch was washed with distilled water in the centrifuge until it was free from acid, shaken with aqueous methanol overnight then stored under aqueous methanol until required. A sample was removed and dried for measurement of iodine uptake, the remainder was fractionated using the method of selective precipitation. Thymol was used for the initial precipitation, and butan-1-ol for the recrystallisation of the amylose. The amylopectin was obtained from the thymol-supernatant by freeze-drying, after the thymol had been extracted twice with ether.

An estimation of the amount of granule solubilised by the acid was obtained by suspending starch (0.5 gm.) in 0.2M-hydrochloric acid (25 ml.) at 45°C under nitrogen. After 5 hours the suspension was centrifuged, the supernatant reduced in volume and, after hydrolysis with 3N-sulphuric acid at 100°C, the glucose estimated by the method of Lampitt, Fuller and Coton (93). The residual starch granules were dried to constant weight in a sintered glass crucible.

The granule after acid-treatment was examined chromatographically. Starch (2 g.) was treated with 0.2M-hydrochloric acid at 45°C under nitrogen for 5 hours. After centrifuging, the starch was gelatinised at 70°C in water (400 ml.) for 1 hour under nitrogen,

and amylose precipitated with butan-1-ol (40 ml.). The precipitate was removed and the supernatant evaporated to dryness. The residue was extracted with 50% methanol and examined chromatographically. The solvent used was butan-1-ol - benzene - pyridine - water, 5:1:3:3, top layer, development time 72 hours at 18°C.

The iodine affinity, limiting viscosity number and degree of polymerisation, were obtained as before. Sedimentation determinations were also carried out, the samples being dissolved in 0.2M potassium hydroxide (section I).

The effect of treating wheat starch with 1M-hydrochloric acid at 16°C has also been studied. The results for both sets of experiments are set out below.

RESULTS.

Table 5. **Effect on the Iodine affinity of wheat starch of acid treatment.**

Time of acid treatment (hr.)		0	1	2	4	6	8	24
For 0.2M-HCl at 45°C	Iodine Affinity	5.0	5.2	5.5	4.4	3.8	-	1.
	Amylose (%)*	26.3	27.3	29.0	23.7	20.0	-	7.
For 1M-HCl at 16°C	Iodine Affinity	5.0	4.0	5.3	5.7	-	5.6	4.
	Amylose (%)*	26.3	21.0	28.0	30.0	-	29.5	25.

* Apparent % of amylose calc. assuming iodine affinity of amylose = 19.0%.

Table 6. Properties of the Fractionated Components.

0.2M-HCl at 45°C.

Acid-treatment hr.	Amylose			Amylopectin			
	I.A.[a]	$[\eta]$[b]	$\overline{D.P.}$[c]	I.A.[a]	% Amylose	[d] $[\eta]$[b]	$^{13}s_{20}$[e]
0	19.0	260	1920	0.5	3	140	121
1	10.3	178	1320	0.4	2	80	28
2	11.2	164	1210	0.5	3	60	22
4	12.5	140	1030	0.5	3	-	-
6	14.8	134	990	0.8	4	50	19
24	-	-	-	- *	-	30	5

1M-HCl at 16°C.

Acid-treatment hr.	Amylose			Amylopectin			
	I.A.[a]	$[\eta]$[b]	$\overline{D.P.}$[c]	I.A.[a]	% Amylose	[d] $[\eta]$[b]	$^{13}s_{20}$[e]
0	19.0	260	1920	0.5	3	140	121
2	12.6	235	1740	0.7	4	175	110
4	16.8	212	1570	1.5	8	110	80
8	18.7	188	1390	1.1	6	140	85
24	-	150	1110	0.8	4	160	56

a Iodine affinity

b measured in 1M-KOH

c calculated from $\overline{D.P.} = 7.4\,[\eta]$

d % of amylose impurity, calc. from (iodine affinity/19.0) x 100.

e sedimentation constant in c.g.s. units at c = 0.2 g./100ml.

* the potentiometric iodine-titration curve was too abnormal to allow calculations to be made.

A control experiment was carried out using water at 45°C. Potentiometric iodine titration curves of the samples removed at different times showed no change from the original. Thus any effects occurring are due to the acid and not to leaching.

Estimation of the total glucose in the supernatant from acid-treatment showed that after 5 hours only 1% of the total weight of the granules went into solution, either as glucosans or reducing sugars. The chromatographic examination of the acid supernatant indicated that the amount of reducing sugar present was small.

Potentiometric iodine titration curves showed changes in the iodine affinity of the starch samples. In the case of 0.2N-acid at 45°C there was an apparent increase in iodine uptake for the first two hours, followed by a decrease thereafter. This effect continued over the first four hours with 1N-acid at 16°C. In addition the slope of the linear portion of the curve increased with time. These results are shown in table 5 , and a typical set of curves for 0.2N-acid at 45°C in fig. 8, p. 72a

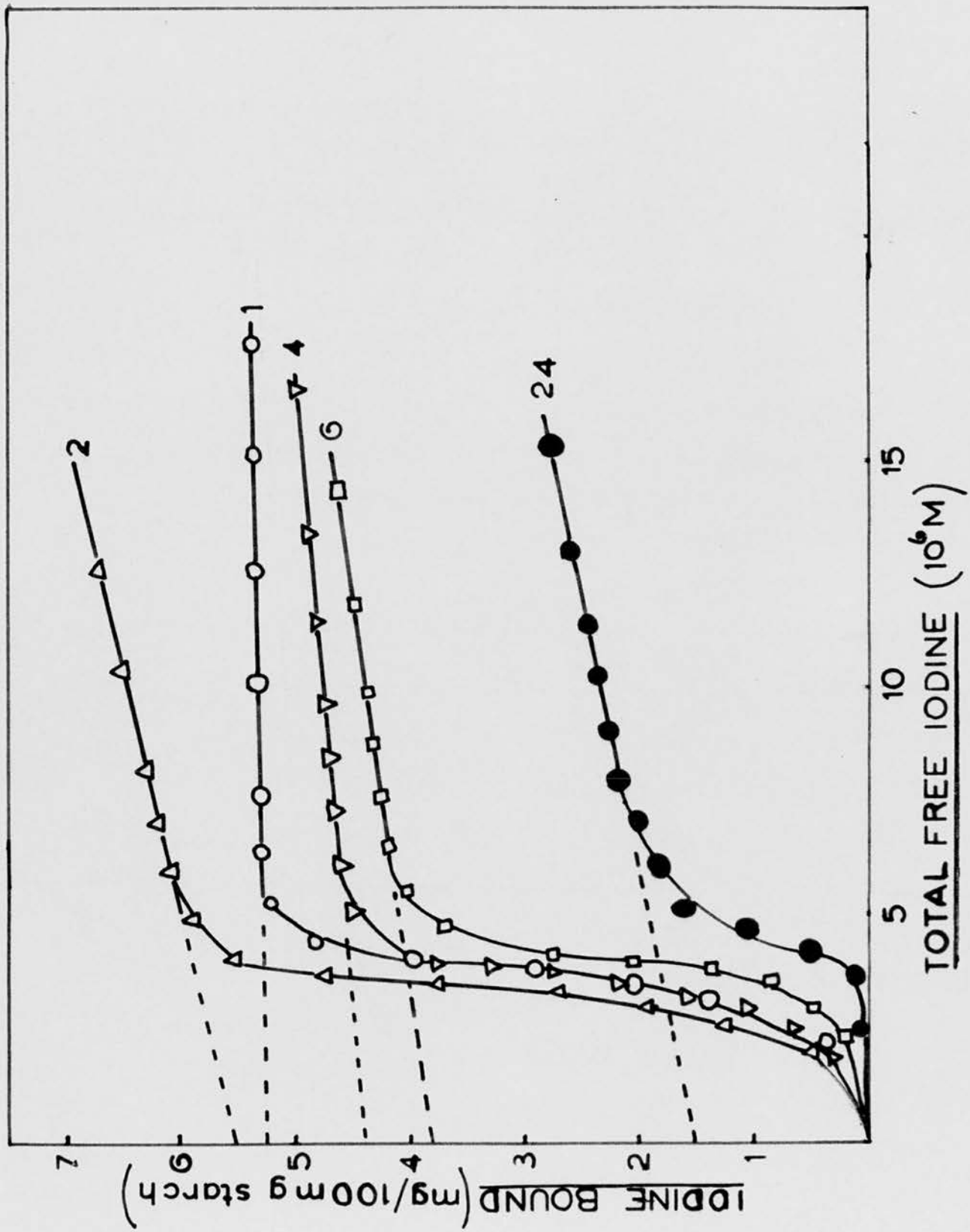
From the viscosity and sedimentation results, the rates of degradation of the two components were calculated. The rate of degradation of the amyloses was expressed as the number of bonds broken per initial molecule per hour.

The number of bonds broken per w gm. of polysaccharide (q) is given by

$$q = \frac{Nw}{Mo}\left(\frac{Mo}{Mt} - 1\right),$$

where Mo and Mt are the number-average molecular weights at times o and t respectively, and N is Avogadro's number.

FIG. 8


IODINE BOUND (mg/100mg starch)
TOTAL FREE IODINE (10^6M)
1
2
4
6
24
1
2
3
4
5
6
7
5
10
15

This can be re-written in the form -

$$q = Nw \left(\frac{1}{Mt} - \frac{1}{Mo} \right)$$

or $$q \propto \frac{1}{M.}$$

Degradation should be followed by number-average methods, but these are insensitive to small <u>initial</u> changes occurring with <u>low</u> degrees of degradation. Weight average methods will provide a sensitive measure of these changes. At a <u>high</u> degree of degradation, however, when only relatively low molecular weight material is present, the methods become insensitive to further changes. Thus in the case of viscosity, the limiting viscosity number obtained will <u>appear</u> to approach a limit.

In the results considered here

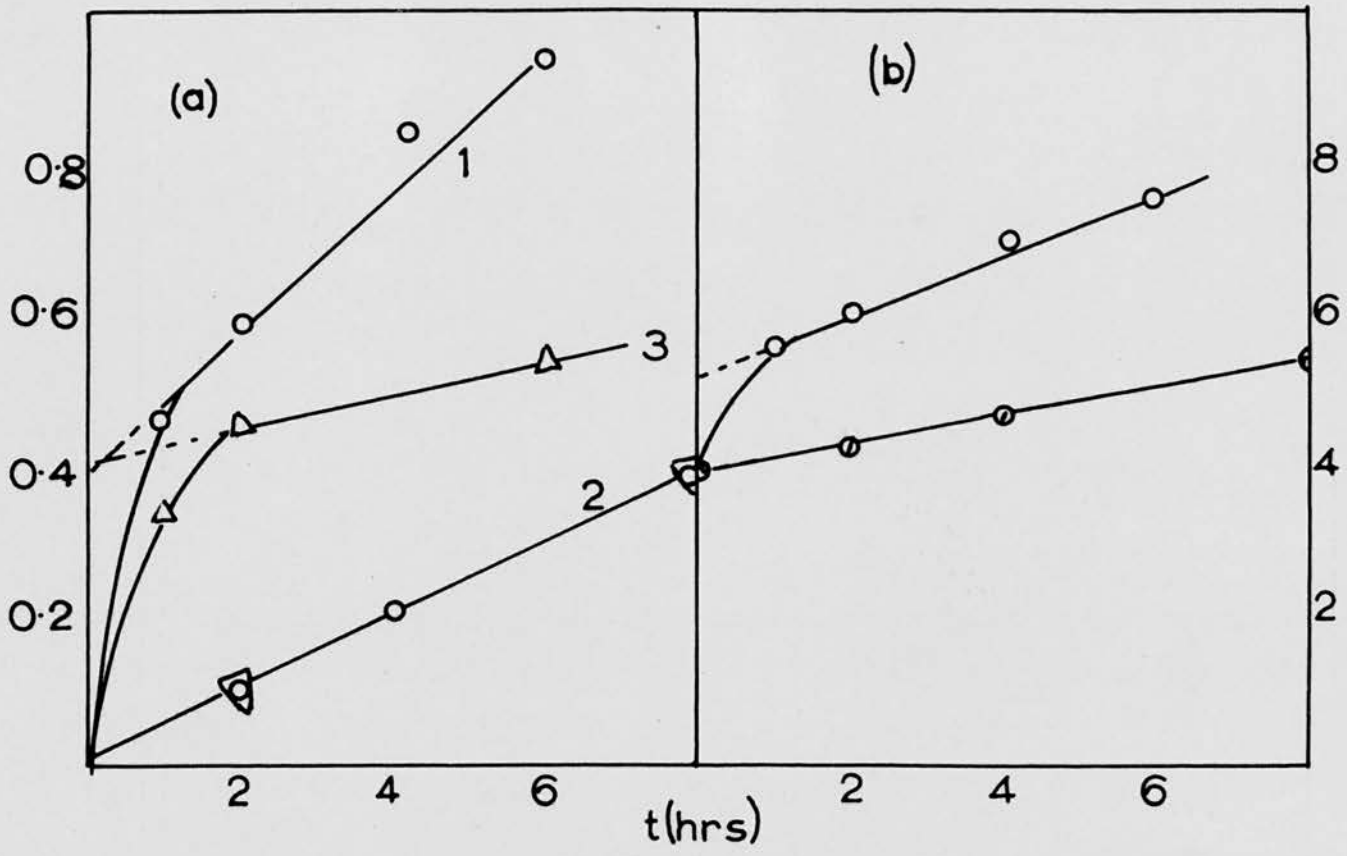
$$M = K[\eta]$$

$$\therefore \quad q \propto \frac{1}{[\eta]}.$$

The correct plot is thus $[\eta]^{-1}$ versus t. This is in agreement with McBurney (147), (the presence of weak bonds will be shown by $\lim_{t \to 0} [\eta]^{-1} \neq [\eta]^{-1}$ for the original polymer).

