University of Edinburgh

PHYSICOCHEMICAL STUDIES ON STARCHES

by

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THESIS

submitted for the degree of

DOCTOR OF PHILOSOPHY

January, 1973
Abstract of Thesis

After a brief Introductory Section, general methods of characterization of starch and its fractions are detailed in Section 2 of this work. The methods of chemical and physical investigation employed in later sections are reported, and some modifications of general techniques proposed.

Section 3 presents two special techniques devised to facilitate later experiments. Firstly, a method of estimation of the starch-content of a cereal grain is described. Starch is extracted from previously macerated plant material by treatment with calcium chloride solution \( d = 1.3 \text{ g/ml} \) at \( 130^\circ - 135^\circ \text{C} \). A complete extraction of all starches, including those of high amylose-content, is achieved, and the extracted starch estimated by a combination of highly-specific enzymes. Secondly, an extensively-modified technique of semi-micro, differential, potentiometric iodine-titration is proposed. A simplified electronic circuit is described utilizing a digital voltmeter as a null detector. Control experiments are described which have established analytical conditions allowing the estimation of the iodine-binding capacity of starch and its fractions to an accuracy of \( \pm 1.5\% \).

The next Section describes a number of experiments on amylomaize starch. After a short review outlining the outstanding problems, a simple method of isolating the anomalous material from this starch is described. A partial characterization of this low-molecular weight material was performed and revealed that some of the branched fraction was fundamentally different from normal maize amylpectin. The starch from the pollen of amylomaize was examined in detail and shown to be similar in nature to the parent endosperm starch.
In Section 5, the biogenesis of starch in general and barley starch in particular is investigated. The character of two barley starch genotypes during growth is detailed and this information related to general theories of biosynthesis. The nature of compound starch granules in varieties of pea and potato is investigated, and a hypothesis concerning the formation of starch granules is advanced.

The final Section of this work, reviews the chemistry of the hydroxyethyl derivative of starch with special relation to its use as a blood plasma expander and cryoprotective agent for human erythrocytes. Serious deficiencies in the present knowledge of this polymer are revealed. The rest of this Section presents some experiments performed to clarify these points of dispute, a model of hydroxyethyl starch is proposed, and the relevance to its intended pharmaceutical use discussed.
Preface

I wish to thank Dr. C.T. Greenwood and Dr. W. Banks for their advice, friendship and encouragement during the period of this research. Some of this work has been published in conjunction with my colleagues and reprints of the relevant papers are inserted at the end of this thesis. My thanks are also due to Dr. B. Lowe for his assistance.

I wish to thank Professor C. Kemball for the provision of laboratory facilities, and the Science Research Council for a maintenance grant during the years 1969-1971, and the Council of the Flour Milling and Baking Research Association and the Office of Naval Research, United States Navy Department, for laboratory facilities and financial assistance in the period October 1971 to December 1972.

My thanks are also due to my many other colleagues for their long-suffering friendship and advice, especially Mr. C. Groves for assistance with the photographic work and Mrs. M. Honour for typing this thesis.
To my parents and Marian
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SECTION ONE

GENERAL INTRODUCTION
General Introduction

Section 1

Starch consists of semi-crystalline, solid granules of density ca. 1.5 g/ml which vary widely in shape and range in size from 1 to over 100 microns. Starch granules occur in all higher plants, where they are laid down in specialized organelles called plastids. In all plants starch acts as an energy reserve, and may be arbitrarily classified into two types:

a) starches with a transient existence, which are regularly metabolized, for example, the starches which are deposited in green leaves during periods of active photosynthesis and are broken down to provide energy during periods of darkness.

and b) starches which retain their integrity over long periods of time, for example endosperm, tuber and rhizome starches; this type of starch remains in the plant storage organs over periods of several months during which the plant is dormant, to be broken down for energy at the initiation of new growth.

Starches have a high nutritive value and plants rich in starch of this latter type are widely cultivated. For this reason, starch and its mode of synthesis have been the subject of academic and commercial research for at least two centuries. Despite huge effort and a voluminous literature on the subject, the biosynthesis of starch is a major unsolved problem, and much is still to be resolved concerning the nature of the components of starch and their organization within the granule.

This thesis represents work by the author on several independent but related topics in starch chemistry. A historical review is not presented separately, as pertinent literature is detailed within each Section. The first two Sections detail some new and modified
experimental techniques which, in conjunction with already established methods, have been applied to later studies. The nature of amylomaize starch is then investigated and some novel conclusions reached. The next Section is devoted to a study of the biogenesis of starch, and experimental evidence is presented which casts doubts on some earlier conclusions reached by other workers in this field. A tentative hypothesis is proposed to accommodate this new data.

The final Section is devoted to a study of the hydroxyethyl derivative of starch, which has become of considerable importance as a potential cryoprotective agent for human erythrocytes and a blood plasma expander.
SECTION TWO

GENERAL EXPERIMENTAL METHODS
Section 2

Summary

Sections 2.1 to 2.3 of this section detail the methods of isolation, assay and fractionation of the starches used in later sections of this work.

Parts 2.4 and 2.5 detail methods of characterization of the starch fractions by enzymic and physical techniques.

Section 2.6 details the methods used to characterize granular starches involving measurement of such properties as microscopic appearance, granule size distribution and gelatinization temperature.
2.1 Isolation of Starch

Starch must be isolated in a high state of purity before fundamental characterization may be carried out. The isolation of cereal starches, the subjects of much of this thesis, presents several problems; for this type of starch occurs firmly occluded in a protein matrix within a well-defined, cellular structure. Isolation of the starch involves initially separating the starch from the cell-wall material then subsequently removing the adhering protein.

The cereal kernels were softened by extensive steeping in 0.01M mercuric chloride, to inhibit enzymic attack and prevent germination. When only a small amount of starch was being isolated, the softened kernels were macerated in a high-speed blender in 0.01M mercuric chloride then screened through 150 μ mesh then 75 μ mesh nylon screen to remove fibre and cell wall debris. An electric meat-mincer was employed to process large quantities of grain when required. The grain was passed through a series of diminishing mincer sizes until a mash of porridge-like consistency was obtained. Fibre was screened off as above. This type of preliminary extraction was shown by Banks and Greenwood (1972) to be superior to dry milling, as little physical damage was inflicted on the starch.

The adhering protein was removed by the method of Greenwood and Robertson (1954). The starch slurry was shaken vigorously by hand for several minutes, with a tenth volume of toluene. The protein was physically denatured at the toluene-water interface and floated into the supernatant toluene layer, whilst the starch readily sedimented in the aqueous phase. It was found that a considerable amount of starch, ca. 50% in the case of some legume starches, was physically entrapped...
in the protein-toluene layer. Recovery was effected by decantation of the toluene layer, dilution with several volumes of water and violent agitation of the mixture with an air stream. Several treatments of the toluene layer were required to remove all of the starch. Repeated shaking of the starch with toluene in this manner eventually yielded a white starch which had a nitrogen content <0.03%, i.e. on the borderline of estimation by the semi-micro Kjeldahl technique employed. In contrast to earlier workers, starches were not subsequently defatted by soxhlet extraction with hydrophilic solvents since this markedly changes many of the granular properties e.g. see Banks (1960) on the leaching of amylose. When necessary, fat was completely removed by dissolving the starch in methyl sulfoxide then precipitating the starch with alcohol. The effectiveness of this technique is discussed in detail in Section 3.3, pp. 76.

Typical analyses for two representative wheat starches isolated by the above technique are shown in Table 2.1. Similar analyses are shown for two commercially-available wheat starches. It is at once obvious that laboratory-isolated starches have much lower levels of physical damage, as measured by the method of Stewart (1966) and considerably lower protein contents. Adkins and Greenwood (1969b) have demonstrated quite convincingly that lower levels of chemical damage are caused by this type of purification procedure than by that used in commercial practice.

2.2 Assay of Purified Starch and its Fractions

Starch may be completely converted to glucose by a careful hydrolysis either by dilute acid, or by a specific enzyme, and the liberated glucose estimated by a large number of techniques. In this work, starch and fractions thereof were routinely estimated by dissolution in methyl sulfoxide, then hydrolyzed enzymically by amylglucosidase to glucose which was subsequently assayed using a coupled glucose oxidase-peroxidase-chromogen system.
Table 2.1

A Comparison of Starches Isolated by the Techniques Detailed in Section 2.1 and Similar Commercially Available Starches

<table>
<thead>
<tr>
<th>Starch</th>
<th>%H$_2$O$^1)$</th>
<th>%N$^2)$</th>
<th>% Damage$^3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat (ex van Joss Cambier)</td>
<td>11.45</td>
<td>0.03</td>
<td>0.8</td>
</tr>
<tr>
<td>Wheat (soft Australian)</td>
<td>9.30</td>
<td>0.02</td>
<td>0.7</td>
</tr>
<tr>
<td>Wheat (commercial English)</td>
<td>12.05</td>
<td>0.08</td>
<td>12.3</td>
</tr>
<tr>
<td>Wheat (commercial Australian)</td>
<td>9.30</td>
<td>0.09</td>
<td>27.3</td>
</tr>
</tbody>
</table>

1) Moisture content, heated for 16 hr at 102°C.
2) Measured by Kjeldahl technique.
Reducing sugar was measured by the alkaline-ferricyanide method. These techniques are detailed below, and some observations on the complete hydrolysis of starch with acid are reported.

(a) Enzymic Hydrolysis

Amyloglucosidase (α-1,4-glucan glucohydrolase) was kindly supplied by Dr. I. D. Fleming, Glaxo Research Ltd., Stoke Poges, Buckinghamshire. It was dissolved in distilled water (1 mg. per ml.) to yield a solution of activity = 14 units per ml. (1 unit = 1 micromole of glucose liberated per minute from soluble starch at pH 4.6 and 37°C), and this stock solution was stored at 2° - 4°C. The starch or fraction thereof was dissolved in methyl sulphoxide and a digest prepared thus:

1) Starch or polysaccharide to give a final glucose concentration ca. 1-2 mg. per ml.

2) Acetate buffer 0.1M, pH 4.8-4.9 such that 0.01M < final buffer concentration <0.025M.

3) Stock enzyme solution to give a final concentration of not less than 0.1 unit per ml.

The digest was stored at 37°C in an incubator overnight and aliquots withdrawn for glucose assay. In cases where the starch fraction was particularly unstable in aqueous solution, a ten-fold increase in amyloglucosidase concentration was used, and the polysaccharide in methyl sulphoxide was added to an already buffered solution containing α-amylase [250 units per ml.; the unit of activity being that defined by Greenwood, MacGregor and Lilne (1965)]. By this technique, the polysaccharide is rapidly reduced to a low degree of polymerization where stability in solution is assured.

(b) Estimation of Reducing Sugar

Several different methods of estimating reducing sugars are available, but the method of Ackins et al (1969) has proved in routine
use to be reliable and of high accuracy. In this technique, reducing sugar is reacted with alkaline ferricyanide for 15 minutes in a boiling water bath. The ferrocyanide so formed is then titrated in acid conditions with ceric sulphate using xylene cyanol FF indicator. The method was calibrated with solutions prepared from anhydrous glucose and maltose monohydrate (Kerfoot, Biochemical Reagents). Linear calibration curves were obtained in the range 0.5 - 3 mg glucose per ml and the corresponding range for maltose. The following calibration constants, identical to those of Adkins et al (1969) were determined and used in all estimations of reducing power.

Glucose : 0.329 mg/ml 0.01 N Ceric Sulphate
Maltose : 0.425 mg/ml 0.01 N Ceric Sulphate

In agreement with Adkins et al (1969), methyl sulphoxide to 1% (v/v) in the original sample was found to have no effect on the reducing values obtained.

(c) Specific Estimation of Glucose

Many of the enzymic techniques detailed later in this section depend on the specific estimation of glucose in the presence of other polyglucans including maltose. This work utilises some of the methods detailed by Banks and Greenwood (1971d), based on the principles suggested by Kesten (1956) that the coupled enzyme system of glucose oxidase-peroxidase acting in the presence of a suitable dyestuff was suitable for the specific micro estimation of glucose.

The enzyme glucose-oxidase specifically catalyses the oxidation of β-D-glucopyranose to δ-gluconolactone which spontaneously hydrolyses to gluconic acid. Glucose oxidase is a flavo-protein, containing the flavine adenosine dinucleotide (FAD) moiety. This is reduced by the abstraction of two hydrogen atoms from carbon C1 on the glucose ring,
and subsequently reacts with dissolved atmospheric oxygen to generate hydrogen peroxide. In the presence of the enzyme peroxidase, this hydrogen peroxide is used to oxidize a colorless solution of O-dianisidine dihydrochloride to a chromophore which is spectrophotometrically estimated. This scheme is shown below:

\[
\text{Glucose} \rightarrow \delta\text{-gluconolactone} \rightarrow \text{gluconic acid}
\]

\[
\text{FAD} \rightarrow \text{FADH}_2
\]

\[
\text{FADH}_2 + O_2 \rightarrow \text{FAD} + \text{H}_2\text{O}_2
\]

\[
\text{H}_2\text{O}_2 + \text{O-dianisidine dihydrochloride} \rightarrow \text{peroxidase} \rightarrow \text{chromophore.}
\]

In this work, the following enzyme/buffer/chromogen system was employed: Glucose oxidase (35 mg; Type II; Sigma Chemical Company), peroxidase (3.0 mg; Type II; Boehringer Corp.) and O-dianisidine dihydrochloride (20.0 mg; "Analar" reagent) were dissolved in tris-glyceral buffer as detailed by Banks and Greenwood (1971d). This buffer system is designed to inhibit the maltase activity present as a contaminant in commercial preparations of glucose oxidase, and allows the estimation of glucose in the presence of maltose.

An aliquot (1.0 ml) of the glucose solutions containing 5-55 μg of glucose was incubated with the enzyme-chromogen mixture (2.0 ml) for 80 minutes at 37°C in hard-glass stoppered tubes. The reaction mixture was cooled in ice-water and sulphuric acid (4.0 ml, 24 N) added. The resulting color was estimated in a "Hilger" Spekker, using 1 cm cells and filter no. 605, i.e. 5500 μ.

The system was calibrated using standard glucose solutions and was always found to yield a linear relation between amount of glucose and color produced for solutions containing 5-55 μg glucose. The linear graph passed through the origin, but was found to vary by a few percent (~ 5%) over a period of several days. A standard glucose solution was
(d) The Hydrolysis of Starch with Dilute Acid

The observation of Marshall and Whelan (1970) that certain preparations of purified amyloglucosidase did not effect total hydrolysis of starch, necessitated the examination of new preparations of that enzyme before routine use. Soluble starch (potato, acid modified) was hydrolysed with sulphuric acid (1.5 N; 2 hours; boiling water bath), and the glucose determined using glucose-oxidase as detailed in the previous section. A similar hydrolysis was carried out using the amyloglucosidase preparation, and glucose was estimated in the same manner. In all cases, recovery of starch, by acid hydrolysis to glucose, was significantly less (96.5 - 97.5%) than that recovered by enzymic means. This at first sight seemed to support the observations of Pirt and Whelan (1951) and Marshall and Whelan (1970) that a significant amount of (1.5 - 4.0%) glucose is destroyed by acid hydrolysis of starch. However, when the sugar was assayed by estimation of reducing power, an apparently complete recovery (99 - 100%) was observed, in agreement with Adkins et al (1969). To resolve this apparent anomaly, the following experiments were performed.

(i) Maltose monohydrate (1.81 mg/ml) in H₂SO₄ (1.5 N) was hydrolyzed on a boiling water bath for a period of four hours. Samples were withdrawn at various time intervals, and glucose was estimated by the specific glucose-oxidase technique, after a suitable dilution with buffer and addition of a predetermined amount of potassium hydroxide to bring the pH to ca. 6.0.

The following result was found:

<table>
<thead>
<tr>
<th>Hydrolysis Time (hours)</th>
<th>1.5</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>% recovery of maltose</td>
<td>99.3</td>
<td>99.5</td>
<td>99.8</td>
<td>99.0</td>
</tr>
</tbody>
</table>
It is clear that no significant destruction of maltose or glucose occurs between 1.5 and 4 hours, and under these conditions hydrolysis is effectively complete.

(ii) Linear amylose (ca. 0.11g, Tapioca; {β}l = 99%) was dissolved in methyl sulfoxide (3.0 ml) overnight to yield a gel-free solution. This solution was made to 100 ml and 1.5 N with respect to H₂SO₄, then hydrolyzed on a boiling water bath. At various time intervals, samples (2 ml) were removed, and cooled rapidly by transferance to a thin-walled glass test tube immersed on an ice bath to freeze the reaction. A sample of cooled digest (1 ml) was added to an appropriately buffered solution ([10% v/v), acetate buffer 0.1 M, pH 4.8], containing the required amount of NaOH for neutralization. Glucose concentration was then estimated enzymically as detailed in part 2(c) of this section. A sample of the amylose solution had been extracted before commencement of hydrolysis and was estimated by enzymic means, after hydrolysis with amyloglucosidase as detailed in part 2(a) of this section. The recovery of amylose was expressed as a percentage of that recovered by enzymic hydrolysis and is shown as a function of hydrolysis time in Figure 2.1. It is clear that hydrolysis is complete in less than 90 minutes and that there is excellent agreement between enzymic and acidic techniques for hydrolysis of amylose.

(iii) Freeze-dried amylopectin (ca. 1.05 mg/ml, potato) was hydrolyzed as above by the enzymic method, and by acid hydrolysis, the acid hydrolysis being monitored by glucose production. A curve of percent conversion as a function of hydrolysis time is shown in Figure 2.2. The curve is divided into two distinct regions; an initially-rapid hydrolysis resulting in 95% conversion (almost identical to that of amylose) within 60 minutes, followed by a slow secondary process which reached total conversion in 180 minutes. At 120 minutes, the standard hydrolysis time of
Figure 2.1 acid hydrolysis
Figure 2.2 acid hydrolysis

PERCENT HYDROLYSIS (to glucose)

HYDROLYSIS TIME (MINUTES)

- AMYLOPECTIN
- GLYCOGEN
- DEXTRAN
Adkins et al (1969), only 97.5\% conversion had been achieved. This experiment was repeated and an almost identical result was obtained with 97\% conversion at 120 minutes. It would seem that under the conditions employed in this hydrolysis, since destruction of glucose had been ruled out, and complete hydrolysis of polymers consisting of residues linked by only \( \alpha-1:4 \)-bonds confirmed, that the \( \alpha-1:6 \)-bond, present in the branched material was significantly more resistant to acid hydrolysis.

Hydrolysis curves for glycogen (ca. 1.0 mg/ml, Oyster Liver B.D.H. Biochemical Reagent) with approximately 8\% \( \alpha-1:6 \)-linkages and dextran (MW 100,000) with approximately 10\% \( \alpha-1:6 \)-linkages are also shown in Figure 2.2. These curves confirm the suggestion that the \( \alpha-1:6 \)-link in branched glucans is much less susceptible to hydrolysis with 1.5N \( \text{H}_2\text{SO}_4 \) than the \( \alpha-1:4 \)-glycosidic bond. To prove this hypothesis, the following experiments were performed. Amylopectin was hydrolyzed under the conditions detailed above for 90 minutes, by which time, in amylose, all the \( \alpha-1:4 \)-links have been hydrolyzed. The hydrolyzate was neutralized with barium carbonate and excess barium ion removed by treatment with cation exchange resin (I.R.4, H\( ^+ \) form). The salt-free solution was reduced in volume, ten-fold, on a rotary evaporator and the concentrate freeze-dried. The white product was dried at 70\° C in vacuo overnight.

The material was tri-methyl silylated, in pyridine, by the method of Sweeley et al (1963) and the sample analyzed by gas-liquid chromatography. Analysis conditions were:

- **Chromatograph**: "Pye" 104; flame ionisation detector.
- **Column**: 14', glass, 1/8" I.D., packed with "Chromosorb W", acid washed, DCM/S treated, coated with silicon gum rubber (3\% S.E. 52, available "Phase Separations", Deeside Industrial Estate, Queensferry, Flintshire).
Carrier gas: Nitrogen 55 ml/min.

Samples: 5 µl of solution of sample dissolved in pyridine.

Temperature Programme: 1) isothermal at 186°C for 10 minutes
2) rapid heating ca 4.0°C per min to 252°C
3) isothermal at 252°C.

This temperature programme was found by experiment to give an excellent separation of maltose and isomaltose standard compounds. A typical chromatogram is shown in Figure 2.3 for a standard mixture of glucose, maltose and isomaltose. Superimposed is the trace for the partial hydrolysate of amylopectin. No maltose was detected, but a small amount of isomaltose was present. This isomaltose may have arisen from one of two sources:

1) as residual α-1:6-linked glucose units from the branch points of the original polymer, or
2) by acid reversion i.e. by recombination of glucose residues under acid catalysis to form isomaltose de novo.

Since, under the conditions employed in this work, no destruction of glucose was evidenced in the hydrolysis of maltose, it seems certain that the isomaltose detected in the latter stages of hydrolysis was residual from the original polymer structure. It was however necessary to explain why the method of Adkins et al (1969) gave apparently complete recovery of starch, whilst enzymic assay and gas chromatography have shown that, after 2 hours hydrolysis on a boiling water bath, the conditions employed by Adkins et al (1969), some 2.5 - 3.5% of the polymer remains unhydrolysed as isomaltose. A very likely explanation lies in the method of estimation of the reducing sugar. The alkaline-ferricyanide reducing agent employed in this reaction is highly non-stoichiometric and, in addition, the stoichiometry varies between glucose and maltose. Consider
Figure 23

SOLVENT

GLUCOSE

MALT Traffic

ISOMALT Traffic

STANDARD SUGARS

AMYLOPECTIN HYDROLYSATE
a mixture of 97% glucose and 3% isomaltose, and assume that isomaltose
does not differ very greatly from maltose in its stoichiometry in the
alkaline ferricyanide reaction. Using constants found in part 2(b).

a) 1 mg glucose = 3.04 ml 0.01 N ceric sulphate.
b) 0.97 mg glucose = 2.95 ml 0.01 N ceric sulphate
    0.03 mg maltose = 0.07 ml 0.01 N ceric sulphate
    i.e.0.97 mg glucose + 0.03 mg maltose = 3.02 ml 0.01 N ceric
    sulphate.

The difference between a) and b) is 0.02 ml or 0.6%. This is
very close to the limits of accuracy of the ferricyanide technique (ca
± 0.5%) and explains the apparently complete hydrolysis of starch. In
this present work, a hydrolysis time of 3 hr was invariably used when acid
hydrolysis was used to completely hydrolyze starch to glucose.

It is noteworthy that this work confirms the observations of Adkins
et al (1969) that negligible destruction of glucose occurs during this mild
hydrolysis. This technique would seem to offer some distinct advantage
over those methods employed by Marshall and Whelan (1970) and by La Berge
and Meredith (1970) where considerable glucose loss (ca 4%) was said to
occur. In the method employed in this work, the polysaccharide was always
put into neutral solution, either by boiling in water in the case of freeze-
dried amylopectin or, in the case of amylose, as the butanol-complex, or
by dissolving the starch in methyl sulphoxide then diluting with water,
before acid hydrolysis was carried out. This method involves no initially-
heterogeneous system which has high local concentrations of polysaccharide -
a condition known to promote acid-catalysed recombination of glucose.

2.3 Fractionation of Whole Starches

Detailed studies of the fine structure of starch fractions require
complete separation of the fractions from the parent starch with as little
modification of the native polymer structure as possible. Slight modification
affecting on average only one residue in ten amylose molecules 
(Degree of polymerisation $-10^4$), have been shown by Banks et al. 
(1959) to occur if precautions are not taken to exclude oxygen from 
fractionation mixtures. Such artificial barriers introduced in 
fractionation may seriously depress the susceptibility of the polymers 
to attack by exo-enzymes.

Two methods of fractionation have been employed in this work — 
aqueous leaching and aqueous dispersion. The former method, shown by 
Cowie and Greenwood (1957) to yield pure amylose from potato starch, is 
used in Section 4 to obtain samples of anomalous material from amylomaize 
starch, and is detailed in the appropriate part of this work. All other 
starch fractions were obtained by aqueous dispersion techniques. These 
involve separating the starch components by complete dissolution then 
preferentially complexing the amylose component.

Whilst tuber starches, such as potato and tapioca, readily 
dissolve in boiling water to yield molecular dispersions, the cereal 
starches do not. A specific pre-treatment using liquid ammonia e.g. 
Hodge et al (1948) or methyl sulphoxide e.g. Banks and Greenwood (1967b) 
is necessary to disrupt the granular structure before complete dispersion 
attained. Both methods are equally effective, but methyl sulphoxide 
pre-treatment is preferred only where small quantities (< 5g) are being 
fractionated, since impractically-large volumes of solvent would otherwise 
be required. Pretreatment was carried out as detailed below:

a) Liquid ammonia pretreatment

As described by Hodge et al (1948), liquid ammonia (100 ml) was 
added to the starch (10-15 gm) in a Dewar flask. After standing some 
30 minutes, the mixture was poured into ethanol (500 ml), and allowed to 
stand overnight in order to permit evaporation of ammonia. The starch
was removed by filtration, and washed with ethanol several times. The resulting "non-granular" starch was used in aqueous dispersion.

b) Methyl-sulphoxide pretreatment

As described by Banks and Greenwood (1967b), the starch was dissolved by stirring in methyl sulphoxide (concentration ca. 2%, adjusted to yield a viscosity such that mild shear occurred between the granules). Ethanol (3 vols.) was added to precipitate the starch, and the precipitate removed by low-speed centrifugation. Repeated tituration with ethanol yielded a finely-divided "non-granular" starch which was subsequently used in fractionation.

The non-granular starch, prepared as above, was then separated into predominantly branched, amylopectin, and predominantly linear, amylose, fractions. Although this separation may be achieved by selective retrogradation (Naquenne and Roux (1905)), electrophoresis (Samec et al (1941)), adsorption chromatography (Fisher and Settele (1954), Ulmann and Wendt (1954)), the method suggested by Schoch (1942) of selective complex formation and precipitation is the most successful yet devised. Polar organic substances complex readily with amylose to form insoluble complexes, which are readily recrystallized from water to yield pure amylose fractions. If, however, primary separation is not complete, the branched amylopectin fraction is not freed from linear contaminant and subsequent treatment is usually futile. To reduce this effect, Greenwood and Robertson (1954) used thymol as the initial precipitant. Fractionation in this work was carried out as follows:

"Non-granular" starch was slurried in a little cold water, and added to boiling deoxygenated water to give a concentration of ca. 0.5%. After boiling 1 hr, in a nitrogen atmosphere, the solution was allowed to cool to 60°C, and powdered thymol (1 gm per litre solution) was added.
After standing 72 hr at room temperature, the amylose-thymol complex was removed by centrifugation \((2.5 \times 10^3 \text{ g}, 20 \text{ minutes})\) and recrystallised three times from hot, saturated butanol solution. The supernatant fluid, obtained after removal of the thymol-complex, was extracted, in a separating funnel several times, with diethyl-ether to remove the residual thymol. The aqueous layer was reduced ten-fold in volume on a rotary evaporator and the concentrate freeze-dried.

Amylose was stored as the butanol-complex, and amylopectin as the lyophilized solid. All fractions prepared as above were readily soluble in hot water to yield clear stable solutions.

2.4 The Enzymic Characterisation of Starch and its Fractions

It is well established that most native starches may be regarded as heterogeneous mixtures of two quite distinct polymer species. These are an essentially-linear material, generally known as amylose, and an essentially-branched fraction known as amylopectin.

Amylose was first shown to be essentially linear by the methylation studies of Meyer et al. (1941), a fact established by showing that the degree of polymerisation \((\bar{M}_n)\) of maize, and later potato, amylose was the same when measured by both end-group assay and osmotic pressure. Hess et al. (1940,a,b) in similar studies, suggested that potato amylose was slightly branched, but Hassid and McCready (1943) confirmed the original observations of Meyer and his co-workers. Potter and Hassid (1951), using a periodate oxidation technique of somewhat limited sensitivity, suggested that some amyloses may be branched.

The use of the exo-enzyme \(\beta\)-amylase apparently proved the linear nature of amylose - treatment of the polysaccharide with this enzyme resulted in quantitative conversion to maltose. As this enzyme degrades amylose from the non-reducing end of the molecule by the stepwise removal of maltose units, a linear chain of \(\alpha-1,4\)-linked glucose residues
appeared to be the only possible structure. Peat et al (1949) reported that crystalline \( \beta \)-amylase from sweet potato converted only ca. 80\% of amylose into maltose. These workers (1952 a, b) then showed the presence, in soya-bean \( \beta \)-amylase, of an impurity which they called \( Z \)-enzyme, that rendered amylose susceptible to complete hydrolysis of purified \( \beta \)-amylase. The nature of \( Z \)-enzyme became a matter of some dispute, for whilst Peat and co-workers (1952c, 1953) claimed it to be a \( \beta \)-glucosidase, Hopkins and Bird (1953) suggested that \( Z \)-enzyme was merely a weak \( \alpha \)-amylase. The problem was solved by Banks et al (1960) who selectively inhibited \( \beta \)-amylase in a mixture containing \( Z \)-enzyme, and showed that the action pattern of \( Z \)-enzyme was indistinguishable from that of \( \alpha \)-amylase. Subsequent work by Banks and Greenwood (1967a) has shown that the limited degradation of native amyloses by purified \( \beta \)-amylases is almost certainly due to the presence of long-chain branches linked by \( \alpha \)-1:6-branch points to the main amylose chain. The extent of hydrolysis (\( \beta \)-limit) of an amylose has been shown by Greenwood and Thomson (1962b) and Geddes et al (1965) to depend on the maturity of the starch from which the amylose was obtained. This may be a factor of some significance in the biosynthesis of starch.

Whilst the fine-structure of amylose is now well defined, that of the branched starch fraction, amylopectin is still somewhat obscure. Methylation studies, i.e. methylation followed by acid hydrolysis to the monomer units and subsequent identification thereof, established that amylopectin was composed of \( \alpha \)-D-glucose units linked by both \( \alpha \)-1:4- and \( \alpha \)-1:6-linkages. The average chain-length was established as ca 25.

The isolation of small quantities of isomaltose by Montgomery et al (1949) and panose by Thomson and Wolfson (1951) from partial acid-hydrolysates of amylopectin established a structure composed of short chains (\( CL_n = 25 \)) of \( \alpha \)-1:4-linked glucose residues branched through carbon-6 by \( \alpha \)-1:6-bonds.
structure is quite possible within the present-knowledge experimental data.

Pfafen (1949) has pointed out that departure from the ideal
predicted by these models are untestable.

On this basis both of the models has an R^2 change ratio close to 1/fit for RNA, and the ideal predicted by the theory of the ideal standard error and its distribution shows that the ideal

The empirical data structure (10.4%) was close to that expected for a random matrix model and the expected value of all these chains was the

The combined weight of the other two chains were residual to the

The data and tryptophan studies of the crystal and amino-acids within the

A catabolic and aminotransferase and other polypeptides were obtained.

By reverse and separated the product of the amino acid or amides with

Peel et al. (1952)÷(1956) determined the 8-limit dextrans of amylodextrin with observation that 8-amylase converted Gm of the molecule to amyllose

To advance a more realistic model which was consistent with the structure. The advent of enzymatic analysis allowed Heyer and Bertrand

To prepare 127(1977) proposed a heritable one, molecular weight, Sandusky and Ishimura (1979) proposed a heritable one, By comparison of the viscosity of amylodextrin and cellulose of the same

Intended to be a complete representation of the molecule (1977)÷(1979) to explain the results of metatization analyses, but was not shown in Figure 2.9. The matured structure was proposed by Heyer

The several ways in which amylodextrin chains may be arranged:

- a free reducing-end group
- a coreptide group end group
- a glycopeptide end group
- a c-polypeptide chain to which other chains are attached and which is
- a polypeptide chain to which the reducing-end group to an adjacent chain

In a polypeptide chain is tied to the molecule only by the reducing-end group

These chain types are shown in Figure 2.4. The proposed structure has three types of chain

Myrick and Sillen (1974) suggested that these three types of chain
Figure 2.4

A-CHAIN

B-CHAIN

C-CHAIN

key:

○ GLUCOSE UNIT

● NON-REDUCING END-GROUP

● REDUCING END-GROUP

α-1:4- BOND

α-1:6- BOND
Figure 2.5  amylopectin structure

Haworth et al

Staudinger & Husemann

Meyer & Bernfeld

Gunja-Smith et al

key:
- non-reducing end-group
- \( \alpha-1:4 \) bonds
- \( \alpha-1:6 \) bond

24.
and in addition suggests that some chains may terminate within the interior portions of the molecule giving rise to "buried" chains resistant to enzymic attack. [French (1957)].

Recently, the validity of the "Meyer" type structure has been questioned by Gunja-Smith et al (1970a). These workers reduced the A-chains and external portions of B-chain to uniform two-unit stubs by the consecutive action of muscle phosphorylase and \( \alpha \)-amylase. The molecule was then debranched by an isoamylase from Cytophaga known to be unable to remove \( \alpha \)-maltosyl residues linked 1-6 to the parent chain. [Gunja-Smith et al (1970b).] This type of action on the Meyer structure can be seen by inspection of Figure 2.5 to yield glucans which are resistant to the subsequent action of \( \beta \)-amylase. This was not found to be the case, since 44% of the debranched limit-dextrin was hydrolysed by \( \beta \)-amylase to maltose.

Gunja-Smith et al (1970a) have proposed another type of branched structure consistent with these results and shown in Figure 2.5. These same authors have also examined the unit-chain profile of glycogen, amylopectin and a synthetic polysaccharide bearing some similarity to amylopectin. All three were found to be quite dissimilar - a surprising observation in view of the widely accepted concept that only the average chain-length distinguishes the structure of glycogen from amylopectin.

It would seem that the fine-structure of amylopectin is still a matter of some considerable doubt, but, for the purposes of this work, the widely accepted Meyer structure with dichotomous branching and the modification thereof by Gunja-Smith et al (1970a) are regarded as being of equal validity.

From the foregoing description of the structures of amylose and amylopectin, it is apparent that any characterization of the starch fractions should if possible:
1) describe the relative amounts of amylose and amylopectin
2) determine the amount of totally-linear material present
3) estimate the amount of long-chain branching of the amylose
and 4) describe the branched structure of the amylopectin.

All these may be done by utilizing the following enzymic techniques:-

A. Measurement of Hydrolysis by β-Amylase

The extent of hydrolysis of a starch fraction by crystalline
β-amylase is a measure of external chain-length, for this enzyme removes
maltose units by stepwise degradation from the non-reducing chain-ends
to within either two or three units of the first branch-point encountered.

1) Estimation of β-amylase activity

The unit of β-amylase activity used in this work was that defined
by Hobson et al. (1950) i.e. the amount of enzyme which when incubated
for 30 minutes at 35°C will liberate 1 mg of maltose from a standard
digest prepared as follows: soluble starch solution (0.6%, 25 ml),
acetate buffer (pH 4.6, 4 ml) and enzyme solution (1 ml). The enzyme
concentration was adjusted so as to produce 10-20 mg. of maltose during
the period of incubation.