The graphs of (<u>bonds broken) versus t</u>, and <u>$[\eta]^{-1}$ versus t</u> for the <u>amylose</u> are shown in Fig. 9, p.73a. For the products isolated after treatment with 0.2M-acid at 45°C it may be observed that neither curve extrapolates through the origin. Similar results have been obtained for potato amylose (148). The linear portion of the curve

FIG 9



(a) No. of bonds broken per molecule per hour

1 Amylose 0·2 M at 45°C

2 Amylose M at 16°C

3 $\left(\frac{S_o}{S_t} - 1\right).10^{-1}$ Amylopectin ∇ at 45°C

∇ at 16°C

(b) $[\eta]^{-1}10^3$ v t

o Amylose 0·2 M at 45°C

⊙ Amylose M at 16°C

in Fig. 9 (a) corresponds to a rate of 0.1 bonds broken per initial molecule per hour. This is 4 - 5 times slower than for potato amylose. For the 1M-acid at 16°C, degradation was very limited, ca. 0.05 (units as above). Under these conditions the functions gave straight lines which extrapolated to the origin, indicating there was little preferential breakdown.

Degradation of the amylopectin was observed from the plot of 1/s versus t. The samples isolated after treatment with 0.2M-acid at 45°C showed an initial preferential breakdown which was much larger than that for amylose. Due to the difficulty of obtaining absolute molecular weights the degradation could not be calculated unambiguously for the amylopectins. The rate of degradation can be calculated from the plot of (So/St - 1) versus t, however, where So and St are the sedimentation constants at times o and t respectively, Fig. 9(a). This assumes that the diffusion constant is unlikely to vary considerably, hence the value obtained is likely to be a minimum one, since any disproportionate increase in this constant with time of acid-treatment will increase the slope of the linear portion. The linear portion of this curve corresponded to a rate of about 0.4 (units as above) again slower than for the corresponding potato component. On extrapolation the linear portion did not pass through the origin. At 16°C the M-acid caused little degradation ca. 0.07, although more than for the corresponding amylose. There was in contrast to the results at 45°C only very limited preferential breakdown.

DISCUSSION OF RESULTS.

The fact that there was no microscopic evidence of swelling in the acid-media would seem to indicate that this treatment preferentially affects the amorphous rather than the crystalline portion of the granule. Similar effects were noted by Meyer and Bernfeld (143).

Potentiometric iodine titration results indicated an increase in the apparent amount of linear material in the granule. This can be explained by assuming that amylopectin is preferentially solubilised. The amount of reducing sugar in the acid supernatant liquor was small, however, as shown by chromatographic examination. It is of interest to note that Schoch and co-workers (61) found a lowering of the iodine affinity when studying acid-modified maize starches.

From the iodine affinities of the components, it will be observed that fractionation of the acid-treated starches proved more difficult than for the starch. Kerr (137) found a similar behaviour when fractionating acid-treated maize starch granules. In the early stages the iodine affinity of the amylose was low, but increased with time of acid treatment. Here also the same effect was observed. In addition the amylopectins from the 1 and 2 hour treatment with 0.2N-acid at 45°C had to be redissolved and precipitated in order to free them from contaminating amylose.

The curves of rates of degradation show that at 45°C amylopectin is degraded considerably faster than amylose, 0.4 bonds broken per

initial molecule per hour for amylopectin, as compared with 0.1 for amylose. As indicated this rate must be a minimum for the amylopectin. As it has been reported (16) that α-1:6 bonds are stronger than α-1:4, these facts indicate that the amylopectin is preferentially degraded so must be more accessible than the amylose. This may well represent attack on amylopectin on the outer surface or amorphous regions.

Since there is no evidence for the existence of amylose preferentially at the surface, the outer layer of the granule must be predominantly amylopectin since it makes up approximately 75% of the granule. Hence the evidence here is not absolute proof of the existence of a true amylopectin outer layer.

The amylopectin is also likely to form the amorphous region of the granule, and swelling would be associated with these regions. Meyer has suggested (16) that outer branches might align themselves to form small crystallites, or link up to amylose in the crystalline region. Short-chain amylose might also form part of the amorphous region.

Although the curves do not extrapolate to the origin, the heterogeneous nature of the reaction makes for caution in interpreting this as indicating the presence of weak linkages. By comparison with cellulose chemistry, where in heterogeneous hydrolysis, the decrease in the rate of cleavage is a real effect ascribable to the crystalline-amorphous ratio, acid modification probably occurs in two stages. There is the rapid attack on the amorphous regions,

followed by the slower attack on the more crystalline regions. Although this happens to both components, the results indicate that initially there is preferential breakdown of amylopectin.

Both Kerr (137) and Ulmann (141) concluded on the basis of their experiments that the amylopectin is preferentially degraded and this is in agreement with the results obtained here. This is contrary to Meyer and Menzi's view that both components are degraded at the same rate.

The results from the acid treatment support the concept of granular structure put forward in the introduction. Due to the limited swelling at the temperature of the experiment, attack must be on the surface of the granule. The more accessible outer chains would be attacked, and at the same time attack occurs on amylose and amylopectin in the amorphous regions. With increased time of acid treatment diffusion into the granule occurs, resulting in degradation throughout the granule. The rate of degradation of the crystalline regions is lower than for the amorphous regions.

The results obtained at 45°C are similar to those for potato starch, but the calculated rates of degradation for both components are, however, slower than for potato. This effect may be related to wheat starch possessing a more compact structure than potato, and hence an increased resistance to dispersion and fractionation as has been found in Section II. The very limited accessibility of the granule is also emphasised by the relatively negligible effect of M-hydrochloric acid at 16°C on the molecular size of the components.

SECTION IV.

THE EFFECT OF ACID ON POTATO AMYLOPECTIN.

INTRODUCTION.

In Section II, 2, various methods of fractionating cereal starches were investigated. None of the methods used yielded a highly purified amylopectin; in the most successful (complete dispersion and selective precipitation) there still remained an appreciable amount of amylose contaminant in the amylopectin. In view of this difficulty in fractionation, and since it was desired to study the effect of acid on the pure amylopectin component, attention was turned to potato amylopectin. This can be obtained in good yield and high purity ($< 1\%$ amylose) (129). The effect of 0.2 M-hydrochloric acid at temperatures between 35° and $54.3^{\circ}C$ on the molecular size of the component was then studied.

EXPERIMENTAL METHODS.

FRACTIONATION.

The method used was that of complete dispersion, and selective precipitation of the amylose from the aqueous solution with thymol (see Section II.2.). The amylopectin solution obtained was extracted twice with ether to remove thymol, then freeze-dried. This procedure gave a good yield of amylopectin containing less than 1% of amylose impurity [by potentiometric measurement of iodine uptake (see Section I).]

ACID TREATMENT OF THE AMYLOPECTIN.

Potato amylopectin (1 g.) was suspended in water (160 ml.) which was then heated on a boiling water bath for 5 mins., under nitrogen and with gentle stirring. The reaction vessel was then transferred to a bath which was thermostatically controlled at $45^{\circ}C \pm 0.1^{\circ}$, and stirring continued overnight. This method ensured complete solution of the sample. Before addition of the acid a sample (20 ml.) was withdrawn to give the zero-time readings. Concentrated hydrochloric acid (3 ml.) was then added, giving an acid strength in the reaction medium of approximately 0.2M. Samples (20 ml.) were then removed at the required times and neutralised with a known volume of potassium hydroxide. The samples were never isolated, but stored at $2^{\circ}C$ until required. A calculated volume of potassium chloride was added to the original sample in order to produce the same ionic conditions as in the other samples.

SEDIMENTATION MEASUREMENTS.

To an aliquot of each sample was added sufficient potassium hydroxide to give an 0.2M solution. From this a dilution series was made and sedimentation measurements were carried out. (See Section I.) The concentration of the solutions was obtained by the alkaline-ferricyanide method (see Section I).

CHROMATOGRAPHIC EXAMINATION.

Potato amylopectin (125 mgs.) was dissolved in water (20 mls.) by heating on a boiling water bath for 2 minutes and stirring overnight at 45°C. A control sample (1 ml.) was removed, and then hydrochloric acid (3.8 ml., 2M) added. Samples were then removed at the required times and chilled in ice to stop any reaction. Without neutralisation, the samples were spotted on chromatographic paper, the solvent being ethyl-acetate-pyridine-water 10:5:3. The papers were developed overnight, then dried and sprayed with aniline oxalate. Chromatograms were also set up with standard sugars (glucose, maltose, maltotriose) dissolved in 0.2M-hydrochloric acid both singly and in mixtures.

MEASUREMENT OF LIMITING VISCOSITY NUMBERS.

Samples after acid treatment were neutralised, and then made normal with respect to potassium hydroxide; the required volume of alkali being obtained by direct experiment on an aliquot, using phenolphthalein as indicator. The limiting viscosity number of each sample was obtained as detailed in Section I. Similar experiments were carried out at 35°C and 54.3°C.

β -AMYLOLYSIS.

The samples were neutralised with a known volume of potassium hydroxide, phosphate buffer (pH 4.6, 10 ml.) was added and the solution made up to 25 ml. in a standard flask. Aliquots were removed and the amount of glucose and maltose determined by the alkaline-ferricyanide method. Purified soya-bean β -amylase (0.1 ml. was added and the whole incubated at 35°C for 24 hours (see Section I). From the final concentrations of glucose and maltose, and taking into account the blank obtained, the β -amylolysis limits were obtained.

PERIODATE OXIDATION.

This was carried out by the method of Potter and Hassid (20) as described in Section I, on samples removed at the required times and precipitated with alcohol from solution after neutralisation.

RESULTS.

The results of viscosity measurements are shown in figure 10, p.82a and table 8.

TABLE 8.

Acid Treatment (hrs.)	Temperature of Treatment (°C) 35 $[\eta]$ [a]	45 $[\eta]$ [a]	54.3 $[\eta]$ [a]
0	210	210	210
1/2	-	189	148
1	-	168	112
2	190	138	77
3	-	-	60
4	174	108	50
6	164	-	-
8	-	80	-
12	132	-	-
24	104	36	-

a Limiting Viscosity Number in 1M-KOH.

In degradation studies where the reaction has been followed by measurement of the viscosity of the polymer degradation products, the rate of degradation has very often been expressed as the decrease in viscosity with time. The graph of limiting viscosity number $[\eta]$ versus time (t) is shown in fig. 11(a), p.83a. In Section III, however, it was indicated that in following degradation processes viscometrically, the degradation rate constant is obtained from the plot of $[\eta]^{-1}$ versus t, and that the presence of any weak or anomalous linkages is shown by $\lim_{t\to 0} [\eta]^{-1} \neq [\eta]^{-1}$ for the original polymer, when $M = K[\eta]$ (see also ref. 149). Accordingly this function was plotted. The results are tabulated in Table 9, and the graph is on Fig. 11(b).

FIG 10

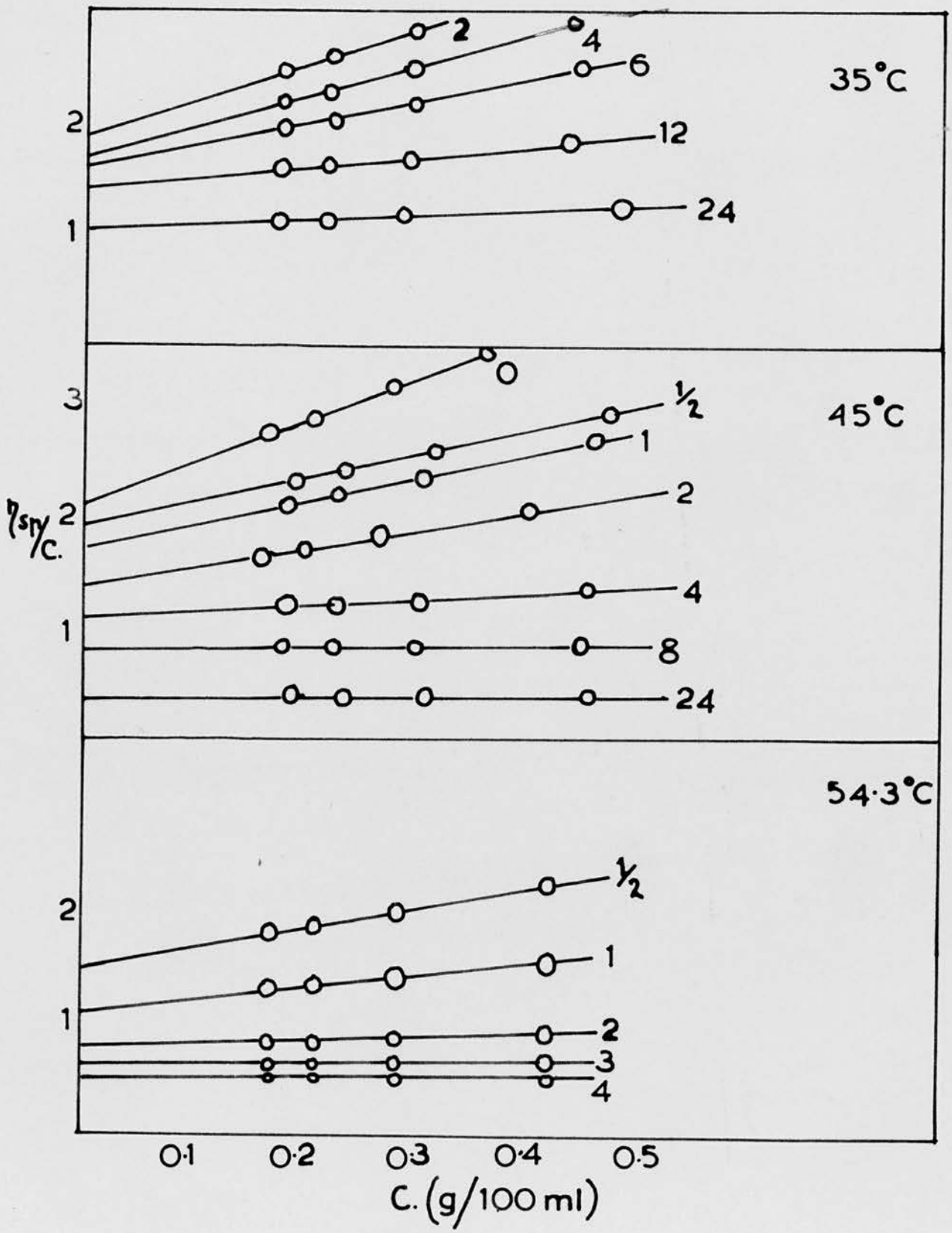

35°C
2
4
6
12
24
1
45°C
0
½
1
2
4
8
24
3
2
1
54·3°C
½
1
2
3
4
2
1
ηsp/C.
0·1
0·2
0·3
0·4
0·5
C. (g/100 ml)

Table 9. $[\eta]^{-1}.10^3$ for Potato amylopectin 0.2M-HCl.