2) Estimation of β-amylase limit

A digest was prepared as follows: acetate buffer (2.5 ml,
pH 4.8, 0.1M, β-amylase (0.1 ml; 10,000 units/ml), polysaccharide
solution in methyl sulfoxide (3 ml; 0.7%) and distilled water to 25 ml.
After incubation overnight at 37°C, the reducing power of the liberated
maltose was estimated by the alkaline ferricyanide method. Aliquots
(1 ml), in triplicate, of the master digest were hydrolyzed with acid
as detailed in Part 2(d) of this section, and the liberated glucose
estimated by the alkaline ferricyanide technique. The β-limit may
then be calculated from:
\[
\{\beta\}_\text{lt} = \frac{\text{wt. of polymer liberated as maltose} \times 100\%}{\text{total wt. of polymer as maltose in digest}},
\]
or using the appropriate calibration factors for the alkaline ferri-cyanide technique.

\[
\{\beta\}_\text{lt} = \frac{\text{titre (ml) of digest} \times 100 \times 1.375\%}{\text{titre (ml) of digest after acid hydrolysis}}
\]

B. Measurement of hydrolysis by \(\beta\)-amylase and \(Z\)-enzyme

Banks, Greenwood and Jones (1960) established that \(Z\)-enzyme was a weak \(\alpha\)-amylase, and demonstrated that the concurrent action of \(\beta\)-amylase and \(Z\)-enzyme was to completely hydrolyze natural amylose, with long-chain branches, and \(\beta\)-limits of less than 100\%, to maltose. Since amylopectin is not significantly attacked by low concentrations of \(Z\)-enzyme, and its \(\beta\)-limit is identical to its \(\{\beta + Z\}\) limit (i.e. extent of hydrolysis by concurrent action of \(\beta\)-amylase and \(Z\)-enzyme), it is possible to compute the amount of amylopectin contaminant in an impure amylose as follows:

\[
\% \text{ amylopectin impurity} = \frac{100 - \{\beta + Z\\} \text{ limit of mixture}}{100 - \{\beta + Z\\} \text{ limit of amylopectin}} \times 100\%
\]

A convenient source of \(Z\)-enzyme is to be found in crude, cereal \(\beta\)-amylase. To measure \(\{\beta + Z\}\) limits, a digest was prepared identical to that for the measurement of the \(\beta\)-limit but with the addition of \(ca\). 5 mg of crude cereal \(\beta\)-amylase. The digest was incubated and assayed by the same procedure employed for measurement of the \(\beta\)-limit. At this concentration of \(Z\)-enzyme, no measurable increase in the \(\beta\)-limit of amylopectin could be detected.

C. Extent of Conversion by \(\beta\)-amylase and Pullulanase

Pullulanase is a debranching enzyme which hydrolyses the \(\alpha\)-1:6-bond in amylopectin, glycogen and pullulan (Manners (1971)). When used concurrently with \(\beta\)-amylase, it allows the complete hydrolysis of amylaceous polymers, containing only \(\alpha\)-1:4- and \(\alpha\)-1:6-links, to maltose. It was found convenient to check the efficiency of \(\beta\)-amylolysis...
by routinely estimating the extent of conversion of the starch fractions by concurrent action of $\beta$-amylase and pullulanase. Digests were prepared by taking aliquots of solution used for assay of $\beta$-limit and adding pullulanase ($0.05 \text{ ml; units per ml, where unit is defined by Gunja-Smith et al (1970a) }$) and incubating this digest for 36 hr at $37^\circ C$.

Maltose was estimated as above. If the ($\beta + P_u$) limit (i.e. extent of conversion by concurrent action of $\beta$-amylase and pullulanase) was significantly different from 100%, the enzymic assays were examined in detail for experimental errors.

D. Measurement of Average Length of Unit-Chain

If the assumption is made that branched, starch polysaccharides have a random distribution (i.e. equal numbers) of odd and even lengths of chain, then the average chain-length may be found by examining the products of the concurrent action of $\beta$-amylase and the debranching enzyme pullulanase. Pullulanase has been shown by Abdullah et al (1966) to specifically lyse the $\alpha-1\rightarrow 6$-bonds in amylopectin, rendering the whole polysaccharide molecule susceptible to the action of $\beta$-amylase. At high concentrations, the $\beta$-amylase will convert (1) chains having an even number of D-glucose residues into maltose, and (2) chains having an odd number of units into maltose and one molecule of D-glucose. By utilizing the method of Banks and Greenwood (1971d), this glucose may be specifically estimated, in the presence of maltose, and hence the average length of unit-chain may be readily determined.

Chain-lengths were estimated by preparing digest as follows: polysaccharide solution in methyl sulphoxide ($0.10 \text{ ml; 5-9\%}$), $\beta$-amylase ($0.35 \text{ ml; 10,000 units/ml}$), glycerol-acetate buffer ($75\%$ glycerol; $25\%$ acetate buffer pH $4.8$, $0.1M; 0.60 \text{ ml}$), pullulanase ($0.05 \text{ ml; 8 units/ml}$), water ($0.90 \text{ ml}$). The digestes were incubated for 36 hours at $37^\circ C$, and liberated glucose was determined by the glucose oxidase-
peroxidase technique described in Part 2(c). An aliquot of the master soln. (1.0 ml) was diluted with water to 50 ml and a digest prepared as follows: diluted polysaccharide solution (2.5 ml), acetate buffer (1.0 ml; pH 4.8, 0.1 M), amyloglucosidase (0.2 ml; 16 units/ml) and water to 10 ml. This digest, which provided the total polysaccharide concentration in the original solution, was incubated for 48 hrs at 35°C, and the glucose liberated was estimated enzymically as above.

The chain length, $\overline{CL}_n$, was calculated from:

$$\overline{CL}_n = \frac{\text{total weight of polysaccharide}}{\text{weight of glucose liberated}} \times 2$$

E. Detection of Short-Chain, Linear Material

Banks and Greenwood (1980) have devised a scheme where linear material may be detected in a heterogeneous mixture of branched and unbranched starch fractions. The basis of this method is that $\beta$-amylolysis of a branched starch fraction can yield only maltose and a residue of high molecular weight, whilst $\beta$-amylolysis of a linear amylose results in complete hydrolysis of the material. If the amylose has a random distribution of odd and even chains then, as in chain-length determination, glucose will be produced from half of the amylose chains, i.e. those initially containing an odd number of glucose residues.

Linear material was detected by determining whether glucose was liberated in the following digests: polysaccharide solution in methyl sulfoxide (0.20 ml; ca 10%), $\beta$-amylase (0.35 ml; 10,000 units/ml), glycerol buffer (75% in acetate buffer pH 4.8, 0.1 M; 1.60 ml) and distilled water (0.95 ml). These digests were incubated for 48 hrs at 37°C. Any glucose liberated and the total polysaccharide concentration were measured as in the chain-length determination.
2.5 Physical Characterization

In this work, the molecular size of starch fractions has been estimated by viscosity measurements in dilute solution. Although the enzymatic techniques detailed in the preceding section give much information on the branching structure of starch fractions, no information is given of molecular size or shape.

The viscometric technique is based on the property of a macromolecule that when dissolved it greatly increases the viscosity of the system, and this increase is proportional to the volume the molecule occupies in solution. The experimentally-available characteristics used were the specific viscosity \( \eta_{sp} \) and the viscosity number \( \eta_{sp/C} \).

Because of the non-ideal behaviour of starch fractions in solution, the viscosity number is a function of concentration. This concentration dependence is overcome by using the limiting viscosity number \( \{\eta\} \), which is the value of the viscosity number extrapolated to infinite dilution i.e.

\[
\{\eta\} = \lim_{C \to 0} (\eta_{sp/C})
\]

or since the specific viscosity is related to the relative viscosity \( \eta_{rel} \) by the following relation:

\[
\eta_{sp} = (\eta_{rel} - 1) = (\eta / \eta_0) - 1 = \left( \frac{\eta - \eta_0}{\eta_0} \right)
\]

where \( \eta \) and \( \eta_0 \) are the viscosities of the solution and solvent respectively, then

\[
\{\eta\} = \lim_{C \to 0} (\ln \eta_{rel})/C
\]

The limiting viscosity number is related to the molecular weight of the polymer by the empirical Mark-Houwink relation

\[
\{\eta\} = K M^a
\]
where $M$ is molecular weight and $K$ and $\alpha$ are experimentally determined constants.

The interpretation of the experimentally-derived values for $K$ and $\alpha$ has been possible, for linear polymers, by the application of a mathematical treatment based on the theory of the "random or drunkards walk" (see Flory (1953)). This treatment regards the polymer as a series of connected vectors with random direction and provides the relation that the mean square end-to-end separation of the chain ends is proportional to the number of steps, i.e. degree of polymerization of the chain. This treatment regards the polymer molecule in a highly simplified way since mathematical vectors may be self-intersecting, whilst the polymer segments may not occupy space points already occupied by polymer segments. In reality, the polymer occupies a larger volume than that of its mathematical counterpart, as a result of this excluded volume effect.

The volume a molecule occupies in solution depends on its shape, the degree of branching, and solvent-polymer interaction. Before any information may be gained above these properties, it is necessary to arrange experimental conditions such that the "excluded volume effect" is cancelled and the polymer behaves as its mathematical counterpart. This is done by working in a Theta-solvent ($\Theta$-solvent). In such a solvent, polymer-polymer attractive forces exceed polymer-solvent attractive forces, the molecule contracts, and the excluded volume effect is cancelled.

In a $\Theta$-solvent, certain conclusions may be drawn from the exponent in the Mark-Houwink relation, i.e.

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

If $\alpha = 0.5$ the molecule is an ideal random coil;
if $\alpha < 0.5$ the molecule is branched to some degree and;
if $\alpha > 0.5$ the molecule either deviates from statistical behaviour or is "free draining". Rod-like molecules such as cellulose exhibit this type of behaviour.

In "good" solvents, polymer-solvent interaction is favoured, the molecule expands and the observed value of $\alpha$ increases.

Various treatments are available dealing with the interpretation of hydrodynamic data obtained in good solvents, but their validity had recently been questioned by Banks and Greenwood (1971a).

The behaviour of starch fractions in various solvent systems has been well documented by Banks and co-workers. The Mark-Houwink equations for amylose in various solvents given by Banks and Greenwood (1968a, 1968b) are

$$
{\eta}_\text{LiSO} = 1.51 \times 10^{-2} \frac{M_w}{M_w}^{0.62} \\
{\eta}_\text{KCl} = 1.15 \times 10^{-1} \frac{M_w}{M_w}^{0.50} \\
{\eta}_\text{KOH} = 8.36 \times 10^{-3} \frac{M_w}{M_w}^{0.77}
$$

where $\{\eta\}_\text{LiSO}$ = limiting viscosity number in methyl sulphoxide

$\{\eta\}_\text{KCl}$ = limiting viscosity number in 0.33 M KCl

$\{\eta\}_\text{KOH}$ = limiting viscosity number in 0.15 M KOH

and $M_w$ is the weight-average molecular weight.

Thus the molecular weight of a linear amylose may readily be established by viscosity measurement in one of a number of solvents. Banks (1960) has also established a relation for branched amylose fractions between viscosity and molecular weight. As would be expected, for a similar molecular weight the branched amylose has a lower viscosity. In this work, viscosity measurements have been used for the intercomparison of series of samples, and where a molecular weight of a branched fraction was required this was obtained by light scattering measurements carried out in collaboration with Dr. J. Banks.
Experimental Procedure

A modified Ubbelohde viscometer of the type shown in Figure 2.6 was used. This viscometer has a side arm at the end of the capillary which breaks the liquid flow to form a "suspended level" and reduces kinetic energy corrections. The pressure head is independent of the volume of liquid in the viscometer, and so a bulb incorporated at the bottom of limb I (Figure 2.6), enables dilutions of the solution to be made directly in the viscometer.

In a capillary viscometer, the absolute viscosity is given by

\[ \eta = A \cdot d \cdot t - b \cdot \frac{d}{t} \]

where \( d \) is the density of the liquid and \( t \) is the flow time for a given volume. \( A \) and \( B \) are constants. The term \( B \cdot \frac{d}{t} \) is a correction for the energy remaining in the liquid as it leaves the capillary with finite velocity. This was shown by the methods of Greenwood (1964) to be negligible for the viscometers used in this work.

If kinetic energy corrections are negligible, for solvent

\[ \eta_s = A \cdot d_0 \cdot t_0 \]

and for solution \( \eta_l = A \cdot d \cdot t \) where \( \eta_s \), \( d \) and \( t \), with the appropriate subscripts, are the viscosity, density and flow-time for solvent and solution. For dilute solutions i.e. \( C < 0.005 \) g/ml \( d = d_0 \) and it follows that

\[ \eta_{sp} = \frac{t - t_0}{t_0} \]

\( \eta_{sp} \) can be readily evaluated from two flow times. The viscometers used in this work had an average shear-rate of 200 sec\(^{-1}\) (Banks et al (1968)).

Viscometers and solutions were placed in thermostat baths at 25°C ± 0.05°C. All apparatus was washed with chromic acid and dried with dust-free solvents. The viscometer was loaded with dust-free solvent, filtered through fritted glass (GL), directly into the bulb on limb I.
Figure 2.6 modified ubbelohde viscometer
Limb 2 was stoppered and liquid was pushed into bulb A by applying slight positive pressure on limb 1. When liquid was past the upper meniscus \(M_1\), pressure was released, the stopper removed from limb 2 and the flow of liquid between the menisci \(M_1\) and \(M_2\) timed to the nearest 0.05 sec using a 10 sec sweep stopwatch. Determinations in practice were reproducible to 0.1 sec. A dilution series was obtained by adding by pipette polymer solution through limb 2. For each dilution, \(\eta_{sp}\) and \(\eta_{sp}/C\) were determined, and \(\eta\) was evaluated by a plot of \(\eta_{sp}/C\) as a function of concentration. No difficulties were encountered in the measurement of viscosity in methyl sulphoxide or dilute (0.15 M) potassium hydroxide solution. The concentration of polysaccharide was measured by enzymic hydrolysis to glucose and subsequent glucose determination by methods described in detail in part 2 of this section.

2.6 Granular Characteristics

(a) Microscopic Appearance

Certain precautions must be made if true and representative photomicrographs of starch granule populations are required. The starches investigated in this work ranged in size from 40 \(\mu\) in the case of some barley samples to ca 1 \(\mu\) in the case of pollen starch from amylomaize. In addition, some mature samples of barley starch had granule size distributions spanning this range.

Representative samples were obtained by thoroughly mixing the starch granules under examination with a glycerol-water (1:1, v/v) mixture to restrict Brownian motion, then spotting a drop of this homogenous suspension on a microscope slide.

Granule size distributions were obtained by measuring directly starch granule size from enlarged photomicrographs. The magnification factor was obtained from a similarly enlarged photomicrograph of a calibrated slide. Light microscopy at the magnification required (ca.
500 diam.), suffers from the defect of having limited depth of focus. This meant that if the largest granules in a population (>40 μ in some cases) were in focus the smallest granules (<5 μ) were completely out of focus. This difficulty was overcome by taking two photographs of the same field with the small and large granules alternatively in focus.

To overcome potential errors due to the uneven stretching of photographic prints during the drying process, a calibrated slide was enlarged and printed by the standard technique, and then printed at right angles. This method allowed detection of any errors due to printing manipulation. Photomicrographs were processed by courtesy of Mr. C.H. Groves, F.I.S.T., I.R.S.H.

(b) Gelatinization Temperature

Starch granules exhibit optical anisotropy when viewed between crossed polars. The birefringence cross seen under such conditions is typical of spherulitic crystallisation, and the negative sign of birefringence implies that the bodies which scatter light have their major axis of refractive index arranged in a radial manner. As a suspension of starch is heated in water, the starch granules swell, and over a range of several degrees the birefringence dissappears. This is due to the disruption of the ordered regions which cause light to be scattered. Whilst the exact nature of this gelatinization process is unknown, it may be regarded to a first approximation as a "melting point".

Gelatinization temperature was measured on a Kofler hot-stage microscope by the method of Watson (1964). A starch sample was slurried in water at a 0.1 - 0.2% concentration. A small drop of this suspension was spotted onto a microscope slide, and surrounded by a continuous and contiguous ring of high-viscosity mineral oil. A cover slip was then dropped on in such a way that the aqueous drop was completely surrounded.
by an oil barrier with no bubbles under the cover glass. The oil barrier prevents the escape of water and stops the penetration of air channels under the cover slip. A uniformly-distributed field of about 100 granules was viewed, and heating commenced at a rate of 2°C per minute. The number of granules having lost their birefringence was estimated as a function of temperature. The Birefringence End Point (B.E.P.) was taken as the temperature at which fifty percent of the granules were no longer birefringent. Samples were evaluated by triplicate determinations and agreement was within 1°C.

(c) Scanning Electron Microscopy

The technique of scanning electron microscopy, developed by Oatley and Smith (1955), provides a convenient method of examining the surface detail of starch granules and has the merit of providing photographs with great depth of focus.

The scanning electron microscope is based on the principle that a beam of electrons, emitted by a heated tungsten filament, is focussed by condenser lenses into a fine electron beam which is projected onto the specimen. A schematic representation is shown in Figure 2.7. The electron beam is scanned on a raster in a similar manner to that of a television set. Electrons reflected and scattered by the specimen are collected and recorded by a scintillation-photomultiplication system and the signal is amplified and produces an image on a cathode ray tube. In normal operation, the electrons reflected from the specimen may be divided into two classes - primary electrons, with energies near to that of the primary beam which arise by reflection and secondary electrons with energies < 50 volts which are emitted from the specimen surface by excitation. In this work, images were produced using secondary electron emission. The nature of this secondary emission is such that the specimen must be completely coated by a conducting layer, or a build up of charge occurs causing intense secondary emission which results in poor specimen images.
Figure 2.7 scanning electron microscope
Starch granules, by nature of their shape, present several problems when coating with a conducting layer. These problems were overcome by using a technique developed by Evers and Green (1972). The starch was deposited from water on a standard aluminium stub, and coated with gold-palladium using vacuum deposition. The starch was then transferred using double-sided adhesive tape to another stub. This operation ensured that the underside of the starch granules on the stub were conducting. A second layer of gold-palladium was then deposited, on what originally had been the underside of the starch granule, to give a continuous conducting layer. The samples were then examined using a "Steroscan III" instrument (made by "Cambridge Scientific Instruments Ltd") at the Wolfson Institute of Interfacial Science, University of Nottingham.
SECTION THREE

SPECIAL EXPERIMENTAL TECHNIQUES

IN STARCH CHEMISTRY
Section 3.1

Summary

This section describes two special techniques which have been developed to allow further information to be gained concerning the nature of starch and its biogenesis.

In the first part, a new technique is described which allows the determination of starch-content in small amounts (~ 20 mg) of biological material. The method is completely general, being applicable to all types of starch i.e. from the waxy varieties to those of high amylose-content.

In the second part, an extensively modified apparatus is described for the measurement of the interaction of starch and its components with iodine. The experimental conditions governing the reaction have been examined in some detail, and a technique is proposed which allows the iodine-binding capacity of the amylaceous polysaccharide to be measured with a hitherto unattainable degree of accuracy and reproducibility.
Section 3.2

Analysis of Starch in Biological Material

Introduction

As a cereal grain matures, the amount of starch laid down increases over a fairly short time interval. The synthesis and deposition of reserve starch has been observed in various cultivars, for example, in dent corn by Wolf et al. (1948), in sweet corn by Maywald et al. (1955), in barley by Harris and MacWilliam (1958) and in rice by Rosario et al. (1968). The increase in starch-content would appear to be a useful measure of the maturity of the cereal grain.

A survey of existing techniques detailed in extensive reviews in Radley (1953), Kerr (1950) and in the "Official Methods of Analysis of the Association of Official Agricultural Chemists" (1965) failed to reveal a method which satisfied the criteria demanded in this present work.

Several novel problems arose and it was necessary that:

(1) the estimation of starch be applicable to the genetic mutants of barley, pea and maize characterised by their apparently high amylose-contents, and

(2) the estimation be applicable on a semi-micro scale, i.e. on 7.5 - 20 mg of cereal flour (allowing, when required, the assay of the starch content of single berries).

The method of Clendenning (1945), based on the polarimetric assay of starch extracted by calcium chloride from flour, was not applicable on a semi-micro scale. Adkins and Greenwood (1966d) have shown that the method of Hassid and Neufeld (1964), by which starch is
extracted by perchloric acid, selectively precipitated with iodine as the starch-iodine complex, hydrolysed with acid and subsequently estimated as glucose, is inferior in the case of starches of high amylose-content to a careful, physical extraction of starch on a quantitative basis. The methods of Thivend et al. (1965) and Donelson and Yamazaki (1965; 1969), which employ a hot-water treatment to solubilize starch prior to estimation, are also inapplicable to the starches of high amylose-content since these starches only go into complete solution in water at temperatures in excess of 100°C.

To overcome these defects, a technique has been devised which allows the assay of small samples of biological material with facility and accuracy. A complete extraction of starch from a flour was achieved by treatment with hot calcium chloride solution. The extracted starch was selectively and completely degraded to glucose by amylglucosidase, and the liberated glucose was estimated by the highly specific coupled glucose oxidase-peroxidase system of Banks and Greenwood (1971d).

**Experimental Methods**

(1) **Sample Preparation**

Whole, sound, representative berries of the cereal were ground in an agate mortar and pestle to pass a 40 mesh per inch sieve. The flour was carefully mixed to ensure a homogeneous distribution of endosperm and husk, then dried overnight (20-26 hr) at 70°C in a thermostat vacuum oven (Townson and Mercer).

(2) **Soluble Sugar Extraction**

Dried flour (7.5 - 20.0 mg) was accurately weighed (to 0.05 mg) into a graduated, hard-glass centrifuge tube (10 ml). Flour particles
adhering to the sides of the tube were carefully washed down with cold ethanol (ca. 3 ml) and the wetted flour compacted by centrifugation. The ethanol-wet residue was extracted three times with hot 80% aqueous ethanol (50°C-60°C; 5 ml) to remove soluble sugars.

3) **Calcium Chloride Extraction**

Calcium chloride dihydrate was dissolved in water to give a solution with a density of 1.30 g/ml at 20°C as measured by hydrometer. The solution was centrifuged until crystal clear and glacial acetic acid was added to adjust the pH to 2 (B.D.H. universal paper).

This solvent (1.0 ml) was added to the centrifuged flour residue, and the mixture was stirred very carefully with a glass rod to give a uniformly-fine suspension. After adding a few porous chips for even boiling and a little cetyl alcohol to prevent foaming, the centrifuge tubes were immersed in an oil bath at 130-135°C for a period of 15 minutes from the time that the mixture started to boil. During this boiling time, material around the sides of the tubes was periodically scraped down with a glass rod, and a constant volume was maintained by the addition, dropwise, of distilled water.

4) **Enzymic Digestion of Extracted Starch**

After cooling in air to 20°C, potassium hydroxide (0.05M, a predetermined volume ca. 2.4 ml) was added to bring the pH of the mixture to 4.6 - 4.8. Amyloglucosidase (14 units per ml, 0.5 ml) and α-amylase (250 units per ml, 0.1 ml) were added, and after gentle stirring, the tubes were immersed in a water bath at 47-48°C for a period of 3 hours with occasional stirring. After completion of the enzymic digestion in this time, the contents of the centrifuge tube were carefully transferred, with copious washings of distilled water, into a 250 ml graduated flask, and made up to volume with distilled
water. The solution was then filtered through a "Whatman" No. 1 filter paper, the first 30 ml of filtrate being discarded, and 20 ml portions were collected for glucose assay.

(5) Glucose Assay

A modification of the glucose assay technique employed in Section 2, was required because the sulphuric acid used to protonate the chromogen formed insoluble calcium sulphate in the presence of calcium chloride. The following procedure was adopted:

Portions of the enzymic digest of starch extract (containing 10-55 µg of glucose) were pipetted into glass-stoppered, boiling tubes and glucose-oxidase/peroxidase/chromogen mixture (2 ml), prepared as detailed in Section 2, was added and the contents of the tube thoroughly mixed by shaking. The digests were incubated at 35°C for 80 minutes. Hydrochloric acid \(\{7\text{M}\}\), prepared by diluting concentrated acid (150 ml) to 250 ml with distilled water \(\{4\text{ml}\}\) was added, the mixture shaken and the optical density of the resulting solution measured in a spectrophotometer \(\lambda = \text{ca.} 5500 \text{ Å}\) in 1 cm cells. The amount of glucose present was determined from the absorbance by means of a previously constructed calibration curve.

(6) Calculation

\[
\text{Percentage Starch} = \left(\frac{\text{O.D.} \times \text{C.F.} \times \text{D.F.} \times 0.90 \times 100}{\text{Sample weight (mg)} \times 1000}\right)
\]

or if dilution is 250-fold,

\[
\text{Percentage Starch} = \left(\frac{\text{O.D.} \times \text{C.F.} \times 22.5}{\text{Sample wt. (mg)}}\right)
\]

where

- O.D. = optical density of solution
- C.F. = calibration factor (in µg)
- D.F. = dilution factor.
Control Experiments

Donelson and Yamazaki (1968), Anderson and Uhl (1963) and Fraser and Hoodless (1963) have all emphasised that a certain critical size is required for efficient extraction of starch. In cereal flours of large particle size (> 50 μ) the starch is embedded in a protein matrix and this exercises a protecting effect by slowing down diffusion of the starch-solvating agent. It was confirmed that, unless the cereal flour was ground to pass a 40 mesh sieve, starch extraction was incomplete.

The optimum temperature for the extraction was established at 130 °C. Microscopic examination of carefully washed (hot water) residue, stained with iodine, revealed blue-staining matter below this temperature, whilst at temperatures much in excess of 130 °C, it was difficult to avoid spurting from the analysis tube with a subsequent loss of material.

In agreement with Clendenning (1945), a pH of 2.0 was found to be optimal for extraction. Above pH 3.0, the efficiency of the extraction was variable and occasionally incomplete. Below pH 2.0, no adverse effects were observed, but difficulties were experienced in buffering solutions.

As shown in Tables 3.1(a), (b), recovery of a standard starch (potato) was optimal when a heating time of 15-20 minutes was employed.

At this optimum time of 15-20 min at (at pH 2.0 and 130 °C) recovery of glucose (Kerfoot; biochemical reagent grade) in four separate analyses was 99.3%, 101.8%, 99.6% and 99.8%. This was convincing evidence that a negligible destruction of glucose occurred in the analysis under the specified conditions.
Table 3.1(a)

Analysis of Standard Potato Starch
(Courtesy of Mr. H.V. Hart, F.R.I.C.)

<table>
<thead>
<tr>
<th>Component</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture</td>
<td>13.6%</td>
</tr>
<tr>
<td>Ash</td>
<td>0.26%</td>
</tr>
<tr>
<td>Protein (N x 5.7)</td>
<td>0.04%</td>
</tr>
<tr>
<td>Ether extract</td>
<td>0.04%</td>
</tr>
<tr>
<td>Purity</td>
<td>86%</td>
</tr>
</tbody>
</table>

Table 3.1(b)

<table>
<thead>
<tr>
<th>Heating Time</th>
<th>Starch Recovery</th>
<th>Percentage Theoretical Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 min</td>
<td>82.6%</td>
<td>96.3%</td>
</tr>
<tr>
<td>15 min</td>
<td>85.7%</td>
<td>99.8%</td>
</tr>
<tr>
<td>20 min</td>
<td>85.0%</td>
<td>99.0%</td>
</tr>
<tr>
<td>30 min</td>
<td>83.8%</td>
<td>97.4%</td>
</tr>
<tr>
<td>40 min</td>
<td>80.6%</td>
<td>94.0%</td>
</tr>
</tbody>
</table>
To establish the time required for complete degradation of starch to glucose, a sample of barley flour was extracted as above, and the extract was treated with the mixture of hydrolytic enzymes for varying periods of time. As can be seen in Figure 3.1, amylolysis was complete within 130 minutes. For routine assay, an incubation time of 180 minutes was chosen.

An alternative procedure may be used if a rapid assay is not required. After cooling the calcium chloride extract to room temperature, the solution was transferred quantitatively to a standard flask (250 ml) with distilled water and amyloglucosidase (1.5 ml; activity 14 units/ml) and acetate buffer (25 ml; 0.1M; pH 4.8) were added. The pH of this digest was adjusted to pH 4.6 - 4.8, where necessary, by the addition of KOH (0.05M). The solution was then incubated at 35°C for 15 hours and after filtration the glucose was estimated as detailed earlier.

Maltose (Kerfoot, biochemical reagent grade) and soluble potato starch (B.D.H. analytical grade) gave recoveries of 99.6% to 99.7%. The concentration of an amylopectin solution was determined, by the analytical technique of Adkins et al. (1969), involving acid hydrolysis and estimation of reducing power, the result so obtained was found to be identical to that given by this new assay.

Comparison with Established Techniques

The semi-micro assay was compared with the 2.5g-scale technique of Clendenning (1945) and the 200 mg scale assay of Hassid and Neufeld (1964).

A calcium chloride extract was prepared as detailed by Clendenning (1945) and the optical rotation of the extract measured
Figure 3.1  amylolysis
using a "Perkin-Elmer" Automatic '141' polarimeter. 10 cm, water-
jacketed cells were used to measure the rotation at $\lambda = 5890 \text{ R}$ of
the extracted solutions. The specific rotation, $\{\alpha\}_D^{20}$ for a number
of starches isolated by the method outlined in section 2, was
measured, using an enzymic technique to assay starch concentration.
Results for four starches are shown in Table 3.2. These experimental
values were used for calculations in the polarimetric method.

Reducing sugar was measured by the alkaline ferricyanide
method (see section 2.3) in the procedure of Hassid and Neufeld
(1964).

Results and Discussion

The starch content of a variety of cereal flours was measured
by the semi-micro technique. Typical results are shown in Table
3.3. Individual results showed a reproducibility of $\pm 1.5\%$.

A comparison of established techniques with the semi-micro
method is included in Table 3.3. The perchloric-acid method was not
applicable to amylomaize starch (Adkins and Greenwood (1966)) but
otherwise the agreement between this method and the semi-micro tech-
nique was good, notwithstanding the tedious and time-consuming nature
of the perchloric-acid method.

In the case of the polarimetric assay, many of the results
were appreciably higher than those obtained with the semi-micro method.
Enzymic assay of the solutions used in the polarimeter eliminated this
discrepancy, suggesting the presence of a non-amylaceous dextro-
rotatory material.

The semi-micro method developed here may be used to assay
native starch in almost any biological material with a high degree
of accuracy.
Table 3.2

The Specific Rotation of Pure, Laboratory-
Isolated Starches in Calcium Chloride Solution

<table>
<thead>
<tr>
<th>Starch</th>
<th>Specific Rotation 1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barley (var. Ymer)</td>
<td>+ 200°</td>
</tr>
<tr>
<td>Regular Maize</td>
<td>+ 204°</td>
</tr>
<tr>
<td>Potato (var. Pentland Crown)</td>
<td>+ 204°</td>
</tr>
<tr>
<td>Amylomaize (Apparent amylose 70%)</td>
<td>+ 200°</td>
</tr>
</tbody>
</table>

1) Specific Rotation \( \left( \frac{\alpha}{D} \right)^{20} = \frac{\alpha \times 100}{1 \times c} \)

where \( \alpha \) is the observed angular rotation in degrees

\( l \) is the cell length in decimeters

\( c \) is concentration of starch in grams per 100 ml.
Table 3.3

Representative Results for the Starch-Content (%) of Various Cereal Flours Obtained by the Semi-Micro Method and a Comparison with Other Assay Procedures

<table>
<thead>
<tr>
<th>Cereal Grain</th>
<th>Assay Method</th>
<th>Semi-micro 1</th>
<th>(a)D 2</th>
<th>Enzymic 3</th>
<th>HClO₄ 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barley (var. Ymer)</td>
<td></td>
<td>59.3</td>
<td>65.2</td>
<td>60.2</td>
<td>59.5</td>
</tr>
<tr>
<td>Regular Maize</td>
<td></td>
<td>65.5</td>
<td>69.3</td>
<td>65.8</td>
<td>66.7</td>
</tr>
<tr>
<td>Wheat</td>
<td></td>
<td>64.0</td>
<td>67.0</td>
<td>63.8</td>
<td>63.3</td>
</tr>
<tr>
<td>Amylomaize (70% amylose)</td>
<td></td>
<td>60.0</td>
<td>58.6</td>
<td>58.0</td>
<td>n.a. 5</td>
</tr>
</tbody>
</table>

1) Enzymic assay method described in text.
2) Clendenning (1945) method - polarimetry on aqueous CaCl₂ extract
3) Solutions from polarimetry assayed enzymatically
4) Hassid and Neufeld (1964) method
5) Method not applicable; reference Adkins and Greenwood (1966a)
Section 3.3

The Starch-Iodine Interaction

Introduction

Starch and its fractions interact with iodine in a very complex manner. The amount and nature of the complex formed depends not only on the relative concentrations of the polysaccharide and iodine, but also on the nature of the polymer, the reaction temperature, the pH of the solution and the total ionic strength of the solution. Extensive reviews by Kerr (1949), Whistler (1964) and Richter et al. (1968) have appeared, detailing the chemistry and nature of the starch-iodine complex.

The radically different behaviour of linear and branched components of starch has been used to allow characterisation of native starches. Amylose, the essentially linear component of starch, interacts with iodine to form a deep-blue coloured-complex with an absorption maximum ca. 630 nm. Hanes (1937) suggested that the polysaccharide-complex was a helical structure and was supported by Freudenberg et al. (1939), who suggested that a helical amylose complex supported the existence of the Schardinger dextrins, cyclic \( \alpha-1,4\) - glucans of six to ten residues. This model was confirmed by Rundle and French (1943) by X-ray diffraction measurements on crystalline amylose-iodine complexes. In the solid crystalline form, dichroism was displayed which was attributed to the alignment of the long axes of the helices. A similar alignment of the helical complex was shown by Rundle and Baldwin (1943) who carried out measurements of streaming birefringence in solutions of amylose-iodine complexes.

More recently Banks and Greenwood (1971) demonstrated quite
unambiguously that a conformational change occurs when iodine is added to a solution of amylose. This supports the concept of amylose in a random coil form in neutral solution undergoing a change in hydrodynamic volume to form a helical complex.

The nature of the iodine in the centre of the amylose helix is still a matter of some doubt for, whilst Gilbert and Mariott (1948) by potentiometric measurements at varying reactant concentrations, suggested a predominance of $I^-_8$ ion, Greenwood (1968), in unpublished work, has found that interpretation of experimental data obtained by this technique is somewhat ambiguous.

Like many other aspects of the chemistry of amylopectin, its interaction with iodine is not well documented. A red-purple complex is formed with an absorption maximum at ca. 540 m, but whether this has a similar helical structure to that of amylose or is due to an absorption mechanism is not known. However, whilst amylopectin binds little iodine at low free-iodine concentrations, both Higginbotham (1949) and Foster and Smith (1953) showed that at high free iodine concentrations considerable quantities of iodine are bound, and the former author showed that under certain conditions amylopectin could bind as much iodine as amylose, partly by a helical mechanism and partly by adsorption.

These major differences in both type and amount of complex formation of the starch fractions have been widely exploited as diagnostic tools. Conditions are generally chosen where the branched fraction shows little interaction whilst amylose reacts freely, i.e. at low free iodine concentrations. The relative proportions of each component polymer may then be readily calculated.

Experimental methods may be conveniently classified into techniques based on:
spectrophotometric determination of the intensity
of the colour of the iodine-complex,

(ii) potentiometric titration of the starch with iodine,

or

(iii) a similar amperometric titration.

Experience has shown that although spectrophotometric methods
provide a convenient and rapid method of analysis, they are not
sufficiently sensitive for accurate measurements on the semi-micro
scale, particularly for assay of small quantities of branched mater-
ial in the presence of large amounts of amylose. Similar
criticisms may be levelled at amperometric methods, and there is
little doubt that the most satisfactory method of measuring the
iodine-binding characteristics of a starch, or of its components,
is by potentiometric titration and, as was emphasised by Gilbert
and Mariott (1948), the accuracy of the direct titration method
introduced by Bates et al. (1945) may be considerably improved by
using a differential potentiometric technique.

Anderson and Greenwood (1955) and latterly Adkine and
Greenwood (1966a, 1966b) have demonstrated the usefulness of this
technique in starch chemistry using a laboratory-built electrometer
to cope with the original high impedance circuit. This work describes
a redesigned apparatus of considerably simpler form utilised in con-
junction with a readily available detector. This new apparatus
necessitated the establishment of suitable reaction conditions and
new methods of sample preparation. These are described in detail in
later parts of this section.

Principle of Differential Potentiometric Titration

The differential technique involves two iodine/iodide half-
cells, each with a bright platinum electrode, which are connected by

54.
a liquid bridge. The test half-cell, $t$, contains the starch sample in a buffered iodide solution and the control half-cell, $c$, contains buffered iodide at an identical concentration. This experimental set-up results in an iodine concentration cell without transference i.e.

$$I_2 (a_{I_2}^-) / I^- (a^-) / I_2 (a_{I_2}^+)$$

where $a$ represents activity. For one electrode, the potential $E_v$ is given by $E = E_0 - {RT \over F} \ln \left\{ (a_{I_2}^+) / a^- \right\}$, where $R$, $T$ and $F$ are the Gas Constant, Temperature and Faraday Constant, respectively.

The overall potential of the cell is:

$$E = -{RT \over 2F} \ln \left\{ (a_{I_2}^-) / (a_{I_2}^+) \right\}$$

The apparatus is used as a null detector for when $E = 0$ i.e. no potential

$$(a_{I_2}^-)_{c} = (a_{I_2}^+)_{t}$$

or since the activity coefficients are assumed equal

$$(I_2^-)_{c} = (I_2^+)_{t}$$

In use, iodine is added to the test half cell, and the potential so produced is cancelled by the addition of iodine to the control half-cell. At the point of zero potential, the concentration of total free molecular iodine in the test solution corresponds to that added to the control solution, whilst the amount of iodine bound by the polysaccharide is given by the difference between this and the original amount added to the test cell. Titration curves are obtained by plotting "bound" iodine as a function of "free" iodine.

**Apparatus**

The apparatus consisted of two, one-litre, round-bottomed, flasks, connected by an easily-removed, liquid bridge. Additional
necks were provided for the introduction of electrodes and small necks for the addition of iodine. A diagram is shown in Figure 3.2 the salient features being:

1. stirring gland,
2. the electrode, and
3. the liquid bridge.

A separate diagram of the electrode is shown in Figure 3.3. A pulley system operated the stirrers continuously during the course of the titration.

The Liquid Bridge

The liquid bridge consists of a U-tube, having a middle arm which can be sealed by means of a glass stop-cock. Since solution inside the bridge plays no role in the reaction, the volume of the bridge never exceeded 8 ml, which represented a change of 3% per half-cell volume. In general practice, a capillary bridge (internal volume ca. 2 ml) was found to be practical. By elimination of fritted-glass discs and salt-bridges, junction potentials were no longer relevant and the impedance of the electrical circuit considerably lowered. The bridge was easily filled without air bubble introduction by opening the stop-cock on the side arm, applying sudden suction by means of a rubber bulb, and closing the stop-cock as soon as the bridge was filled. No siphoning occurred, as shown by the use of dye solution, if the liquid levels in the two half-cells were arranged to be identical to the liquid surface in the thermostat bath.

Electrodes

The electrodes were constructed of platinum foil (1" x 1" x 0.005") welded to platinum wire, sealed through soft glass, and silver-soldered to a silver rod (see Figure 3.3). It was imperative that
1 STIRRER ASSEMBLY
2 ELECTRODE
3 LIQUID BRIDGE
Figure 3.3

electrode

- Silver Rod
- Platinum Wire
- Soft Glass Seal
- Platinum Foil
all joints were strain free — they were carefully tested to eliminate small, random junction-potentials which gave rise to detector instability. The electrodes were connected directly to the detector which was in turn efficiently earthed. The electrodes were cleaned by immersion in concentrated nitric acid, then repeatedly washed, first with water, and then with buffer, until a potential of less than 0.01 mV was observed when the two electrodes were placed in the same iodine/iodide solution. It was also necessary that the electrodes responded rapidly to changes in iodine concentration. This was tested by removing one electrode, rinsing in distilled water, then replacing it. For satisfactory results, the originally-observed potential should be reattained in less than 20 seconds.

Electronic Detector

A digital voltmeter was used directly in this low-impedance circuit, it could however be applied with equal facility to the high impedance circuit of Anderson and Greenwood (1955). A "Solartron" digital voltmeter Model 1450 supplied by Solartron Electronic Group Ltd., Farnborough, Hampshire, England, was found to be suitable. Readings were available to 0.01 mV on the 20 mV range. The instrument was used on automatic display (50 readings per second) with a 60 dB filter in operation to alternate series mode interference from 50 Hz to 120 Hz.

Thermostat

The cells were supported by clamps in an ethylene glycol/water thermostat bath. Temperature control was maintained ± 0.05°C by the use of a "Circotherm" thermostat control and circulatory unit.
Control at temperature below ambient was achieved by the use of a "Grant" refrigerator unit (running continuously) in conjunction with the appropriate setting on the "Circotherm".

By lagging the copper bath with 2" of polyurethane foam, condensation was prevented at low temperatures.

**General Analysis Conditions**

The extent to which a completely-dispersed, pure starch, or starch component, binds iodine depends on:

(1) the concentration of iodide and other ions present,
(2) the pH, and
(3) the temperature.

These variables must be fixed for routine analysis.

**Iodide Ion Concentration**

A molarity of 0.01 for the iodide concentration in the supporting electrolyte was used, for, in agreement with the spectrophotometric results of Kuge and Ono (1960), it was found that maximum binding occurred at this molarity.

**pH**

To repress hydrolysis of iodine, the titration was performed in acid media; Phosphate buffer, pH 5.8 was used.

**Temperature**

Adkins and Greenwood (1966a) demonstrated that iodine-binding capacities of amylomaize starches varied considerably with temperature. Investigations at two temperatures, 20°C and 20°C, were routinely carried out to obtain maximum information.

**Concentration of Starch**

The optimum amount of polysaccharide (per 840 ml) in this semi-micro technique was:

60.
Amylose = 3-6 mg
Amylopectin = 12 - 24 mg
Starch = 5 - 25 mg

Sample Preparation

In order to obtain meaningful values for the iodine-binding capacities of starches, three criteria must be fulfilled:

(i) the starch must be in a true molecular dispersion,
(ii) all interfering substances must be removed, and
(iii) the amount of starch used must be assayed accurately.

Two methods have been successfully utilised for the complete dispersion of native starches, liquid-ammonia pretreatment after Hodge et al. (1948) and methyl-sulphoxide pretreatment after Banks and Greenwood (1967b). Methyl-sulphoxide pretreatment was utilised in this work.

Anderson and Greenwood (1955) demonstrated the interfering effects of protein and lipid impurities on the iodine-binding curves of starches. Protein was reduced to a very low level (N < 0.03%) by a careful application of the physical technique of Greenwood and Robertson (1954). Lipids were removed by repeated extraction of the starches in, or by prolonged soxhlet extraction with, polar solvent systems e.g. 85% dioxane. The third criterion was satisfied by enzymic assay of the starch as detailed in Section 2.

Hydrous starch (i.e. starch granules which had been stored under water to equilibrium) was dissolved in methyl sulphoxide (DMSO) to give a 1-4% solution dependent on the character of the starch. As Adkins (1966) observed, hydrous starch dissolved most easily in DMSO under conditions of mild shear. This was achieved by altering the concentration of starch to provide a suitable viscosity, and
applying low-speed stirring. In general, it was observed that waxy starches had a much higher viscosity potential than starches of high-amylose content and thus a lower concentration was used for waxy starches in this procedure.

The criterion used in this work for the attainment of complete solution was that on centrifugation at $2 \times 10^3$ g for 20 minutes, no gel was obtained. The starch was precipitated with ethanol (3 volumes), a small quantity of saturated sodium chloride solution being added to induce flocculation when colloidal dispersions were formed. On centrifugation, the starch was carefully washed with ethanol to ensure complete removal of DMSO. By repeated titration, under changes of ethanol, a finely-divided precipitate, free from DMSO, was obtained. The starch was dried overnight at 65°C in vacuo. The dry, non-granular starch was dissolved in DMSO, with mild stirring, and used in iodine titration.

**Titration Procedure**

203 ml 0.1M potassium iodide and 20 ml 0.2M phosphate buffer (pH 5.8) were diluted to 2 litres with distilled water to provide the supporting electrolyte. 830 ml electrolyte were added to each half-cell, and the cells were placed in the thermostat bath so that the liquid level in each was the same as that in the thermostat bath. The liquid bridge was placed between the half-cells and filled by the procedure detailed earlier, the electrodes were placed in the cells, and stirring was started and maintained at a rate sufficient to give rapid mixing without causing undue turbulence.

A stock solution of the sample for titration was prepared by diluting the polysaccharide - DMSO solution (1 ml) with distilled water (11 ml). A blank, omitting only the polysaccharide, was prepared.
in a similar manner. Blank and sample solutions (10.0 ml) were added to their respective half-cells by pipettes (plugged with cotton-wool to prevent inadvertent introduction of salivary amylase to the sample). Thirty minutes were allowed for temperature equilibrium to be attained before commencing the titration. During this period, small random-potentials were observed on the detector. These were not significant, as the detector was effectively on open circuit.

An aliquot, usually 5 "Agla" units, (50 "Agla" units = 1 ml) of iodine solution (0.005 M I₂ in 0.01 M KI) was added to the sample half-cell by means of an "Agla" micrometer syringe (Wellcome Reagents Ltd., Beckenham, Kent, England), and five minutes allowed for equilibrium to be achieved. Iodine solution was then slowly added to the blank half-cell until zero potential (less than 0.01 mV) was indicated on the detector. At this point, the concentration of molecular iodine in the two half-cells was equal, and hence the amount bound by the polysaccharide was given by the difference in volumes added to both sides. This addition and balancing process was repeated until the complete curve of iodine bound as a function of free iodine was obtained.

**Determination of Polysaccharide Concentration**

The concentration of the stock solution was determined by taking an aliquot (1.0 ml) of the remaining polysaccharide DMSO-water stock solution, hydrolysing the polysaccharide to glucose using amyloglucosidase, and the glucose estimated by the specific enzymic technique detailed in Section 2.

It was imperative that the aqueous DMSO-polysaccharide solution was used for both iodine titration and concentration estimation immediately upon its preparation. Whilst this precaution was unnecessary for the
majority of starches, it was mandatory in the case of amylomaize starch, and its fractions, because incipient retrogradation, which affects both the iodine uptake, and the concentration determination, occurred if the solution was allowed to stand for several hours before use.

**Calculation**

If \( I_t \) and \( I_c \) are the amounts of iodine (in "Agla" units) added to the test and control half-cells, respectively, the amount of bound iodine, \( I_b = I_t - I_c \).

The values of total bound iodine = \( \Sigma I_b \), and the total free iodine = \( \Sigma I_c \), are evaluated consecutively.

\( I_b \) is then expressed as milligrams iodine bound per 100 mg polysaccharide i.e.

\[
\text{weight of iodine} = \frac{\Sigma I_b \times I_2 \text{ normality} \times 254 \times 10^3 \text{ mg}}{50 \times 2 \times 10^3} = \frac{V_I}{I_2}
\]

\( \text{weight of amyllose} = \frac{V_A}{I_2} \)

Percentage of iodine bound = 100 \( \frac{V_I}{V_A} \)

\( \Sigma I_c \) is converted to an iodine concentration \( \{I_2\} \) (moles per litre), i.e.

\[
\{I_2\} = \left( \Sigma I_c \times I_2 \text{ normality} \right) \frac{50 \times 2 \times 840}{100 \frac{V_I}{V_A}} \text{ moles per litre}
\]

100 \( \frac{V_I}{V_A} \) is graphed as a function of \( \{I_2\} \).

**Results and Discussion**

Typical titration curves are shown in Figure 34 for potato starch, and its component amyllose and amylopectin fractions. It has been customary to define iodine binding capacity of a starch in one of two ways:
Figure 3.4 Iodine binding curves

T = 204°C

- • POTATO AMYLOSE
- ○ POTATO STARCH
- O POTATO AMYLOPECTIN

BOUND IODINE PERCENT POLYSACCHARIDE

FREE IODINE x 10^6 MOLAR
(i) as the point at which the extrapolated maximum and minimum slopes meet (shown in Figure 3.4 by \(X\)). This extrapolation is based on the concept of two independent binding processes occurring consecutively; that is a completely different mechanism occurs after initial adsorption of iodine into amylose helices.

(ii) as the extrapolation of the line obtained at high free-iodine concentrations to zero free-iodine concentration. This is based on the concept that binding, other than in helices, occurs by an absorption process at all levels of free iodine. (This method is demonstrated by the point \(Y\) in Figure 3.4). Both techniques are equally arbitrary and in this work the second concept is applied.

Generally the difference between the two methods of extrapolation was small (with the exception of amylo maize starch, which is discussed in detail in a later section of this thesis).

The Equilibrium Time for Potentiometric Titration

In this apparatus, the short detector response time allowed direct visualization of the time required to form the amylose-iodine complex. Equilibrium was not instantaneous after physical mixing had been completed, implying that the starch helix was not preformed in solution. It was found that although the initial portions of the binding curves were time dependent, the final, linear portion of the curve was independent of time. Reproducible results were obtained by standardization of the titration procedure, allowing five minutes for equilibrium after each addition.

Factors Influencing Iodine Binding Capacity

1. The presence of methyl sulphoxide

Mature potato starch (var. Pentland Crown) was dispersed in 0.2M KOH at 2°C with shaking, neutralised to pH 5.8 with 0.15 M
phosphoric acid, and an aliquot (10.0 ml) taken for iodine titration. The experiment was repeated twice, adding

a) 1.0 ml DMSO, and

b) 2.0 ml DMSO

The three curves so obtained were identical (blank and 1.0 ml DMSO are shown in Figure 3.5), in agreement with the results of Adkins and Greenwood (1966a,b).

It was noted, however, that some slight interaction between iodine and DMSO occurred, which lead to erroneous results if there was a difference in DMSO content between the half-cells. In all experiments, therefore, the DMSO concentration in the sample solution and the blank was arranged to be identical.

(2) Variation in buffer concentration

The effect on the iodine binding capacity of mature potato starch of varying the concentration of phosphate buffer (pH 5.8) in the half-cells is demonstrated in Table 3.4.

Increasing the buffer concentration has little effect on the iodine binding capacity - increasing the former twenty-fold, decreased the latter by 4.9%. Small differences in concentration between various batches of buffer did not cause any measurable change in the iodine binding capacity of starches.

In agreement with the spectrophotometric observations of Kuge and Ono (1960), it was noted (Figure 3.6) that as the buffer concentration was increased, the polysaccharide bound iodine at progressively lower concentrations of free iodine.

(3) The potential oxidizing action of iodine

The reversibility and extent of iodine binding would be altered if the iodine oxidized or degraded the polysaccharide. Under the
Figure 3.5 Iodine binding curves

FREE IODINE $\times 10^6$ MOLAR

BOUND IODINE PERCENT POLYSACCHARIDE

$T = 20.4 ^\circ C$

O CONTROL

CONTROL PLUS DMSO
### Table 3.4

The Iodine-Binding Capacity of Mature Potato Starch (var. Pentland Crown) as a Function of Phosphate Buffer Concentration

<table>
<thead>
<tr>
<th>Phosphate Buffer Concentration</th>
<th>Iodine-Binding Capacity (mg I₂ bound/100 mg Starch)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.95 x 10⁻⁴ M</td>
<td>4.58</td>
</tr>
<tr>
<td>1.98 x 10⁻³ M</td>
<td>4.54</td>
</tr>
<tr>
<td>4.95 x 10⁻³ M</td>
<td>4.50</td>
</tr>
<tr>
<td>9.90 x 10⁻³ M</td>
<td>4.37</td>
</tr>
</tbody>
</table>
Figure 3.6  Iodine binding curves

T = 20.4°C

- Sample
- Buffer concentration x 10⁻³ molar

0.495
1.98
9.90

FREE IODINE x 10⁻⁶ MOLAR

BOUND IODINE PERCENT POLYSACCHARIDE
experimental regime employed in this work, no such reaction occurred, for the titration of an equivalent weight of glucose directly, or the addition of glucose to an amylose-iodine system at equilibrium, caused no change in the observed potential.

(4) The procedure for dissolution of starch

As detailed by Banks and Greenwood (1967b) and Adkins and Greenwood (1966a), methyl sulphoxide was found to be the most satisfactory general solvent for starches. To a large extent, the type of starch governs the ease of solution; for example, starches of high amylose-content dissolve to yield a gel-free solution in under one hour whilst waxy starches often required overnight stirring for the same result. Tuber starches also tended to be less readily soluble than cereal starches. The dependence of iodine binding capacity on time of solution was investigated for a mature potato starch in DMsO. A sample of starch was slurried vigorously in DMsO and stirring at a moderate rate of shear continued. Samples were removed at time intervals and the iodine binding capacity measured. The results are shown in Table 3.5. It is clear that complete solution of potato starch occurred in 6-7 hr. In addition, the starch DMsO-solution retained its stability for prolonged intervals of time. The iodine titration of this potato starch was repeated after a storage period of approximately six months, the iodine binding capacity was 4.55 mg iodine bound/100 mg starch.

It was noted that these results showed a significant, and reproducible, increase of some 5% over those reported previously by Greenwood and Thomson (1962a). In that technique, the starch granules were dissolved in potassium hydroxide and the resulting solution neutralized with phosphoric acid prior to titration. The effect of
<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Iodine Binding Capacity (mg I₂ bound/100 mg Starch)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.5</td>
<td>4.38</td>
</tr>
<tr>
<td>6</td>
<td>4.47</td>
</tr>
<tr>
<td>7</td>
<td>4.58</td>
</tr>
<tr>
<td>10.5</td>
<td>4.58</td>
</tr>
<tr>
<td>12.0</td>
<td>4.52</td>
</tr>
<tr>
<td>24.0</td>
<td>4.54</td>
</tr>
</tbody>
</table>
varying the method of dispersion of potato starch granules on iodine binding capacity is shown in Table 3.6.

The highest iodine binding capacity was observed using DMSO as the dispersing agent. Variability was low using DMSO (ca.± 1% of the mean iodine binding capacity). KOH was a much less satisfactory solvent, with a variability of ± 3% in a number of experiments. Increasing the concentration of KOH used as dispersant led to enhanced iodine binding capacities, in agreement with the work of Adkins and Greenwood (1966a) on amylo maize starch. Similar results were obtained with normal maize starch.

Banks and Greenwood (1967b) and Adkins et al. (1970) reported that to completely destroy the structure of a mature cereal starch-granule, it was necessary to precipitate the starch from DMSO solution, then redissolve it in DMSO. The effect of repeated solution of various cereal starches defatted by the procedure of Schoch (1942) on the measured iodine-binding capacity is shown in Table 3.7.

The iodine binding capacity tended to increase as a result of a second precipitation from DMSO. The increase did not, however, justify the extra labour involved. In some cases, a drop in iodine affinity was observed on the second precipitation. This was always found to be due to incomplete precipitation of starch. Addition of a few drops of saturated sodium chloride solution to the precipitation mixture facilitated flocculation and prevented incomplete precipitation of starch.

DMSO was also a better solvent for non-granular starch than 0.2M KOH. Non-granular starch was the term applied to the polysaccharide obtained from alcohol precipitation of a solution of starch in DMSO.
Table 3.6


<table>
<thead>
<tr>
<th>Reagent for Dispersion of Granules</th>
<th>Iodine Binding Capacity (mg I₂ bound/100 mg starch)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>4.55</td>
</tr>
<tr>
<td>0.2M KOH</td>
<td>4.27</td>
</tr>
<tr>
<td>0.5M KOH</td>
<td>4.41</td>
</tr>
</tbody>
</table>
Table 3.7

The Iodine Binding Capacity of Cereal Starches as a Function of the Dissolution and Precipitation Procedure

<table>
<thead>
<tr>
<th>Procedure</th>
<th>I</th>
<th>II</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Barley (var. Ymer)</td>
<td>5.66</td>
<td>5.76</td>
</tr>
<tr>
<td>Barley (var. Lethbridge)</td>
<td>5.55</td>
<td>5.60</td>
</tr>
<tr>
<td>Barley (var. Pentlandfield)</td>
<td>8.15</td>
<td>8.20</td>
</tr>
<tr>
<td>Wheat (var. Capitole)</td>
<td>5.40</td>
<td>5.51</td>
</tr>
</tbody>
</table>

1) **I** = Defatted starch dissolved in DMSO, precipitated with ethanol, dried and re-dissolved in DMSO.

**II** = Defatted starch dissolved in DMSO, precipitated with ethanol, dissolved in DMSO, precipitated with ethanol, dried and re-dissolved in DMSO.
(5) **Defatting Technique**

Fat interferes with the iodine-binding mechanism of starch in two ways:

(i) by absorbing iodine directly at unsaturated bonds, and

(ii) by preferentially complexing with amylose.

Fat may be extracted by Schoch's (1942) method employing a hydrophilic fat-solvent such as 85% aqueous methanol or 80% aqueous dioxane. These methods were time consuming and difficult to apply to small quantities (< 20 mg.) of starch and some doubt may be cast on their efficiency since Rogols et al. (1969) have demonstrated that the lipid extracted from starch depends on the particular solvent system employed.

Since both DMSO and ethanol are solvents for fats, the solution procedure detailed above should provide an efficient and rapid method of removing fat from starches (Table 3.8). This effect was demonstrated by taking the DMSO-ethanol supernatant from the precipitated starch and removing the ethanol by distillation under reduced pressure. The residual DMSO was then added to the corresponding starch-DMSO solution, and the iodine-binding capacity of the mixture was measured. A marked depressant effect was observed (see Figure 3.7) in all cases, confirming that ethanol-soluble material was responsible for the low iodine-binding capacities obtained using untreated starches. The material, although not specifically isolated, was almost certainly a fatty-substance.

(6) **The effect of temperature**

Adkins and Greenwood (1966a, b), in observations on the effect of temperature on the iodine-binding capacity of starch and its components, reported that for normal amylose the change in the amount of bound-
The Effect of Defatting Procedure on the Iodine-Binding Capacity of Cereal Starches

<table>
<thead>
<tr>
<th>Procedure</th>
<th>I (mg I₂ bound/100 mg starch)</th>
<th>II (mg I₂ bound/100 mg starch)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barley (var. Pentlandfield)</td>
<td>5.35</td>
<td>8.20</td>
</tr>
<tr>
<td>Barley (var. Ymer)</td>
<td>3.73</td>
<td>5.66</td>
</tr>
<tr>
<td>Wheat</td>
<td>4.22</td>
<td>5.40</td>
</tr>
<tr>
<td>Rye</td>
<td>4.70</td>
<td>5.51</td>
</tr>
<tr>
<td>Maize</td>
<td>n.d. 2)</td>
<td>5.33</td>
</tr>
</tbody>
</table>

1) I = Iodine-binding capacity of undefatted starch  
2) II = Iodine-binding capacity of starch precipitated from DM3O by the addition of ethanol.  
2) n.d. = not determined.
Figure 3.7 Iodine binding curves

[Graph showing iodine binding curves for different samples: Barley, Starch, AC 38. The temperature is 20.4°C.]

- Control
- Control plus Ethanol Extract
iodine was negligibly small in comparison to the change which occurred for the degraded polysaccharide.

The variation in iodine-binding capacity has been measured for normal amylose and various starches. These are shown in Table 3.9 for pea starches at 1.5°C and 20.5°C. The amylose contents of the starches were calculated by:

\[
\% \text{ amylose} = \left( \frac{\text{iodine-binding capacity of starch at } T \degree C}{\text{iodine-binding capacity of amylose at } T \degree C} \right) \times 100
\]

The iodine-binding capacity of amylose increased with decreasing temperature, as did the iodine-binding capacities of the various starches. For the starches examined, there was good agreement at both temperatures.

Amylomaize was an exception to the rule, in that its apparent amylose content increased with decreasing temperature. This has been suggested by Banks et al. (1971a) by analogy with the behaviour of synthetic amylose oligomers, to be a simple diagnostic test for short chain material (30 < chain length < 150 glucose residues) in starch, or its fractions. This point is examined in detail later in this thesis.

Conclusions

This new technique enabled reproducible and highly accurate results to be obtained for the semi-micro determination of the iodine-binding capacity of starch and its components. In the case of amylopectin, amylose impurities of the order of 0.5% were readily detected.

The experimental technique for the complete dispersion of starch granules prior to titration was formulated using DMSO to give accurate and reproducible results. Iodine-binding capacities of starches recorded in this work tend to be significantly higher than those reported by Greenwood and Thomson (1962a).
### Table 3.9

The Iodine-Binding Capacity of Amylose and Various Pea Starches Measured at Two Temperatures, and the Calculated Amylose-Contents of the Starches

<table>
<thead>
<tr>
<th>Sample</th>
<th>Temperature</th>
<th>I.B.C. 1)</th>
<th>% amylose</th>
<th>I.B.C. 1)</th>
<th>% amylose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.5°C</td>
<td></td>
<td>20.5°C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amylose</td>
<td></td>
<td>22.2</td>
<td>100</td>
<td>19.5</td>
<td>100</td>
</tr>
<tr>
<td>Pea (var. Superb)</td>
<td></td>
<td>8.3</td>
<td>37</td>
<td>7.0</td>
<td>36</td>
</tr>
<tr>
<td>Pea (var. Early Onward)</td>
<td></td>
<td>15.3</td>
<td>69</td>
<td>14.1</td>
<td>72</td>
</tr>
<tr>
<td>Pea (var. Little Marvel)</td>
<td></td>
<td>15.9</td>
<td>72</td>
<td>13.8</td>
<td>71</td>
</tr>
<tr>
<td>Pea (var. Dwarf Defiance)</td>
<td></td>
<td>18.6</td>
<td>84</td>
<td>15.9</td>
<td>82</td>
</tr>
</tbody>
</table>

1) I.B.C. = Iodine-Binding Capacity (mg iodine bound/100 mg polysaccharide).
It is suggested that this can be attributed to three effects:

(i) DMSO treatment more effectively defats granular starch than extraction with hydrophilic, fat solvents.

(ii) DMSO-treatment completely disrupts granular structure and increases the availability of amylose to bind iodine.

(iii) The enzymic method for determining polysaccharide concentration is more accurate than the gravimetric methods used previously.

These higher values are more representative of the "true" iodine-binding capacity of the starches.
SECTION FOUR

AMYLOMAIZE STARCH.

THE NATURE OF THE 
BRANCHED MATERIAL
Section 4.1

**Studies on Amylomaize Starch**

**Introduction**

Considerable academic and commercial interest has been shown in the three cultivars, pea, maize and barley, known to have reserve starches with apparently-enhanced amylose-contents. Academic interest centres on the pathways involved in the biosynthesis of starch, for any proposed biosynthetic scheme must take into account the formation of the unusual, mutant starches. Commercial interest centres on amylose, the essentially unbranched component of starch, and its film-making properties.

Langlois and Wagoner (1967) reviewed the commercial preparation and use of amylose and pointed out that amylose films are edible, only slightly permeable to gases and capable of being greaseproofed. This type of film is an ideal container for foodstuffs, in a pollution-conscious world, as it presents no waste disposal problems. Many commercial processes have been devised to separate amylose from normal starches, but these are lengthy and expensive, since the potential yield is only ca. 26%. The possibility of obtaining a native starch with a high amylose-content thus became the subject of much commercial investigation.

Senti and Dimler (1959), discussing the technological requirements of amylose films, suggested that a minimum of 80% for the amylose-content of a starch was required to give desirable film characteristics. Barley starch (43% apparent amylose) and pea starch (ca 70% apparent amylose) have amylose-contents too low for useful direct exploitation as film making materials, but varieties of maize have, however, been reported with apparent amylose-contents as high as 85%. These amylomaize strains are the result of an extensive breeding programme, in which the expression of the amylose-extender gene was enhanced by combination with a number.
of modifier genes (Zuber (1965)). Surprisingly these starches of apparent high-amylose content have not become a commercial success, for there are major and unanticipated differences between these and other maize starches. Granular form is quite different as shown in Plates 4.1(a) and (b), and 4.2(a) and (b). Normal and waxy maize starches are virtually identical in granular form, being an admixture of irregularly-shaped, round and angular granules. Both starches exhibit optical anisotropy and show characteristic birefringence crosses of similar intensity. However, as the apparent amylose-content increases to over 50%, the shape of the starch granules alters drastically from irregular, round granules in 'Ainylon 50' to a spectrum of strange shapes, as shown in Plate 4.2(b), for 'Mylon 70'. (Note: 'Ainylon' is the Trade Name for commercially available amylomaize starches supplied by National Starch Inc. The number following the name is reputed to be the amylose-content as a percentage of the starch.) It should be stressed that all the starches examined are pure, having little protein present (<0.1%), and virtually no bound lipid. All the granules of the amylomaize mutants stain blue-black with iodine, and the strangely-shaped particles shown in Plates 4.2 are without doubt, starch granules.

Amylomaize starch has a completely-different swelling pattern from the other maize starches, best exemplified by subjecting the starch to a cooking cycle in water. Figure 4.1 shows the pasting behaviour of maize starches of apparent amylose-contents of -1%, 28% and -55% as measured by a "Brabender" Visco-amylograph. (Brabender Corp. Duisburg, Germany.) Slurries of the starches, as 9% solids, were heated from 50°C at a rate of 1.5°C per minute until a temperature of 95°C was attained, and the starches were held at this temperature for 30 minutes. As the starch granules are heated, gelatinization occurs, they swell and impinge upon each other, imparting a thickening effect to the paste. The paste viscosity was recorded continuously by the
PLATE 4.1 (a) Typical angular granule of normal maize starch. Scale 1 cm = 5.3 microns.

PLATE 4.1 (b) Rounded and angular granules of waxy maize starch similar to normal maize. Scale 1 cm = 5.3 microns.
PLATE 4.2 (a) Granules of "Amylon 50" starch, with unusual rounded shapes. Scale 1 cm = 3.2 microns.

PLATE 4.2 (b) Granules of "Amylon 70" starch, with numerous, long, tendril-like granules. Scale 1 cm = 3.2 microns.
Figure 4.1 \ Brabender Amylograms

- Waxy maize starch
- Normal maize starch
- 'Amylon' 50 starch

TIME minutes

BRABENDER VISCOSITY UNITS
Visco-Amylograph, a device by which the viscous drag of a constantly stirred paste is measured by the rotational displacement of a moveable head. On reaching their gelatinization temperature, waxy and normal maize starches swell and produce considerable paste viscosities. As shown in Figure 4.1, under identical conditions 'Amylon 50' imparts no measurable thickening action. Such behaviour on normal cooking, has led to the commercial failure of amylomaize starches in many outlets in the food industry.

A detailed examination reveals other significant differences between amylomaize starch and other starches, even those of as high, apparent amylose-contents. The iodine-binding curves of waxy-maize, normal-maize, Amylon 50, Amylon 70 and, for comparison purposes, wrinkled-seeded pea starch, at 20.4°C are shown in Figure 4.2. In agreement with the observations of Adkins and Greenwood (1966a), the curves for amylomaize starch are unusual, for the iodine binding isotherms are inflectionless in the range of "free" iodine concentrations usually associated with normal starches (e.g. see Section 3.3), and are not capable of extrapolation to yield an unambiguous value of apparent amylose-content. Greenwood and Mackenzie (1966) and Adkins and Greenwood (1966a) have found similar results and attribute this behaviour to the presence of linear material of short unit-chain length. Banks et al (1970a,b) have shown that a range of both linear and branched materials of unit-chain length, 20-60 units, exhibit similar unusual iodine-binding isotherms. No conclusions of a definitive nature may be drawn from iodine-binding curves alone, and resort must then be made to investigations of the polymeric components of amylomaize starch, in order to explain the unusual characteristics of these materials.

An extensive review has been published by Adkins and Greenwood (1966c) detailing the results of investigations on the nature of the amylostarches. Their conclusions may be summarized as follows:
Figure 4.2 Iodine Binding Curves

starches

\[ T = 20.4 \, ^\circ \C \]

- amylomaize band 7
- amylomaize band 5
- wrinkled-seeded pea
- normal maize

BOUND IODINE PERCENT POLYSACCHARIDE

TOTAL FREE IODINE \times 10^6 \text{ molar}
1) The amylose component of amylomaize is similar to that obtained from normal maize.

2) Intermediate types, between the commonly accepted models (see section 2.4) for amylose and amylopectin may exist.

3) The "amylopectin" component is radically different from that of normal maize. Interpretation of its structure is contradictory.

It is worthwhile examining briefly the evidence for the structure of the branched component. This abnormal starch fraction has five unusual and distinctive properties:

a) The branched material is, to varying degrees unstable in aqueous solution.

b) Potentiometric titration of the branched material yields unusual iodine-binding curves.

c) A spectrum of the iodine complex is quite distinctive and cannot be explained on the basis of mixtures of "normal" maize fractions.

d) The action of crystalline β-amylase yields maltose in greater amounts than is usually encountered for amylopectins.

e) The average chain-length, measured by a variety of methods, is considerably greater than that of "normal" amylopectin.