Acid Treatment (hrs.)	Temperature of reaction (°C). 35	45	54.3
0	4.762	4,762	4.762
1/2	-	5.291	6,757
1	-	5.952	8.929
2	5.263	7.246	12.99
3	-	-	16.77
4	5,747	9.259	20.00
6	6.098	-	-
8	-	12.50	-
12	7.576	-	-

The slopes of the graphs of $[\eta]^{-1}$ versus t allow the degradation rate constants to be calculated, whence

$$k_{35} = \underline{6.5 \cdot 10^{-8} \text{ sec}^{-1}}.$$

$$k_{45} = \underline{31.70 \cdot 10^{-8} \text{ sec}^{-1}}.$$

$$k_{54.3} = \underline{115 \cdot 10^{-8} \text{ sec}^{-1}}.$$

The temperature coefficient of the reaction rate can be obtained from the Arrhenius equation -

$$k = Ae^{-E/RT} \quad \ldots \quad (1)$$

where k is the reaction rate, A the frequency factor, and $e^{-E/RT}$ the Boltzmann factor. E is the activation energy, R the

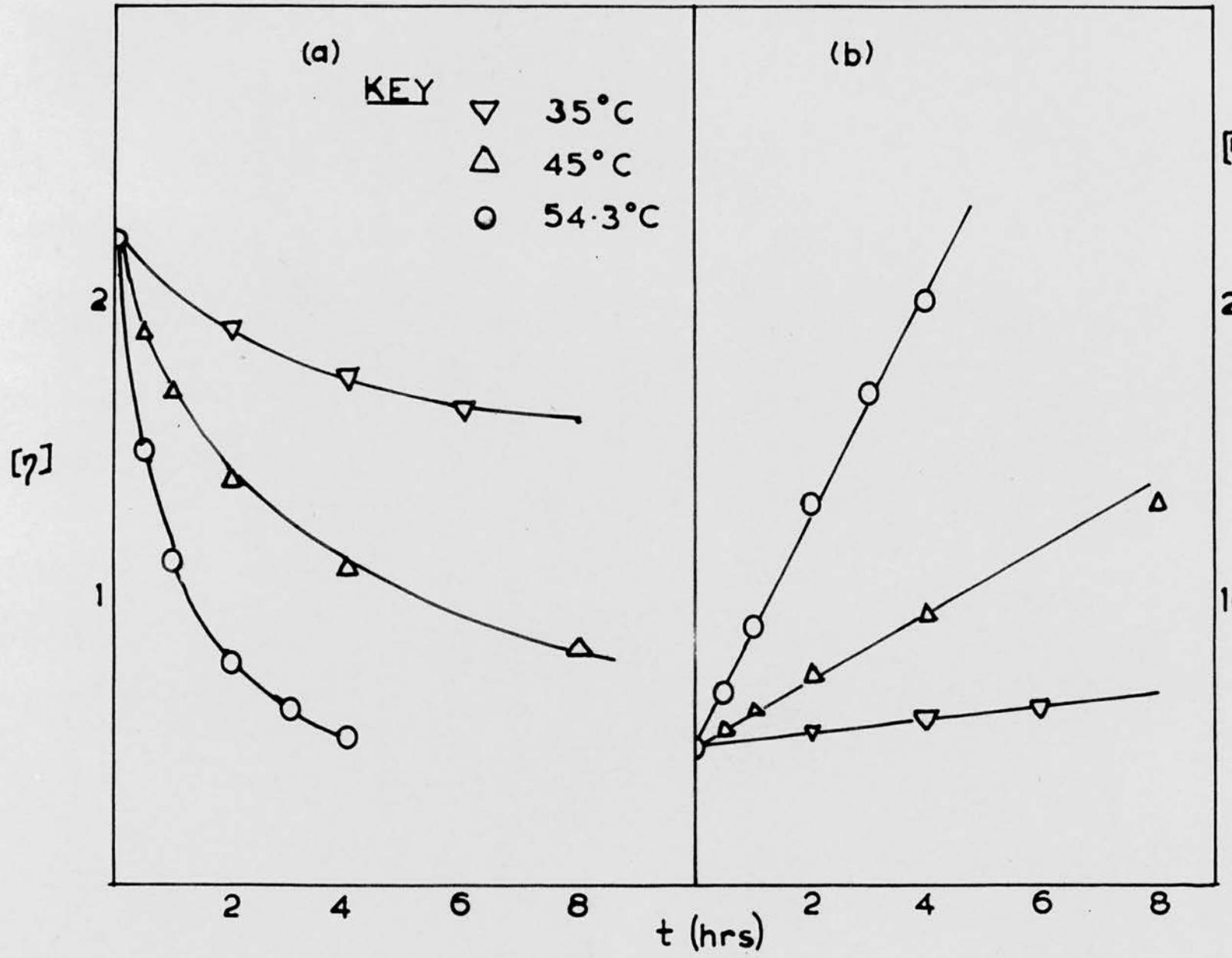


FIG 11

molar gas constant (1.987 cal. degree^{-1} mole^{-1}), and T is the absolute temperature.

Taking logarithms of equation (1)

$$\log K = \log A - {}^{E}/_{2.303.R.T.} \quad \ldots \quad (2),$$

thus the graph of log k versus $^{1}/_{T^{o}K}$ should be a straight line, intercept log A and slope $^{-E}/_{2.303.R.}$ This is shown in Fig. 12, p. 84a

From the graph -

$$E = \underline{29.44 \text{ K cal.}}$$

CHROMATOGRAPHIC INVESTIGATION.

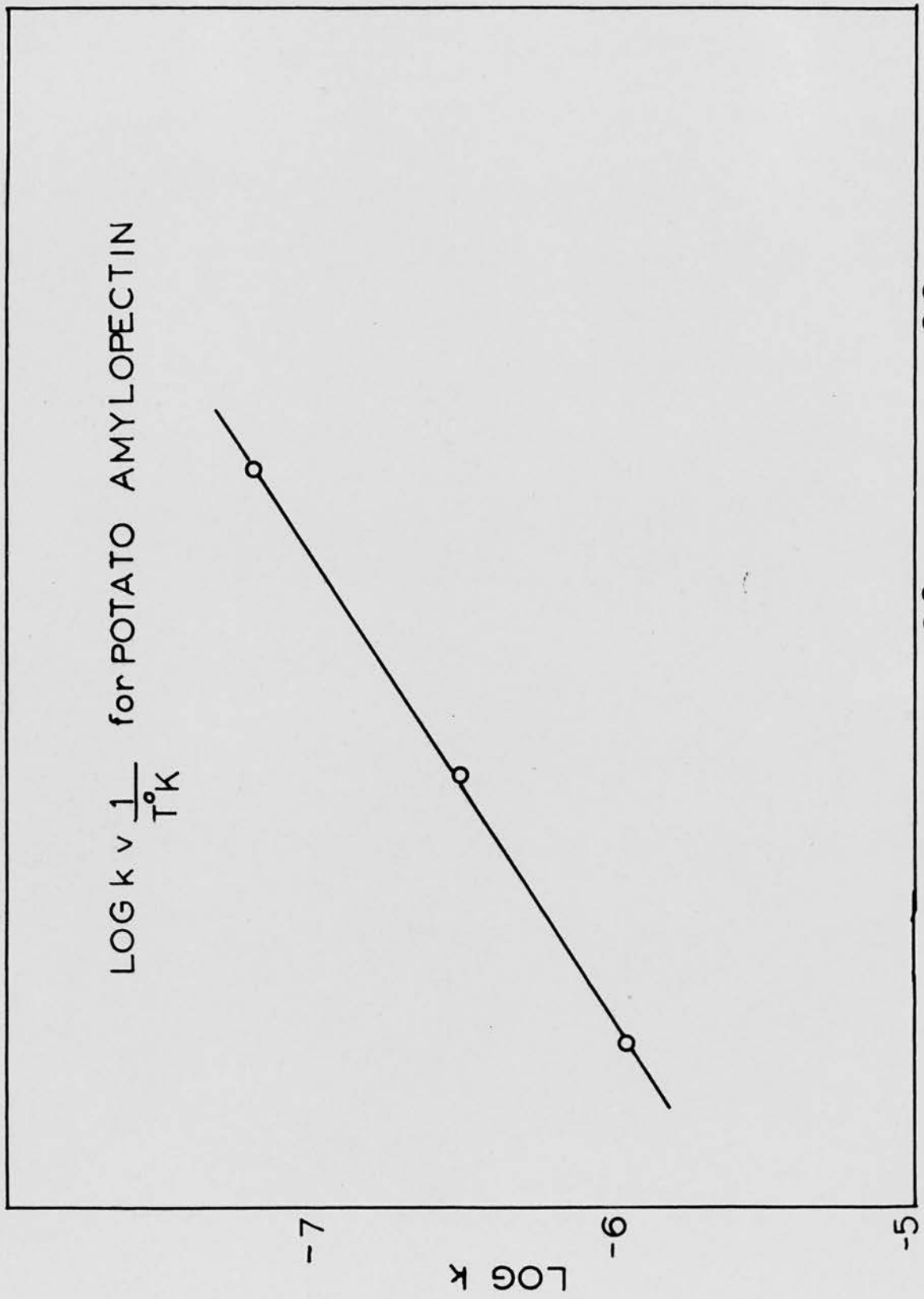
The control chromatograms showed that running in an acid solvent allowed satisfactory development and separation of glucose, maltose and maltotriose. The untreated sample showed no trace of small sugars. Samples were removed every 15 mins. over the first two hours reaction, thereafter half-hourly. After 15 mins. maltose appeared but no other sugars, and in all the subsequent papers maltose was present, but even after 30 hrs. reaction there was no evidence of glucose or any other small sugars.

The results of β -amylolysis and periodate studies are summarised below:

Acid Treatment (hrs.)	0	½	1	2	4	8
Conversion Limit [a]	55	55	52	56	53	58
Average length of unit chain	25	24	26	24	25	25

[a] expressed as % conversion into maltose.

FIG 12


LOG k v $\frac{1}{T°K}$ for POTATO AMYLOPECTIN
LOG k
-7
-6
-5
3·0
3·1
3·2
3·3
$\frac{1}{T°K} \cdot 10^3$

SEDIMENTATION MEASUREMENTS.

Fig. 13, p.85a, shows the curves obtained by plotting the sedimentation constant (S) versus concentration (c). By extrapolation the sedimentation constants at infinite dilution (S_o) were obtained. Table 10 shows these results, and values of $[S_o]^{-1}$ and $(S/S_t - 1)$ calculated from them.

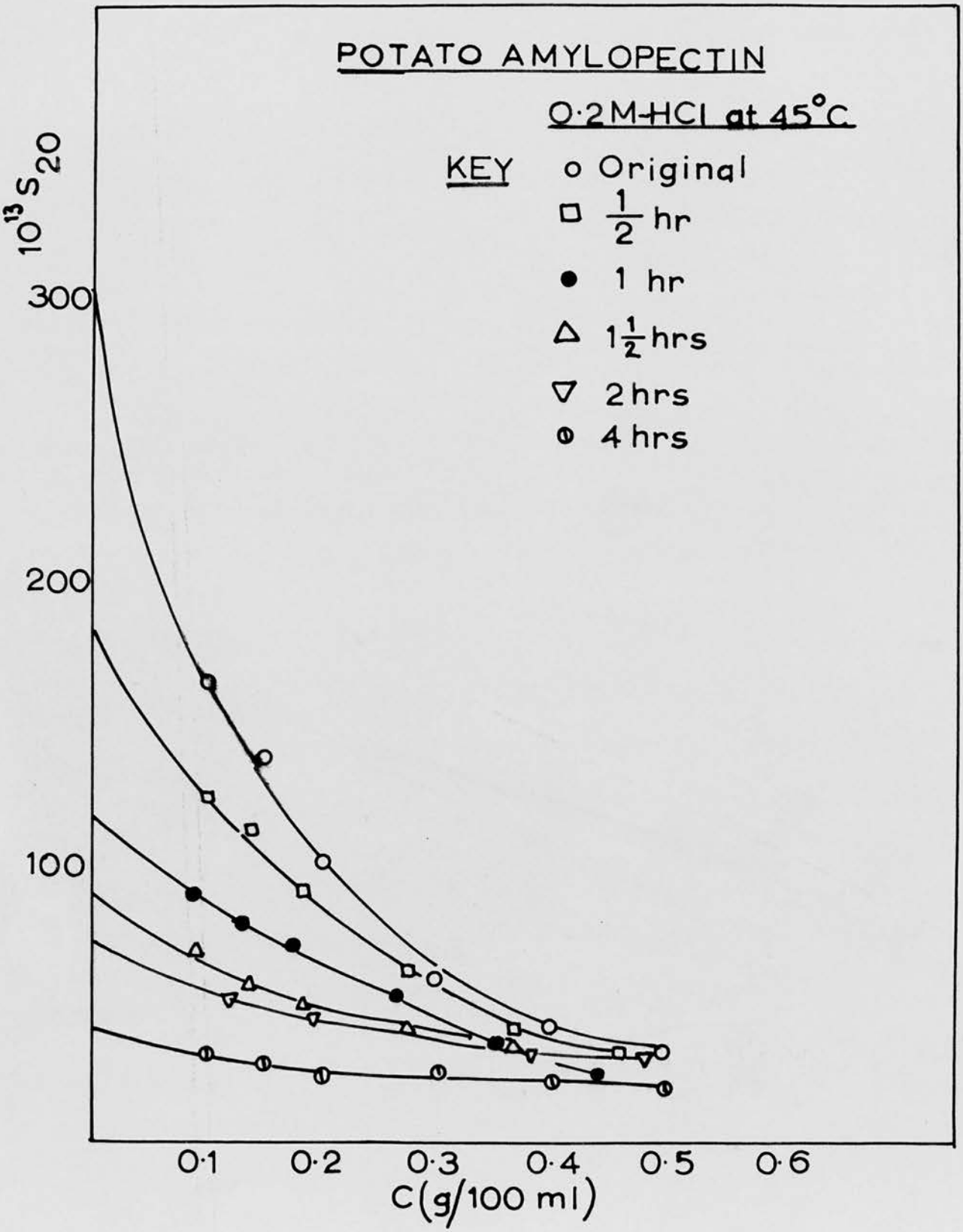
TABLE 10.

Acid Treatment (hrs.)	S_o [a]	$[S_o]^{-1}.10^3$	$(S/S_t - 1)$ [b]
0	300	3.33	0
1/2	180	5.56	0.67
1	112	8.93	1.68
1½	86	11.63	2.49
2	72	13.89	3.17
4	40	25.00	6.50

a - Sedimentation constant in c.g.s. units x 10^{13}
b - $S = S_o$ for the original amylopectin
$S_t = S_o$ at time t.

Fig. 14, p.86a, shows the graph of S_o versus c, fig. 15, p.86b the graphs of (a) $[S_o]^{-1}$ and (b) $(S/S_t - 1)$ versus t. From the graph of $(S/S_t - 1)$ versus t the number of bonds broken per initial molecule per hour is 1.6.

FIG 13


POTATO AMYLOPECTIN
0·2M-HCl at 45°C
KEY
o Original
□ ½ hr
● 1 hr
△ 1½ hrs
▽ 2 hrs
◍ 4 hrs
$10^{13} s_{20}$
300
200
100
0·1
0·2
0·3
0·4
0·5
0·6
C (g/100 ml)

DISCUSSION.

The effect of acid on potato starch granules has been previously studied (148). The conditions used here enabled the hydrolysis of potato amylopectin to be studied in homogeneous solution, when the effects due to granular inhomogeneity will be removed, and all bonds should be equally accessible. Thus reaction rate measurements should serve to prove the presence or absence of 'weak' or anomalous linkages.

The chromatographic evidence that only small amounts of maltose are formed, even after 30 hours of reaction, indicates that under these conditions the lower sugars will only be formed after very extensive degradation of the amylopectin, possibly to the dextrin. These results are similar to those obtained by Ulmann (141). Further the results obtained from β-amylolysis conversion limits and length of unit chain, indicate that the reaction occurring is purely random, since the figures obtained are relatively constant.

At the three temperatures examined the rate of degradation, as measured by plotting $[\eta]$ versus t (Fig. 11a), decreases and the limiting viscosity number appears to be reaching a limiting value. This could be interpreted as indicating that 'weak' linkages were present. On this basis, the curve obtained shows an initial rapid rate due to preferential scission of these, followed by a decreased rate of degradation to a limiting value. In Section III it was indicated that the plot should be not $[\eta]$ but $[\eta]^{-1}$ versus t. This is shown in Fig. 11b and at all three temperatures the graphs are straight lines which pass through the value of $[\eta]^{-1}$ for the original polymer, i.e. $\lim_{t\to 0} [\eta]^{-1} = [\eta]^{-1}$ for the original polymer.

FIG 14

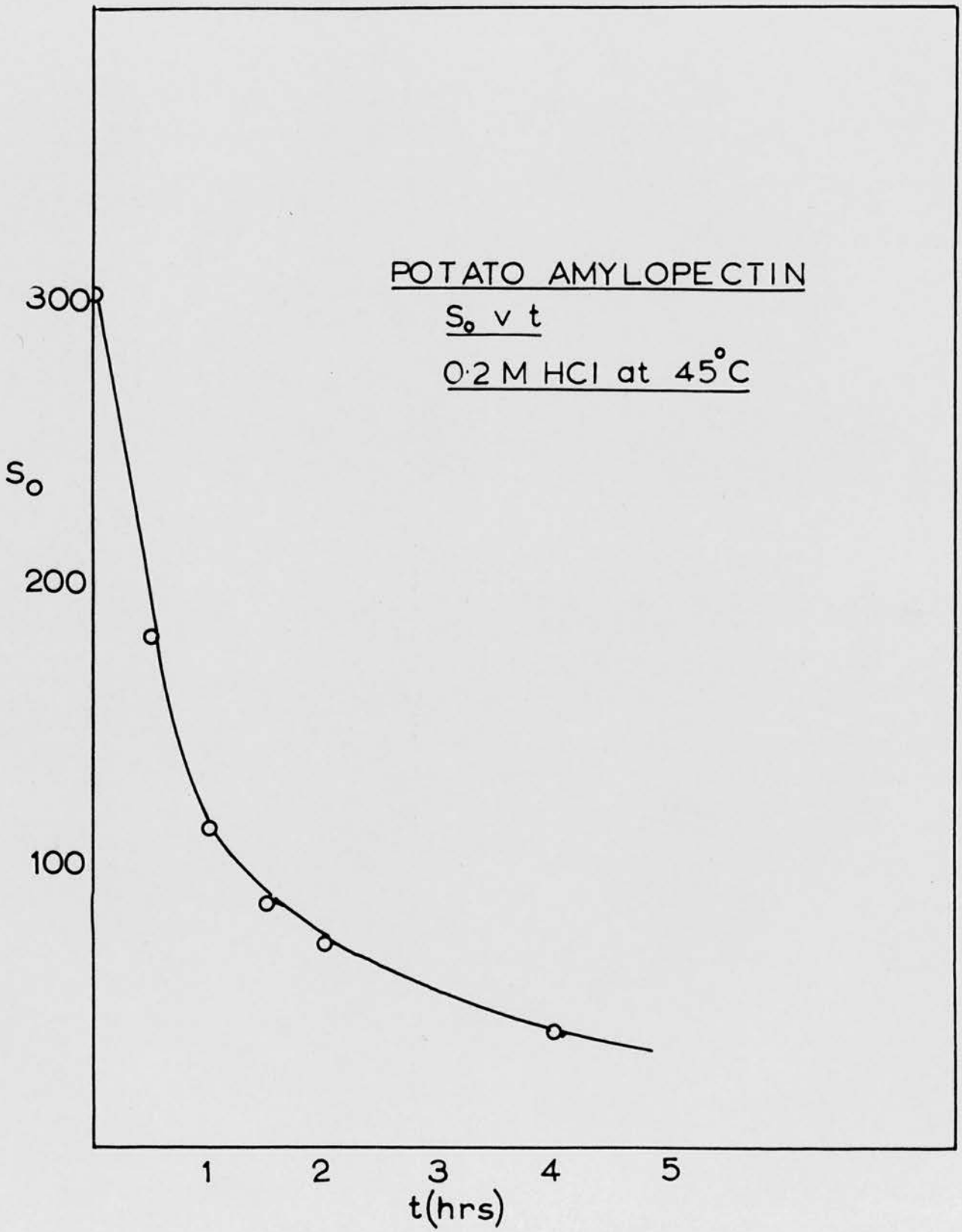
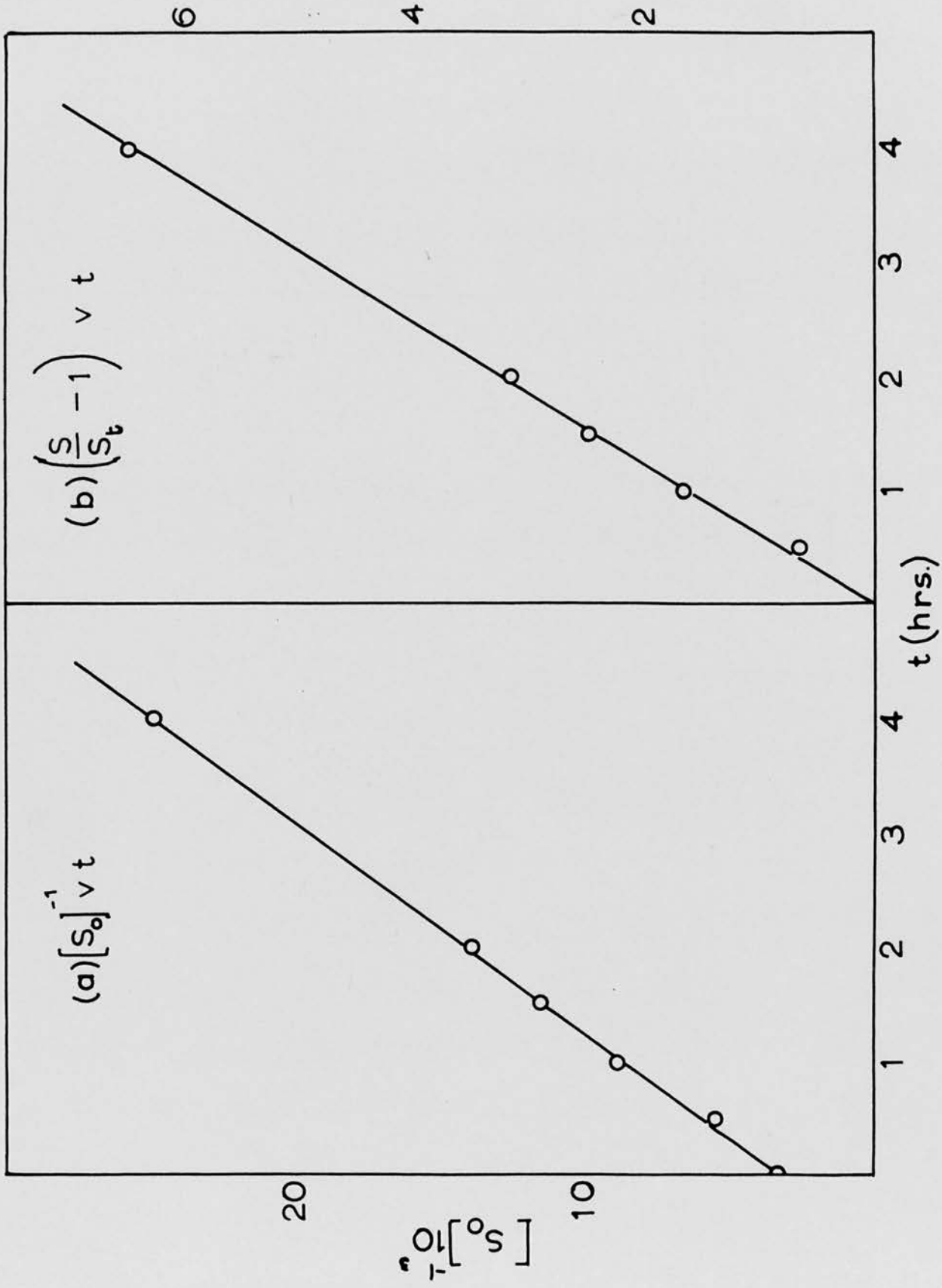

POTATO AMYLOPECTIN
S_o v t
0·2 M HCl at 45°C
300
200
100
S_o
1
2
3
4
5
t(hrs)

FIG 15


(a) $[S_o]^{-1}$ v t
$[S_o]^{-1} 10^2$
20
10
1
2
3
4
t (hrs.)
(b) $\left(\frac{S}{S_t} - 1\right)$ v t
6
4
2
1
2
3
4

There is therefore no evidence for the presence of any weak or anomalous linkages in amylopectin. From Philipp, Nelson and Ziifle's work (150) on the hydrolysis of crystalline and amorphous cellulose, it can be deduced that hydrolysis occurs much more rapidly in the amorphous region of the fibre. By analogy, in the case of acid-treatment of potato starch granules, the results were attributed to a two stage attack (148). In homogeneous solutions this difficulty in interpretation does not arise. The rate of degradation is a constant over the whole course of the reaction measured, and at all three temperatures. Thus there does not appear to be preferential attack at any weak or anomalous linkage but rather a random hydrolysis. This is supported by β-amylolysis and periodate studies. [The results obtained here are in agreement with those obtained by replotting results described in the literature for the degradation of cellulose by various methods (149)(147)] . A similar treatment of the results from sedimentation measurements is shown in Figs. 14 and 15a. Again the plot of S_o versus t results in an apparent approach to a limiting value of S_o, whereas the plot of $[S_o]^{-1}$ versus t shows that the rate of degradation is in fact constant. The value of the number of bonds broken per initial molecule per hour - 1.6 - is higher than that obtained for the heterogeneous degradation. For the reasons already stated in Section III this rate is probably a minimum value.

The activation energy calculated for the reaction is 29.4 K cals. This value is comparable with the figure of 29.0 K cal. which was obtained by Freudenberg and co-workers (151) for the α-1:4 -D bonds

in starch, as a result of reducing end-group measurements on hydroly of starch by very strong acid. It is also of the same magnitude as the corresponding figures obtained for the hydrolysis of normal glycosidic linkages obtained by Moelwyn-Hughes (152). This then indicates that the reaction predominantly involves random hydrolysis of α-1:4 bonds. Swanson and Cori (153) studied the rates of acid hydrolysis of various polysaccharides, including maltose, amylose and amylopectin, and from the fact that maltose and amylose were hydrolysed at the same rate, they concluded that the α-1:4 bond between two glucose units has a characteristic stability which is not dependent on the length of the chain in which it is situated. Amylopectin, although being branched and a more complex structure, had the same rate of reaction, which they took as indicating that all the α-1:4 bonds are equally susceptible to acid attack. From the fact that the limiting sedimentation constants obtained here indicate large changes, it can be concluded that inter-unit bonds are also being attacked. Since, however, the rate curves are uniform, these bonds at the branch points must either be hydrolysed at the same rate as the α-1:4 bonds or, if hydrolysed at a differen rate, then they must be too few to have a marked effect. Swanson and Cori were in fact able to show by use of bacterial-dextrans, that the α-1:6 linkage is less readily hydrolysed than the α-1:4 one.