Three models have been proposed to explain these unusual properties. A polysaccharide with longer-than-average internal and external chains in the branched structure was suggested by Wolff et al (1955). Their evidence is based on the points mentioned above, with the exception that their "amylopectin" had a conversion of 58% to maltose with β-amylase. On this basis, the amylomaize "amylopectin" had inner and outer chain lengths of 13 and 23, respectively. The possibility of some contamination by either low degree of polymerisation ($Dn$) amylose or amylopectin was not discounted, as precipitation techniques showed some evidence of this, and measurements of chain-length also pointed to a polydisperse system.
Different and rather conflicting models have been proposed by Montgomery and co-workers and Greenwood and co-workers, for, whilst both sets of workers are agreed that the "amylopectin" from amylomaize has a β-limit considerably in excess of the value of 54-58% normally associated with amylopectin, and an average chain-length in excess of 35, interpretation of these results is a matter of some controversy. Montgomery et al (1964) maintain that the polysaccharide is basically homogeneous and therefore has normal internal chains, and long external chains, whilst Greenwood et al e.g. see Adkins et al (1970) have suggested that the anomalous amylopectin is in fact a heterogeneous mixture of short chain linear material and normal, high-molecular-weight amylopectin. This unusual mixture of polysaccharide is attributed to alpha-amylolytic activity during the formation of amylomaize starch.

That the amylomaize starch contains short-chain linear material has been conclusively demonstrated by Banks and Greenwood (1968c). If linear material, of low degree of polymerisation, is present in considerable quantities a measurable amount of glucose will be produced from chain ends (see Section 2.4 for details) on β-amylolysis since, statistically, half the chains will have an odd number of glucose residues. The relevant experiment did, in fact, result in the detection of appreciable quantities of glucose, showing that Montgomery et al (1964) are in error.

Notwithstanding the very strong support for the presence of short-chain, linear material in amylomaize starch, a satisfactory model for the branched component is yet to emerge. This present work demonstrates a new, rapid and simple technique for the isolation of a considerable portion of the anomalous component of amylomaize starch and reports on subsequent partial characterisation of that material. In the last part of this section, the starch from the pollen of amylomaize is examined.
Section 4.2

Isolation of Anomalous Material from Amylomaize

Amylomaize is exceedingly difficult to solubilize, temperatures in excess of 125°C being required to achieve maximum viscosity potential, and on cooling the solution of starch there is extreme instability, resulting in massive retrogradation. To overcome these problems during fractionation, complex schemes have been devised, for example Adkins and Greenwood (1969a) dissolved the starch in methyl sulphoxide and added a mixture of buffer and butan-1-ol. A complex formed within 30 minutes and was easily removed by low-speed centrifugation. This and similar schemes, e.g. Greenwood and Mackenzie (1966); Montgomery et al (1961), yield an "amylopectin" which is a heterogeneous mixture of short-chain, linear material and branched material. Adkins and Greenwood (1966b; 1969a) achieved some separation by preparative ultracentrifugation, and complex formation with iodine, but both methods were laborious and of somewhat variable reproducibility (Banks (1970)).

It was noted that amylomaize starches lost their birefringent character whilst being heated in water to temperatures over 85°C. This was indicative of some irreversible loss of molecular order and suggested that it would be possible to leach some material from the starch.

Amylomaize starch was slurried in distilled water (c = 0.5% w/v) and held at 98°C on an electrically-heated, thermostatted 'isomantle'. Constant stirring and sparging with oxygen-free nitrogen were required to prevent uneven boiling. Samples were withdrawn at various time intervals, quickly centrifuged (2 x 10^3 g) to remove particulate matter, and the polysaccharide concentration in solution, estimated by acid hydrolysis, and estimation of reducing power as detailed in Section 2.2. Figure 4.3 shows the percentage solubilization of two amylomaize starches of different apparent amylose-contents as a function
Figure 4.3 Leaching of Amylomaize Starch at 100°C

- AMYLON 50
- AMYLON 70
of time of heating in boiling water. In both cases, an initial, rapid solubilization of material is observed, followed by a slower secondary process. The amount of starch solubilized is a function of apparent amylose-content, a larger amount being extracted from starch of lower amylose-content.

The starch ("Amylon 50") was then extracted, in a separate experiment, for one hour in boiling water, cooled rapidly to room temperature, and the residual starch granules removed by low-speed centrifugation. The supernatant liquor was saturated with butan-1-ol and allowed to stand at room temperature (20°C) for 2 hr. The resultant polysaccharide butan-1-ol complex was removed. Potentiometric titration and enzymic assay showed this to be amylose. (Iodine binding capacity = 19.1 mg I₂ bound/100 mg polysaccharide; β + Z limit = 98%). The supernatant liquor was concentrated on a rotary evaporator, and ethanol (3 vol) was added. A copious precipitate was obtained, which was washed repeatedly with ethanol, triturated to a fine powder, then dried in vacuo. The iodine-binding curve was measured at 20.4°C as detailed in Section 3.3, and is shown in Figure 4.4.

This non-complexible fraction, comprising some 15% of the total starch, exhibited iodine-binding characteristics associated with the abnormal fraction of amylomaize \((\text{Adkins and Greenwood (1966a)})\). When an aqueous solution was subjected to ultracentrifugation (average force-field = 50,000 g) for 2.5 hr, treatment shown to remove > 95% of a potato amylopectin from solution, the supernatant liquor contained, within experimental error, all the polysaccharide originally present.

The non-complexible fraction exhibited instability when stored in a 0.1% solution, at room temperature, for four days, behaviour shown, by \text{Adkins and Greenwood (1966f)}, to be a marked feature of the anomalous material found in amylomaize.
Figure 4.4 Iodine Binding Curves

- x amylose
- • non-complexible fraction

T = 20.4°C

BOUND IODINE PERCENT POLYSACCHARIDE

TOTAL FREE IODINE $\times 10^6$ molar
It was concluded that this leaching and complexing technique provides a rapid and simple method of obtaining large quantities of anomalous material from amylomaize, free from conventional "amylopectin"-type polysaccharide.

The above experiments were performed using a commercial sample of "Amylon 50" supplied by National Starch Inc. The experiments were repeated using a sample of starch isolated from greenhouse-grown amylomaize by the techniques detailed in Section 2.

The iodine-binding curves at 1.2°C are shown for the parent starch, leached non-complexible function (N.C.P) and the residue in Figure 4.5 and the characteristics are very similar to those of the sample obtained from commercial starch, showing that the original observations are valid. It is noteworthy that the residual starch, after leaching ca 50% as amylose, still has a very high apparent amylose-content and displays the typical anomalous behaviour of the parent starch.
Figure 4.5 Iodine Binding Curves

Greenhouse Grown
Band Five Amylomaize

![Graph showing iodine binding curves with different markers for starch, residue, and non-complexible fraction.](image)

- **Starch**
- **Residue**
- **Non-complexible fraction**

**Conditions:**
- $T = 1.8^\circ C$

**Axes:**
- **Y-axis:** Bound iodine percent polysaccharide
- **X-axis:** Total free iodine $\times 10^6$ molar
Characterization of the Non-Complexible Fraction

All of the following characterization measurements were made using "Amylon 50", kindly supplied by National Starch Corp. The starch was leached by the procedure detailed in the previous section, then fractionated by three complexing techniques.

i) The supernatant liquid containing the leached material was warmed to 65°C, powdered thymol (0.1% w/v) added, and the solution vigorously stirred. After cooling to room temperature (20°C) and storage at this temperature for 24 hours, a copious precipitate formed. The thymol complex was removed by centrifugation (~ 2,500 g) and the clear supernatant allowed to stand at 0°C for a further 24 hours. During this time a slight precipitate formed, which was subsequently identified as thymol. Thymol was removed from solution by repeated extraction with diethyl ether, the resulting solution concentrated eight-fold in volume on a rotary evaporator, and the polysaccharide therein precipitated by the addition of ethanol (3 vol.). The precipitate, NCF(a), was washed repeatedly with ethanol then dried in vacuo.

ii) To the leach supernatant at room temperature (20°C), butanol (8% v/v) and sodium chloride (0.1% w/v) were added. A copious precipitate formed within ten minutes. After storage at 20°C for 24 hours, the butanol-complex was removed by low-speed centrifugation (~ 2,000 g) and the polysaccharide remaining in solution was precipitated with ethanol as above, to yield NCF(b).

iii) The liquid from a leaching experiment was treated as for NCF(b), but only 30 minutes was allowed for complex formation with butanol. After treatment as above NCF(c) was obtained.
Results and Discussion

In the preceding part of this Section, it has been established that the material, obtained by leaching amylomaize starch in water at 98°C, exhibits the anomalous properties associated with the parent starch. The preparation procedures detailed above were designed to show the influence of the complexing agent on the material left in solution i.e. the non-complexible fraction. Differences do appear, but as will be shown later, this is in degree rather than kind. Waxy maize amylopectin, whose properties are well documented (see for example Banks et al. (1971b), was used as a control standard for the behaviour of 'normal' branched starch fractions.

Ultracentrifugation

Adkins and Greenwood (1966e) used ultracentrifugation as a means of achieving separation in whole maize starch. Under the conditions described in Part 2 of this Section, little material sedimented from solution at 50,000 g. In a 60,000 g force-field for three hours, however, small quantities of material sedimented from all the anomalous fractions as shown in Table 4.1. Aliquots of solution (1.0 ml) of the non-complexible fractions in methyl sulfoxide (ca. 0.75% w/v polysaccharide) were diluted with water in a graduated flask (10 ml). The polysaccharide concentration was estimated, after hydrolysis with amylglucosidase as detailed in Section 2, before and after centrifugation (average force-field 60,000 g; 3 h; 25°C). The difference in the solution concentration was a measure of the amount of material which sedimented from solution. It is apparent that only a minor fraction of the total material has been sedimented in the case of the amylomaize fractions under conditions in which 'normal' amylopectin is almost completely removed. The sediments were not intensively investigated since they compose a very small proportion of the total starch (<3%), but are not 'normal' amylopectin as shown by:
Table 4.1

The Weight-Percentage of Material After Ultracentrifugation from Various Starch Fractions

<table>
<thead>
<tr>
<th>Sample</th>
<th>NCF(a)</th>
<th>NCF(b)</th>
<th>NCF(c)</th>
<th>Wx(1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Residue(2)</td>
<td>90%</td>
<td>85%</td>
<td>80%</td>
<td>4%</td>
</tr>
</tbody>
</table>

(1) \(wx = \) waxy maize amylopectin.

(2) Residue in solution after centrifugation at 60,000 g average force-field for 3 hours.
1) The iodine-binding curve of $1^2$, which had almost high molecular-weight material is shown at $1.8^\circ C$ before and after ultracentrifugation in Figure 4.6. The slight difference in iodine-binding behaviour could not be brought about by the removal of normal amyllopectin.

2) As the efficiency of the complexing procedure used in sample preparation increases, the amount of material sedimented decreases, implying that the sedimented material may complex under certain conditions. Such material is neither, by definition, amyllose or amyllopectin, and corresponds to the intermediate types of material of Proctor (1965) or Banks and Greenwood (1959a). As this material comprised only a minor portion of the total sample, no further effort was made to characterize it.

Solution Stability

The solution stability of non-complexible fractions was estimated by measuring the turbidity of the polysaccharide (0.075% w/v) in a DMSO-water mixture (10% v/v) using a 'Spekker' spectrophotometer (550 m\(\mu\) filter; 1/2 cm cell). DMSO-water (10% v/v) was used as the reference blank.

The non-complexible fractions were all found to be stable in aqueous solution at $30^\circ C$ for periods in excess of 52 h (at a concentration of 0.75% w/v). However, either increasing the concentration of polysaccharide or decreasing the temperature, greatly increased the instability of the solution. The effect of decreasing solution stability is shown in Table 4.2 for dilute solution at $4^\circ C$. Under such conditions, considerable precipitation occurs - at 52 hours, heavy, visible precipitates are obvious in all three non-complexible fractions. In contrast, waxy-maize amyllopectin is quite stable under identical circumstances. Once a measurable turbidity had been produced in solutions of the non-complexible fractions, heating could not reduce it
Figure 4.6 Iodine Binding Curves

Non-Complexible Fraction

'C'

• before centrifugation

× after centrifugation

T = 1.8°C
Table 4.2

The Solution Stability of the Non-Complexible Fractions at 4°C

<table>
<thead>
<tr>
<th>Sample</th>
<th>Absorbance at time t (h)</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>6</td>
<td>22</td>
<td>52</td>
</tr>
<tr>
<td>NCF(a)</td>
<td>0.00</td>
<td>0.00</td>
<td>0.40</td>
<td>0.77</td>
<td>0.92</td>
<td>ppt</td>
</tr>
<tr>
<td>NCF(b)</td>
<td>0.00</td>
<td>0.00</td>
<td>0.04</td>
<td>0.59</td>
<td>0.87</td>
<td>ppt</td>
</tr>
<tr>
<td>NCF(c)</td>
<td>0.00</td>
<td>0.00</td>
<td>0.77</td>
<td>1.00</td>
<td>1.01</td>
<td>ppt</td>
</tr>
<tr>
<td>wx (2)</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.01</td>
<td>0.00</td>
</tr>
</tbody>
</table>

(1) ppt = heavy visible precipitate
(2) wx = waxy maize amylopectin
prolonged immersion in a boiling water bath had no significant effect on the turbidity. This phenomenon, usually known as retrogradation and associated with the amylose component of starch, is due to a crystallization process, and is governed by a complex interaction between such variables as concentration, temperature, pH, molecular size, and molecular size distribution. Retrograded amylose is virtually insoluble in boiling water; in aqueous caustic alkali, it apparently dissolves to give clear solutions, but neutralization of these solutions usually leads to massive precipitation. For this reason, DMSO, which does not give rise to such phenomena, is the only reliable reagent for solubilization of retrograded amylose. The precipitates of the non-complexible fractions could apparently be dissolved in aqueous KOH, but neutralization led to immediate turbidity - rather as though nuclei for the crystallization process had been retained in alkaline solution (see for examples, in the synthetic polymer field, Banks and Sharples (1963b)). Once however, the retrograded material had been dissolved in DMSO, the results in Table 4.2 could be reproduced, showing that all nuclei had been destroyed. The analogies with the retrogradation behaviour of amylose, lead to the conclusion that the non-complexible fractions crystallize by a similar mechanism.

It should be noted that the three samples follow quite distinct retrogradation patterns i.e. minor changes in the isolation procedures gave rise to very great differences in solution stability. In each sample there is an apparent lag-time, about 90 minutes, during which no precipitation could be detected. However, it is likely that the crystallization is described by the Avrami (1939) equation:

\[ 1 - \left( \frac{x_t}{x_\infty} \right) = \exp(-kt^n) \]

where \( x_t \) is the crystallinity at time \( t \), \( x_\infty \) is the limiting value of the crystallinity, and \( k \) is a rate constant. The integer \( n \) takes values \( 1 < n < 4 \), including fractional values and interpretation thereof.
has been shown to be quite ambiguous \(\text{Banks and Sharples (1963a)}\). Irrespective of the value of \(n\), the form of the above equation is such that \(x_t\) is finite for all values of \(t\); if, therefore, the Avrami equation is applicable to the crystallization of the non-complexible fractions, as appears probable, the lag-time before precipitation is real, and reflects only the fact that the magnitude of the rate constant \(k\) in the above equation is rather low. The conclusions to be drawn from these instability measurements are that:

i) the mechanism by which the material retrogrades is similar to that for amylose, and

ii) the process is best described as a polymer crystallization phenomenon.

**Iodine Interaction**

The interaction of starch and its fractions has been shown in Section 3.3 of this work, and by \text{Banks et al (1970a, (b); 1971a)}, to be a powerful diagnostic tool. Short-chain amyloses, amyllopectin with long external chains and glycogen with long external chains, all have characteristic iodine-binding curves. The technique is, however, empirical, and it must be stressed that only in the presence of other structural data, derived from enzymic measurements, may any meaningful conclusions be reached. The iodine-binding curves for the non-complexible fractions are shown for \(1.8^\circ\text{C}\) and \(20.4^\circ\text{C}\) in Figures 4.7 and 4.8, respectively. The curves are inflectionless over the range of 'free' iodine concentrations, at which normal starch and its fractions bind in two distinct steps (compare with Figure 4.2), and the anomalous material binds iodine to high levels of 'free' iodine. Extrapolation of the 'linear' portions of the curves gives the data recorded in Table 4.3. \text{Banks et al (1971a)} have shown that the ratio of two iodine affinity measurements made at \(20^\circ\text{C}\) and \(2^\circ\text{C}\) is a measure of the average degree of polymerization of linear amyloses in the range \(36 < \text{DP} < 134\).
Figure 4.7 Iodine Binding Curves

BOUND IODINE PERCENT POLYSACCHARIDE

TOTAL IODINE FREE
$\times 10^6$ molar

NCF. (a)
NCF. (b)
NCF. (c)

$T=1.8^\circ C$
Figure 4.8  Iodine Binding Curves

$T = 20.4^\circ C$

BOUND IODINE PERCENT POLYSACCHARIDE

TOTAL FREE IODINE $\times 10^6$ molar

- NCF (a)
- NCF (b)
- NCF (c)
Table 4.3

The Iodine Affinities of the Non-Complexible Fractions

<table>
<thead>
<tr>
<th>Sample</th>
<th>NCF(a)</th>
<th>NCF(b)</th>
<th>NCF(c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I.A.(^{(1)})</td>
<td>6.2</td>
<td>8.3</td>
<td>9.1</td>
</tr>
<tr>
<td>I.A.(^{(2)})</td>
<td>12.1</td>
<td>13.5</td>
<td>14.8</td>
</tr>
<tr>
<td>R(^{(3)})</td>
<td>0.51</td>
<td>0.62</td>
<td>0.62</td>
</tr>
</tbody>
</table>

(1) mg iodine bound per 100 mg polysaccharide at 20.4°C
(2) mg iodine bound per 100 mg polysaccharide at 1.8°C
(3) Ratio 1): 2)
Using their data, the non-complexible fractions in Table 4.3, correspond to linear materials with average lengths of unit chain in the range 50-70 units. The material could not, however, be compared with amylopectin with long external-chains, but it should be emphasized that it is unlikely that the material under examination had the same distribution of unit-chain lengths, and this may invalidate the comparisons made.

**Enzymic Characterization**

Extensive enzymic measurements were made on the non-complexible fractions as detailed in Section 2 and the results are presented in Table 4.4.

Complete conversion by the concurrent action of crystalline $\beta$-amylase and pullulanase (99-102%) confirms that only $\alpha$-1:4- and $\alpha$-1:6- linkages are present in the material. Beta-amylolysis limits are very much higher than those generally accepted for normal amylopectin, as are the beta + Z amylolysis limits. A point of considerable interest was that the beta + Z limit could be increased considerably by the addition of more enzyme and prolonged incubation - conditions which had no significant effect on the waxy maize amylopectin. It may be concluded from this evidence, that the branched fraction of the anomalous material is more susceptible to the action of $\zeta$-enzyme than is normal amylopectin, and this implies (1) either that the internal chain length of the material is longer than normal, or (2) that it has a much smaller molecular size.

All samples had linear material present as an impurity, as shown by the technique of Banks and Greenwood (1968a), and either the amount of material or its nature changed with the complexing technique.

The average length of unit chain ranged from 35-42 units, in agreement with general observations on this anomalous type of material, representative of the amylomaize starches.
<table>
<thead>
<tr>
<th>Sample</th>
<th>$\beta$ 1)</th>
<th>$\beta + Z$ 2)</th>
<th>$\beta + P_u$ 3)</th>
<th>$\frac{4}{CL}$</th>
<th>$L.M.$ 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(i)</td>
<td>(ii)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NCF(a)</td>
<td>66</td>
<td>70</td>
<td>81</td>
<td>99</td>
<td>35</td>
</tr>
<tr>
<td>NCF(b)</td>
<td>72</td>
<td>73</td>
<td>84</td>
<td>99</td>
<td>38</td>
</tr>
<tr>
<td>NCF(c)</td>
<td>74</td>
<td>76</td>
<td>84</td>
<td>102</td>
<td>42</td>
</tr>
<tr>
<td>wx(6)</td>
<td>58</td>
<td>57</td>
<td>58</td>
<td>100</td>
<td>20</td>
</tr>
</tbody>
</table>

1) Percent conversion in to maltose by crystalline $\beta$-amylase.

2) Percent conversion in to maltose by concurrent action of crystalline $\beta$-amylase and Z-enzyme:
   (i) after 24 hours.
   (ii) after 48 hours plus excess Z-enzyme.

3) Percent conversion in to maltose by concurrent action of crystalline $\beta$-amylase and pullulanase.

4) Average-length of unit-chain measured as in Section 2.

5) Average-length of unit-chain of linear material (L.M.) calculated assuming all material is linear.

6) as in Table 4.1.

7) n.a = not applicable; no linear material detected.
Intensive efforts were made to separate the linear material from the branched material in the non-complexible fractions; as has already been shown ultracentrifugation was ineffective; the iodine-complexing procedure of Adkins and Greenwood (1969a) yielded no separation, and at pH 12.0 in the presence of salt, conditions known to complex amylose of low molecular weight, a complete cross-section of material was removed from solution. It was concluded from these experiments that the branched material had the ability to complex with certain reagents to a limited, but significant extent. The following calculations were then applied to the data in Table 4.4.

The average degree of polymerization of the linear amylose present in a sample was assumed, values compatible with the iodine binding behaviour as shown in Table 4.3 being selected. The weight per cent present in the sample was calculated in the mixture of linear and branched material from data in Table 4.4:

\[
\% \text{ linear material} = \frac{CL_{\text{assumed}}}{CL \text{ if all linear}} \times 100\%
\]  

(4.1)

From this the average length of unit chain of the branched material is computed:

\[
\frac{x}{CL(x)} + \frac{y}{CL(y)} = \frac{1}{CL(n)},
\]

(4.2)

where \(x\) = weight fraction of linear material calculated from equation (1), \(y = (1-x)\) = weight fraction branched material; \(CL(x)\) and \(CL(y)\) are the respective chain-lengths; \(CL(n)\) is the measured average chain length from Table 4.4. As the beta-amylolysis limit of linear amylose is 100%, once the amount of such material is known from equation 4.1, the beta-amylolysis limit of the branched material is easily derived:

\[
(x \times \{\beta\}_x) + (y \times \{\beta\}_y) = \{\beta\},
\]

(4.3)

where \(x\) and \(y\) are the respective weight fractions of linear and branched material and \(\{\beta\}\) is the beta-amylolysis limit.
Table 4.5 shows the computed inner and outer chain lengths of the branched material and the weight percentage assuming only the chain length of the linear material, for samples NCF(a) and NCF(c); (values for NCF(b) are intermediate between the values of the other fractions.) The \((\beta + 2)\) amylolysis-limit of the branched material was assumed to be 58, and the weight percent linear material calculated as in Section 2. Two methods of computation of the weight per cent linear material are in agreement, if the linear material has average unit chain-lengths in the range 65-70, a range compatible with the iodine-binding data presented in Table 4.3. It should be noted that the internal chain length of the branched material lies between 11-13 units, i.e. double the value for waxy maize amylopectin, and that this measurement is independent of the value assumed for the amylose chain-length. This fact is substantiated by the evidence presented earlier with respect to susceptibility to the action of \(\beta\)-enzyme, and was further corroborated by measurement of the iodine-binding curves of the \(\beta\)-amylolysis limit dextrin of NCF(b). Figure 4.9 shows the iodine binding curves of this material at 20.4°C and at 1.8°C. The dextrin binds no iodine at the higher temperature, but considerable quantities at the lower one, and, by comparison with the data of Banks et al (1971a), the weight average chain length is in the range 20-26, a value quite comparable with a number-average value \textit{ca} 12 units. These calculations confirm that the internal chain length of the branched material in amylo maize is very much longer than that of normal amylopectin. The exact nature of the outer chains has not been proven, but a considerable body of evidence suggests that the amylose chain-length is \textit{ca} 70 units and in consequence that of the external chains must be \textit{ca} 18-20 units. All the evidence presented herein suggests that amylo maize starch contains, in addition to large amounts of linear material of short chain length, a branched material quite distinct in its structure from normal amylopectin.
The Computed Properties of the Branched Material from the Anomalous Fractions.

<table>
<thead>
<tr>
<th>Sample</th>
<th>( L^1 )</th>
<th>( \text{wt}^2 )</th>
<th>( \beta^3 )</th>
<th>( \text{CL}^4 )</th>
<th>int</th>
<th>ext</th>
<th>( \text{wt}^5 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCF(a)</td>
<td>50</td>
<td>19</td>
<td>58</td>
<td>33</td>
<td>11</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td></td>
<td>70</td>
<td>26</td>
<td>54</td>
<td>30</td>
<td>11</td>
<td>19</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>34</td>
<td>49</td>
<td>27</td>
<td>11</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>NCF(c)</td>
<td>50</td>
<td>34</td>
<td>61</td>
<td>39</td>
<td>13</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td></td>
<td>70</td>
<td>48</td>
<td>50</td>
<td>31</td>
<td>13</td>
<td>18</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>62</td>
<td>32</td>
<td>23</td>
<td>13</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Wx</td>
<td></td>
<td></td>
<td>58</td>
<td>20</td>
<td>6</td>
<td>14</td>
<td></td>
</tr>
</tbody>
</table>

1) the assumed chain-length of the linear material (L.M.)
2) calculated weight percent linear material from equation 4.1
3) calculated beta-amylolysis limit of branched material from equation 4.3
4) calculated average unit-chain length from equation 4.2
5) calculated weight of linear material from \((\beta+Z)\) - amylolysis limit (see text)
6) as in Table 4.1

112.
Figure 4.9  Iodine Binding Curves
beta limit dextrin
N.C.F(b)

- $T=1.8^\circ$
- $T=20.4^\circ$
The branched material has an internal structure with chain lengths **double** that of normal amylopectin and it is likely that the outer chains are considerably longer than usual.

**Summary and Conclusions**

The preceding parts of this section have described the major differences between amylomaize starch and other starches, and have shown that this is due to components of an unusual composition. A method was devised to successfully isolate a representative portion of the anomalous material in large quantities, and further studies have provided a partial characterization. Two major points have emerged

1) Previous observations of Greenwood and co-workers, concerning the presence of short chain linear material have been corroborated, and this material shown to have a likely chain length ca 70 units and,

2) the nature of the branched component, a matter of some dispute, has been shown definitively to have a much longer than average inner unit-chain length, and considerable evidence suggests that the outer chains are also correspondingly larger.

Amylopectin is known to be the structure-forming component of normal starches (see Section 5 for a discussion of this point) and, since predominantly linear material is leached by the technique devised in this work, there is no reason to believe that the branched material is not also the principal structural material in amylomaize starch. As the average chain-length has been shown in this work to be longer by a factor of two, than that of normal amylopectin, it is not surprising that this material can crystallize more readily. Thus the granular structure would be expected to be much more stable and the branched material more prone to retrogradation - the experimentally observed characteristics of this unusual type of starch.
Further work is however necessary to define the exact nature of the outer chains of the branched material and the differences, if any, of the residual material left after leaching. The significance of this present work is that it demonstrates for the first time that the amylomaize starches are not merely admixtures of degraded starch fractions from normal maize, but contain branched material of a distinctive type, intermediate between linear material i.e. amylose and the branched material generally known as amylopectin.
Section 4.4

Pollen Starch from Amylomaize

The extensive use of starch, in a variety of widely different commercial fields, has led to plant breeding programmes designed to give cultivars with endosperm starch of a particular type. In many instances, plants with the required starch characteristics have quite unsuitable general properties for growth on an economic scale. Intense effort has been expended, especially in the case of maize, to incorporate genes for particularly desirable starch types into the genetically stable background of well-established, high-yielding, and disease-resistant varieties.

The waxy and the high-amylose starch mutants are well defined genotypes which have attracted considerable attention. Although Radley (1968) points out that waxy starch, composed entirely of the branched starch component, amyllopectin, has been detected in many plant sources, only rice, sorghum, maize, millet and barley are of commercial importance. The amylose-extender gene, giving starches with high proportions of linear material, would at present, seem to be limited to varieties of pea, maize and barley.

In the case of the waxy allele, it was noted by Parnell (1921) that the pollen starch of glutinous or waxy rice exhibited similar iodine-staining properties to those of the endosperm starch from the same plant. Pollen starch from waxy rice stained red with iodine, that from normal rice starch blue and the pollen from the F1 hybrid generation exhibited chemical dimorphism with equal numbers of red- and blue-staining pollen grains. It appeared, therefore, that the starch characteristics of the gametophytic generation mirrored those of the sporophytic generation and Parnell (1921) concluded that the waxy gene (wx) was a simple recessive to the normal starchy gene (Wx).

Similar experiments were carried out by Demerec (1924) on waxy maize and normal maize. Similar results to those of Parnell (1921)
were observed, with the gametophytic generation having identical starch to the parent and, once again, 1:1 segregation was observed in the F1 heterozygote by means of iodine staining. Karper (1933) investigated the pollen starch of a third waxy cereal, sorghum and, once again, the pollen starch was shown to be similar to the parent endosperm starch, separating in the case of heterozygotes into differentiated pollen types, governed by the haploid characteristics. It was clear that, in three waxy cereals, there was a close correlation between the starch characteristics of the gametophytic generation controlled by the haploid number of genes and those of the homozygous sporophytic generation controlled by the diploid number of genes.

The segregation of starch types in the gametes promised to be a most useful property for, by simple examination of the pollen starch, it was possible to identify heterozygotes without lengthy programmes of selfing or backcrossing. Longley (1924) successfully applied this principle to evaluate the success of crosses between maize, euchiena and coix by using one waxy parent and observing the ratio of red- and blue-staining pollen in the hybrid.

It was all the more surprising, therefore, when Vineyard et al. (1958) and Zuber et al. (1960) reported that there was no significant correlation between the amylose contents of the pollen starch and the endosperm starch of amylomaize. This implies that the starch of the gametophytic generation differs significantly from that of the sporophytic generation. A rather more complex genetic system would therefore govern amylomaize starch synthesis than in the case of the waxy mutant.

This work presents an analysis of the pollen starch of two amylomaize varieties, homozygous for the amylose extender gene, but influenced in starch type by differing modifier genes.
Experimental Methods

(i) Sample Collection and Starch Extraction

Two amylomaize varieties, band 5 and band 7 were kindly supplied by Dr. R.P. Bear of the Bear Hybrid Corn Co. Decatur, Illinois, U.S.A. and grown by Dr. J.T. Walker, Rothwell Plant Breeders Ltd, Rothwell, Lincoln, England in a controlled glasshouse environment. Pollen was collected from bagged tassels, and the excess, after self-fertilisation was accomplished, was subjected to analysis. After collection, the pollen was immersed in HgCl$_2$ solution (0.01M) to inhibit enzyme action, and later extracted for 2 minutes in a high-speed mechanical blender, once more in the presence of HgCl$_2$. The resultant slurry was screened (75 μ nylon mesh), and the residue re-extracted in a similar manner. After eight such extractions, the residue no longer stained blue with iodine and was discarded. The combined filtrates were allowed to sediment for 24 hours at room temperature. Gentle suction was applied to remove the supernatant from the sedimented material. The sediment was suspended in dilute aqueous saline, and shaken with toluene (one-eighth volume) overnight. The starch was allowed to sediment, the toluene layer removed, added to water (10 volumes) and air bubbled vigorously through the mixture to release starch granules physically entrapped in the toluene layer. After standing several hours, the toluene and aqueous layer were removed from the sediment and rejected. The starch sediments were combined, suspended in aqueous saline and the extraction with toluene repeated. A total of 15 such extractions were necessary to achieve a clear toluene layer, signifying the removal of all contaminating protein. Finally, differential sedimentation was employed to remove a small amount of macerated pollen tissue which had survived the screening procedure. The starches were pure-white in colour.
(2) Dissolution of Starch

The starch (ca. 50 mg) was dissolved in methyl sulfoxide (DMSO) with gentle mechanical stirring to give a 2.5%(w/v) solution. Ethanol (3 vols.) was added to precipitate the polysaccharide, which was titrated with repeated washings of ethanol to a finely divided powder, and finally dried overnight, at 65°C in vacuo. The non-granular starch was dissolved in DMSO (2.0 ml), and an aliquot (0.5 ml) taken for measurement of iodine binding capacity.

(3) Determination of Iodine Binding Capacity

The iodine binding capacity and apparent amylose contents of the pollen starches were measured by the new technique detailed in Section 3.3.

Results and Discussion

(1) Microscopic Appearance of the Starches from Amylomaize Pollen

Scanning electron micrographs of starch granules from the pollen of both Band 5 and Band 7 amylomaizes are shown in Plates 4.3 and 4.4, respectively. The granules are small ranging from 0.5 - 2.5 µ and appear to be predominantly prolate ellipsoids having a major axis 1-2 µ and a minor axis 0.2 - 0.5 µ. Both samples of starch appeared similar in morphology, no noticeable differences being observed in either size or shape. When viewed by light microscope between crossed polars no birefringence could be detected.

Both pollen starches stained deep blue when treated with dilute iodine/potassium iodide solution. Although red-staining starch is diagnostic of the waxy type, blue-staining is observed equally in all normal starches and cannot be used to differentiate between starches containing 20% amylose and 85% amylose.
PLATE 4.3 (a) Pollen starch from "Amylon 50". Scale 1 cm = 1.5 microns.

PLATE 4.3 (b) Small spherical pollen starch granules from "Amylon 50". Scale 1 cm = 0.4 microns.
Plate 4.4 (a) Pollen starch from "Amylon 70". Scale 1 cm = 1.7 microns.

Plate 4.4 (b) Typical long granule of starch from the pollen of "Amylon 70". Scale 1 cm = 0.4 microns.
(2) **Iodine-Binding Capacity**

The iodine binding curves of the amylomaize pollen starches are shown in Figures 4.10 and 4.11 at 20.4°C and 1.8°C respectively. The curves are extrapolated to zero free iodine concentration as detailed in Section 3.3 to give the iodine-binding capacities. The results are summarized in Table 4.6. The apparent amylose-contents of the respective starches were calculated on the basis that the iodine-binding capacity of amylose is 19.5 mg% at 20.5°C and 22.4 mg% at 1.5°C. Banks *et al* (1971a) have shown that the difference between amylose contents measured at two temperatures by this technique is significant only when material which has a fairly small chain length \( (C.L._{n}) \) i.e. \( 30 < C.L._{n} < 150 \) is present. This type of material, which forms the subject of Section 4.3 has previously been found only in appreciable quantities in the endosperm starch of amylomaize.

Contrary to the findings of Vineyard *et al* (1958) and Zuber *et al* (1960), the apparent amylose-contents of both pollen starches are considerably in excess of that reported for the endosperm starch of normal maize (ca. 27% amylose). In addition, the interaction with iodine at two temperatures, with a significant change in iodine-binding behaviour, suggests very strongly that anomalous material, of the type found in mature amylomaize endosperm starch, is present.