SECTION V.

STUDIES ON WAXY MAIZE STARCH AND GLYCOGEN.

INTRODUCTION.

Most starches contain 15 - 27% amylose and hence study of the pure amylopectin component necessitates a suitable fractionation procedure. There are, however, a group of naturally occurring starches, the so-called waxy or glutinous starches, which contain very little amylose. They are in fact naturally occurring amylopectins, giving a red colour with iodine, and a potentiometric iodine titration curve typical of an amylopectin. In order to compare such a natural amylopectin with the samples previously obtained by fractionation, a sample of waxy maize starch was isolated.

At the same time the acid-hydrolysis of glycogen, whose structure is similar to that of amylopectin (see p.8) was studied for comparison. A commercial sample (National Biochemical Corporation) was used, because of its relatively narrow molecular weight distribution compared to laboratory extracted material (154).

EXPERIMENTAL.

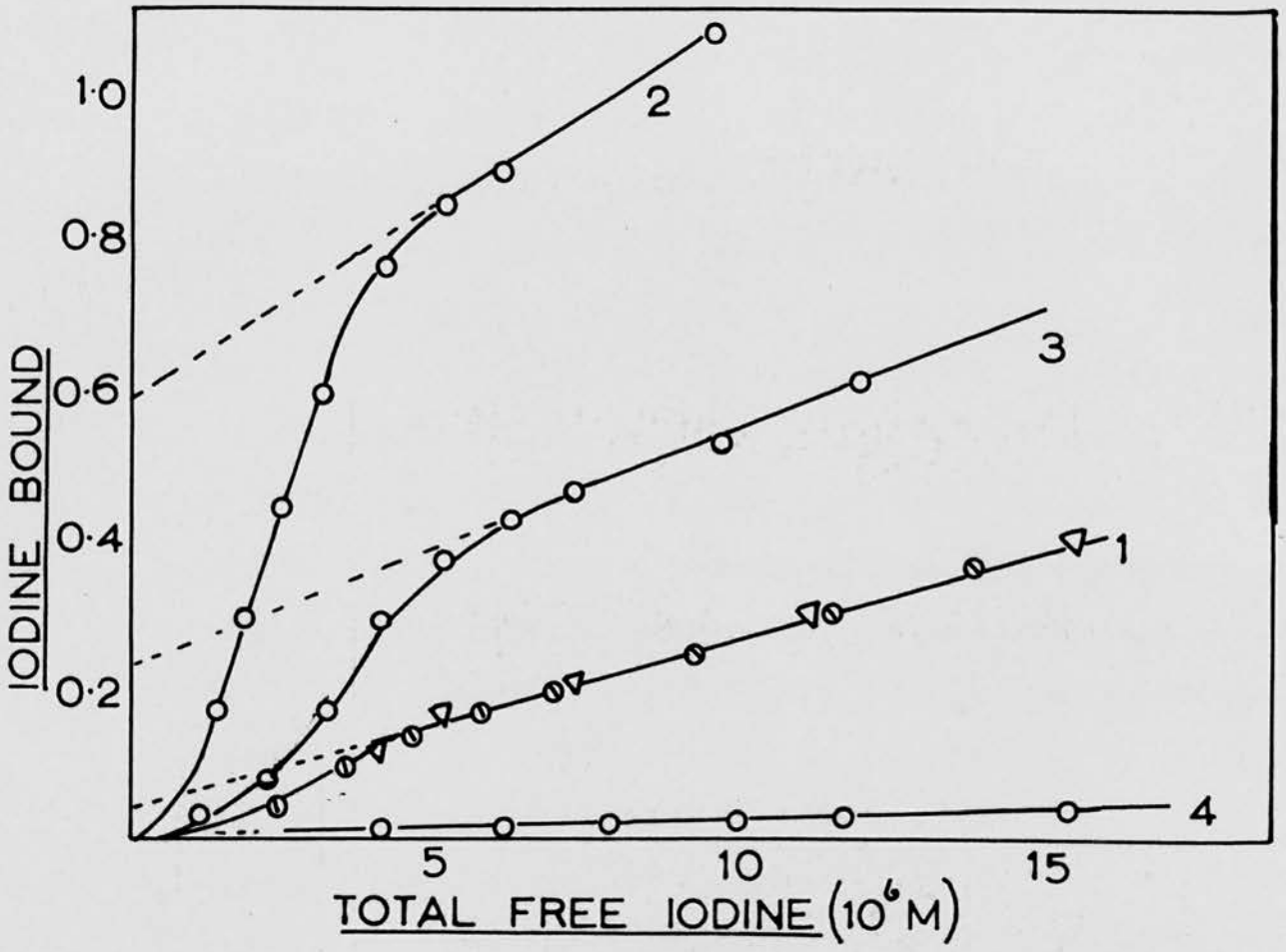
ISOLATION AND PURIFICATION OF THE STARCH SAMPLE.

The maize was lightly-milled and then allowed to stand at room temperature for 48 hrs. in 0.01 M-mercuric chloride to swell it, with a layer of toluene on top. Mercuric chloride has been shown by Peat, Whelan and Turvey (155) to prevent amylolytic degradation occurring during aqueous extraction. Starch was then obtained by shaking with 0.001 M-mercuric chloride and filtering the extract through a double layer of muslin. The material remaining was extracted a further three times until yields of starch became negligible. The extracts were then combined and the starch removed by centrifugation.

The method used to remove protein was as for cereal starches in Section II, and resulted in a product containing less than 0.1% protein. The purified waxy maize starch was exhaustively defatted with 80% methanol in a Soxhlet extractor for 48 hours, then stored under aqueous methanol at 0°C until required.

Potentiometric iodine titration on the purified, defatted starch gave a typical amylopectin type of curve, see Fig. 16, p.90a. For comparison this also shows wheat and potato amylopectins and glycogen. The curve shows that there was still a slight preferential uptake of iodine by linear material, presumably contaminating amylose, although some long branches in the amylopectin cannot be entirely excluded. Extrapolation of the linear portion of the curve indicated an iodine affinity of 0.05 which corresponds to less than 1% amylose. The limiting viscosity number in M-potassium hydroxide - 145 - is of the

FIG 16



1 WAXY MAIZE STARCH ○ before boiling
▽ after boiling

2 WHEAT AMYLOPECTIN

3 POTATO AMYLOPECTIN

4 GLYCOGEN

same order as the amylopectins from fractionated starches. Since the iodine titration curve showed the presence of linear material, attempts were made to fractionate the waxy maize starch.

The method used was that described in Section II involving complete aqueous dispersion, by boiling for 1 hour in a nitrogen atmosphere, followed by selective precipitation of the amylose with thymol. After 3 days the thymol-saturated solution was centrifuged and any precipitate removed. The supernatant after two extractions with ether was freeze-dried, and characterised by (1) limiting viscosity number, and (2) potentiometric iodine uptake.

This method proved unsuccessful for fractionation. On the small scale of experiment carried out (2 g.) no precipitation occurred, and the potentiometric iodine titration curves of the sample before and after boiling were identical. [Fig. 16.] The limiting viscosity numbers obtained - 145 and 148, Fig. 17, p.91a - also show no variation. The failure of this method to remove the small amount of linear material present is in agreement with the difficulty encountered in the removal of traces of contaminating amylose from the amylopectin-product of a starch fractionation (16). The amount of linear material present is so small that it is unlikely to affect any studies on waxy maize starch.

THE EFFECT OF ACID ON WAXY MAIZE STARCH AND GLYCOGEN.

Introduction.

The first experiments describe the effect of normal sulphuric acid on waxy maize starch, the method being essentially that of Bourne and Peat (156). The second set describes the effect of 0.2 M-hydrochloric acid on both waxy maize starch and glycogen, these conditions being chosen to allow comparison with potato amylopectin (Section IV).

Experimental

(1) Treatment of Waxy Maize Starch with sulphuric acid.

Waxy maize starch (20 g.) was dispersed in 0.1% sodium chloride (2L) and boiled for 1 hour, under nitrogen and with stirring. It was then allowed to cool to room temperature and any insoluble material was removed on the centrifuge. Powdered thymol (4 g.) was then added to the supernatant, which was then kept at 30°C for 48 hours in a water bath. Sufficient concentrated sulphuric acid was then added slowly to give an approximately molar solution, ahd the whole kept at 30°C for a further 72 hours. Any precipitate was then removed in the centrifuge (1600 r.p.m., 15 mins. M.S.E.).

The well-packed solid was washed with water (100 mls.) saturated with thymol, three times with aqueous alcohol (100 ml., 30% water), and triturated with alcohol. The solid was then dissolved in water and freeze-dried for later work.

The precipitated product was then characterised by (1) β-amylolysis

(2) limiting viscosity number $[\eta]$ in M-potassium hydroxide and

(3) potentiometric iodine titration.

The supernatant was neutralised with sodium hydroxide, and any salts which precipitated were removed on the centrifuge. A portion was then dialysed and freeze-dried.

(2) Treatment with hydrochloric acid.

(a) Waxy maize starch (1 g.) was suspended in water (160 ml.) and heated on a boiling water bath for 5 minutes, under nitrogen and with slow stirring. The reaction vessel was then transferred to a bath whose temperature was controlled to $45^{\circ}C \pm 0.1^{\circ}$ and left stirring overnight. A control sample was first removed, then concentrated hydrochloric acid (3 ml.) was added. Samples were removed at noted times, neutralised and stored at $0^{\circ}C$ until required. A small amount was removed from each sample before neutralisation and spotted directly on chromatographic paper [control sugars, solvent and development were as for potato amylopectin, Section IV].

The samples were studied by means of the ultracentrifuge. Potassium hydroxide was added to each sample to give a 0.2 M-solution, and from this a dilution series made in 0.2 M-potassium hydroxide. The graph of sedimentation constant versus concentration was used to obtain a value for the sedimentation constant at infinite dilution.

(b) Glycogen was treated in a similar fashion with hydrochloric acid. The size of the products was examined by means of the ultracentrifuge as above, and in addition by light scattering measurements. With Mr. I. G. Jones absolute turbidities (τ) were determined with a

Brice-Phoenix Photometer (157) at wavelength 546 mμ. The dissymmetry ratio ($I_{45}°/I_{135}°$) was also measured. The solvent, 0.2 M-sodium chloride, was filtered through sintered glass (G.5) directly into the scattering cell. The glycogen solutions were filtered through sintered glass (G.4), and successive aliquot parts were added to a weighed amount of solvent in the scattering cell to form a concentration series in the range 3 - 20 x 10^{-5} g./ml. [concentrations were obtained by the alkaline ferricyanide-ceric sulphate method, Section I]. Molecular weights (M) were calculated from the equation:

$$\frac{Hc}{\tau} = \frac{1}{M(P_{90}°)}$$

$$\text{where} \quad H = \frac{32\,\pi^2 n^2 (dn/dc)^2}{3\,\lambda^4 N.}$$

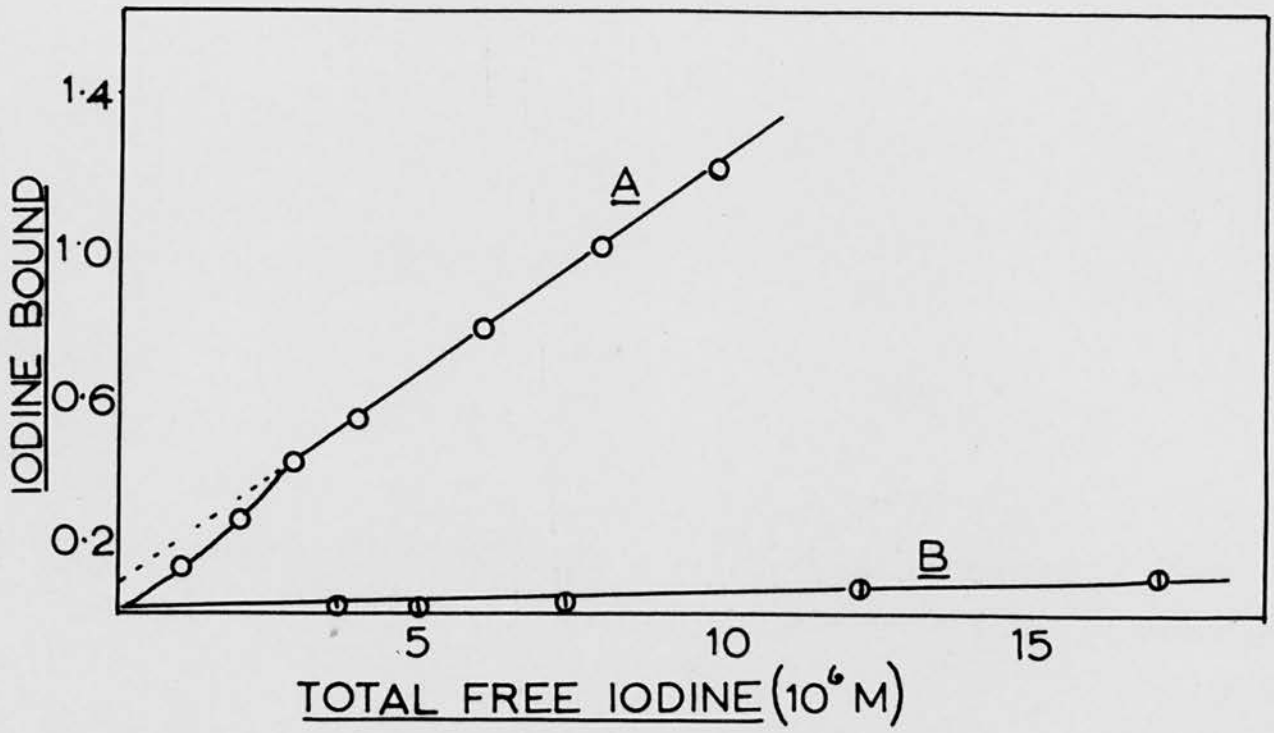
($P_{90}°$) is the particle scattering factor (158); N - Avogadro's Number; λ the wavelength; c the concentration, and n the refractive index of the solvent. Within experimental error (Hc/τ) was independent of c for the range of concentrations studied.

RESULTS AND DISCUSSION.

(1) Sulphuric acid on Waxy Maize Starch.

After solution of the starch had occurred, a sample was removed and freeze-dried, the iodine binding power of this giving that of the original material (A). The iodine binding power of the supernatant after the fractionation (B) was also determined. These results are shown in Fig. 18, p.95a, while Fig. 19, p.95b, shows the iodine titration curve of the precipitated material (C). The yield of (C) from an initial 20 g. of starch was 130 mg., which corresponds to less than 1% of the total. The iodine affinity of (A) which was 0.08 indicates the presence of less than 1% of amylose, using the value 19.0 as the maximum iodine binding power. This figure 19.0 was the maximum iodine binding power obtained for pure amylose from cereal starches (Section II), and is used here to allow comparison between the yields of (C) and the percentage of amylose originally present. The two figures thus obtained are in good agreement. The graphs of the iodine titration curves of (A) and (B) indicate that some measure of fractionation has occurred, and that of (C) further shows that the precipitate is enriched in amylose. The curve, however, is similar in nature to those obtained from acid treated wheat starch, and for comparison one of these 0.2N-HCl at 45°C for 24 hours is shown, Fig. 19 D. The abnormal nature of these curves makes it difficult to calculate the purity. Rough extra-:polation to zero free iodine concentration, however, gives an iodine affinity of 12.5, corresponding to 66% amylose (calcd. from (I.A. x 100)/19.0.)

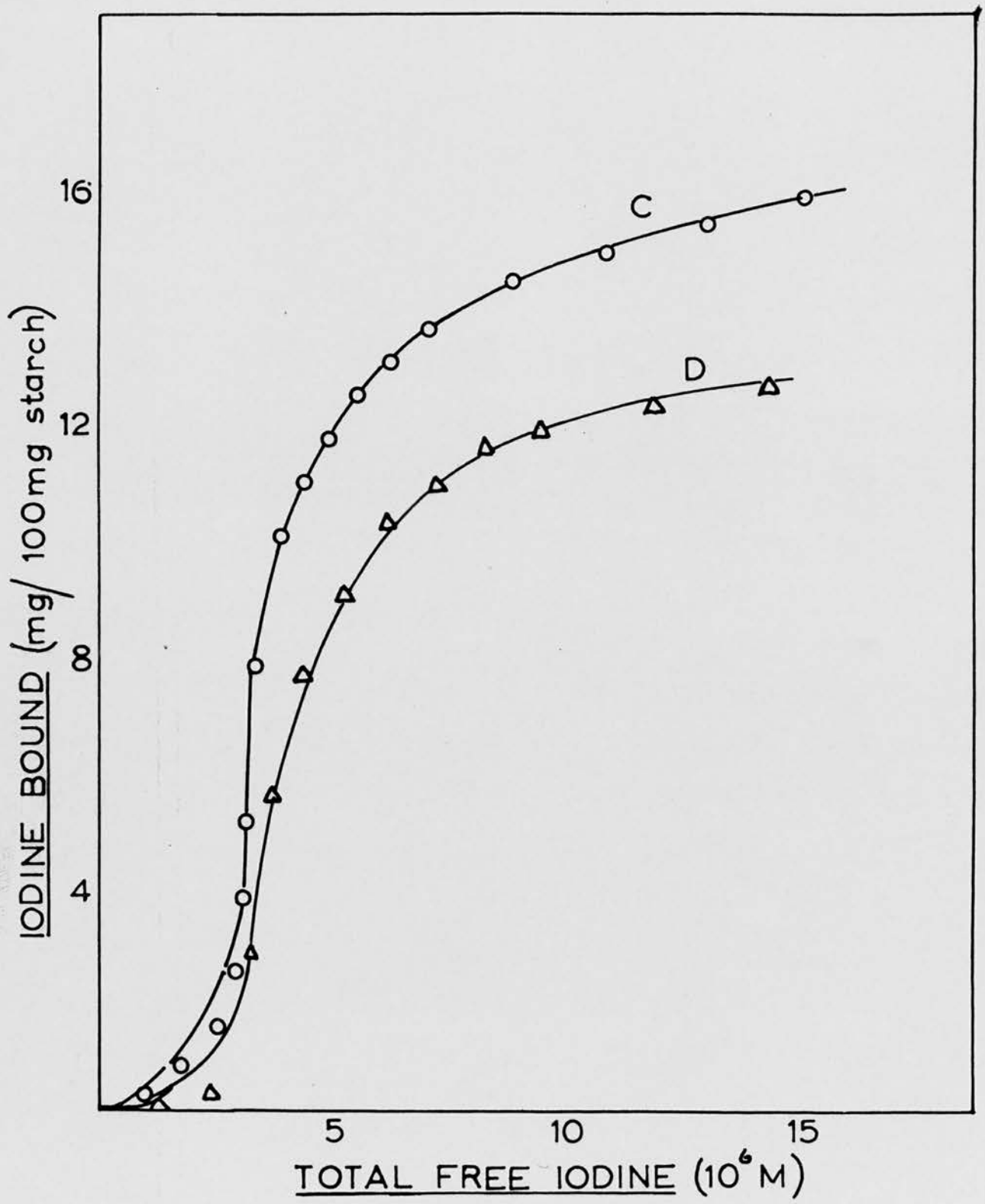
FIG18



A ORIGINAL WAXY MAIZE STARCH

B MATERIAL in SUPERNATANT from THYMOL-H_2SO_4 TREATMENT

FIG 19


16
12
8
4
C
D
5
10
15
IODINE BOUND (mg/ 100mg starch)
TOTAL FREE IODINE (10^6 M)

The β-amylolysis experiments were carried out using (i) pure soya-bean β-amylase and (ii) barley β-amylase, which contains β-amylase and Z-enzyme, as described in Section I. The results obtained were for (i) 71% and for (ii) 85% conversion limits, expressed as % conversion into maltose. Assuming that conversion of the amylopectin impurity into maltose is 56%, the percentage conversion of the amylose using pure β-amylase is 78%, while using β-amylase plus Z-enzyme it is 100%. These figures are comparable to those obtained for cereal and potato starches (Tables 3 and 4, Section II).

The limiting viscosity number of (C) is 45 (Fig.16) which is low in comparison with the values obtained for other fractionated amylose indicating that quite extensive degradation must have occurred. The extent cannot be obtained due to the difficulty of obtaining (C) by a method which does not involve degradation.

Thus a product is obtained similar to the amyloses obtained by fractionation of other starches, as indicated by iodine titration curve and β-amylolysis limits. The low limiting viscosity number and the type of titration curve obtained, however, indicate that extensive hydrolytic degradation has occurred.

(2) <u>0.2 M-hydrochloric acid at 45°C</u>.

The results of these experiments are summarised in Table 11 (p.97).

Since the results obtained for both waxy maize starch and glycogen are similar they will be treated together.

It has already been indicated (Section IV) that any 'weak' or

TABLE 11.

Effect of 0.2 M-hydrochloric acid at 45°C on Waxy Maize Starch and Glycogen.

Sedimentation Measurements.

Waxy Maize Starch

Acid treatment (hrs.)	s_o [a]	$[s_o]^{-1}.10^3$	$(s_o/s_t - 1)$ [b]
0	500	2.0	0
½	280	3.6	0.80
1	178	5.6	1,80
1½	126	7.9	2.95
2	98	10.2	4.10
4	56	17.9	7.95
8	30	33.3	15.5

Glycogen

Acid treatment (hrs.)	s_o [a]	$[s_o]^{-1}.10^3$	$(s_o/s_t - 1)$ [b]
0	69.5	14.4	0
½	64	15.6	0.09
1	61.5	16.3	0.13
1½	57	17.5	0.22
2	54.5	18.4	0.28
4	47	21.3	0.48
8	35.5	28.2	0.96

[a] Sedimentation constant at infinite dilution measured in c.g.s. units x 10^{13}.

[b] S_o and S_t are the sedimentation constants at infinite dilution at times o and t hours.

Light Scattering Measurements.

Glycogen.

	0	½	1	2	4	8
$M.10^{-6}$ [c]	5.4	4.5	4.2	3.4	2.8	1.8
$[M]^{-1}.10^6$	0.19	0.22	0.24	0.29	0.36	0.56

[c] M = molecular weight.

anomalous linkages will be indicated from the plot of $[S_o]^{-1}$ versus t. Extending this to light scattering measurements the plot of $[M]^{-1}$ versus t should show the presence of weak bonds. Waxy maize starch shows a large concentration dependence making extrapolation of the sedimentation results for the untreated sample extremely difficult. A value of 500, however, allows some idea to be obtained of the rate of degradation. In the case of the glycogen sample, a straight line relationship was found to hold for S versus c over the concentrations studied (0.6 - 0.2 g./100 ml.).

The graphs of S_o versus t and $[S_o]^{-1}$ for waxy maize starch are shown in Fig. 20, p.98a, while that of $(S_o/S_t - 1)$ is on Fig. 21, p.98b Fig. 22, p.98c shows the graphs of (i) S_o versus t and (ii) $[S_o]^{-1}$ versus t and (iii) $(S_o/S_t - 1)$ versus t for glycogen, while the results from light scattering measurements are shown graphically in Fig. 23, p.98d

It will be observed that for glycogen and waxy maize starch, S_o versus t (and M versus t for glycogen) indicates a very rapid initial decrease in the sedimentation constant, after which the curve tends to reach a limiting value. That this is not indicative of weak bonds breaking at a faster rate than the rest of the bonds is shown by the fact that plotting the reciprocal of these functions versus t gives straight lines. Hence there is here for both waxy maize starch and glycogen, as there was for potato amylopectin, no evidence of any 'weak' or anomalous linkages being preferentially broken at a rate which is greater than for the rest of the molecule.

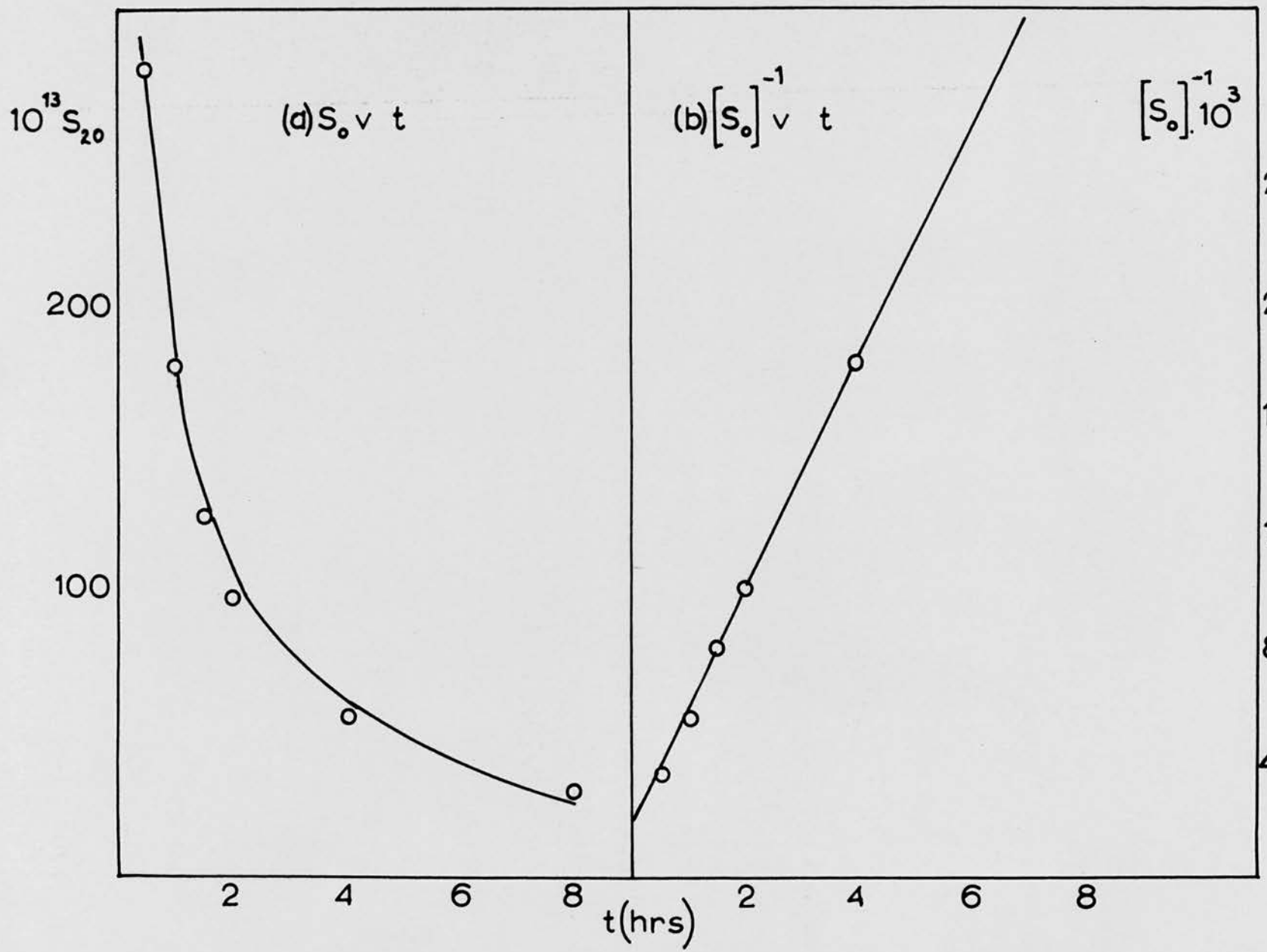

$10^{13} S_{20}$
(a) S_o v t
200
100
2
4
6
8
t(hrs)
(b) $[S_o]^{-1}$ v t
$[S_o]^{-1} 10^3$
24
20
16
12
8
4
2
4
6
8