Certain anomalies exist, however, in the present data. In the case of measurements carried out at room temperature:

(a) the apparent amylose-contents of the pollen starches are in the reverse order to those of the endosperm starches, and

(b) the shapes of the titration curves are not those expected of amylomaize starches (see for example curves in Figure 4.2), where a linear extrapolation of the curve is not possible. Notwithstanding these facts, the slopes of the curves are considerably greater than
Figure 4.10 Iodine Binding Curves

pollen starch

T = 20.4°C

○ amylomaize band 5
○ amylomaize band 7

BOUND IODINE PERCENT POLYSACCHARIDE

TOTAL FREE IODINE \times 10^6 \text{ molar}
Figure 4.11  Iodine Binding Curves

pollen starch

BOUND IODINE PERCENT POLYSACCHARIDE

0  4  8  12
TOTAL FREE IODINE  \times 10^6 \text{ molar}

T=1.8^\circ C

○ amylomaize band 7
○ amylomaize band 5
Table 4.6

The Iodine Binding Capacities, and Apparent Amylose Contents of two Amylomaize Pollen Starches, measured at 20.4°C and 1.8°C.

<table>
<thead>
<tr>
<th>Amylomaize Type</th>
<th>20.4°C</th>
<th>1.8°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I.B.C.</td>
<td>% amylose</td>
</tr>
<tr>
<td>Band 5</td>
<td>9.2</td>
<td>48</td>
</tr>
<tr>
<td>Band 7</td>
<td>8.1</td>
<td>42</td>
</tr>
</tbody>
</table>

1) I.B.C. = iodine binding capacity (mg I₂ bound/100 mg starch)
2) % apparent amylose calculated from 100 x I.B.C. $\div$ 19.5 (see text)
3) % apparent amylose calculated from 100 x I.B.C. $\div$ 22.4 (see text)
those encountered in normal maize starches, and this is especially marked in the case of the pollen starch from Band 7 amylo maize. At low temperature, however, this anomaly is resolved, for at any concentration of free iodine, Band 7 amylo maize pollen starch binds more iodine than the starch from the Band 5 amylo maize pollen. As emphasized in Section 4.3, the extrapolations necessary to yield an apparent amylose percentage for the abnormal starches from amylo maize are arbitrary and should be interpreted as a qualitative rather than strictly quantitative measure of the starch type.

There is an irreconcilable discrepancy between the present data and that previously reported for the starch from amylo maize pollen by Vineyard et al (1958) and Zuber et al (1960). It has been shown in Section 3.3 that interfering substances, such as protein and lipid, seriously depress the iodine binding capacity of starch. Because pollen is especially rich in protein and nucleic acids, it would not be surprising that a rigorous purification of the starch is required if low and erroneous values of the iodine-binding capacity are to be avoided.

Conclusions

It is concluded that despite the fact that a much wider range of genotypes and determinations would be required to determine a statistical correlation, the starches from the pollen of amylo maize differ from those of the endosperm in degree, rather than in type. The differences in iodine-binding behaviour shown in Figures 4.8 and 4.9 almost certainly reflect differences in the distribution of anomalous material present in both starches. This type of material, characteristic of amylo maize, is discussed in detail in Section 4.3.

Although the starch-purification stage of this analysis is both time-consuming and tedious, this does provide a method of examining the
gametophytic generation of the amylo maize plant, and it is not inconceivable that this property could, as in the case of waxy maize, be utilized to identify heterozygotes without resort to the traditional methods of selfing or back-crossing to a homozygous parent.
SECTION FIVE

THE BIOSYNTHESIS

OF STARCH
Section 5.1
The Biosynthesis of Starch

Introduction

In recent years, a number of detailed reviews have appeared by Manners (1968), Geddes (1969) and Badenhuizen (1969) dealing with the biogenesis of starch. These reviews examine in detail the enzymic systems which have been detected at the site of starch synthesis, the nature of the starch granule, and changes in granule morphology that occur during maturation. It is apparent, however, that no satisfactory theory for starch biosynthesis in vivo exists, and the major outstanding problems are outlined in this section.

The mode of biosynthesis of starch has been attacked on three fronts. Botanists seem to have concentrated on the morphology of starch granules and their relation to other components in the starch-synthesizing systems. They have established that starch is laid down in specialized organelles called plastids. Whilst it is said that all plastids accumulate starch at some stage of their life cycle, in specialized cases, for example in chloroplasts, starch deposition is transient. Plastids in which reserve starch accumulates are generally known as amyloplasts, and the stroma or ground-substance of these amyloplasts is suggested to contain localized concentrations of starch-synthesizing enzymes and complex membrane-structures, the necessary biosynthetic material for starch formation.

Biochemists have examined this material and have established that several enzymes capable of metabolizing starch are present. Three enzymes have been detected which synthesise α-1:4- bonds.

(1) Phosphorylase can act in both degradative and synthetic modes, and catalyzes the reaction:
Glucose 1-phosphate + (glucose)_n → (glucose)_{n+1} + inorganic phosphate,
where (glucose)_n represents a glucose oligomer with n ≥ 4 units.
(2) The **amylose synthetases** catalyze the incorporation of glucose into linear chains of amylose, amylopectin, or low molecular-weight oligomer substrates. The reaction may be represented as:

\[
\text{UDP- or ADP- glucose + (primer)}_n \rightarrow \text{UDP or ADP + (primer)}_{n+1}
\]

(3) **D-enzyme** isolated from potatoes catalyzes disproportionation reactions between oligosaccharides. The reaction for example on maltotriose is:

\[
\text{Maltotriose} \leftrightarrow \text{maltopentaose} + \text{glucose}.
\]

If glucose is removed, as for instance by specific conversion to the phosphate by enzymic means, the reaction may be forced to the right, and amylose with a degree of polymerisation ~50 units has been made in this way. The principal role of this reaction is, however, more likely to be the production of low molecular-weight oligomers.

Only one branching-enzyme is at present known to be important in starch synthesis. This enzyme, known as **Q-enzyme**, catalyzes a transglucosylation in which an \(\alpha-1:4\)-bond is hydrolyzed in a donor molecule, and the liberated fragment is linked to an acceptor molecule by an \(\alpha-1:6\)-bond. It has been shown that the donor substrate, if linear, should be at least 40 units long, and that suitable branched molecules should have outer chains of at least 14 units.

It is generally accepted that Q-enzyme may act in conjunction with a chain-extending enzyme to form the branched macromolecule, amylopectin. The physical properties of amylopectin almost certainly reflect the mode of its synthesis, but, as pointed out in Section 2, until the amylopectin structure is clarified the exact role of these enzymes must remain the subject of speculation.

Chemists have played their part, in the investigation of starch biosynthesis by devizing suitable methods of fractionation and characterization of starch. These techniques have been discussed in Section 2, and show that starch normally has at least two quite-distinct, polymer species. The characteristics of these fractions were discussed in detail in Section 2, but for the sake of coherence...
will be summarized here. The major starch component comprising ca 75% of the starch molecule is branched, some 4-5% of the molecule being involved in branch points. This component has molecular weights ranging from a minimum of ca $5 \times 10^5$ to $5 \times 10^8$, the upper accessible limit of molecular weight measurement. These molecular weights have been shown to be true values, for no difference could be detected when they were measured in disaggregating solvents (Banks et al (1972)). The minor component, amylose, comprising some 25% of the starch, is essentially linear and ranges in molecular weight from ca $10^5$ to $5 \times 10^6$.

The problem now remains of devizing a suitable model-system to describe the formation of the molecular components of starch and their accretion in the granule. This section of thesis describes a series of experiments which attempt to elucidate some of these outstanding problems. The first part of this section investigates the molecular properties of barley starch during growth. The following part describes the changes occurring in granule morphology concurrent with these changes in molecular properties, and the final part of this section proposes a hypothesis for biosynthesis consistent with the experimentally-observed results.
Section 5.2

The Properties of Starch Components in Barley During Growth

It is well-established that:

1. Normal starches contain a heterogeneous mixture of both branched and linear material. The fractionation techniques described in Section 2 allow isolation of these widely-different components from native starch, and the methods proposed in Section 3 allow measurement of the relative proportions of these components in the starch granule.

2. As the site of reserve-starch synthesis matures, the starch deposited alters in properties. It has been shown that, in wheat (Bisco et al. 1945), maize (Wolf et al. 1948; Erlander 1960), barley (Harris and MacWilliam 1958), tobacco leaves (Matheson and Wheatley 1962), peas (Greenwood and Thomson 1962b), and potato tubers (Geddes et al. 1965), the amount of iodine bound by the starches increases dramatically as growth proceeds.

These observations are usually interpreted to show that the relative amount of the essentially-linear component, amylose, increases as the site of starch deposition matures, and the amount of reserve starch increases. The fraction of amylose is calculated from the ratio of iodine-binding capacity of the starch to that of the pure amylose component, and a value of 19-20 mg iodine bound per 100 mg polysaccharide is assumed for the latter. Banks et al. (1971a) have shown that, as chain-length or the degree of branching of an amylose varies, concomitant changes occur in the iodine-binding characteristics of the amylose. The suggestions of Matheson (1971), and the observations of Wood (1960) and Briones et al. (1968), that the amylose component varies in its nature with maturity, then assumes some significance.

The present work was undertaken to attempt to elucidate, the mechanism by which the components of starch are simultaneously formed, and to investigate the cause of the apparent increase in iodine-binding.
power of the starch granule on maturation. A unique opportunity to do this arose when a series of samples of two barley cultivars of the same variety and differing only in starch-type were made available by Dr. J.T. Walker. Merritt and Walker (1969) reported that they found, in the six-rowed, semi-smooth-awned, spring variety Glacier, two distinct genotypes. Banks et al (1971b) confirmed this observation, and showed that Glacier (CI 9676) had a typically-normal starch with an apparent amylose-content of 28%, whilst Glacier (AC 38) had a starch with an apparent amylose-content ca 42%.

Seeds of these varieties were grown in the field in 1969, and samples of grain were collected as a function of the time from anthesis (i.e. fertilization). Two comparable series of barley grain were thus obtained which differed only in their starch. These starches were then examined in detail.

Experimental Methods

Ears from CI 9676 and AC 38 were dated at anthesis, which occurred in the period ca 7th July 1969. Samples of the required number of ears were collected on the appropriate data after anthesis. Within one hour of harvesting, the grain was removed from the ear, and the awns and pales discarded. The remainder of the grain consisting of the pericarp, testa, germ, and endosperm was weighed and immersed in 0.01M mercuric chloride to inhibit enzymic attack and prevent bacterial growth. Since each inflorescence on the same ear anthesed at different times, over a period of two to three days, the average date of anthesis was recorded.

The starches were extracted, purified, fractionated, and characterized by the techniques detailed in Sections 2 and 3 of this thesis.

Results

The average weight of grain per ear and the starch content as a percentage of the total dry matter are shown for CI 9676 and AC 38 in Figures 5.1(a) and 5.1(b), respectively. In both cases, the weight
FIGURE 5.1A Cl. 9676

DAYS FROM ANTHESIS

DAYS FROM ANTHESIS
FIGURE 5.1B  AC 38

**Top Diagram:**
- **Y-axis:** DRY WEIGHT PER EAR [G]
- **X-axis:** DAYS FROM ANTHEsis
- Data points showing an increasing trend in dry weight over days from anthesis.

**Bottom Diagram:**
- **Y-axis:** STARCH % DRY MATTER
- **X-axis:** DAYS FROM ANTHEsis
- Data points showing an increasing trend in starch percentage over days from anthesis, followed by a plateau.

134.
of grain per ear increases over the period of 5 to 45 days after
anthesis. The starch deposition appears to occur principally between
8 and 30 days after anthesis. During this period, (a) starch is laid
down at a faster rate than other material, and (b) the major changes
in starch character occur. It is of interest to note that in AC 38
the final starch content at maturity is 6% lower than that of CI 9676.
Greenwood and Thomson (1962b) noted that varieties of pea with apparent
amylose-contents of 37% and 69% differed in starch content in a similar
manner, although in their case the difference was almost 30%.

The apparent amylose-contents of both series of starches are
present as a function of maturity in Table 5.1. Employing the semi-
micro, differential, potentiometric technique described in Section 3.3,
the apparent amylose-contents were found to be constant, within experi-
mental error, when measured at 20°C and 2°C. This test, suggested by
Banks et al (1971b), shows that in neither starch is there an appreciable
quantity of material other than 'normal' amylose or amylopectin.

In both varieties, starches isolated from AC 38 and from CI 9676,
the apparent amylose-content increases with maturity, despite the large
difference in absolute values. This similarity in their behaviour
suggests some common mechanism being involved in the synthesis of starch,
or in the deposition of the starch granule.

The properties of the components isolated from the starches of
CI 9676 are shown in Tables 5.2(a) and 5.2(b). The amylose fractions
were all free from amylopectin as they were completely converted to
maltose by the concurrent action of β-amylase and α-amylase. The extent
of conversion to maltose by crystalline β-amylase differed markedly as
the starch matured. This decrease in β-amylolysis limit reflects an
increase in long-chain branching of the molecule (Banks and Greenwood
(1959b)). Contrary to the results of Wood (1960) on the development of
wheat starch, and Briones et al (1968) on the development of rice starch,
Table 5.1

The Apparent Amylose-Contents of the Starches from CI 9676 and AC 38 as a Function of Maturity.

<table>
<thead>
<tr>
<th>Maturity&lt;sup&gt;1)&lt;/sup&gt;</th>
<th>CI 9676</th>
<th>AC 38</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>15.8</td>
<td>n.d.</td>
</tr>
<tr>
<td>14</td>
<td>19.1</td>
<td>22.5</td>
</tr>
<tr>
<td>20</td>
<td>23.2</td>
<td>28.6</td>
</tr>
<tr>
<td>27</td>
<td>26.2</td>
<td>36.4</td>
</tr>
<tr>
<td>32</td>
<td>29.2</td>
<td>43.4</td>
</tr>
<tr>
<td>46</td>
<td>28.3</td>
<td>45.0</td>
</tr>
</tbody>
</table>

<sup>1)</sup> Number of days from the average date of anthesis.

<sup>2)</sup> From iodine-affinity at 20.4°C, as described in text.

Table 5.2(a)
The Properties of the Amyloses from the Starches of CI 9676

<table>
<thead>
<tr>
<th>Sample</th>
<th>Maturity</th>
<th>$\beta$</th>
<th>$\beta+Z$</th>
<th>Iodine Affinity ($%$)</th>
<th>$\eta$ (ml/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>14</td>
<td>100</td>
<td>101</td>
<td>19.4</td>
<td>215</td>
</tr>
<tr>
<td>3</td>
<td>20</td>
<td>100</td>
<td>99</td>
<td>19.5</td>
<td>248</td>
</tr>
<tr>
<td>4</td>
<td>27</td>
<td>97</td>
<td>100</td>
<td>19.6</td>
<td>268</td>
</tr>
<tr>
<td>5</td>
<td>32</td>
<td>91</td>
<td>101</td>
<td>19.3</td>
<td>403</td>
</tr>
<tr>
<td>6</td>
<td>46</td>
<td>83</td>
<td>101</td>
<td>19.6</td>
<td>391</td>
</tr>
</tbody>
</table>

1) No. of days from average date of anthesis.
2) % conversion to maltose by action of crystalline $\beta$-amylase
3) % conversion to maltose by concurrent action of crystalline $\beta$-amylase and Z-enzyme
4) Iodine affinity in mg iodine bound per 100 mg polysaccharide at 20.4$^\circ$C
5) Limiting viscosity number in 0.15M KOH
The Properties of the Amylopectin from the Starches of CI 9676

<table>
<thead>
<tr>
<th>Sample</th>
<th>Maturity</th>
<th>{β}</th>
<th>(β+P)$_{14}$</th>
<th>η</th>
<th>L \times 10^6</th>
<th>IA</th>
<th>Int CL</th>
<th>Ext CL</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>14</td>
<td>57</td>
<td>102</td>
<td>148</td>
<td>50</td>
<td>0.69</td>
<td>20.2</td>
<td>6.2</td>
</tr>
<tr>
<td>3</td>
<td>20</td>
<td>58</td>
<td>101</td>
<td>141</td>
<td>100</td>
<td>0.99</td>
<td>21.5</td>
<td>6.5</td>
</tr>
<tr>
<td>4</td>
<td>27</td>
<td>56</td>
<td>99</td>
<td>152</td>
<td>250</td>
<td>1.10</td>
<td>20.2</td>
<td>6.4</td>
</tr>
<tr>
<td>5</td>
<td>32</td>
<td>56</td>
<td>100</td>
<td>143</td>
<td>250</td>
<td>1.06</td>
<td>20.7</td>
<td>6.6</td>
</tr>
<tr>
<td>6</td>
<td>46</td>
<td>57</td>
<td>101</td>
<td>141</td>
<td>250</td>
<td>0.42</td>
<td>20.8</td>
<td>6.6</td>
</tr>
</tbody>
</table>

1) As in Table 5.2(a)

2) % conversion into maltose under the concurrent action of β-amylase and pullulanase

3) Limiting viscosity number in methyl-sulphoxide (DMSO)

4) Weight average molecular weight from light scattering

5) Average length of unit chain, i.e. the no. of glucose residues per non-reducing chain-end

6) Internal chain-length; calculated from: \( \text{Int CL} = \overline{CL} - \text{Ext CL} \)

7) External chain-length; calculated from: \( \text{Ext CL} = (\overline{CL}) \times \{β\} + 2.5 \)

138.
the iodine-binding capacities of the amyloses do not vary with maturity. In the light of the supporting evidence presented here that the amyloses are of high purity, it seems likely that the previous observations that amylose iodine-binding characteristics are a function of maturity are in error, and are due to incomplete separation of the branched fraction. This work also provides conclusive evidence that the observed increase in iodine-affinity of the total starches is a true reflection of the increase in amylose-contents. The limiting viscosity number of the amylose fractions increased considerably with maturity, almost doubling in the period under examination. This viscosity measurement was reproduced within 3% on a duplicate fractionation of a mature starch, and in consequence a fair degree of confidence may be placed in this observed increase in molecular size.

The amylopectins isolated from CI 9676 have the properties shown in Table 5.2(b). The iodine affinities show that these fractions have a maximum impurity of 5% linear material, assuming that the amylopectin itself makes no contribution to iodine-binding. It is apparent, therefore, that the branched fractions are adequately pure. The extent of conversion to maltose by crystalline β-amylase is constant within experimental limits, and the efficiency of this measurement may be judged by the complete conversion to maltose by the concurrent action of β-amylase and pullulanase. This is in contrast to the findings of Geddes et al (1965) who found that the β-amylolysis limit of potato amylopectin decreased with increasing maturity. On the other hand Thomson (1961) had reported earlier that the β-amylolysis limit of potato amylopectin increased with increasing maturity. Also in contrast to the findings of Geddes et al (1965) and Thomson (1961), the amylopectins isolated from CI 9676 were found to have no variation in chain length, within experimental error. The average internal and external chain lengths were calculated and found to be 6.5 and 14.2 respectively. These values
agree well with those of Banks et al (1971b) for the mature starch. The molecular-weights of the amylopectins increased five-fold during the period under examination, and paralleled the increase in molecular size of their respective amylose counterparts. The limiting viscosity numbers of the amylopectins did not vary significantly, but this merely reflects the insensitivity of viscosity measurements when applied to branched molecules of very-high molecular-weight.

Table 5.3(a) and 5.3(b) present the comparable data for the starches isolated from AC 38. Here it is surprising to find that, despite the obvious disparity in granular properties and amylose-content of the starches from CI 9676 and AC 38, a strikingly similar pattern emerges. For the barley of high amylose-content, the major changes are (1) a dramatic increase in the size of the molecular components and (2) a decrease in the extent of conversion of the amylose to maltose by the action of crystalline $\beta$-amylase. This pattern of growth is identical to that of CI 9676, despite the significant difference in the properties of the branched fraction. In agreement with Banks et al (1971b) the average lengths of unit chain of the amylopectin fractions from AC 38 were found to be higher than those of the branched fractions from CI 9676.

Conclusions

This work has demonstrated that the amylose content of barley starch is a function of maturity. It has also shown that, although the molecular components of starches from AC 38 and CI 9676 are quite dissimilar in character, the pattern in which starch is laid down is remarkably alike in both genotypes. Although these barley starches are laid down in a manner quite unlike that of potato tuber starch or pea starch, there are again quite remarkable analogies. It is clear that for a particular genotype, or even cultivar, the properties of the starch during growth are a function of maturity. The significance of this finding and of some of the other observations in this section is discussed in Part 4 of this section.
Table 5.3(a)

The Properties of the Amyloses from the Starches of AC 38

<table>
<thead>
<tr>
<th>Sample</th>
<th>Maturity</th>
<th>1) (β)</th>
<th>1) (β+Z)</th>
<th>Iodine 1) Affinity</th>
<th>1) (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>14</td>
<td>100</td>
<td>98</td>
<td>20.1</td>
<td>n.d.</td>
</tr>
<tr>
<td>3</td>
<td>20</td>
<td>98</td>
<td>101</td>
<td>19.2</td>
<td>200</td>
</tr>
<tr>
<td>4</td>
<td>27</td>
<td>95</td>
<td>100</td>
<td>20.0</td>
<td>242</td>
</tr>
<tr>
<td>5</td>
<td>32</td>
<td>90</td>
<td>100</td>
<td>19.5</td>
<td>n.d.</td>
</tr>
<tr>
<td>7</td>
<td>46</td>
<td>92</td>
<td>99</td>
<td>n.d.</td>
<td>370</td>
</tr>
</tbody>
</table>

1) As in Table 5.2(a)

2) n.d. = not determined
Table 5.3(b)

The Properties of the Amylopectin from the Starches of AC 38

<table>
<thead>
<tr>
<th>Sample</th>
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1) As in Table 5.2(a)

2) As in Table 5.2(b)
Section 5.3

The Properties of the Starch Granules During Growth

Introduction

In the preceding part of this section, it has been demonstrated that the properties of the components of two barley starches, widely different in type, exhibit very similar changes as the plant matures. This section describes the characteristics of the starch granules during the same period, and attempts to correlate these two sets of properties.

Experimental Methods

The starches used were those isolated from the barley varieties AC 38 and CI 9676, and used for the work detailed in Section 5.2. At no time had these starches been subjected to chemical treatments or temperatures in excess of 25°C, and in all cases the starches had never been dried.

Microscopic measurements of barley starches are complicated by the irregularity and diversity of shape of the granules. The granules in both cases were approximated by oblate ellipsoids having a major axis twice that of the minor axis. It was assumed therefore that the density of all the starch granules was equal, and then by use of the relation:

\[ \text{weight} = (\text{density}) \times (\text{volume}) \]

where

\[ V = \frac{2}{3} \pi x^3, \]

and \( V \) = volume of the starch granule,

\( a \) = size of major axis of the granule,

the weight of the granule was estimated. All curves were normalized by expressing the number and weight of the granules within a size-range as a percentage of the total.

To ensure that a representative sample was obtained at least 2000 granules were measured from each group, and photomicrographs of at least four different fields, of the same sample, containing at least

143.
500 granules each, were taken. Measurements of granule size from individual fields usually agreed within $\pm 5\%$, and in no instance was the deviation greater than $\pm 10\%$. The sampling and photographic conditions were held constant throughout the series of experiments, and although the absolute values of granule size may be subject to a limited error, the relative sizes are directly comparable for both series of measurement.

**Results**

The curves of frequency-distribution and weight-distribution as a function of granule size are shown in Figures 5.2 and 5.3 for CI 9676 and AC 38 respectively. These data, when combined with those in Figure 5.1, yields the curves shown in Figures 5.4 for the development of starch granules within low, middle and upper size classes. The weight of starch granules within a size range was chosen as the important property of the total starch, for this parameter, not the number within a size-range, is directly related to the major properties - swelling-behaviour, amylose-content of the starch.

Quite striking differences appear between the two sets of curves - in the case of CI 9676 progressively larger granules appear as the plant matures, whilst in contrast, small granules appear to be the major population throughout maturation of AC 38, and relatively more are produced as the plant develops. The results for CI 9676 follow broadly the findings of Hay and Buttrose (1959) and MacGregor et al (1971) for other normal barley starches, but are in contrast to the findings of Herritt and Walker (1969) for starch from the same barley variety. As shown in Figure 5.2, a binodal distribution of granule sizes becomes apparent for CI 9676 at around 27 days from anthesis. Two types of granules, confusingly named "A type", i.e. those granules over 15 microns, and "B type", i.e. those granules less than 10 microns, form quite distinct groups. In mature starch,
FIGURE 5.2B  CI 9676

WEIGHT-SIZE DISTRIBUTION

14 DAYS

20 DAYS

27 DAYS

32 DAYS

46 DAYS

146.
FIGURE 5.3A  AC 38

NUMBER - SIZE DISTRIBUTION

14 DAYS

32 DAYS

20 DAYS

46 DAYS

147.
FIGURE 5.3B  AC 38

WEIGHT - SIZE DISTRIBUTION

14 DAYS

20 DAYS

32 DAYS

46 DAYS

148.
Figure 5.4

Grams starch per ear vs. days from anthesis for two wheat cultivars: CI 9676 and AC 38. The graph shows the starch content at different stages of growth, categorized by size classes (0-10 μ, 10-20 μ, >20 μ, 10-15 μ, 15-20 μ) for each cultivar. The data is presented for different time periods ranging from 0 to 40 days from anthesis.
the "B-type" granules form over 80% of the starch by number, but under 5% by weight. May and Buttrose (1959) proposed that the "A-type" granules are formed by successive apposition of starch material on a small number of nuclei, and at about 14 days after anthesis, "B-type" granules are initiated and form in vesicles between the, by then large, "A-type" granules. The work of MacGregor et al (1971) substantiates the appearance of a secondary population, which in their case occurs at 21 days from anthesis. In this present work, no samples were analysed in detail before 14 days from anthesis, because up to this date, considerable quantities of pericarp starch derived from maternal tissue are present in samples of the whole grain. The limited quantity of material available precluded dissection of the grain to yield well-characterized fractions. Notwithstanding this limitation, the results in Figure 5.4 do suggest that, after 14 days from anthesis, there is little overall change in the number of "B-type" granules, and that the large granule population increases at the expense of granules in the intermediate ranges. This would be consistent with a mechanism in which no new granules in the "A-type" population were born and those already present grew by apposition.

The starch of high amylose-content from AC 38 shows a completely different pattern, for the mature starch appears to have only a single distribution of granule sizes (see Figure 5.3) and the average granule size is very much less throughout the growth period. Small starch granules play a dominant role, as they appear to increase in relative numbers after the larger granules have ceased to grow. Although the results presented by Merritt and Walker (1969) for AC 38 are erratic and somewhat haphazard, they do record a relatively large influx of small granules at the later stages of growth. The dominance of small granules in the populations of AC 38 is well exemplified by Plates 5.1 - 5.5, where scanning-electron micrographs (SEM) of samples of starch with increasing maturity are presented. Plates 5.6, of CI 9676 at maturity, is provided
PLATE 5.1 Starch from AC38, 9 days after anthesis when pericarp starch is still prevalent. Scale 1 cm = 13.5 microns.

PLATE 5.2 Starch from AC38, 14 days after anthesis; predominantly endosperm starch. Scale 1 cm = 13.5.
PLATE 5.3  Starch from AC38, 27 days after anthesis; granules are fairly large on average. Scale 1 cm = 13.5 microns.

PLATE 5.4  Starch from AC38, 32 days after anthesis; note influx of smaller granules. Scale 1 cm = 13.5 microns.
PLATE 5.5. Mature starch from AC38 with many small granules, 46 days after anthesis. Scale 1 cm = 13.5 microns.

PLATE 5.6. Mature starch from CI 9676, 46 days after anthesis with typical large and small types of granules. Scale 1 cm = 13.5 microns.
for comparison purposes, and shows the typical bi-nodal granule population.

Microscopic examination of the starches from CI 9676 and AC 38 did not reveal the major changes in granule morphology exhibited by wheat starch during growth, e.g. Sandstedt (1946) and latterly Evers (1971). In agreement with Badenhuizen (1969), many irregular- and kidney-shaped granules were observed at early stages of growth of CI 9676 - see Plate 5.7(a) and (b) - and were not significantly in evidence at maturity - Plate 5.8. Caution must, however, be exercised in interpreting the relative scarcity of kidney shaped granules in the mature starch, since there are ca 10 times as many granules in this size class at maturity compared to the period 14 days after anthesis, and consequently the relative abundance of this type is much less.

In AC 38 the starches are very little different in appearance at 14 days after anthesis and at maturity. Plates 5.9(a) and 5.9(b) show this point quite clearly for, although there is a factor of three in the magnification, the external appearance of the granules is very similar.

Summary

The growth patterns for the starches from CI 9676 and AC 38 are quite different, for, in normal barley the overall starch properties are controlled by the largest granules in the population, which increase in size with maturity, whilst in barley starch of high amylose-content, small granules assume the dominant role and become progressively more important as maturity approaches.
PLATE 5.7 (a) Micrograph of starch from CI 9676, 14 days from anthesis, kidney shaped granules are arrowed. Scale 1 cm = 16.5 microns.

PLATE 5.7 (b) S.E.M. of starch from CI 9676, 14 days after anthesis, with many unusual shaped granules. Scale 1 cm = 3.7 microns.
Light micrograph of starch from CI 9676 at maturity, 46 days after anthesis; the typical binodal distribution is seen with large "A" type granules and very much smaller "B" types. Scale 1 cm = 16.5 microns.
PLATE 5.9 (a) Starch from AC38, 14 days after anthesis. Scale 1 cm = 2.4 microns.

PLATE 5.9 (b) Starch from AC38, 46 days after anthesis. Scale 1 cm = 7.2 microns.
Section 5.4

Interpretations and Conclusions

The preceding Parts of this Section have shown that, as the barley plant matures, there are significant changes in the amylose-content and granule morphology of the endosperm starch. The relation between the amylose-content and the average size of the granules, calculated on a weight basis is shown in Figure 5.5, in which data, from the work of MacKenzie (1964), is also presented for comparison purposes. Although the starches from normal barley (CI 9676) and potato show a fairly linear increase in amylose-content with increasing granule size, in contrast, there is no significant correlation between these properties for starches of high amylose-content from AC 38. Before any attempt may be made to interpret these observations, it is desirable to examine in some detail the mechanism by which both amylose and amylopectin are formed simultaneously, and the way in which these polymers are subsequently deposited within the starch granule.

Three model systems have been proposed for the simultaneous formation of linear and branched material. Whelan (1963) has proposed a system whereby amylose and amylopectin are synthesized independently within the plastid. Amylose synthetase is localized on one side of a membrane, whilst Q-enzyme and amylose synthetase occur together on the other side. The membrane, for which amylopectin is proposed as a suitable material, allows penetration of sugar nucleotides only, and effectively separates compartments synthesizing individual components. The major fault in this postulate is that no experimental evidence for such a membrane with highly selective properties exists, but in view of the ready permeability of the whole starch granule to small molecules, e.g. NaIOn, it is possible that amylopectin performs this role in situ.

Geddes and Greenwood (1969) propose that linear material is formed prior to being branched by Q-enzyme. Saturation of Q-enzyme
by intense synthesis of linear material at the granular surface is then suggested to occur, and the amylose then precipitates before it may be branched. It is suggested that such a system may explain the increase in amylose-content of starch granules with maturity.

Erlander (1958) postulated a new debranching enzyme for starch synthesis from phytoglycogen, a material shown by Greenwood and Das Gupta (1958) to be similar in structure to highly-branched, mammalian muscle- or liver-glycogen. Phytoglycogen was proposed to act as the common precursor of both branched and linear material, since debranching yielded a less highly branched structure, amylopectin, and the linear material so liberated in this process was fused enzymically to form amylose. Although phytoglycogen is present at maturity in certain varieties of maize (Laywald et al. 1955), there is no evidence that it is normally present at the site of starch synthesis in other cultivars. This fact, and the absence of a detectable enzyme with suitable debranching properties, casts doubts on the validity of this hypothesis.

Of the above theories none are at all satisfactory, and only the scheme of Geddes and Greenwood (1969) is capable of any rational modification to account for the experimental observations of this Section. The problem of amylose formation may be solved if (a) branching activity is restricted, either by having fewer sites for branching than for chain extension, or sites where the efficiency of branching is low and (b) if a suitable mechanism for the isolation of linear material so formed is available.

This latter postulate stresses the inadequacy of the schemes for concurrent synthesis of amylose and amylopectin, for without this restraint no rational explanation may be advanced. As discussed in Part 1 of this Section, the branching enzyme is highly specific, but this specificity alone does not suffice to explain amylose formation in its presence. For example if the branching enzyme is able to accept
chains with a minimum unit length, then the lower limit of unit-chain length is easily defined. In practice, it is more difficult to conceive of the restriction on the upper limit for branching, which is unlikely to be specified by size alone, since amylose is converted to a branched molecule by Q-enzyme isolated from potatoes (Haworth et al. 1944).

To render the linear chains inaccessible to branching, upon reaching a certain size, two in vitro methods are possible - complex formation or retrogradation. Retrogradation is unlikely, for amylose is easily leached from granular starch, and as amylose readily forms complexes with protein and lipid a mechanism of the first type is more likely.

All native granular starches contain some tightly-bound lipid, released only after complete dispersion of the starch (see for example Section 3.3 of this work), while the same starches may be purified to an essentially protein-free state. Amylose-lipid complex formation is thus a possible method by which the molecule is rendered inaccessible to branching enzymes. Such a mechanism is unlikely to be of high efficiency, and this might explain why (1) some long chains are present in amylopectin, (2) some branch points occur in amylose, and (3) a small amount of material of intermediate character is present in all starches. It is interesting to note that while Q-enzyme will branch amylopectin with outer chains larger than 14 units (Larner 1953), it is slow to act on linear molecules with less than 40 units in the chain (Peat et al. 1953), about the level at which amylose starts to form inclusion complexes (Banks et al. 1971a). The following tentative scheme for starch biosynthesis is therefore proposed:

(1) In most starches, Q-enzyme is much less efficient than chain-extension mechanisms, and pools of linear material are so formed. Upon reaching DP ~40, the amylose becomes capable of forming inclusion
complexes with lipids, to become less accessible to Q-enzyme activity; and (2) since branched material becomes a substrate for Q-enzyme, when its outermost chains are about one third of the length of a correspondingly accessible linear chain, a system, once initiated as in (1) above, will tend to become self-perpetuating (provided that chain extension proceeds at equal rates in both linear and branched molecules).

The above scheme is not intended as a complete representation of the biosynthetic mechanism for starch, but is capable of extension to explain the changes observed in growth for all the starches examined to date. Since the branching enzyme specificity does not change during maturity - the amylpectins from CI 9676 and AC 38 do not change in nature with growth - a relative decrease in its activity would account for the observed increase in amyllose-content of the starches.