FIG 20

FIG 21

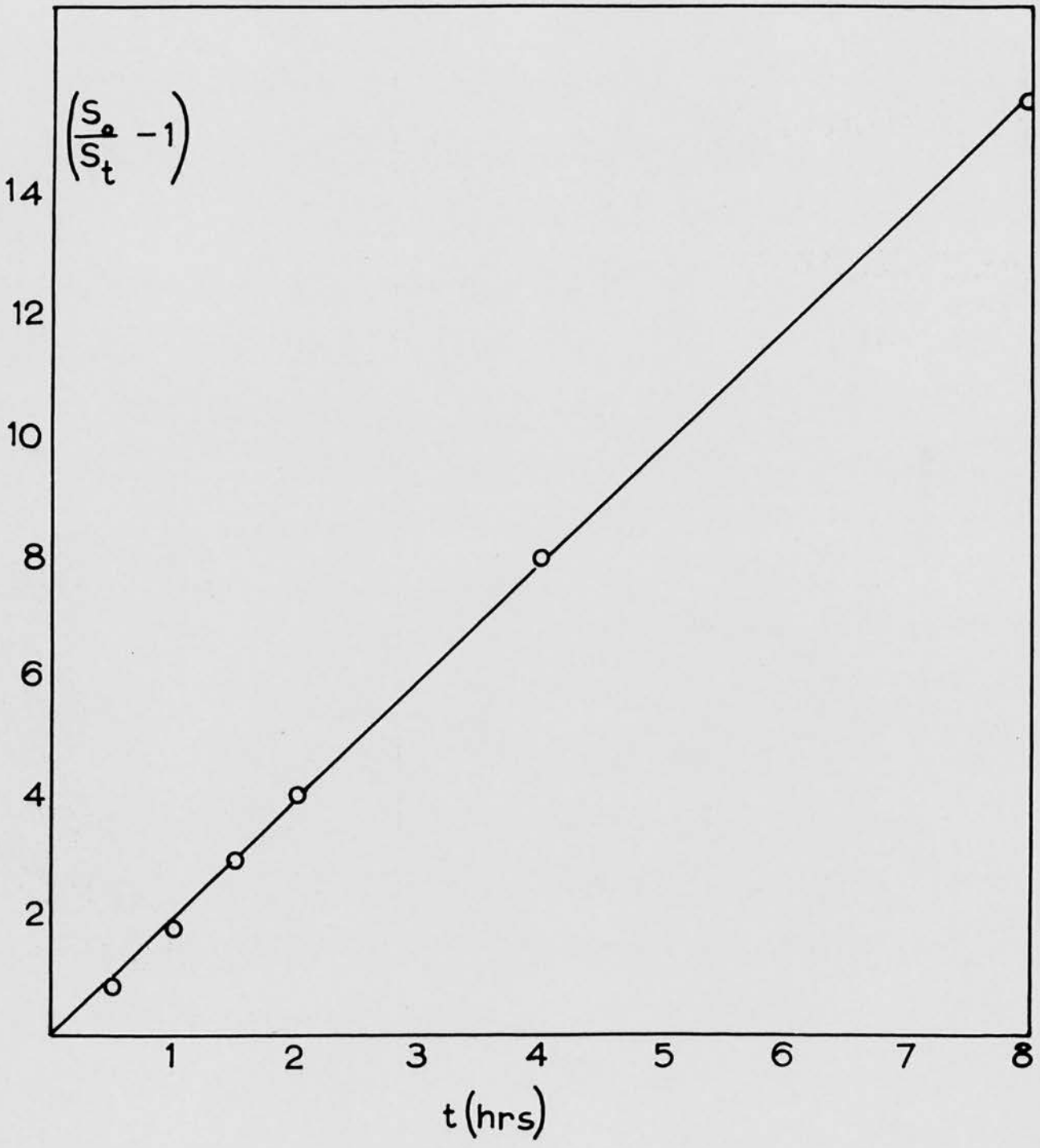


FIG 22

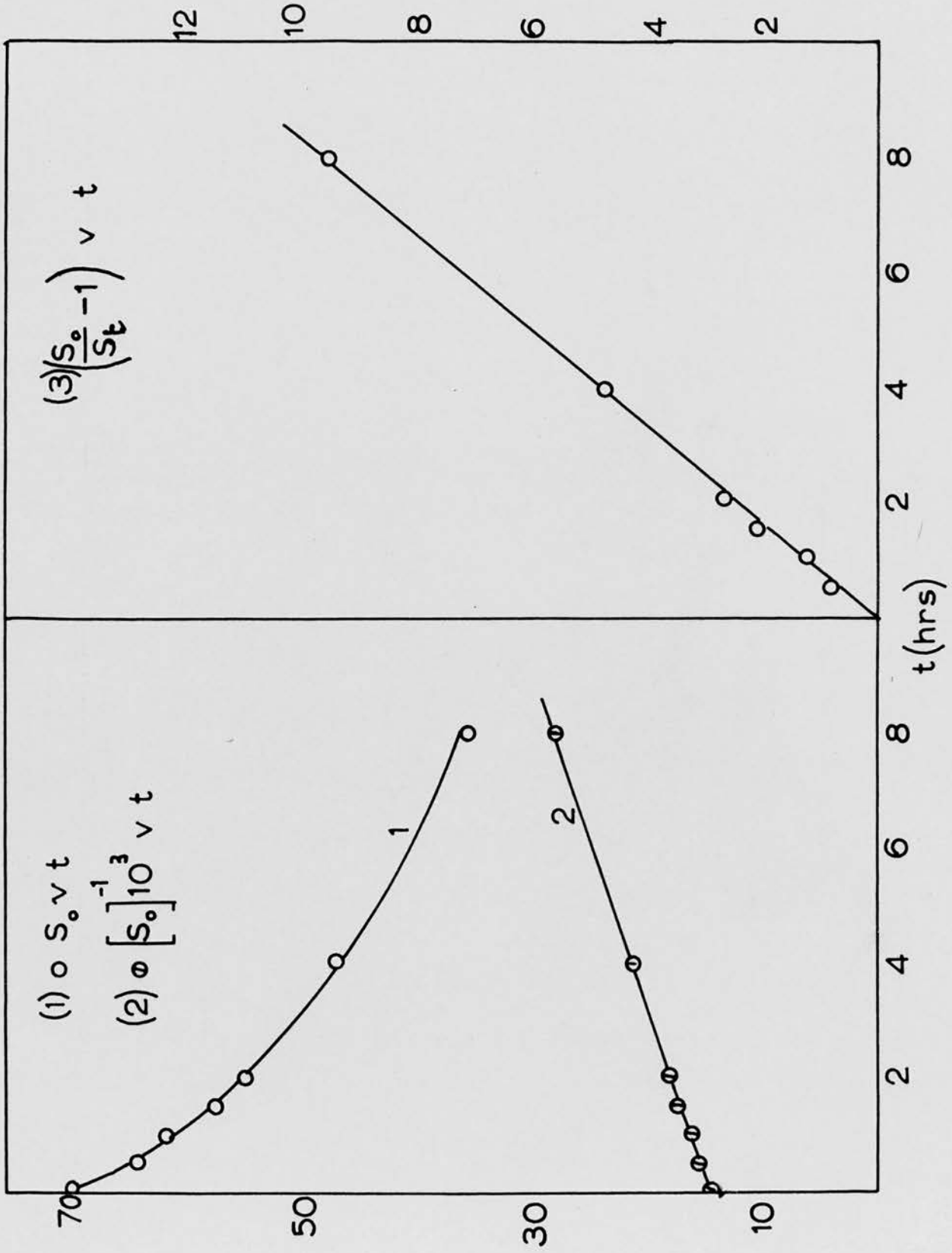
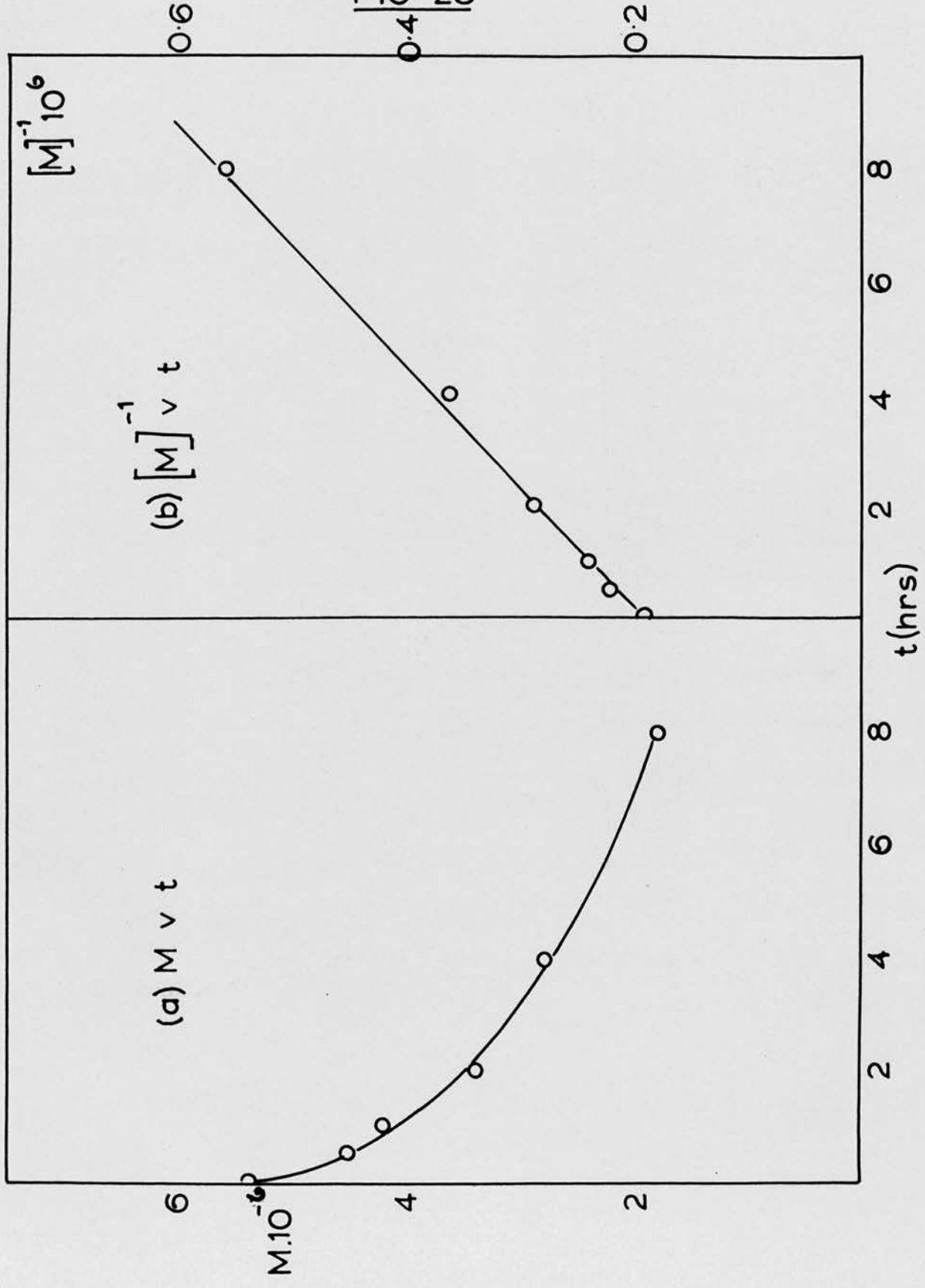

(1) o S_o v t
(2) o $[S_o]^{-1}10^3$ v t
(3) $\left(\frac{S_o}{S_t}-1\right)$ v t
1
2
70
50
30
10
12
10
8
6
4
2
2
4
6
8
t(hrs)

FIG 23



The chromatographic evidence obtained from the waxy maize starch sample is also very similar to that obtained for potato amylopectin. The original sample showed no evidence of any glucose or maltose, but the 30 minute and subsequent samples were all shown to have maltose present but no glucose. Although this appearance of maltose occurred in both potato amylopectin and waxy maize starch, there does not at present appear to be any reason why this should occur. In both cases, the amounts did not appear to increase markedly as the reaction proceeded. It is of interest to note that in a preliminary experiment, using 1 M-hydrochloric acid at 45°C, chromatographic examination of the products indicated that glucose was not formed until 48 hours had elapsed, although after 6 hours the sedimentation constant at approximately 0.2% concentration had fallen to $9 \times 10^{13} s_{20}$.

From Fig. 21 the number of bonds broken per initial molecule per hour is 1.9. This figure is slightly greater than that of 1.6 obtained for potato amylopectin under the same conditions.

From Fig. 22(b) it can be calculated that the rate of glycogen degradation is only 0.1 bond broken per initial glycogen molecule per hour. A similar result (0.2) is obtained from fig. 23(a) M versus t, using the expression no. of bonds broken = $(M_o/M_t - 1)$ where M_o and M_t are the molecular weights at times o and t respectively. Thus glycogen, although similar in general structure to amylopectin, must have fine structural differences. The slower rate is probably due to its more compact nature, the inter-chain linkages being so closely arranged as to be less accessible to hydrogen ions.

HYDROLYSIS OF METHYLATED WAXY MAIZE STARCH.

INTRODUCTION.

The acid-hydrolysis of various methylated starches in aqueous methanol solution containing oxalic acid has been reported (38)(39). These authors have suggested that the conditions causing disaggregation in the methylated starch did not appear to be suitable for the free starch, where it appeared that hydrolysis of the repeating units themselves occurred. To examine what would happen in the case of the pure methylated amylopectin component, attempts were made to methylate waxy maize starch.

EXPERIMENTAL.

METHOXYL DETERMINATIONS.

These were carried out by the modified Zeisel method (159)(160). The method consists of splitting of the methoxyl group as the alkyl halide, by heating it with hydriodic acid. The alkyl iodide is absorbed in a solution of bromine, sodium acetate, and glacial acetic acid in water, where it reacts forming methyl bromide and iodine bromide. The last is then oxidised to iodate, the sodium acetate neutralising the hydro-bromic acid formed, and the iodine liberated on addition of potassium iodide estimated by titration with standard sodium thiosulphate solution. Determinations were done in duplicate. The theoretical methoxyl content for tri-O-methylamylopectin is 45.6%.

The methylation of waxy maize starch has been reported (161). In the initial experiments this method, essentially that of Hirst and Young (38), was used. Waxy maize starch (20 g.) was made into a cream with water (250 ml.), and sodium hydroxide (175 ml.; 30%) slowly added with vigorous stirring. The resulting viscous paste was dispersed by the addition of water (100 ml.). Sodium hydroxide (400 ml.; 30%) and dimethyl sulphate (200 ml.) were then slowly added from dropping funnels into the reaction vessel. Vigorous stirring was continued throughout and a constant stream of nitrogen bubbling through the solution ensured oxygen-free conditions and prevented degradation. The reaction was allowed to proceed overnight and a further addition of reagents made. The mixture was then partially neutralised with sulphuric acid, and gently heated on the steam bath.

The resultant layer of partially methylated starch was removed and dissolved as far as possible in acetone (400 ml.), and remethylated with sodium hydroxide (350 ml.; 30%) and dimethyl sulphate (140 ml.) a further five times. The acetone was then removed under pressure, the partially methylated starch, obtained as a rubbery mass, removed and remethylated as above. Samples were removed at intervals during the methylation. These were dissolved in chloroform and dried over anhydrous sodium sulphate. After filtering this, most of the chloroform was removed under pressure until a viscous solution remained. This was then slowly poured into petroleum ether (b.p. 100 - 120°C) with stirring. The flocculent white precipitate which separated was filtered and dried <u>in vacuo</u> at 70°C.

No. of methylations	2	7	10	13	20
Methoxyl Content %	24.2	32.2	37.6	38.0	39.6

A second sample after eight additions of dimethyl sulphate and sodium hydroxide at 40°C gave a product containing OMe 34.2%. Further methylation of this product was carried out using Purdie and Irvine's method (162). Dried partially methylated starch (7.3 g.) was dissolved in neutral methyl iodide (200 ml.) and heated on a water bath till the solution was boiling gently. The condenser was fitted with a calcium chloride guard tube to prevent entry of water. Freshly prepared silver oxide (100 g.) was then added in batches of 2.5 g. every 15 minutes. The addition was made over 10 hours and the reaction left overnight. The flask was then allowed to cool and the contents

filtered. While most of the methylated material was obtained in the filtrate, a small amount remained absorbed on the oxide. This was removed by extracting the oxide with hot chloroform and filtering, the extraction being repeated.

No. of methylations with Ag_2O/MeI	1	2	3
Methoxyl content %	36.0	36.7	37.4

Methods making use of agents which cause swelling of polysaccharides have been suggested for methylation. The modification of the liquid ammonia method of Freudenberg, Boppel and Meyer-Delius (163) by Hodge, Karjala and Hilbert (164) was investigated.

The essential requirements are that the liquid ammonia should be dry, and a reaction temperature of -30°C, the boiling point of liquid ammonia be maintained. Waxy maize starch (2 g.) was stirred with dry liquid ammonia in a reaction vessel as described by Robertson (165). Sodium was then added in small pieces, the rate of addition being so controlled that the amount of free sodium present in the reaction mixture did not exceed the amount which would react within the next 15 minutes. The sodium reaction time was 1 hour, after which methyl iodide was added and allowed to react for 1 - 1½ hours. After four treatments the liquid ammonia was evaporated off under anhydrous conditions in vacuo, and the solid remaining dialysed (3 days), then freeze-dried. The methylation was repeated, the isolation and purification process being repeated after every three treatments. The final product was extracted in a Soxhlet extractor with light

petroleum (b.p. 60 - 80°C) and the extracts discarded. The solid was dissolved in chloroform and the methylated material precipitated with light petroleum (b.p. 40 - 60°C).

		Methoxyl Content %
Found:-	10 additions Na/MeI	27.6
	16 additions Na/MeI	32.8.

The above series of experiments indicate that the sample studied here could not be fully methylated. It is thought that this may be due to the resistance of the granular structure to any swelling agents, or the very large molecular weight as indicated by sedimentation measurements. Rigorous exclusion of oxygen, thus preventing degradation, could be an assisting factor.

For comparison with the free component results obtained previously by Forsyth (161) for the acid hydrolysis, under the conditions of Hirst and Young (38), of a fully methylated waxy maize starch were critically re-examined. The results obtained in Forsyth's work is shown in Fig. 24, p.103a, as η_{sp}/c versus t, while the graph of $\left[\eta_{sp}/c\right]$ versus t is shown on Fig. 25, p.104a, for the two temperatures of the experiment.

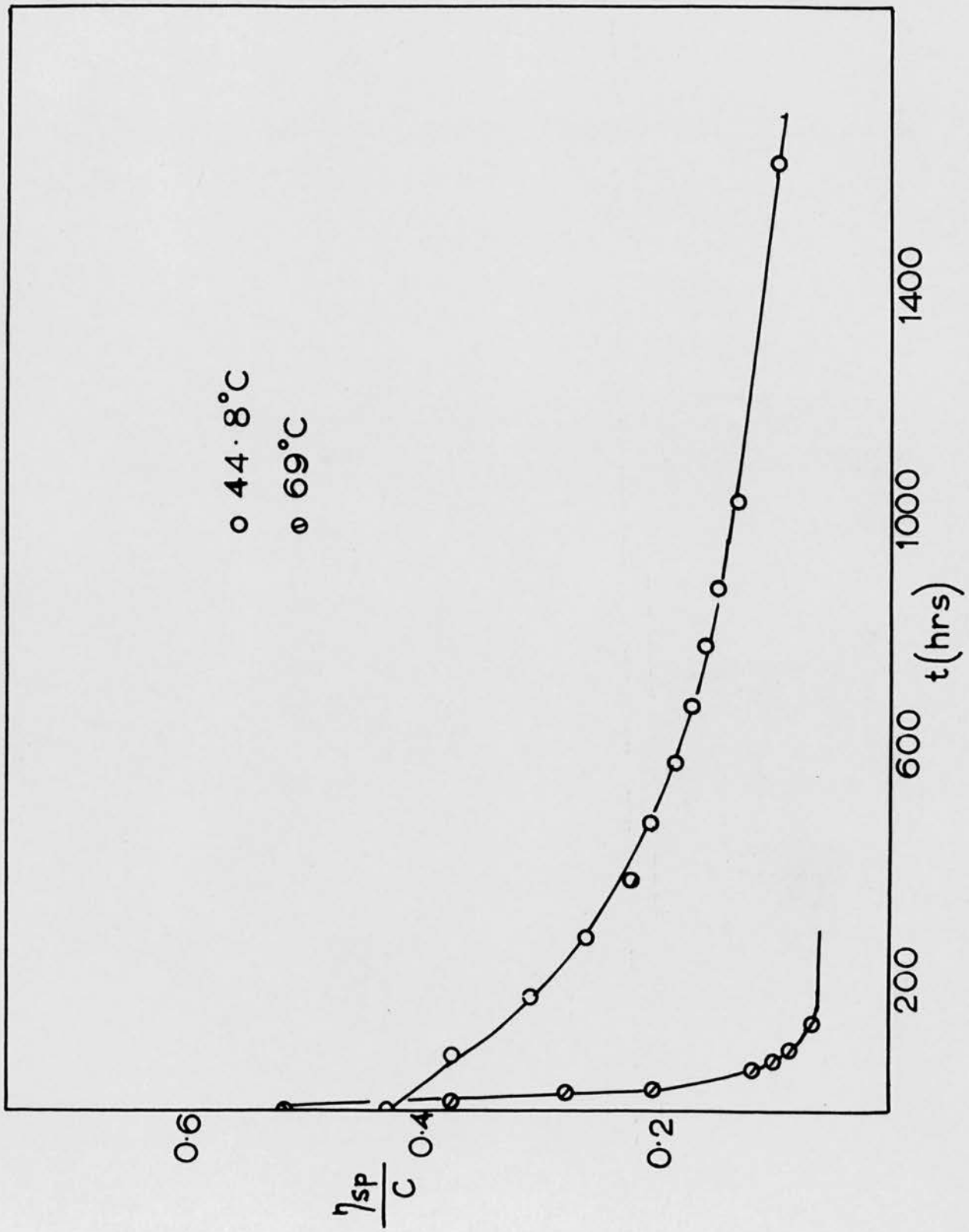
From the slopes of the graphs in Fig. 25, it is possible to calculate the velocity constant of each reaction,

$$k_{44.8^\circ C} = 13.6 \cdot 10^{-7} \cdot \text{sec}^{-1}$$

and $$k_{69.0^\circ C} = 26.9 \cdot 10^{-6} \cdot \text{sec}^{-1}$$

Using these values the activation energy of the reaction can be

FIG 24



obtained from the expression

$$E = 2.303 \cdot R \cdot \log \frac{k_2}{k_1}\left(\frac{T_2 - T_1}{T_1 T_2}\right)$$

where k_1 and k_2 are the rates at absolute temperature T_1 and T_2 and R is the gas constant.

Then - $E = 26.6$ K cals.

DISCUSSION

The conditions used for the disaggregation of methylated waxy maize starch have been made comparable to the original experiments on methylated starches. Although the viscosity results indicate an initial rapid rate of degradation and first order kinetics are not obeyed until 100 hours have elapsed, the plot of $\left[\eta_{sp}/c\right]^{-1}$ versus t is a straight line passing through the value obtained for the original sample. This suggests that there are in fact no weak or anomalous linkages in methylated waxy maize starch.

It was found by Hirst and Young (38) that the size of the final product ($\overline{D.P.}$ = 100), as measured by viscosity, was independent of the starch source. For waxy maize starch a similar result was found by Forsyth, the final product having a $\overline{D.P.}$ of 110. These units suggest that a separation of repeating units is occurring, and support from this comes from the high yield of disaggregated product and its apparent homogeneity. However for such low molecular weight products, viscosity measurements are insensitive and provide no true measure of molecular size. A limiting viscosity is then fallacious and a number average method should be used (149).

On the basis that α-1:6 bonds are the only inter-unit bonds, it is difficult to explain why some of these bonds break while others of the same nature do not. The presence of bonds other than the normal α-1:6 could furnish an explanation. An approach to this problem has been made through periodate oxidation studies (76). The method involves periodate oxidation of amylopectin followed by hydrolysis and examination of the hydrolysate chromatographically. If a cross-

linkage occurs through c_2 or c_3 of a D-glucose unit in any chain, this unit will not be attacked by periodate and will appear as D-glucose on hydrolysis of the polyacetal. The amount of D-glucose which has been reported varies (76)(77)(78), and its absence after oxidation has also been reported (79)(80), hence the evidence on this point is conflicting.

The fact that the percentage of end-group for methylated and disaggregated product is the same, although suggesting that there is no reduction in the chain length might also be due to the fact that the percentage increase in the number of terminal groups is so small as to be within experimental error.

The value of the energy of activation (26.6 K cal.) of the methylated waxy maize starch from the graphs of $\left[\eta_{sp}/c\right]^{-1}$ versus t for two temperatures agrees with that obtained on recalculating Forsyth's data (26.0 K cal.). This value is still of the same order as that for the hydrolysis of normal glycosidic linkages, although it is lower than that obtained for the activation energy of the hydrolysis of the unsubstituted potato amylopectin (30 K cals.).

In conclusion, the results obtained here indicate that methylated amylopectin, like the free components, contains no 'weak' or anomalous linkages, which are hydrolysed first and give an initial reaction rate which is greater than that for the normal linkages.

SUMMARY.

Physical methods have been used to study the properties of various starch products, with regard to both purity and molecular size.

Oat and wheat starches have been isolated and purified under conditions likely to reduce inadvertent degradation. The behaviour of these, and a sample of commercial wheat starch, on fractionation by dispersion and aqueous leaching has been critically examined. The efficiency of the procedures has been followed by measurements of the purity and molecular size of the resultant components. The method of fractionation involving pre-treatment with alkali, followed by conventional fractionation using precipitants, appears to be the most satisfactory. Aqueous leaching at various temperatures caused sub-fractionation of the amylose. The uniformity of these different amylose fractions has been investigated.

Wheat starch has been treated with 0.2 M-hydrochloric acid at 45°C, and M-hydrochloric acid at 16°C, and the effect on the granular structure of the starch and molecular size of the fractionated components examined. Fractionation of acid-treated samples proved more difficult than for the original starch. Both the amylose and amylopectin components were degraded, the amylopectin preferentially. The results are also discussed with regard to the structure of the granule.

The degradative effect of 0.2 M-hydrochloric acid on the molecular size of potato amylopectin has been examined at three temperatures (35, 45 and 54.3°C). The homogeneous conditions of the reaction allows

the presence of 'weak' or anomalous linkages to be detected. Under none of the conditions studied was there any indication of such bonds. The activation energy for the reaction, 29,500 cals., is of the same order as for normal glycosidic linkages.

The degradation of waxy maize starch, a naturally-occurring amylopectin, and a similar α-1:4 branched glucosan-glycogen - have been studied under the same conditions as for potato amylopectin. In neither case was any evidence found for the presence of weak bonds. The rate of degradation of waxy maize starch is similar to that for potato amylopectin, while the rate for glycogen is much slower, indicating a more compact structure.

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