Geddes and Greenwood (1969) suggest that the enzymes in starch synthesis are regulated by being attached to the granule surface. At the time that work was presented, this postulate had two major advantages over other suggestions. Firstly it explained the change in amyllose-content of potato starch granules with size and secondly it allowed the formation of planar amylpectin molecules. This latter concept was advanced by Greenwood (1956) to explain the hydrodynamic behaviour of the polysaccharides, e.g. Banks et al (1972), and to rationalize the ability of a branched molecule to form crystalline structures within the starch granule. The experimental evidence in Figure 5.5, however, shows for AC 38 at least, that amyllose-content is a function in general of maturity rather than size. A simple modification of Greenwood's hypothesis, by localizing the starch-synthesizing enzymes on the amyloplast-membranes, allows the formation of amylpectin with a two-dimensional structure, and would explain the observed changes in starch during growth, if, as the amyloplast developed, the relative number of sites for branching decreased. This present
theory has certain advantages:

(1) Greenwood's hypothesis requires that as the molecules are synthesized, they are laid down tangentially to the granular surface - a condition which seems to be unlikely by evidence presented later [Frey-Wyssling (1940)] and

(2) if the starch-synthesizing enzymes are on the granule surface then, as the granule increases in size, the enzymes must recognize this property to vary the amylose-amylopectin ratio. In contrast, if the starch-synthesizing enzymes are localized on the plastid membrane surface, then the plant itself controls the regulatory mechanisms, but it is also necessary to postulate that starch granules are formed in two distinct and separate stages, i.e. (1) amylose and amylopectin material are synthesized by processes outlined above, and (2) subsequently these polymers crystallize from a heterogeneous solution to form the starch granule.

The observed changes in granule properties with maturity are compatible with this hypothesis, but two quite different alternatives are possible:

(1) as the plant matures, the amyloplasts change in size and in their relative contents of branching enzyme or,

(2) during development, new amyloplasts are born with, inherent in them, relatively fewer sites capable of branching.

The evidence presented in Figure 5.5 favours the second alternative as a general case, for by this means it is possible to have a completely different type of granule formed as the plant matures and all the starches in Figure 5.5 could be accommodated within one scheme. This point will be discussed further later in this Section, but before doing so, it is desirable to examine what is known about the detailed structure of the starch granule and its mode of formation.
The organization of molecules within the starch granule may be examined on two levels:

(1) on a molecular level, x-ray measurements, e.g. Katz (1937), reveal a complex diffraction pattern, which has been interpreted to show that the molecules within the starch granule exist in a partially crystalline form, as a number of different helical configurations (for a review of this topic see Sterling (1968)). Various types of x-ray diffraction pattern are observed, but there appears to be little correlation between such patterns and other observed granular properties.

(2) on a supra-molecular level, starch granules are optically anisotropic and display a marked birefringence pattern (see Plate 5.10b). This type of pattern has been interpreted (Frey-Wyssling (1940)) to show that the optical axes with higher refractive indices are arranged in a radial fashion. Caution must be exercised in interpretation of this phenomenon, for although the major axis of the polymer and the major optical axis coincide when the molecule is in an extended form, it is less easy to predict the relation between overall molecular orientation and optical symmetry if the polymer is in a helical state - as in the crystalline areas of the starch granule.

Despite the lack of precise knowledge of the granular structure of starch, the granule has many similarities to a polymer spherulite (Geddes and Greenwood (1969), but although there is much information available on the nature of polymer spherulites, little attempt has been hitherto made to relate this data to that on starch granules. On consideration of the extensive review articles by Price (1959), Lindenmeyer (1963), Handalkern (1964) and Keith (1963) the following concensus opinion on polymer spherulite appears - when the polymer crystallizes it does so from primary nuclei; as the process of crystallization proceeds, progressive layers of material are deposited
on these nuclei, imperfections or defects being excluded and pushed outwards into the melt or solution. Often defects or dislocations change the orientation of the crystal, and cause branching or twinning. Where such defects occur frequently, the mode of crystallite extension is dominated by branching and a radial skeleton quickly develops with large amounts of imperfect material, rejected from the crystalline fibrils, incorporated in the overall structure.

It is at once apparent that analogies to this form of structure exist in the starch granule. Amylopectin is the basic structural component of starches - 'waxy' starches containing no amylose show identical birefringence (Baker and Whelan (1950)) and x-ray diffraction patterns to their 'normal' counterparts - and the branch-points may provide the defects necessary for frequent branching of the growing crystallites necessary for spherulitic growth. Radiating fibrils of material, resistant to fracture by mechanical manipulation, have been observed in potato starch by Sterling (1971) using scanning electron microscopy, though this work gives no indication of the nature or orientation of the molecules within the fibrils. A model for starch granules of radiating, para-crystalline fibrils of amylopectin, the interstices of which contain predominantly amylose, is consistent with many of the experimental observations on starch and conforms to the typical spherulite structure. This model is substantiated by the gentle leaching experiments of Banks (1960), where amylose is preferentially leached from potato starch at its gelatinization temperature, and by the work of Cowie and Greenwood (1957), and by the data presented in Section 4 of this thesis, where extensive leaching of granular starch at elevated temperatures leaves a residual starch structure rich in the branched component. Several methods have been suggested by which starch granules may be derived.
from concentrated solutions of the polymeric components, and a
close examination of these is necessary before any conclusion may
be made regarding starch structure and changes during growth.
Nucleation is generally agreed to occur from coacervates -
coacervation is a process in which phase separation occurs in
hydrophilic sols to yield colloid rich droplets (the coacervate)
in a medium replete of co-lloid. The polymer within the coacervate
droplet crystallizes to form embryonic starch granules (Lackiasters
et al (1946) and Erlander (1961)). Subsequent growth is possible
by a number of different processes :-
(1) by the successive apposition of layers of material in con-
centric shells,
(2) by progressive crystallization from a nucleus outwards, i.e.
on extended form of the process invoked for initial nucleation or,
(3) by growth inwards from a shell, a process known as intussusception.
The third type of mechanism is ruled out as the general case,
because radio-chemical experiments by Badenhuizen and Dutton (1956)
and Yoshida et al (1958) indicate that starch granules grow from nuclei
outwards. Of the other modes of granule formation, growth by
successive apposition of layers is generally favoured, e.g. Badenhuizen
The only major argument in favour of growth by apposition of successive
layers of starch seems to be the occurrence of concentric rings or
lamellae in some starch granules. May and Buttrose (1959) and
Buttrose (1960) associate such rings in wheat starch with diurnal
variations in growth. They showed that wheat starch isolated from
plants grown under constant illumination showed no ring structure,
whilst starch from plants of the same variety grown in natural day-
night regimes showed definite lamellae. Latterly Evers (unpublished
experiments, 1972) has confirmed these observations, but has also shown
that major differences in grain morphology occur e.g. in protein content. Other points are that (1) the ring structure does not become apparent by light-microscopy until the starch is treated either with amylolytic enzymes or by concentrated acid; (2) potato starch, which exhibits a definite ring structure without chemical modification (see Plate 5.10a) does so whether or not the plant is exposed to continuous illumination; and (3) the ring structure produced after chemical treatment of wheat starch is concentric, a fact difficult to reconcile with the proposed growth stages of Sandstedt (1946) and Evers (1971). By the apposition theories of these authors, one would expect considerable irregularity in the lamellae corresponding to early stages of growth.

The anomalies in a general hypothesis of appositive growth are however overshadowed by one major objection - the occurrence of compound starch granules.

A compound starch granule is one which is characterized by several birefringence nuclei when examined by crossed polars. Two examples with relatively high numbers of compound starch granules are examined in this work - potato and wrinkle-seeded pea starch, though compound granules have been observed in very limited numbers in wheat starch (Gough 1972) and maize starch.

Although potato starch is not usually thought to have compound granules, some photomicrographs in the literature clearly show their existence (Seidermann (1966) and Schoch (1961)]. A wide survey of potatoes revealed a source rich in compound starch granules in a potato tuber var. Pentland Dell. Plates 5.10(a) and 5.11(a) show simple granules with distinct rings structures and compound granules from the same starch sample. It is emphasized that these starch granules are derived from one tuber. Note the appearance of the triplet granule, where there are three distinct nuclei when viewed by crossed polars - each nucleus
PLATE 5.10 (a)  Light micrograph of typical potato starch granules with rings. Scale 1 cm = 10 microns.

PLATE 5.10 (b)  Same field as Plate 5.10 (a) viewed between crossed polars, demonstrating typical birefringence patterns. Scale 1 cm = 10 microns.
PLATE 5.11 (a) Micrograph of potato starch from same sample as in Plate 5.10 (a), showing compound granules. Faint rings are discernible within the segments of the triplet granule. Scale 1 cm = 9.5 microns.

PLATE 5.11 (b) Field of Plate 5.11 (a) viewed between crossed polars demonstrating the multi-nucleate nature of the compound starch granules. Scale 1 cm = 9.5 microns.
is surrounded (see Plate 5.11(a)) by faint but discernible rings. It is quite inconceivable that this granule grew by apposition in the sense implied by May and Buttrose (1959).

The existence of compound granules in wrinkle-seeded pea starch is disputed by Badenhuizen (1969) who insists: "The impression of a compound granule is created because the mature starch is subdivided into segments by radial fissures. By definition, a compound starch granule is formed from several granules, originating in one amyloplast and growing together,..." Plates 5.12 and 5.13 show typical photo-micrographs of wrinkled-seeded pea starch, which may be interpreted in this way. However using scanning electron microscopy, Plate 5.14 of a general field, and Plates 5.15 to 5.19 show clearly that a wide range of compound granules do occur, with three to over seven clearly defined segments per granule. It is improbable that such granules could occur merely by shrinkage of a spherical granule under conditions of dehydration. Badenhuizen (1965) also states quite categorically that: "each (wrinkled-seeded pea) granule has one polarization cross only". Plate 5.20(a) shows the field from Plate 5.12 between crossed polars. It is difficult to see why each granule has only one polarization cross [compare with Plate 5.10(b)], though an unusually symmetrical granule with four segments in centre field could be described in this manner. The other granules with a larger number of segments most definitely do not have simple birefringence patterns. This point is best seen in Plate 5.20(b), where the field in 5.20(a) has been photographed with crossed polars and with an interference plate (Red I) at 45° angle to the polarization planes. It is unnecessary to examine the optical theory producing such patterns, for the effect is merely to enhance the clarity of birefringence patterns obtained without an interference plate.
PLATE 5.12  Light micrograph of compound starch granules from wrinkled seeded pea. Scale 1 cm = 10 microns.

PLATE 5.13  S.E.M. of starch granule from wrinkled seeded pea, with radial cracks. Scale 1 cm = 3.6 microns.
PLATE 5.14  S.E.M. of general field of starch from wrinkled-seeded pea, with an admixture of compound and simple granules. Scale 1 cm = 14 microns.

PLATE 5.15  S.E.M. of wrinkled-seeded pea starch granule with three distinct segments. Scale 1 cm = 1.8 microns.
PLATE 5.16  S.E.M. of wrinkled-seeded pea starch granule with four segments and central hole. Scale 1 cm = 3.6 microns.

PLATE 5.17  Five segmented granule of wrinkled-seeded pea starch. Scale 1 cm = 1.8 microns.
PLATE 5.18  S.E.M. of different types of compound starch granules from wrinkle-seeded pea. Scale 1 cm = 3.6 microns.

PLATE 5.19  Wrinkle-seeded pea starch granule with over eight segments and small spherical granule resting in central depression. Scale 1 cm = 3.6 microns.
PLATE 5.20 (a)  Granules of wrinkled-seeded pea starch from field of Plate 5.12 between crossed polars in light microscope. Scale 1 cm = 10 microns.

PLATE 5.20 (b)  Light micrograph of wrinkled seeded pea starch from field of Plate 5.20 (a), with crossed polars and Red 1 plate at 45°. Scale 1 cm = 10 microns.
The above work shows that compound starch granules do occur in some varieties of the pea and the potato. In both cases, growth of the granules by successive apposition on nuclei leads to conceptual difficulties, especially to accommodate the hole in the centre of compound pea-starch granules as shown in Plate 5.19. A more reasonable suggestion would be to consider spherulitic growth in one continuous step from several simultaneously initiated nuclei within a large coacervate droplet. Compound spherulites with sharp, linear boundaries would be formed as in the case of the typical polymer melt (see for example Sharples (1966)), and would have optical properties similar to those observed in Plates 5.11(b) and 5.20(a) and (b).

Ringed structures are common in synthetic-polymer spherulites, and have been explained in theories by Price (1959) and Keller (1959) not involving successive layers of material. Both authors interpret the ring phenomena as due to twisting of crystalline fibrils during growth of the spherulite.

The above evidence suggests that pea and potato starch granules may form by spherulitic crystallization in a continuous step, but it is rather more difficult to justify such a hypothesis in the cases of the barley starches examined earlier in this Section, and of the wheat starch examined by Evers (1971). Such a theory would, however, account for the predominance of different types of granule during growth, without invoking complex theories of successive apposition in a highly specific manner. It is also then simple to conceive of the dramatic changes in overall amylose-content of a starch during maturation without individual starch granules being required to display heterogeneity from the centre to the periphery – in accord with other observations of Badenhuizen (1956). Using the model of spherulitic growth, different granules could be laid down at different stages of growth, and hence, as all the starches shown
in Figure 5.5 demonstrate, the total starch properties could be a function of maturity. The idea of changes in the type of plastid formed is in agreement with the above evidence, and for that presented earlier with respect to the observed variations in amylose-content.
SECTION SIX

HYDROXYETHYL STARCH.

ITS STRUCTURE WITH

SPECIAL RELATION TO

ITS USE AS A BLOOD

PLASMA VOLUME EXPANDER
6.1 Introduction

The hydroxyethyl derivative of starch has been known since at least 1920 when German Patents were issued describing the reaction of alkaline-starch with ethylene oxide. In the past two decades, HES has assumed considerable commercial importance as a sizing agent in the textile and paper industries. The magnitude of its use may be judged from the production figures for 1964, when in the United States of America alone, some 45,000 tons were produced.

The last decade has seen an upsurge of interest in HES as a potential blood-plasma expander and cryoprotective agent for erythrocytes. The starch component amylopectin, bears a considerable resemblance in structure to the mammalian, reserve polysaccharide, glycogen. It is, however, highly susceptible to the action of serum amylases, and its intravascular persistence is so low as to provide no efficient therapeutic effect. The introduction of substituent groups into starch makes the material less susceptible to the action of enzymes. With this in mind, Wiedersheim (1957) tested HES as a potential plasma expander and found that it was relatively non-toxic.

This work was subsequently confirmed by Walton and co-workers, e.g. Thompson et al (1962, 1964a, 1964b, 1970) and Bogan et al (1969). These workers have expanded the work of Wiedersheim (1957) to show that HES produces no more deleterious effects in experimental subjects than the widely used bacterial polysaccharide, dextran. In addition, HES exhibits superior resistance to precipitation under extreme conditions designed to promote crystallization. (Shields et al (1965)). Granath et al (1969) have set forth some opposition to the claimed non-toxicity of HES though their work has been challenged by Brake (1970).

(cf Granath et al (1970)).
Knorpp et al (1967) have suggested that HES may be used as an extracellular agent to protect erythrocytes from freezing damage and subsequently Knorpp et al (1971) reported promising results for the use of HES in this role.

The present work deals with the chemistry, and physical chemistry of HES - subjects which have not been considered in detail by those dealing with the pharmacological use of the material. The next part of this section presents a review of the current literature.

6.2 A Review of the Current Literature Concerning Hydroxyethyl Starch

(a) Formula for HES

The basic repeat unit of starch has the structure:

\[
\begin{align*}
(5) & \quad \text{OH} \\
(4) & \quad \text{OH} \\
(3) & \quad \text{CH}_2 \text{OH} \\
(1) & \quad \text{O} \\
(2) & \quad \text{H} \\
(6) & \quad \text{C} \\
\end{align*}
\]

where the carbon atoms are numbered as shown. The reaction of ethylene oxide with starch may be represented as:

\[
\text{STARCH} + \text{CH}_2 = \text{CH}_2 \rightarrow \text{ROCH}_2\text{CH}_2\text{OH}
\]

It is at once apparent that this reaction may occur at three different sites in starch, on carbon atoms C(2), C(3) or C(6) or on any combination of these three sites. In addition, the newly generated hydroxyethyl group has a reactive hydroxyl group which may itself react:

\[
\text{ROCH}_2\text{CH}_2\text{OH} + \text{CH}_2 = \text{CH}_2 \rightarrow \text{ROCH}_2\text{OCH}_2\text{CH}_2\text{OH}
\]
to form side chains of poly-(ethylene oxide). This complex behaviour requires that a general formula for HES be given by:

\[
\text{HO-} \ (\text{CH}_2\text{CH}_2\text{O})_x \text{-OH}
\]

\[
\text{C} \quad \text{O} \quad \text{H}
\]

\[
\text{H} \quad \text{C} \quad \text{H}
\]

\[
\text{C} \quad \text{O}
\]

\[
\text{H}
\]

\[
x, y, z = 0, 1, 2 \ldots \infty
\]

It is necessary, not only to specify the fraction of each type of site on the polymer backbone which is substituted, but also to indicate the amount of polymeric substitution which occurs as side-chains. This is best done by assigning four separate rate constants \( k_2, k_3, k_6 \) and \( k_p \) to the substitution reactions at \( \text{C}(2), \text{C}(3), \text{C}(6) \) and the newly-introduced hydroxyl group \( \text{C}(p) \) and describing the final polymer structure in terms of these four rate constants.

If, following the principles of Spurlin (1939), it is assumed that the introduction of a substituent at any one of the three available hydroxyl groups in the glucose residue does not affect the reactivities of the remaining hydroxyls, and that the substitution at each of the three functionalities is kinetically of the first order, then

\[
S_2 = 1 - \exp (-k_2 t) \quad (1)
\]

\[
S_3 = 1 - \exp (-k_3 t) \quad (2)
\]

\[
S_6 = 1 - \exp (-k_6 t) \quad (3)
\]

where \( S_2, S_3 \) and \( S_6 \) are the fraction of hydroxyl groups substituted at positions \( \text{C}(2), \text{C}(3) \) and \( \text{C}(6) \) at time \( t \). (These relationships are derived in detail in part 3 of this section). The rate of formation of polymeric side-chains \( S_p \) is given by

\[
\frac{d S_p}{dt} = k_p (S_2 + S_3 + S_6) \quad (4)
\]

180.
where $k_p$ is the rate constant for the polymerisation reaction. The relative values of these four rate constants $k_2$, $k_3$, $k_6$ and $k_p$ govern the final product. If, for instance, $k_6 > k_2$, $k_3$ and $k_p$, then the resultants HES will, to a first approximation, consist of a series of anhydro-glucose residues substituted solely at C(6). On the other hand, if $k_p > k_2$, $k_3$ and $k_6$, the HES would have relatively few glucose units substituted, but each one that was would carry a long side-chain of poly-ethylene oxide.

There is, at present, no a priori method by which values may be assigned to the various rate constants. Rather, it is necessary to determine experimentally the substitution pattern at a given molar substitution and fit a suitable set of values for $k_2$, $k_3$, $k_6$ and $k_p$ to these results.

With such complexity, one parameter such as molar substitution ($\mathbf{MS}$), defined as the mole-fraction of hydroxyethyl groups per anhydro-glucose residue, is not sufficient to define the nature of the polymer.

Mathematically $\mathbf{MS} = \frac{\text{wt. % hydroxyethyl group}}{100 - \text{wt. % hydroxyethyl group}} \times \frac{162}{44}$

It has been customary to define degree of substitution as one third of $\mathbf{MS}$, since there are three potential sites per anhydro-glucose residue. In the case where mono-substitution is predominant, this definition is adequate, but, in the present case, this is no longer so. In this work, degree of substitution (DS) is defined as the fraction of anhydro-glucose residues substituted (in any way, at any of the three potentially-reactive sites). Both $\mathbf{MS}$ and the proportion of anhydro-glucose residues unsubstituted must be measured before DS can be evaluated. The DS may then be obtained from:

$$\text{DS} = 1 - \frac{\text{wt. of anhydro-glucose "free"}}{162 \times \text{wt. of polymer}} \times (1.0 - \mathbf{MS}) 162 + 206 \mathbf{MS}$$
These two characteristics, US and DS, are quite distinct and, in a reaction system where multiple substitution or side-chain formation occurs, together yield much valuable information. Since the object of this work was primarily to provide a suitable model for HES being used in biological systems, this particular definition of DS assumes some relevance. The major attribute of the hydroxyethyl group in these systems is to block enzymic attack and, since a glucose residue is unavailable for attack if substituted in any way the most important characteristic of the polymer is the fraction of unsubstituted anhydroglucose i.e. \(1 - DS\).

To amplify this, not hitherto appreciated, point two polymers with equivalent US and quite different DS may be formed. In one case, mono-substitution is prevalent and DS is close to US in value, whilst in the other polymeric side-chain formation occurs. Here DS is very much less than US. The polymer with the lower DS will be more susceptible to attack by amylolytic enzymes and will thus have different properties as a pharmacological agent.

(b) Preparation of HES

Although HES may be formed by reacting a solution of starch in alkali with ethylene oxide, most commercial processes carry out the reaction with the starch in its granular form. This is done by reacting starch with gaseous ethylene oxide under pressure. (Kesler and Hjermstad (1950a, 1950b)), often in the presence of catalysts such as inorganic salts (Kesler and Hjermstad (1950b)), inorganic alkalies (Kesler and Hjermstad (1950b, 1950c)) and certain organic bases (Kerr and Tancette (1956); Broderick (1954)). Granular swelling is restricted, by limiting the amount of water present in the reaction system (Kerr and Tancette (1956); Caldwell and Martin (1957)), by the addition of inorganic salts, or by
carrying out the reaction in certain alcoholic media (Broderick (1960); Kesler and Hjemstad (1958)). Mimii and Gokoyama (1970) pre-gelatinised starch, then reacted the material in pyridine, with 2-choro-ethanol, and a claimed high efficiency. 2-choro-ethanol was again used as hydroxyethylating agent by Gayer (1950), who specifically claimed formation of the uniformly 2-substituted HES by reaction in pyridine with the starch alkoxide. A graft co-polymer of ethylene oxide and starch has been produced by Tahan and Zilkha (1969a). Recently, Srivastava et al (1971) have used ethylene carbonate to hydroxyethylate starch and claim some advantages over homogeneous reaction in aqueous alkali with ethylene oxide.

With such a large variety of reaction conditions it would not be surprising if HES with different substitution patterns were formed. Indeed, Tahan and Zilkha (1969 a, b) have obtained a product in which \( k_2 > k_3 \) \( k_6 \) and which has chains of poly-(ethylene oxide) of some considerable length. This point seems to have been overlooked by those studying the structure of HES and will be dealt with later in this section.

(c) Measurement of Molar Substitution

The MS of a sample of HES is perhaps the single most important characteristic of the material. It is measured by cleavage of the substituent hydroxyethyl group by hydroiodic acid and subsequent estimation of the hydrolysis products. The method of Morgan (1946) and a subsequent modification by Lortz (1956) utilizes constant-boiling hydroiodic acid to cleave the ether linkage and liberate a mixture of ethyl iodide and ethylene which are estimated by volumetric techniques after reaction with alcoholic silver nitrate and bromine in acetic acid respectively.

Van der Bij (1967) obtained ethyl iodide as the sole reaction product by using 70% hydroiodic acid under pressure. This worker extracted the ethyl iodide so formed with tri-chloroethylene, and estimated it by gas-liquid chromatography.
Infra-red spectroscopy was used by Anderson and Zaidi (1963) to estimate the trapped reaction products obtained by use of the method of Morgan (1946).

A rapid and simple secondary method of analysis has been devised by Tai et al (1964). Acetaldehyde produced by the controlled pyrolysis of HES was estimated in a specially designed gas-liquid chromatograph and the technique was standardised against the method of Lortz (1956).

The methods listed above have a somewhat limited accuracy since the conditions of reaction are quite extreme. Some secondary products are formed which Anderson and Duncan (1961) attribute to the oxidation of the polymer backbone. These give rise to high sample blanks, and reduce the accuracy of the estimation. Errors quoted are ± 3-5%, a fact which does not seem to have been taken into account in many cases when values of MS are quoted to the second decimal place.

(d) The Substitution Pattern of HES

Various attempts have been made to characterise HES. Experiments on the polymer itself have yielded limited information and hydrolysis with acid to the monomer units gives an unusually complex mixture. Several characterisations of this nature have been made and an attempt will be made to review the relevant information available.

Husemann and Kafka (1960) examined the properties of three samples of hydroxyethyl amylose (0.3 < nS < 1.1) and found that the extent of reaction of sodium meta-periodate with the polysaccharide decreased during the substitution reaction. Periodate ion cleaves only 1,2 diols in starch i.e. the C(2) - C(3) bond in glucose, producing aldehyde groups on C(2) and C(3). Substitution of either the C(2) or C(3) hydroxyl group by the hydroxyethyl ether prevents cleavage of this bond.
If, as Husemann and Kafka (1960) found, susceptibility to cleavage decreased with increasing MS, substitution must occur predominantly at C(2) or C(3). This conclusion was supported by tritylation reactions. Tritylation, i.e., formation of the tri-phenyl-methyl ether, occurs preferentially at primary hydroxyl groups and thus only one hydroxyl at C(6) on starch is readily substituted. Formation of the hydroxyethyl ether at carbon C(2) or C(3) introduced a new primary hydroxyl group and thus if hydroxyethylation was to occur initially at either C(2) or C(3) then the polymer would be susceptible to increased tritylation. This was observed, and on this basis Husemann and Kafka (1960) concluded that hydroxyethylation occurred predominantly at the secondary hydroxyl groups in amylose and only at high MS did substitution occur to any great extent on C(6). No consideration was made in this work of the possibility of side-chain formation.

Schoch (1965) gives, without any experimental details, the following values for the substitution pattern of HES with MS - 0.9: 60% reaction at carbon C(6), 20% reaction at C(2) and, 10% reaction at C(3). The implication is that $k_6 > k_2 > k_3$, a conclusion quite different from that of Husemann and Kafka (1960). In addition, Schoch (1965) stated that there was no evidence for complex substitution having occurred - this implying that $k_f$ was very small or zero. It should be noted, however, that Schoch's analytical data only accounts for 90% of the substituent hydroxyethyl group found to be present, and it is possible that the remainder is to be found in the form of polysubstituted products.

A series of hydroxyethyl amyloses (0.23 < MS < 1.03) was characterized by Lott and Brobst (1966). These investigators examined by gas-liquid chromatography the acid hydrolysates of the polymer. Although some difficulty was experienced in the separation of certain tri-methyl-silyl ethers e.g. that of 3-O-(2-hydroxyethyl)-β-D-glucose
from that of 6-O-(2-hydroxyethyl)-D-glucose, the substitution patterns corresponded to rate constants in the order $k_2 > k_3 > k_6$. They also showed that with increasing MS large proportions of the hydroxyethyl content was to be found as complex derivatives, which are probably attributable in part to the presence of poly-(ethylene oxide) side chains.

A similar approach was used by Bollenback et al (1969). These workers characterised the acid hydrolysate of a HES (MS = 0.6) by separation using paper chromatography and subsequently estimated the sugars by the reaction with hypoiodite [Miller and Burton (1959)]. They concluded that their results were consistent with a kinetic scheme for the hydroxyethylat ion process in which $k_2 > k_6 > k_3$ and added there was apparently only a low level of disubstitution. This conclusion is a little suspect since Shyluck and Timell (1956) have demonstrated the difficulty in separating the mono- and di-hydroxyethyl glucose by this analytical technique.

De Belder and Norman (1969) investigated the substitution pattern of hydroxyethyl-starch and hydroxyethyl-dextran by techniques similar to those employed by Lott and Brobst (1966). Using samples of low MS (0.2), these authors showed that $k_2 > k_6 > k_3$. It is of interest to note that for dextran, a predominantly $\alpha$-1:6-linked glucan, the relation $k_2 > k_4 > k_3$ holds.

Srivastava and Ramalingam (1967) applied a Smith degradation to a commercial sample of HES (MS = 0.1). The HES was oxidised by periodate, the resulting dialdehyde groups then reduced by sodium borohydride to the corresponding alcohols and the polymer hydrolysed with dilute acid. The fragmentation products were characterised by paper- and adsorption-chromatography. The results were consistent with
a kinetic scheme in which $k_2 > k_6 > k_3$. No mention was made of complex derivatives - at the low level of substitution employed in this work, the amount of such products would escape detection by the analytical techniques employed.

Anionic graft co-polymers of poly-(ethylene oxide) and starch were prepared by Tahan and Zilkha (1969a, 1969b) over a very wide range of $\text{LiS}$. No detailed investigation of the relative reactivities of the different hydroxyls to grafting was carried out, but periodate oxidation suggested that the reaction occurred preferentially at the secondary hydroxyl group i.e. C(2) and C(3).

The results of these investigations are summarised in Table 6.1, and it seems that the situation is completely confused. With the exception of Schoch (1965), the consensus would favour C(2) as the most reactive hydroxyl group towards etherification. It is unfortunate that Schoch's model seems the most widely accepted for, judging by the diagrams in their papers, Thompson et al (1962), Russell et al (1966) and Cerny et al (1967), that is, those concerned with the medical aspects of HES, are using this model which appears to be incorrect.

However, even when $k_2$ is numerically largest, some doubt exists as to whether $k_3$ or $k_6$ has the smallest value. The situation is further confused by the general lack of appreciation of the possibility of complex derivatives being formed. So slight is the agreement that one is forced to consider that the actual chemical structures differ because of the mode of preparation.

Ramnas and Samuelson (1968) have shown that the relative rate of hydroxyethylation of the hydroxyls in cellulose is grossly dependent on base concentration whilst Roberts et al (1971, 1972) considering the reaction of glucosides with $N,N$-diethylaziridinium chloride have also
Table 6.1

A Summary of the Various Substitution Patterns for HES

<table>
<thead>
<tr>
<th>Authors</th>
<th>Material</th>
<th>MS</th>
<th>Relative order of rate constants</th>
<th>Polysubstitution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Husemann and Kafka (1960)</td>
<td>HEA 2)</td>
<td>0.3 - 1.1</td>
<td>$K_2$ or $K_3 &gt; K_6$</td>
<td>0</td>
</tr>
<tr>
<td>Schoch (1965)</td>
<td>HES</td>
<td>0.9</td>
<td>$K_6 &gt; K_2 &gt; K_3$</td>
<td>-</td>
</tr>
<tr>
<td>Lott and Brobst (1966)</td>
<td>HEA</td>
<td>0.2 - 1.0</td>
<td>$K_2 &gt; K_3 &gt; K_6$</td>
<td>+</td>
</tr>
<tr>
<td>Srivastava and Ramalingam (1967)</td>
<td>HES</td>
<td>0.1</td>
<td>$K_2 &gt; K_3 &gt; K_6$</td>
<td>0</td>
</tr>
<tr>
<td>De Belder and Norrman (1968)</td>
<td>HES</td>
<td>0.2</td>
<td>$K_2 &gt; K_6 &gt; K_3$</td>
<td>0</td>
</tr>
<tr>
<td>Bollenback et al (1969)</td>
<td>HES</td>
<td>0.6</td>
<td>$K_2 &gt; K_6 &gt; K_3$</td>
<td>-</td>
</tr>
<tr>
<td>Tahan and Zilkha (1969a)</td>
<td>HES</td>
<td>0.0 - 5.0</td>
<td>$K_2$ or $K_3 &gt; K_6$</td>
<td>++</td>
</tr>
</tbody>
</table>

1) 0 = not considered or not expected at this level of MS
- = not detected
+ = detected

2) HEA = hydroxyethyl-amylose
shown considerable dependence of reactivity of the various hydroxyls as a function of base concentration. With this in mind, the suggestion that the various workers have examined different types of HES, becomes quite feasible.

(e) The Physical Structure of HES

The molecular size and shape of a polymer in solution can greatly influence the behaviour of the polymer. This is of especial relevance to the use of HES as a cryoprotective agent, for ultimately the colloid must be ejected from the body via the kidneys, and the rate at which this occurs will be determined by the size of the macromolecule.

In Section 2 of this thesis, some mention was made of the possibilities of determining molecular shape and size from a combination of molecular weight and viscosity measurements. Extensive reviews have been published, by for example, Tanford (1961) or Banks and Greenwood (1971a) detailing the theory and applications of such measurements to the elucidation of polymer shape. However, the solution behaviour of HES has not been extensively investigated by such techniques, and only three reports of the shape and size distribution of HES in solution, and one report on hydroxyethyl-amyllose, have appeared.

The most thorough study appears to have been made by Husemann and Resz (1956). They fractionated three samples of hydroxyethyl-amylose by acetone precipitation, measured the average degree of polymerization of the fractions by osmotic pressure measurements, and related these to specific viscosity. These authors used an out of date relation, i.e. \( \eta = K \bar{M} \) to obtain information on polymer shape. If their data is recalculated using the now universally-
accepted Mark-Houwink equations, \( \eta = K \frac{M^a}{M} \) values of the exponent \( a \), vary considerably with MS as shown below:

<table>
<thead>
<tr>
<th>Molar Substitution</th>
<th>( a )</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.31</td>
<td>1.65</td>
</tr>
<tr>
<td>0.60</td>
<td>1.21</td>
</tr>
<tr>
<td>1.08</td>
<td>0.64</td>
</tr>
</tbody>
</table>

Extrapolation of these results to the case where MS is zero would appear to give values of \( a \approx 1.0 \). This corresponds to amylose existing in solution as a totally extended coil, a view quite contrary to the generally-accepted evidence supporting the model of amylose as a flexible Gaussian coil (Banks and Greenwood (1971)).

In the other physical investigations of the solution properties of HES, the branched fraction of starch, amylopectin, has been used. As pointed out in Section 2, there is still some major argument over the nature of amylopectin. In addition, theoretical treatments of hydrodynamic data are somewhat limited for branched polymers in solution.

Greenwood and Hurston (1967) reported the physical characteristics of five samples of HES in the range 0.83 < MS < 0.93, and showed that the viscosity potential of these samples reflected the branched nature of the substrate, being about one fifth of that of dextran, for a comparable molecular weight.

Cerny et al (1967), fractionated HES (MS = 0.85) using acetone and isopropanol, measured the number-average molecular weight by osmometry and the weight-average molecular weight by light scattering, and related these properties to viscosity. They obtained the equations:

\[
\eta = 5.29 \times 10^{-3} M_n^{0.30}
\]

and

\[
\eta = 3.27 \times 10^{-3} M_w^{0.35}
\]
Positive virial coefficients were found for both light scattering and osmotic pressure measurements. The equations indicate the branched nature of the polymer, and the disparity between the number average and weight average results may be due to a difference in molecular weight distribution between samples. A branching-index was calculated, by comparison of the intrinsic viscosity, radius of gyration, and second virial coefficients with those of hydroxyethyl-cellulose and ethylhydroxyethyl-cellulose, and they concluded that it was not possible to estimate solvent-polymer interaction using current theories. The comparison of hydroxyethyl-cellulose and ethylhydroxyethyl-cellulose with HES would seem to be unjustified in obtaining the branching parameter, since the required analogue is hydroxyethyl-amylose and the nature of the glycosidic linkages in the parent amylose and cellulose has been shown to confer quite different properties in the derivatives.

Granath et al (1969) carried out extensive physical characterisations of HES of various molar substitutions. For a sample with MS = 0.6, they obtained the relation

\[ \eta = 2.91 \times 10^{-3} \eta_{\text{w}}^{0.35} \]

The low exponent was attributed to the branched nature of the material, but these authors noted that viscosity increased with MS, and suggested that this may be due to hindered rotation about the glycosidic linkage.

These investigations show that HES exhibits the characteristics of the parent amylopectin in, i.e. low viscosity for a given molecular weight. The exact nature of solvent-polymer interaction is unknown, for whilst the results of Husemann and Resz (1956) could be interpreted to show that polymer-solvent interaction decreases with increasing MS, those of Granath et al (1969) show the diametrically opposite view.
It would seem that further investigation of the hydrodynamic behaviour of hydroxyethyl-amylose will be required to establish the exact nature of the effect of the substituents on the solution behaviour of the polymer.

(f) The Enzymic Degradation of HES

The resistance of HES to exo-amylase attack was used by Ziese (1934; 1935) to differentiate beta- from alpha-amylase. This was confirmed by Scholander and Hyrback (1951) and later by Greenwood and Hourston (1967). Husemann and Resz (1956) carried out a systematic investigation of the attack of α-amylase on HEA. They found that the α-amylase from Aspergillus oryzae attacked HEA at increasingly slower rates as MS increased and was still finite at MS of 1.03. It would seem that the model proposed by Lott and Brobst (1966), where 20% of the glucose residues are unsubstituted at MS of 1.03, is essentially correct, for in this model there would be sufficiently numerous areas of unsubstituted glucose to allow conventional attack by α-amylase.

(g) General Properties of HES

Tahan and Zilkha (1969a) report that, as MS increases from 0.4 to 1.0, HES formed by graft polymerisation becomes more readily soluble in cold water, and at MS = 3.0, the products are thermoplastic, and water- and alcohol-soluble. These polymers would seem to combine the characteristics of both the starch granule and poly-(ethylene oxide). Stavennann et al (1961) reported that, as MS increased in HES, the ability to bind iodine was sharply diminished, reflecting the disrupting effects of the substituent groups on the ability of amylose to form a helix.

(h) Conclusions

Although methods of characterization of HES are well developed, agreement has not been reached on the nature of the substitution.
Little appreciation has been shown of the possibility of side-chain formation, and the full implication of this when relating resistance to \( \alpha \)-amylolysis with DS does not seem to have been considered. The physical structure of HES in solution is a matter of some considerable doubt.

Before the full potential of HES as a pharmacological agent can be utilised, it is necessary to define more closely the chemical structure of HES. Part 3 of this section reports work undertaken to do this, and also reports on a method of measuring DS which allows a useful correlation between extent of substitution and resistance to enzymic attack to be made.
6.3 Preparation and Analysis of HES

Hydroxyethyl starch for medical use has been made commercially by derivatization of the waxy starches from genetic mutants of maize and sorghum. These starches are composed entirely of the branched starch-polymer, amylopectin and, for this reason, HES should precisely be referred to as hydroxyethyl-amylopectin. It was decided in this work to study initially the structure of its linear analogue, hydroxyethyl-amylose, for, as discussed in Section 2, the characteristics of amylose are rather more fully understood than those of amylopectin.

(a) Isolation of Polymer Substrates

Amylose was isolated from a commercial sample of amylomaize starch (Amylon 50, provided by courtesy of National Starch Inc.) using the technique devised in Section 4.2. The amylose was isolated as the butan-1-ol complex, in which form it is readily soluble in warm water, and characterized by measurement of its limiting viscosity number in 0.33 M KCl (found, \( \eta \)) = 65 ml/g) and its iodine affinity (I.A.) (found I.A. = 19.5 mg iodine bound/100 mg amylose at 20.4 C). This latter quantity showed the sample to be free of amylopectin. It should be noted that the viscosity number of this amylose is approximately twice that of commercial preparations of HES, despite the fact that the molecular weight of the former is about two orders of magnitude less than that of HES. This observation emphasized the profound differences in hydrodynamic volume (of which the limiting viscosity number is a measure) between linear and branched macromolecules of similar molecular weight.

The amylopectin required for confirmation purposes in this study of substitution pattern was isolated from waxy maize starch using the methods detailed in Section 2.3.
(b) Preparation of Hydroxyethyl Derivatives

As described in the previous part of this section, many methods of preparation have been used to make the hydroxyethyl derivative of starch. Since material for pharmacological use has, according to Schoch (1965), been prepared by the homogeneous reaction of ethylene oxide with starch in aqueous alkali, the same technique was used to prepare samples employed in this work.

The reaction of starch type polysaccharides with ethylene oxide in the presence of alkali may be represented as nucleophilic substitution:

$$\text{GOH}^- + \text{CH}_2\text{CH}_2 \xrightarrow{\text{base}} \text{GOCH}_2\text{CH}_2\text{CH}_2$$

where $G$ = an anhydroglucose residue.

However, since the reaction occurs in an aqueous system, many side reactions occur, e.g.

$$\text{CH}_2\text{CH}_2 + \text{H}_2\text{O} \xrightarrow{\text{base}} \text{HOCH}_2\text{CH}_2\text{OH}$$ ethylene glycol

$$\text{HOCH}_2\text{CH}_2\text{OH} + n(\text{CH}_2\text{CH}_2) \xrightarrow{\text{base}} \text{HOCH}_2\text{CH}_2\text{O(CH}_2\text{CH}_2\text{O)}_n\text{H}$$ poly-(ethylene glycol)

Because of these side-reactions, an excess of ethylene oxide over that calculated from stoichiometric considerations is always required. A small-scale pilot experiment was used as the most convenient means of ascertaining the required reaction conditions.

The required polymer, amylose or amylpectin, was dissolved in aqueous alkali (1.0 m KOH) at room temperature to give a 1% solution. This solution was sparged thoroughly with nitrogen, and all subsequent operations were performed under an atmosphere of this gas to minimize alkaline degradation of the polysaccharide. The solution was cooled to ca. 2°C and ethylene oxide (also at 2°C) was added by pipette. Working
at this low temperature, below the boiling point of ethylene oxide, greatly facilitates control of the derivatization on a laboratory scale. The ethylene oxide was added by pipette, holding the tip just below the surface of the solution to ensure that none of the reagent volatilized before reaching the alkaline solution. After rapid mixing, the reaction flask was sealed by wiring down the stopper, and immersed in a thermostat bath (40°C) for 16 hours, with constant stirring (magnetic). At the end of this period, the flask was cooled to 2°C, unsealed, and the contents neutralized with dilute hydrochloric acid. After extensive dialysis against distilled water, in order to remove salts and ethylene glycols, the solution was concentrated on a rotary evaporator and freeze-dried. It was found that extensive dialysis of the product is as efficient as repeated solvent extraction for the removal of unwanted side products.

(c) Chemical Characterization of the Hydroxyethyl Derivatives

(i) Measurement of Molar Substitution (M5)

The M5 was measured by the method of Morgan (1946), in which the ether linkage is cleaved by reaction with constant-boiling hydroiodic acid. The products, ethylene and ethyl iodide, are trapped in bromine/acetic acid, and silver nitrate solution, respectively, and estimated titrimetrically. The method was calibrated with 3-O-hydroxyethyl glucose and poly-(ethylene oxide). A reaction time of one hour was found to give optimal conditions. Frequent measurement of reagent blanks, and the use of freshly-distilled hydroiodic acid were required. In agreement with Lortz (1956), it was observed that samples with an appreciable moisture-content gave low results. Because of the poor reproducibility of the technique (3-5% error when 0.4 < M.S. < 1.0), triplicate estimations were performed on all samples.
The above procedure, particularly the titration stage, is somewhat laborious, and therefore the method of Van der Bij (1967) was investigated. The complete conversion of the reaction products under pressure appeared to be adequate, but it was found that extraction of the reaction-mixture with trichloroethylene to remove ethyl-iodide also removed considerable amounts of hydroiodic acid, which led to rapid failure of the column material in the gas-chromatograph. Rather than embark on a lengthy search for an alternative technique, it was decided to persist with Morgan's method.

(2) Measurement of Degree of Substitution (DS)

As was pointed out in the review part of this Section (see p. 181), DS is quite a different parameter from MS, and it is desirable to be able to measure both quantities. To obtain DS and MS, the weight fraction of anhydroglucose units which are not substituted must be known. A suitable, simple and reliable method was devised and is now described:

The material was dried in vacuo at 70°C overnight, and dissolved in sufficient water to give a known concentration in the range 1-3 mg/ml. An equal volume of sulphuric acid (1.5M) was added, and the polysaccharide derivative hydrolyzed for 3 hours on a boiling water bath. This procedure has been shown in Section 2 to achieve complete hydrolysis of the glycosidic bonds in amylose and amylpectin without any concomitant acid reversion of the glucose. (The ether linkages of the derivative are resistant to this relatively mild hydrolysis.) The samples were cooled to room temperature, neutralized by the addition of a predetermined amount of caustic alkali (1.0 M KOH), and diluted in a graduated flask to give a free-glucose content in the range 5-40 μg/ml. An aliquot (1.00 ml) was taken for analysis using the coupled glucose oxidase/peroxidase/chromogen system described in Section 2.2. Control experiments showed that none of the various, mono-substituted, hydroxyethyl derivatives of glucose reacted...
with this particular system. This observation was not surprising in view of the high specificity of the enzyme glucose-oxidase (Keilin and Hartree (1952)). Thus the "free" glucose content of any preparation of hydroxyethyl-amylose or hydroxyethyl-starch may be determined quite unambiguously by acid hydrolysis to the monomer units followed by specific enzymic estimation of the unsubstituted glucose. The DS is then calculated from the measured MS and the amount of free glucose from the relation:

$$\text{DS} = 1 - \frac{W_G \left(162 \times 44 \text{ MS}\right)}{162 \ W_p},$$  \hspace{1cm} (6.2)

where $W_G$ is the weight of anhydroglucose in a polymer sample of weight, $W_p$.

(3) Periodate Oxidation

Sodium metaperiodate specifically oxidises 1,2 diols, which in the case of amylose means that, to an excellent approximation, only the hydroxyl groups on carbon atoms C(2) and C(3) are susceptible to oxidation. (In fact, the terminal units at both the reducing and non-reducing ends of the linear chain are subject to further oxidation, but since the chain is at least 500 units long these end-effects may be neglected. (This is an advantage of using the amylose derivative as a model substance for, in the case of amylopectin, some 4-5% of the glucose units are present as non-reducing ends, and this increased oxidation renders the type of calculation used later in this work of somewhat dubious significance.)

As mentioned in the review part of this Section, substitution at either position C(2) or C(3) will block oxidation, whereas that at position C(6) will not. A measure of the substitution pattern is thus possible, for by measuring the amount of periodate consumed in the reaction, using various samples of hydroxyethyl-amylose (H EA) of different MS, it is possible to determine whether substitution occurs primarily at C(6) or at either C(2) or C(3).
Experimental conditions used were as follows: HEA was dried, weighed accurately, and dissolved in water to give a known concentration in the range (4-8) mg/ml. To this solution (20.0 ml), sodium metaperiodate (5.0 ml; 0.125 iv) was added, and the reaction flask stored in the dark at ca. 3°C in a refrigerator. Samples were withdrawn at 22, 40 and 80 hours, excess potassium iodide was added, and the liberated iodine estimated by titration with thiosulfate. Periodate consumption reached a limiting value in 22 hours, and did not thereafter change appreciably. Hence within this range, time does not significantly affect the results, and therefore the determination was stopped at some time in the period 24-48 hours.

The technique of periodate oxidation is an important diagnostic tool in its own right, but in combination with the determination of free glucose (as described in the previous sub-section) is very much more valuable.

A typical series of hydroxyethyl amylases having 0.54 < MS < 1.21 was characterized by the above techniques and the results are shown in Table 6.2. Control experiments were also carried out on native amylase, and 100% of the residues were found to be totally unsubstituted, and 10% unsubstituted at positions C(2) or C(3). These results demonstrate that considerable confidence may be placed in the values recorded in Table 6.2.

It is clear that at low molar substitution, apparently all the free C(2) - C(3) hydroxyl pairs are to be found on unsubstituted anhydroglucose residues, i.e. substitution occurs predominantly at C(2) or C(3), and as MS increases a small amount occurs at C(6). For the sample 4.1, MS = 0.54, which gives DS = 0.50; the difference between MS and DS is a measure of complex substitution that has occurred. If these
Table 6.2

A comparison of the weight fraction of glucose units totally unsubstituted (obtained from acid hydrolysis, and estimation of glucose with glucose-oxidase) with those unsubstituted at carbon atoms C(2) and C(3) (obtained from periodate consumption).

<table>
<thead>
<tr>
<th>Sample Code</th>
<th>4.1</th>
<th>4.2</th>
<th>4.3</th>
<th>4.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wt. % anhydroglucose</td>
<td>44</td>
<td>31</td>
<td>22</td>
<td>16</td>
</tr>
<tr>
<td>Wt. % unsubstituted at C(2) or C(3)</td>
<td>44</td>
<td>32</td>
<td>26</td>
<td>20</td>
</tr>
</tbody>
</table>
values are accepted, then there is $\frac{0.54 - 0.50}{0.54} \times 100\%$ complex substitution, i.e. approximately 7%. The experimental error in the determination of MS renders this point of less significance since $MS = 0.54 \pm 0.03$, and at this value the difference between MS and DS is not significant. At the highest MS value, the DS can be calculated as 0.79. In this case there is no possible doubt; the difference between MS and DS is so great that polysubstitution must have occurred.

In any mixture of complex i.e. either polysubstituted with side chains, or multiply substituted on any combination of two or three hydroxyl groups, glucose derivatives and simple monohydroxyethyl derivatives, $MS > DS$. To confirm the observations in Table 6.2 a series of HEA and HES samples were prepared in a similar fashion and MS and DS measured. The results are shown in Table 6.3.

As the hydroxyethyl content increases, the divergence between MS and DS also increases in the case of both HEA and HES. There can be no doubt that these figures demonstrate quite unambiguously that the complex glucose derivatives reach a measurable amount when MS exceeds 0.50 and at MS = 0.8 (the material widely used as a blood plasma volume expander has a similar value of MS), some 15% of the hydroxyethyl groups are present as complex derivatives of glucose.

It must be stressed that some samples of both HEA and HES were subjected to repeated extraction with 95% acetone, in case any ethylene glycol or its oligomers had survived the dialysis stage. (Such a mixture would exhibit the same properties as those displayed by the samples in Table 6.3). However, in no case did this treatment lead to any significant change in the values of MS or DS, and hence it is concluded that the results shown in Table 6.3, and the inferences derived therefrom, are real.

The results show (i) that reaction occurs predominantly at C(2) or C(3), the secondary hydroxyls, and (ii) that polymerization to form side chains occurs to a significant extent. The relative magnitudes of
Table 6.3. A comparison of the molar substitution (MS) and degree of substitution (DS) for hydroxyethyl amylose (HEA) and hydroxyethyl starch (HES) of varying hydroxyethyl contents.

<table>
<thead>
<tr>
<th></th>
<th>Hydroxyethyl amylose</th>
<th>Hydroxyethyl starch</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MS</strong></td>
<td>0.45</td>
<td>0.43</td>
</tr>
<tr>
<td></td>
<td>0.60</td>
<td>0.55</td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>0.64</td>
</tr>
<tr>
<td></td>
<td>0.90</td>
<td>0.64</td>
</tr>
<tr>
<td></td>
<td>1.10</td>
<td>0.81</td>
</tr>
<tr>
<td></td>
<td>1.25</td>
<td>0.69</td>
</tr>
<tr>
<td><strong>DS</strong></td>
<td>0.45</td>
<td>0.42</td>
</tr>
<tr>
<td></td>
<td>0.56</td>
<td>0.52</td>
</tr>
<tr>
<td></td>
<td>0.61</td>
<td>0.59</td>
</tr>
<tr>
<td></td>
<td>0.69</td>
<td>0.69</td>
</tr>
<tr>
<td></td>
<td>0.71</td>
<td>0.77</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

202.
and $k_2$ and $k_3$ have still to be resolved and the onset of significant quantities of complex derivatives defined. To do this calculation it was necessary to hydrolyze the polymer to its substituent monomer units and characterize this mixture.

(4) Separation and Characterization of the Sugars

Characterization of the chemical structure of HEA and HES necessitated three stages namely: (i) hydrolyzing the polymer to monomer units, (ii) separating these units on the basis of the carbon atom at which substitution had occurred, and (iii) characterizing them by identifying the position of the substituent hydroxyethyl group.

(i) Hydrolysis

Hydrolysis was carried out using the conditions defined in Section 2.2, which have been shown to give complete hydrolysis of amylopectin with no concurrent reversion of glucose.

Because many neutral salts interfere with chromatographic processes, their removal was necessary. The hydrolysate was neutralized by adding barium carbonate until the solution reached pH 7.0. The precipitated barium sulphate was removed by filtration, and the solution decreased in volume at reduced pressure. More barium sulphate precipitated, and again it was removed by filtration. This procedure was repeated until the concentration of the sugar solution was approximately 10% (w/v). The solution was then passed through an ion-exchange column (Amberlite IR 140, in the hydrogen ion form) to remove residual barium ions. The column effluent was lyophilized, and the resultant solid dried in vacuo at 70°C for 12 hours.

(ii) Separation of the Sugars

Five different techniques were used in an effort to adequately separate the sugars present in the hydrolysates of HEA. These methods were, gel permeation chromatography on a "Sephadex" G-10 column; ion
exchange chromatography using a "Bio-Rad" cation exchange resin (Aminex AG 50 W = X2) in the lithium form (Jones et al. 1960), charcoal adsorption chromatography (Khistler and Durso 1950), paper chromatography, and gas-liquid chromatography. None of the first three methods yielded suitable separations, despite an intensive investigation, and are not therefore further discussed.

Croon and Linberg (1956) and Brownell and Purves (1957) have reported the paper-chromatographic behaviour of the mono-hydroxyethyl ethers of glucose. Using a hydrolyzate of HEA, four spots plus an unresolved streak were obtained on developing a paper-chromatogram by the method of Trevelyan et al. (1950) for reducing sugars. (Descending chromatography, paper developed on the solvent system acetate: acetic acid: water (9:2:2)). Glucose was identified by spraying a chromatogram with the glucose oxidase/peroxidase/chromogen mixture discussed in Section 2; the reagent of Gardiner and Percival (1958) was used to develop spots corresponding to sugars not substituted on position C(2). A combination of these spray-reagents indicated that the major components of the hydrolysate were glucose and 2-O-hydroxyethyl glucose; the other two resolved spots corresponding to the 3-O-hydroxyethyl and 6-O-hydroxyethyl glucose respectively. The conclusions to be drawn from these experiments supported that made in the last section, i.e. the secondary hydroxyl group at C(2) is the most reactive group in starch.

An attempt was made to make the paper-chromatographic separation quantitative by eluting the individual sugars and by estimating them with the phenol/sulphuric acid of Dubois et al. (1956). This proved to be quite inaccurate since the intensity of colour developed by equimolar quantities of glucose and substituted glucose were not identical. For this reason, gas-liquid chromatography was investigated.

The apparatus used was as follows:
Column - glass (15 feet, inside diameter \( \frac{1}{4} \) inch); 3% silicone gum rubber E350 on acid-washed, dichloromethylsilylated, "Chromosorb-\( \frac{1}{4} \)" support (60-80 mesh).

Oven - "Pye" 104 gas chromatograph oven with temperature programming facility.

Detector - Single flame-ionization detector.

Amplifier - "Vibron" electrometer model 33B-2 (Electronic Instruments Ltd, Richmond, Surrey), with shunt, model A-49A (Electronic Instruments Ltd).

Recorder - 1 mV full-scale deflection (Honeywell Controls Ltd).

Integrator - Type 1E-165 (Gas Chromatography Ltd., Boyn Valley Road, Maidenhead).

Experimental conditions - Nitrogen carrier gas, flow rate 55 ml/minute.

To reduce detector sooting, the detector-flame was switched on after the solvent (pyridine) peak has passed.

Temperature programme - The following programme was designed to reduce solvent tailing, and to give optimum peak separation: at injection of sample, and for the next 5 minutes, 150°C, then from 150-188°C at 4°C/minute, and finally isothermally at 188°C.

Various methods are available for the formation of derivatives of sugars suitable for analysis by gas-liquid chromatography. The most useful derivative is the trimethyl silyl ether. This may be formed by the method of Sweeley et al (1963) or by that of Lott and Brobst (1966). Both techniques are simple, but the former was used in this work because it was found to be more reliable. Glucose and 3-O-hydroxyethyl-D-glucose both gave only two major peaks, corresponding to the \( \alpha \) - and \( \beta \) - anomers, with spurious impurity peaks corresponding to less than 1% of the major peak areas. The sample (2-5μl) of the solution of the trimethyl-silyl ethers in pyridine was introduced into the carrier gas by means of a
5 μl calibrated syringe, and the temperature programme detailed above set in operation. A typical trace of various peaks obtained by gas chromatographic separation of the tri-methyl silyl ethers of an acid-hydrolyzate of HSA is shown in Figure 6.1.

(iii) Characterization of the sugars

The number of possible O-hydroxyethyl derivatives of glucose is infinite for, although only three mono-, three di- and one tri-substituted derivatives are possible, the possibility of side-chain formation must not be discounted. The mono-substituted O-hydroxyethyl ethers are well characterized and Shlyuck and Timell (1956) have published techniques for the preparation of these sugars. One of these derivatives 3-O-hydroxyethyl-D-glucose was synthesized. Diacetone glucose was reacted in KOH (1.0M) with ethylene oxide (four-fold excess) to give a mixture of 3-O-hydroxyethyl diacetone glucose, 3-O-ethoxyhydroxyethyl diacetone glucose, and unreacted diacetone glucose. After acid hydrolysis (H₂SO₄; 1.5N; 1 h) to remove the blocking groups, the mixture of sugars was absorbed on granular charcoal and eluted with a linear gradient of ethanol in water. The separation is shown in Figure 6.2. Pure 3-O-hydroxyethyl-D-glucose was obtained. This material gave only two peaks when analysed by G.L.C. corresponding to the α- and β-anomers of 3-O-hydroxyethyl-D-glucose. A mixture of 2-O-hydroxyethyl-D-glucose and 3-O-hydroxyethyl-D-glucose was prepared by a similar reaction of methyl 4,6-O-benzylidene-D-glucose with ethylene oxide in base. An analogous purification procedure was followed. Periodate oxidation and free-glucose estimation has already shown that reaction occurs predominantly at the secondary hydroxyls of glucose in HES. By comparing the peaks (see Figure 6.1) with the prepared derivatives it is clear that 2-O-hydroxyethyl glucose is the predominant derivative formed at low MS. It is then apparent that there is considerable overlap of peaks corresponding to the various hydroxyethyl ethers. In order to allow a quantitative assay, it was necessary to calibrate the
2-heg = 2-O-hydroxyethyl, D-glucose
3-heg = 3-O-hydroxyethyl, D-glucose
6-heg = 6-O-hydroxyethyl, D-glucose

(peaks not to scale)

complex derivatives
Figure 6.2 carbon column chromatography

A = GLUCOSE

B = 3-O-HYDROXYETHYL GLUCOSE

C = COMPLEX DERIVATIVES
G.L.C. This was done using erythritol as an internal standard. Equimolar quantities of erythritol, glucose and 3-O-hydroxyethyl glucose were weighed out accurately and derivatized. The peak areas were measured by planimeter and the relative molar responses calculated. The relative molar responses of all the mono-substituted O-hydroxyethyl-D-glucose derivatives were assumed to be equal. Before analysis of a mixture, an aliquot of erythritol was carefully weighed into a tube and a known weight of deionised, freeze-dried hydrolysate added. This mixture was then derivatized and analyzed by G.L.C. Since only one peak of an anomer of each of the substituted sugars was completely separated, it was necessary to check that the anomer ratios were not variable, or disturbed by the derivatization procedure. Tests on glucose showed that the anomer peak areas did not change relatively by more than ±3%. A quantitative analysis of HES (α3 = 0.45) is shown in Table 6.4. The values are reproducible to ±5%. They fully confirm the previous conclusions: the most reactive hydroxyl group in the starch components is at C(2), and that complex derivatives are present in substantial amounts at comparatively low MS. These results also show that the reactivities at C(3) and C(6) are approximately the same and very much lower than that at C(2).

At this point, it is possible to suggest a mathematical model which describes the substitution pattern of HES as a function of MS.

Each glucose residue in starch or amyllose has three free hydroxyl groups available for reaction with ethylene oxide. If it is assumed that the reaction at any one of these groups is independent of whether or not the other sites on that residue have reacted, and that the rate of substitution is proportional to the number of sites which have not reacted, i.e. a first-order reaction, then:

209.
Table 6.4

Quantitative analysis of the sugars present in a hydrolysate of hydroxyethyl amylose of $\text{LiS} = 0.45$

<table>
<thead>
<tr>
<th>Sugar</th>
<th>% by weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>62</td>
</tr>
<tr>
<td>2-O-hydroxyethyl glucose</td>
<td>25</td>
</tr>
<tr>
<td>3-O-hydroxyethyl glucose</td>
<td>3.5</td>
</tr>
<tr>
<td>6-O-hydroxyethyl glucose</td>
<td>4.5</td>
</tr>
<tr>
<td>Complex derivatives</td>
<td>5</td>
</tr>
</tbody>
</table>
\[ \frac{dS}{dt} = k(1-S), \quad (6.3) \]

where \( S \) is the fraction of available sites which have reacted, and \( k \) is the rate constant for substitution. Integration of equation (6.2), with the constraint that \( S = 0 \) when \( t = 0 \), gives:

\[ \ln (1 - S) = -kt \quad (6.4) \]

This may be expressed in exponential rather than logarithmic form to give:

\[ 1 - S = \exp (-kt), \quad (6.5) \]

or \[ S = 1 - \exp (-kt) \quad (6.6) \]

Each of the three available hydroxyl groups will react in the manner shown by equation (6.6), but there is no reason to suppose that the rate constants for all three reactions will be the same. The reactions may be described by the relations:

\[ S_2 = 1 - \exp (-k_2t), \quad (6.7) \]
\[ S_3 = 1 - \exp (-k_3t), \quad (6.8) \]
\[ S_6 = 1 - \exp (-k_6t), \quad (6.9) \]

where \( k_2, k_3 \) and \( k_6 \) are the rate constants for \( \text{C}(2), \text{C}(3) \) and \( \text{C}(6) \) hydroxyl groups, respectively.

The rate of poly-(ethylene oxide) side-chain formation must be proportional to the fraction of hydroxyl groups already substituted. Thus:

\[ dS_p = k_p (S_2 + S_3 + S_6) \, dt \quad (6.10) \]

Using equations (6.7), (6.8) and (6.9), the terms \( S_2, S_3 \) and \( S_6 \) may be replaced, and equation (6.10) may be written as:

\[ dS_p = k_p \left\{ (1-\exp(-k_2t)) + (1-\exp(-k_3t)) + (1-\exp(-k_6t)) \right\} \, dt \quad (6.11) \]

If terms in \( S_3 \) and \( S_6 \) are, for the moment, ignored and only

\[ dS_p = k_p S_2 \, dt \quad (6.12) \]

\[ = k_p (1-\exp(-k_2t)) \, dt \quad \text{is used, then integration} \]
gives:
\[ S_p = k_p \left( t + \frac{1}{k_2} \exp - k_2 t + C \right), \quad (6.13) \]

where \( C \) is the integration constant. Applying the boundary condition that \( S_p = 0 \) when \( t = 0 \) then:

\[ C = -\frac{1}{k_2}. \quad (6.14) \]

If follows, therefore, substituting for \( C \) in equation \( (6.13) \) that

\[ S_p = k_p t - \left( \frac{1}{k_2} \right) (1 - \exp - k_2 t). \quad (6.15) \]

Returning to equation \( (6.11) \), this may be integrated by an analogous procedure:

\[ S_p = k_p \left( 3t - \left( \frac{1}{k_3} \right) (1 - \exp - k_3 t) + \left( \frac{1}{k_3} \right) (1 - \exp - k_2 t) + \left( \frac{1}{k_6} \right) (1 - \exp - k_6 t) \right). \quad (6.16) \]

On the basis of the results presented earlier in this section values of \( k_3 : k_6 : k_2 : k_p \) were given as 1:1:15:10 and thus

\[ S_3 = 1 - \exp - k t, \quad (6.17) \]

\[ S_6 = 1 - \exp - k t, \quad (6.18) \]

\[ S_2 = 1 - \exp - 15 k t, \quad (6.19) \]

and for poly-(ethylene oxide) side-chain formation:

\[ d S_p = 10k (S_3 + S_6 + S_2) \ dt, \quad (6.20) \]

substituting for \( S_3, S_6 \) and \( S_2 \) in equation \( (6.20) \), and using \( (6.17), (6.18) \) and \( (6.19) \), and integrating with respect to time with the constraint that \( S_p = 0 \) when \( t = 0 \) gives

\[ S_p = 30 k t - 20 (1 - \exp k t) - \frac{8}{3} \]

\[ (1 - \exp - 15 k t). \quad (6.21) \]

The equations \( (6.17), (6.18), (6.19) \) and \( (6.21) \) may then be solved for given arbitrary values of the parameter \( k t \). In Figure 6.2, the variation of \( S_2, S_3, S_6 \) and \( S_p \) are shown as a function of \( k t \). The curve representing \( M_S \) is obtained by summation of \( S_2, S_3, S_6 \) and \( S_p \) at a given value of \( k t \).
The relative values chosen were based on the results of Table 6.4. This particular sample of HEA with $i\Sigma = 0.45$ was chosen as the level of complex derivatives was low and no complex terms were required for disubstituted products. The value of $k_p$ was chosen to fit the data presented in Table 6.3 and must necessarily be approximate. Inspection of the curves presented in Figure 6.3 suggest that even at high levels of substitution, i.e. $i\Sigma > 1.0$, some free glucose is present in accord with the earlier observations. This may explain the susceptibility of these samples to attack by $\alpha$-amylase.

(5) The effect of amylolytic enzymes on HEA

The exo-enzymes (those hydrolyzing the molecule sequentially from the non-reducing end) $\beta$-amylase, phosphorylase and amyloglucosidase have negligible effect on HEA even at low $i\Sigma$. For example, if the typical degree of polymerisation of amylose is taken to be 1000 glucose residues, then at $i\Sigma = 0.1$ less than 1% of the polymer is available to such chain-end degradation. The endo-amylases, or $\alpha$-amylases are not, however, constrained in this manner since their action-patterns are more or less random (Greenwood and Milne (1968)) in the early stages of the hydrolysis. Thus they may effectively by-pass groups (such as substituted glucose residues or branch points) on which they have no action. Their only requirement is that a number of unsubstituted glucose residues (at least two and probably greater) be found in sequence. In order to verify in a qualitative manner the model for the substitution pattern of HEA proposed in this work, HEA of varying $i\Sigma$ was exposed to $\alpha$-amylase.

The resistance of HEA to the action of $\alpha$-amylase was determined by measuring the viscosity number of the polysaccharide during hydrolysis by the enzyme, using a Ubbelohde viscometer as detailed in Section 2.5. The solution (15 ml; 0.15% HEA in 0.9% saline) was placed in a viscometer at 25°C and, after temperature equilibrium, the viscosity number was measured.
Figure 6.3 a mathematical model

![Graph showing a mathematical model with lines labeled M.S., S2, S_p, S_3, S_6 against a scale ranging from 0.05 to 0.10 kt.]
An aliquot (0.10 ml) of a dilute solution of porcine, pancreatic α-amylase [3,000 units/ml, the unit of activity being that defined by Greenwood et al (1965)] was then added, and the solution mixed thoroughly. The viscosity number was re-determined as a function of time. Table 6.5 shows the variation with time of the viscosity numbers of a series of HEA samples of different hydroxyethyl content. HEP was chosen in preference to HES because the viscosity technique is more sensitive to changes in the molecular weight of linear polymers than it is to such changes in a branched polymer.

The results in Table 6.5, show clearly that DS is the dominant factor in determining the resistance of the molecule to α-amylolysis. Even ignoring the values of DS, the fact that samples with MS Values of greater than unity are susceptible to amylolytic attack shows, qualitatively at least, that complex derivatization has occurred. Since α-amylase requires a sequence of unsubstituted residues, probably greater than three, it is necessary to reconcile this fact with the experimental observations. If a minimum "free" segment of three units is accepted, then the probability of finding three unsubstituted glucose residues in sequence when DS = 0.75 is 

\[ \left( \frac{1}{2} \right)^3 \]

or 1 in 64, the probability of finding four or five unsubstituted residues in sequence are 1 in 256, and 1 in 1024, respectively. The number average degree of polymerization of the initial HEA is approximately 500 monomer units, thus the number of bonds accessible to the action of α-amylase varies in the range (8 - 0.5) per HEA molecule, depending on the minimum size of the unsubstituted segment required by the enzyme. With this calculation in mind, the susceptibility of samples with high MS to α-amylolysis provides confirmation of the suggested model for the substitution pattern of HEA.
The viscosity number ($n_{sp/C}$) of hydroxyethyl amylose of different molar substitutions as a function of time of exposure to alpha-amylase at 25°C.

<table>
<thead>
<tr>
<th>Molar substitution</th>
<th>Degree of substitution</th>
<th>$n_{sp/C}$ at t minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>C=0</td>
</tr>
<tr>
<td>0.45</td>
<td>0.45</td>
<td>76</td>
</tr>
<tr>
<td>0.60</td>
<td>0.56</td>
<td>77</td>
</tr>
<tr>
<td>0.75</td>
<td>0.64</td>
<td>84</td>
</tr>
<tr>
<td>0.90</td>
<td>0.69</td>
<td>88</td>
</tr>
<tr>
<td>1.10</td>
<td>0.74</td>
<td>85</td>
</tr>
<tr>
<td>1.25</td>
<td>0.77</td>
<td>85</td>
</tr>
</tbody>
</table>
The interaction of HES with iodine

As was shown in Section 3.3, the interaction of starch with iodine is a distinctive feature of that polymer. The amylopectin component of starch, or waxy maize starch itself, gives a reddish-purple colour with iodine, with a wavelength of maximum absorption (\( \lambda_{\text{max}} \)) of about 540 nm. The absorption spectra of a series of HES samples (0 < MS < 0.7) is shown in Figure 6.4.

This figure shows that, with increasing MS, the intensity of the peak with \( \lambda_{\text{max}} = 540 \) nm diminishes, and \( \lambda_{\text{max}} \) itself moves to the red end of the spectrum. A hitherto, unobserved peak appears at ca. 395 nm, shifting towards slightly higher wavelengths as MS increases. No previous report of such an absorption has been found, and it is concluded that this is a specific result of the reaction of the polysaccharide with ethylene oxide. The interaction of a number of samples of poly-(ethylene oxide) with iodine was investigated, and in all cases an absorption at ca. 400 nm was found. It is therefore tempting to suggest that the complex derivatives obtained an acid hydrolysis of HES and HEA are predominantly poly-substituted rather than multi-substituted. However, the appearance of the peak at \( \lambda = 400 \) nm when MS = 0.06, at which level no complex substitution can be detected by gas chromatographic techniques, suggests that this interpretation is incorrect.

No satisfactory explanation can, at present, be offered for this phenomenon.

It is of interest to note that as MS increases, the main absorption peak ca. 540 nm diminishes in intensity. This presumably reflects hindrance to the formation of helical segments, caused by the introduction of substituent groups.

(7) Conclusions

The work in this section has shown that the previous state of knowledge of HES was somewhat limited. Using HEA as a model substrate,
Figure 64: Iodine interaction spectra

- Wavelength: 400 m\(\mu\) to 600 m\(\mu\)
- Absorbance

Spectra at different concentrations: 0.00, 0.06, 0.18, 0.27, 0.37
the substitution pattern of HEM, formed under conditions similar to those used for preparation of plasma expanders, has been elucidated. It has been demonstrated that polymeric side-chain formation plays an important role in this system, and a method has been devised to measure this type of substitution. A mathematical model has been proposed which describes the substitution pattern quantitatively at low IS, and qualitatively at high IS. This model explains the apparent anomaly that α-amylase will degrade a HEM sample with IS > 1.0. This work demonstrates the non-equivalence of DS and IS, and suggests that DS may be a useful characteristic for determining susceptibility to endo-amylase attack.

The significance of these findings or the use of HES as a blood plasma expander is considerable. Any hydroxyethyl substituent which does not affect enzymic attack, i.e. on a side-chain, is effectively redundant, and, in addition, since there is no known analogue of poly-(ethylene oxide) in biological systems, it is unlikely that the body can metabolize such an entity. This may explain the apparent toxicity of some samples of HES (Granath et al 1969). From a medical viewpoint, it is desirable that as little polymeric substitution as possible occurs, and it is not unlikely that further work will furnish a suitable reaction system.
A.


Badenhuizen, N.P. and R.N. Dutton (1956), Protoplasmatologia, 47, 156.


Calwell, C.G. and I. Martin (1957), U.S. Patent, 2 802 000.


Cerny, L.C., J.D. Granz and H. James (1968), Biorheology, 5, 103.


D.


E.


Erlander, S.R. (1961), Abstracts 140th Meeting Amer.Chem.Soc. 9D.


Evers, A.D. and L. Green (1972), Unpublished Observations.

F.


Flory, P.J. (1953), "Principles of Polymer Chemistry", Cornell.


French, D. (1957), Brewers Digest, 32, 50.


Freudenberg, K., E. Schaaf, G. Dumpt and T. Ploetz (1939), Naturwissenschaften, 27, 850.


G.


Greenwood, C.T. and D.J. Hourston (1967), Stärke, 19, 243.
Greenwood, C.T. and E.A. Milne (1968), Stärke, 20, 139.

H.
Haworth, W.N., S. Peat and E.J. Bourne (1944), Nature, 154, 236.
Hess, K. and E. Kranjc (1940a), Ber., 73, 976.
Hess, K. and E. Steurer (1940b), Ber., 73, 1076.
Higginbotham, R.S. (1949), Shirley Institute Memoirs, 23, 171.

225.


K.
Karper, R.E. (1933), J. Heredity, 24, 259.


Maquenne, L. and E. Roux (1905), Compte rend., 140, 1303.
Matheson, N.K. (1971), Phytochemistry, 10, 3213.

Parnell, F.R. (1921), J.Genet., 11, 209.


S.

230.
Sweeley, C. C., R. Bentley, M. Mokita and W. J. Wells (1963), J. Amer. Chem. Soc., 85, 2497.


Thivend, P., C. Mercier and A. Guilbot (1965), Stärke, 17, 278.

U.

V.
Van der Bij, J. R. (1967), Stärke, 19, 256.

Whelan, W.J. (1963), Stärke, 15, 247.


The Characterization of Starch and its Components

Part 2. *) The Semi-Micro Estimation of the Starch-Content of Cereal Grains and Related Materials

By W. Banks, C. T. Greenwood, and D. D. Muir, Edinburgh (Scotland)

A rapid, semi-micro method has been developed for the estimation of the amount of starch in cereal grains. The method is suitable for samples of flour in the range 7.5—20 mg, and hence the amount of starch in a single grain can be readily determined. The procedure — which is given in detail — involves first solubilizing the starch by hot aqueous calcium chloride, and then subjecting the extract to the concurrent action of α-amylase and amyloglucosidase. A quantitative determination of the resultant glucose is then made by the use of glucose oxidase. Relatively few manipulations are involved.

This technique is applicable to a wide range of cereals, including the high-amyllose-content, genetic mutants of maize. The method has a reproducibility of ± 1.5%.

The new procedure has been compared with methods based on polarimetry and perchloric acid extraction.

(Übersetzung siehe Seite 108; Résumé à la page 108)

Introduction

During our current studies of the biosynthesis of starch in the developing cereal plant, we have had the problem of determining the amount of starch in as little as a single grain of a cereal. For this purpose, we required a method of starch-estimation which satisfied the following criteria:

(i) the method could be used on the semi-micro scale, i.e. on 7.5—20 mg of cereal flour;
(ii) the method would be absolute, and not involve any arbitrary correction factors;
(iii) it would be accurate and easily reproducible;
(iv) the time required would be short, and the number of manipulations involved would be minimal;
(v) the method would be applicable to all types of cereals, particularly the amylomaizes and other starches of high amylose-content.

Many methods for the estimation of starch in cereals have been described in the literature and they have been extensively reviewed [1, 2]. However, none of these techniques met all our requirements; neither did the two procedures described in the “Official Methods of Analysis of the Association of Official Agricultural Chemists” [3]. The latter procedures involve either polarimetry of a calcium chloride extract of the cereal flour [4], or a perchloric acid extraction followed by precipitation of the starch as the iodine-complex, and its subsequent hydrolysis to glucose and the estimation of reducing sugar [5]. In this connection, it should be noted that in an earlier paper [6], we have shown that a careful physical method of extraction of the whole starch granules is more effective for amylo-maize starches than the perchloric acid extraction method [3, 5].

Recently, starch-estimation procedures have been described [7—9] in which the starch in cereals has been gelatinized, and then subjected to an enzymic assay, but again these methods did not meet all our requirements.

In the work described in this paper, we have modified our enzymic technique for the semi-micro estimation of the concentration of starch and its components in solution [10] to deal with the starch present in the cereal grain. We have found that the method described by Clendenning [4] for solubilizing wheat starch from the grain is extremely effective, and can be readily adapted to the semi-micro scale for a wide variety of cereals, including amylomaize.

We have then established conditions whereby the concurrent action of α-amylase and amyloglucosidase results in a rapid and complete conversion of the solubilized starch to
glucose, which is then estimated by glucose oxidase using a procedure similar to that of Fleming and Pegler [11, 12]. This new assay procedure is detailed below. Results obtained by this method for a variety of cereal grains are compared with those from the polarimetric and perchloric acid methods [3—5].

**Experimental Methods**

**Assay Procedure**

**Reagents**

Analytical grade reagents were used throughout.

(i) 80% aqueous ethanol.

(ii) Aqueous calcium chloride.

(iii) Cetyl alcohol.

(iv) Acetate buffer (0.1 M; pH 4.8).

(v) Potassium hydroxide (0.05 M).

(vi) Amyloglucosidase solution.

The enzyme (α-1:4-glucan glucohydrolase) was kindly supplied by Dr. I. D. Fleming, Glaxo Research Ltd., Greenford, Middlesex. It was dissolved in water (1 mg per 1 ml) to yield a solution of activity = 14 units per 1 ml (1 unit = 1 micromole of glucose liberated per minute from soluble starch at pH 4.6 and 37 °C). The enzyme solution was stored at 2 °C.

(vii) Alpha-amylase solution.

A volume of suspension containing 0.5 mg of crystalline porcine pancreatic α-amylase (Sigma Chemical Co.; manufacturer's reputed activity = 500 units/mg) was diluted to 1 ml with distilled water. The enzyme solution was stored at 2 °C.

(viii) Glucose oxidase-peroxidase-chromogen mixture.

Horse-radish peroxidase (Boehringer Corporation (U. K.) Ltd.; 3 mg) glucose oxidase (Sigma Chemical Company; 40 mg), and o-dianisidine dihydrochloride (20 mg) were dissolved in tris-buffer (100 ml) [11]. The mixture was stored in a brown bottle at 2 °C.

(ix) Hydrochloric acid (7 M).

Concentrated acid (150 ml) was diluted to 250 ml.

**Sample Preparation**

The grains of cereal were ground in an agate mortar and pestle to pass through at least a “Number 40 Mesh” (i.e. 40 meshes per one inch) sieve. The flour was then dried at 70 °C in a thermostat vacuum oven (Townson & Mercer) for 24 hr.

**Extraction to Remove Soluble Saccharides**

Dried flour (7.5—20.0 mg) was accurately weighed (to 0.05 mg) into a graduated, hard-glass centrifuge tube (10 ml). The sides of the centrifuge tube were carefully washed down with cold ethanol (ca 3 ml) and the wet flour compacted by centrifugation. The ethanol-wet residue was then extracted three times with hot 80% aqueous ethanol (50—60 °C; 5 ml) to remove soluble saccharides.

**Calcium Chloride Extraction**

Calcium chloride solution (1 ml) was then added to the centrifuged residue, and the mixture was stirred carefully with a glass rod to give a uniform suspension. After adding a few porous chips for even boiling and a little cetyl alcohol to prevent foaming, the centrifuge tubes were immersed in an oil-bath at 130—135 °C for a period of 15 min from the time that the mixture started to boil. During this boiling time, material around the sides of the tubes was periodically scraped down with the glass rod, and a constant volume was maintained by the addition, dropwise, of distilled water.

**Enzymic Digestion of Extract**

After cooling in the air to 20 °C, potassium hydroxide (a pre-determined volume, ca 2.4 ml) and acetate buffer (4 ml) were added to bring the pH of the mixture to 4.6—4.8. Enzyme solutions (0.5 ml amyloglucosidase solution plus 0.1 ml α-amylase solution) were then added, and after gentle stirring, the tubes were immersed in a water-bath at 47—48 °C for a period of 3 hr with occasional stirring. After completion of the enzymic digestion in this time, the contents of the centrifuge tube were carefully transferred with copious quantities of distilled water into a 250 ml graduated flask, and made up to volume. The solution was then filtered through Whatman No. 1 filter paper, the first 30 ml of filtrate being discarded, and 20 ml portions were collected for glucose oxidase assay.

**Glucose Assay**

Aliquots (1 ml; to contain 15—50 microgram of glucose) of the filtered digest were pipetted into glass-stoppered, boiling tubes (14 ml; preheated to 35 °C). Glucose oxidase-peroxidase-chromogen mixture (2 ml) was added, and the tubes were incubated for 80 min at 35 °C. Hydrochloric acid (4 ml) was then pipetted into the tubes, the contents were thoroughly mixed by shaking, and the optical density of the resultant chromophoric complex was measured in a Hilger Spekker spectrophotometer (1 cm cells; Number 605 filter, λ = ca 5500 Å). The amount of glucose present was determined from the optical density by means of a previously constructed calibration curve.

**Calculation**

\[
\% \text{ of starch} = \frac{(\text{Optical density} \times \text{calibration factor (in } \mu \text{g}) \times \text{dilution factor} \times 0.90 \times 100)}{\text{sample weight (mg)} \times 10^6}
\]
or since dilution is 250-fold:

\[
\% \text{ of starch} = \frac{\text{Optical density} \times \text{calibration factor (in } \mu \text{g}) \times 22.5}{\text{sample weight (mg)}}
\]

**Control Experiments**

It has been well-established in the literature [8, 13, 14] that the size of particles in the cereal flour is critical for efficient extraction. We have confirmed that grinding has to be continued until the grain will pass a number 40 -- or finer -- screen.

Preliminary experiments established that 130 °C was the optimum temperature for the extraction with the calcium chloride. Below this temperature extraction was often incomplete as shown by microscopic examination of the iodine-stained, cereal residue, whilst above 130 °C, it was found to be difficult to avoid spouting from the tube, with consequent loss of material.

In agreement with Clendenning's results [4], a pH of 2 for the calcium chloride solution was found to be optimal. Above pH 3, the efficiency of the extraction was variable, and sometimes incomplete. Below pH 2.0, no adverse effects were observed, but there were difficulties in buffering the solution procedure varied from 99.3 to 101.8 °/o, thus demonstrating that no destruction of glucose occurred.

It was then established that the optimum extraction time at 130 °C with the calcium chloride at a pH of 2 was 15 -- 20 min. Recovery of glucose (Kerrfoot; biochemical reagent grade; see ref. [10]) when subjected to the extraction procedure varied from 99.3 to 101.8 °/o, thus demonstrating that no destruction of glucose occurred.

In order to establish the optimum time of amylolysis, samples of barley grain were extracted as above, and they were then incubated with the enzyme mixture for 30, 60, 90, 120, 150, 180, 210 and 240 min. The resultant percentages of starch were:

37.8, 42.3, 58.9, 65.2, 64.7, 66.0, 65.6, and 65.9, respectively. An incubation time of 180 min was chosen for routine assays.

Recovery of maltose (Kerrfoot; biochemical reagent grade; see ref. [10]) was 99.6 to 99.7 °/o using the enzyme mixture, whilst the same recovery was found for soluble potato starch (B. D. H.; see ref. [10], and also for an amylopectin assayed both by the above technique and that involving acid hydrolysis, and alkaline ferricyanide determination of reducing power [10].

**Comparison with Established Techniques**

The semi-micro enzymic assay was compared with the 2.5g-scale calcium chloride/polarimetric method of Clendenning [4,3] and the 200 mg-scale perchloric acid/iodine complex/reducing sugar method of Hassid and Neufeld [5,3].

A Perkin-Elmer Automatic '141' Polarimeter (10 cm cell; 20 °C; \( \lambda = 5890 \) Å) was used to measure the rotation of the calcium chloride solutions. Using our enzymic assay to measure the starch concentrations, the specific rotations, \([\alpha]_{D}^{20}\) for some pure, isolated starches [6] were:

barley = + 200°; regular maize = + 204°; potato = + 204°; amylo maize (AH70) = + 200°.

These experimental values were used for calculations in the polarimetric method.

In the Hassid and Neufeld technique, liberated reducing sugar was estimated by the alkaline ferricyanide method [10].

**Results and Discussion**

We have determined the starch-content of a variety of cereal grains by this semi-micro method. Typical results are shown in Table 1; individual results show a reproducibility of ± 1.5 °/o.

Table 1. Typical Results for the Starch-Content (%) of Various Cereal Grains obtained by the Semi-micro Method; and a Comparison with Other Assay Procedures.

<table>
<thead>
<tr>
<th>Cereal grain</th>
<th>Semi-micro ( \text{[} [\alpha]_{D}^{20}\text{]} )</th>
<th>([\alpha]_{D}^{20})</th>
<th>([\alpha]_{D}^{20})</th>
<th>HCIO4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barley</td>
<td>59.3</td>
<td>65.2</td>
<td>60.2</td>
<td>59.5</td>
</tr>
<tr>
<td>Regular maize</td>
<td>65.5</td>
<td>69.3</td>
<td>65.8</td>
<td>66.7</td>
</tr>
<tr>
<td>Wheat</td>
<td>64.0</td>
<td>67.0</td>
<td>63.8</td>
<td>63.3</td>
</tr>
<tr>
<td>Amylo maize (70% amylose)</td>
<td>60.0</td>
<td>58.6</td>
<td>58.0</td>
<td>n. a.</td>
</tr>
</tbody>
</table>

1) Enzymic assay method described in Text.
2) Clendenning method [3, 4] -- polarimetry on aqueous CaCl2 extract.
3) Solutions as in 2, except that concentrations of the solubilized starch were determined enzymically.
4) Hassid and Neufeld method [3, 5].
5) n. a. = method not applicable; see ref. [6].

A comparison with established assay techniques was also carried out, and the results obtained by the polarimetric- and perchloric acid-extraction methods are also included in the Table.

It can be seen that the perchloric acid method is not applicable to amylo maize starch [6], but that otherwise the agreement between this method and the semi-micro method is good, notwithstanding the tedious and time-consuming nature of the perchloric acid assay.

In contrast, the results from the polarimetric method were nearly always appreciably higher than those from the semi-micro assay. However, this discrepancy was almost entirely eliminated when the starch-concentration in the polarimetric assay were estimated enzymically. We are of the opinion, therefore, that the polarimetric method is assayung dextro-rotatory, non-starch material from certain varieties of cereals.

We would suggest that the semi-micro assay developed here can be used routinely to estimate accurately the amount of starch in any cereal product.
Acknowledgements

This work was supported in part by a Grant from the U.S. Department of Agriculture under P.L. 480. One of us (D. D. Muir) also thanks the Science Research Council for a maintenance grant.

Zusammenfassung


Das neue Verfahren wird mit Methoden verglichen, die auf der Polarimetrie und der Perchlorsäureextraktion beruhen.

Résumé

Caractérisation de l’amidon et de ses constituants. II. Un semi-micro dosage de la teneur en amidon des grains de céréales et des produits similaires. Une semi-micro méthode rapide pour la détermination de la teneur en amidon dans les grains de céréales a été mise au point. La méthode est appropriée pour une quantité d’échantillon de farine comprise entre 7,5 et 20 mg, en conséquence la teneur en amidon d’un seul grain peut être facilement dosée. Le mode opératoire — qui est décrit en détail — consiste d’abord à solubiliser l’amidon dans du chlorure de calcium à chaud, suivi par l’action combinée de l’α-amylase et de l’amylglucosidase sur l’extrait. Le glucose produit est alors dosé quantitativement au moyen de la glucose- oxydase.

La technique implique relativement peu de manipulations, elle est applicable à une large gamme de céréales, les mutants génétiques de maïs à haute teneur en amylose inclus. La reproductibilité de la méthode est de ± 1,5%.

Ce nouveau mode opératoire a été comparé avec ceux basés sur la polarimétrie et l’extraction à l’acide perchlorique.

References


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(Received: March 2, 1970).
The Characterization of Starch and Its Components

Part 3.*) The Technique of Semi-Micro, Differential, Potentiometric, Iodine Titration, and the Factors Affecting It

By W. Banks, C. T. Greenwood, and D. D. Muir, Edinburgh (Scotland)

An improved apparatus for the semi-micro, differential, potentiometric titration of starch and its components with iodine is described. The necessary experimental manipulations are given in detail. The factors affecting the measurement — the amount of dimethylsulphoxide present, the buffer concentration, the method of dissolving the starch, the defatting procedure used on the starch, and the temperature of measurement — are critically examined.

The new technique results in values of iodine binding capacity for starches which are 5—15% higher than previous measurements. The significance of this finding is discussed.

(Zusammenfassung siehe Seite 124; Résumé à la page 124)

Introduction

The interaction of starch and its component amyllose and amyllopectin fractions with iodine is perhaps the most characteristic property of these polysaccharides. On the basis of this interaction, various methods have been devised for determining the amyllose-content of starches. The methods, which have been reviewed in detail [1, 2], can be classified into techniques depending on either (1) potentiometric titration of the starch with iodine, or (2) a similar amperometric titration, or (3) spectrophotometric determination of the intensity of the colour of the iodine-complex.

Experience has shown that although spectrophotometric methods provide a convenient and rapid method of analysis, they are not sufficiently sensitive for accurate measurements on the semi-micro scale, particularly for those on the amyllopectin component. Amperometric methods also appear to have the same disadvantages, and there is little doubt that the most satisfactory method of measuring the iodine binding characteristics of a starch, or its components, is by potentiometric titration. However, as was first emphasized by Gilbert and Marriott [3], the accuracy of the direct titration method introduced by Bates, French, and Rundle [1, 2, 4] can be improved by using a differential, potentiometric technique.

We have found that the semi-micro, differential, potentiometric, iodine titration method is an essential tool in starch chemistry. Our earlier apparatus [5] utilized a laboratory-built electrometer to deal with the high impedance of the original circuit [3], and this complication obviously restricted the general applicability of our technique. The current ready-availability of commercial, electronic equipment with the necessary specifications has now made the setting-up of the detector for a differential titration very much simpler. Using a digital voltmeter, we have now modified our apparatus; it is now easier to use, and its stability and sensitivity have been increased.

In this paper, the new apparatus is described together with a detailed account of the experimental method. Factors which affect the measurement of the apparent iodine binding capacity of a starch are also critically discussed.

General Conditions for the Analysis

The extent to which a completely-dispersed, pure starch or starch component binds iodine depends on (1) the concentration of iodide and other ions present, (2) the pH, and (3) the temperature. For the purpose of routine analysis, these variables have to be fixed.

Iodide ion concentration. We use a molarity of 0.01 for the iodide concentration in the supporting electrolyte, for, in agreement with the results of Kuge and Ono [6], we have found that maximum binding occurs at this molarity.

pH. In order to repress hydrolysis of the iodine, the titration media has to be kept slightly acidic: phosphate buffer, pH 5.8, is used in a fixed amount.

Temperature. To obtain maximum information from the results of the iodine titration, the measurement is best carried out both at 2°C and 20°C [7]. Elsewhere, we shall discuss in detail the effect of these, and other variables, on complex-formation.

Concentration of Starch

The optimum amount of polysaccharide (per 840 ml) in our semi-micro technique is:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amyllose</td>
<td>3—6 mg</td>
</tr>
<tr>
<td>Amylopectin</td>
<td>12—24 mg</td>
</tr>
<tr>
<td>Starch</td>
<td>5—25 mg</td>
</tr>
</tbody>
</table>

Depending on type.

Principle of the Method

The differential technique involves two iodine/iodide half-cells, each with a bright platinum electrode, which are connected by a liquid bridge. The test half-cell, r, contains the starch sample in the buffered iodide solution; and the control half-cell, c, contains buffered iodide at an identical concentration.

This experimental set-up results in an iodine concentration cell without transference, i.e.

\[ I_r[(a_{12})c] = I'(a_{1-}) = L_c[(a_{12})c] \]

where a represents the activity. For one electrode, the
potential, \( E \), is given by 

\[
E = E_0 - \frac{RT}{2F} \ln \left( \frac{[a_{12}]_c}{[a_{12}]_t} \right)
\]

where \( R \), \( T \), and \( F \) are the Gas constant, temperature, and Faraday constant, respectively. The overall potential of the cell is thus:

\[
E = \frac{-RT}{2F} \ln \left( \frac{[a_{12}]_c}{[a_{12}]_t} \right)
\]

The apparatus is used as a null instrument for when there is no potential between the electrodes, then

\[
(a_{12})_c = (a_{12})_t
\]

assuming the activity coefficient is unity.

In use, iodine is added to the test half-cell, and then the potential produced is cancelled out by addition of iodine to the control half-cell. At this point of null potential, the amount of iodine added to the control cell corresponds to the concentration of free molecular iodine in the test solution, whilst the amount of iodine bound by the starch is given by the difference between this amount and the original amount of iodine added.

Titration curves of "bound iodine" versus "free iodine" are then obtained directly.

**Experimental Methods**

**Reagents**

Analytical-grade, inorganic reagents were used throughout.

(i) Stock potassium iodide solution (0.1 M)
(ii) Stock iodine (0.05 M) in potassium iodide (0.1 M)
(iii) Arsenious oxide (0.005 M)
(iv) Starch indicator solution
(v) Sodium bicarbonate solution (saturated)
(vi) Iodine solution for titration (0.005 M I₂ in 0.01 M KI).

Fresh iodine solution was prepared daily by tenfold dilution of the stock 0.05 M I₂/0.1 M KI with distilled water, and this solution was kept in a black, stoppered bottle. The dilute iodine solution was standardised regularly by means of arsenious oxide (0.005 M), the titration being carried out in the presence of sodium bicarbonate, using starch solution as indicator.

(vii) Phosphate buffer (0.2 M KH₂PO₄ and K₂HPO₄; pH, 5.8)
(viii) Amyloglucosidase solution [8].

The enzyme (a-1:4 glucan glucohydrolase) was kindly supplied by Dr. I. D. Fleming, Glaxo Research Ltd., Greenford, Middlesex, England. It was dissolved in water (1 mg per 1 ml) to yield a solution of activity = 14 units per 1 ml (1 unit = 1 micromole of glucose liberated per minute from soluble starch at pH 4.6 and 37 °C). The enzyme solution was stored at 2 °C.

(ix) Glucose oxidase-peroxidase-chromogen mixture [8].

Horse-radish peroxidase (Boehringer Corporation (U.K.) Ltd.; 3 mg), glucose oxidase (Sigma Chemical Company; 40 mg), and o-dianisidine dihydrochloride (20 mg) were dissolved in tris-buffer (100 ml). The mixture was stored in a brown bottle at 2 °C.

(x) Hydrochloric acid (7 M)

Concentrated acid (150 ml) was diluted to 250 ml.

**Sample Preparation**

Hydrous starch (i.e. starch granules which have been stored under water to equilibrium) was dissolved in dimethyl-sulphoxide (DMSO) to give a 1—4 % solution, depending on the character of the starch [9]. The criterion of complete solution being achieved was that on centrifugation at 2 \( \times 10^3 \) g for 20 min, no gel should be obtained. The starch was precipitated with ethanol (3 vol), a small amount of saturated sodium chloride solution being added to induce flocculation in difficult cases. After centrifugation, the starch was washed repeatedly with ethanol to ensure complete removal of DMSO, and dried overnight in vacuo at 65 °C. The dry, non-granular starch was then dissolved in DMSO, and this solution used in the iodine titration as described below.

**Apparatus**

The apparatus is shown diagrammatically in Figure 1. Each half-cell consists of a 1 litre round-bottom flask, equipped with 4 ground glass joints which accommodate (a) the stirring gland, (b) the electrode, (c) the liquid bridge by which the two half-cells are connected, and (d) the "Agla" syringe (Wellcome Reagents Ltd., Beckenham, Kent, England), by which iodine is added.

The stirrers, which operate continuously during the titration, are driven by a pulley system.

The liquid-bridge consists of a U-tube, having a middle arm which can be sealed by means of a glass stopcock. This simple system eliminated junction potentials and proved
particularly effective. (A frittered glass disc in the U-tube was not necessary.) The bridge (internal volume ca. 2 ml) was filled by opening the stopcock on the side-arm, applying sudden suction by means of a rubber bulb, and closing the stopcock as soon as the bridge was filled. (By rapid filling of the bridge in this manner, air bubbles are entirely eliminated.)

The use of coloured solutions has shown that the equalization of liquid levels in the two half-cells may be carried out sufficiently accurately by arranging them both to be identical with the thermostat-bath liquid-surface; no siphoning was observed on filling the connecting bridge.

**Electrodes**

Electrodes are constructed of platinum foil (1 inch × 1 inch × 0.004 inch) welded to platinum wire, sealed through soft glass, and silver-soldered to a silver rod (see fig. 2). All the joints in the electrodes are strain-free, and should be carefully tested to ensure the elimination of small, random junction potentials which can give rise to detector instability. The electrodes are connected directly to the detector. The electrodes were cleaned by immersion in concentrated nitric acid and repeated washing with first water and then ethanol, until no potential (less than 0.01 mV) is observed when the two electrodes are placed in the same iodine/iodide solution.

**Thermostat**

The cells are supported in an ethylene glycol/water thermostat bath. Temperature control at 20 °C ± 0.05 °C is maintained by a "Circotherm" thermostat control and circulating water unit. Control at 2 °C ± 0.05 °C is achieved by use of a "Grant" refrigerator unit (running continuously) in conjunction with the appropriately-set "Circotherm".

Lagging the copper bath with a 2 inch thick layer of polyurethane foam effectively prevents any condensation problems at low temperatures.

**Electronic Detector**

A digital voltmeter can be used directly to deal with the original high impedance circuit [3, 5], or the low impedance set-up described above. We have found that a "Solartron" digital voltmeter Model LM 1450 (Solartron Electronic Group Ltd., Farnborough, Hampshire, England) is ideal. Readings can be made to 0.01 mV on the 20 mV range. The instrument is used on automatic display (50 readings per second) with the 60 dB filter in use (60 dB attenuation of series mode interference from 50 c/s to 120 c/s). Any instrument with equivalent specifications would be suitable for the titration.

**Titration Procedure**

Electrolyte solution was prepared by diluting 203 ml 0.1 M potassium iodide and 20 ml 0.2 M phosphate buffer (pH 5.8) to 2 l with distilled water. 830 ml of this electrolyte solution was then added to each half-cell and the cells placed in the thermostat so that the liquid level in each was the same as that in the thermostat bath. The liquid bridge was then placed between the half-cells as described above and filled; the electrodes were then placed in the cells; and stirring was started and maintained, at a rate sufficient to give rapid mixing without causing undue turbulence.

The stock solution of the sample to be titrated was prepared by diluting the polysaccharide/DMSO solution (1 ml) with distilled water (11 ml). A blank was prepared in a similar manner, omitting only the polysaccharide. Blank and sample solutions (10.0 ml) were added to their respective half-cells by micrometer syringes. Thirty minutes were allowed for temperature equilibrium to be attained before commencing the titration.

An aliquot (usually 5 "Agla" units) of iodine solution (0.005 M, in 0.01 M KI) was added to the sample half-cell by means of an "Agla" micrometer syringe, and five minutes allowed for equilibrium to be achieved. The iodine solution was then slowly added (again by micrometer syringe) to the blank half-cell until zero potential (less than 0.01 mV) was indicated on the detector. At this point, the concentration of molecular iodine in the two half-cells is equal, and hence the amount bound by the polysaccharide is given by the difference in the volumes added to both sides. The addition and balancing process was repeated until the complete curve of iodine bound as a function of free iodine was obtained.

**Determination of Polysaccharide Concentration**

Immediately after adding the aqueous DMSO/polysaccharide solution to the sample half-cell, an aliquot (1.0 ml) of the remaining stock solution was taken for determination of concentration. This was achieved by hydrolysis to glucose using amyloglucosidase (starch solution so diluted that the polysaccharide concentration was 15–40 g/ml), and the glucose estimated by the glucose oxidase/peroxidase technique described previously [8].

It should be noted that the aqueous DMSO/polysaccharide solution is to be used for both iodine titration and determination of concentration immediately upon its preparation. Whilst this precaution is unnecessary for the majority of starches, it is mandatory in the case of amylomaize starch, and its components, because incipient retro-

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Small random potentials may be observed before commencement of iodine addition which are not significant as the electrometer is effectively on open circuit.

$^{50}$ "Agla" units = 1.00 ml.
gradation, which affects both the iodine uptake, and the concentration determination, occurs if the solution is allowed to stand for several hours before use.

**Calculation**

If $I_t$ and $I_c$ are the amounts of iodine (in "Agla" units) added to the test and control half-cells, respectively, the amount of bound iodine, $I_b = I_t - I_c$.

The values of total bound iodine $= \Sigma I_b$, and the total free iodine $= \Sigma I_c$, are evaluated consecutively.

$\Sigma I_b$ is then converted to the weight of iodine and divided by the weight of starch to obtain the milligrammes of iodine bound per 100 mg of starch, i.e.

$$\text{weight of iodine} = \frac{\Sigma I_b \times \text{normality} \times 254 \times 10^3}{50 \times 2 \times 10^3} \text{ mg}$$

$$\text{weight of starch} = \text{Calibration factor (C.F.)} \times \text{optical density (O.D.)} \times \text{dilution factor} \times \text{volume added} \times 0.9 = W_A$$

Thus the percentage of iodine bound $= 100 \frac{W_{I_b}}{W_A}$.

$\Sigma I_c$ is converted to an iodine concentration $[I_c]$ (moles per litre), i.e.

$$[I_c] = \frac{(\Sigma I_c \times \text{normality})}{50 \times 2 \times 840} \text{ moles per litre}.$$  

$100 \frac{W_{I_b}}{W_A}$ is then graphed against $[I_c]$.

**Results and Discussion**

**The Definition of Iodine Binding Capacity**

Typical titration curves are shown in Figure 3 — in this case, for potato starch, and its component amylose and amyllopectin fractions. We define the iodine binding capacity as the weight (in mg) of iodine bound by 100 mg of the polysaccharide at zero free-iodine concentration. Thus to obtain the iodine binding capacity the results obtained over a finite range of free-iodine concentrations have to be extrapolated to zero concentration. The extrapolation is shown in Figure 3 by the broken lines to obtain the values of iodine binding capacity described by the $X$ values.

The extrapolation procedure has no firm theoretical foundation; other workers [1, 2, 4] define iodine binding capacity as the point at which the extrapolated maximum and minimum slopes meet. In Figure 3, this point, denoted by $Y$, is also shown. This procedure, however, is equally as arbitrary as our own. The two extrapolation techniques are based on different concepts. We believe that the uptake of iodine other than in helices (this secondary uptake is probably due to a surface absorption effect) occurs at all concentrations of free-iodine, thus justifying extrapolation to zero free-iodine concentration. The alternative viewpoint is that the secondary process occurs subsequent to the helices being occupied; accepting this argument leads to the second method of extrapolation.

In most cases, the difference between the two methods of extrapolation is quite small, and therefore negligible. This is not the case with amylopoize staches, however, where the limiting slope is rather large, leading to an appreciable difference in the two techniques.

**The Equilibrium Time for Potentiometric Titration**

The direct visualization of potential in the new experimental set-up has enabled absolute observations to be made, for the first time, of the time necessary to form the iodine-complex. These results will be discussed elsewhere, but the salient feature of importance here is that — after physical mixing has been achieved — equilibrium is not instantaneous. This result implies that the starch helix is not preformed in aqueous solution, and that some type of cooperative phenomenon is occurring.

The effect is most pronounced in titrations with amylose, and here both the initial portion and the rising portion of the binding isotherm are time-dependent. However, the final, linear portion of the binding curve is independent of time. Similar effects are found for starches, and these results will be discussed elsewhere.

Reproducible results are obtained by standardization of the titration procedure, i.e. allowing 5 min for equilibrium for each point on the curve.

**Factors Influencing Iodine Binding Capacity**

(1) The amount of dimethylsulphoxide. Mature potato starch (var. Pentland Crown) was dispersed in 0.2 M KOH at 2°C with shaking, neutralised to pH 5.8 with 0.15 M phosphoric acid, and an aliquot (10.0 ml) taken for iodine titration. The experiment was repeated twice, adding (a) 1.0 ml DMSO, and (b) 2.0 ml DMSO, to each half-cell im-
It should be stressed, however, that there is some slight interaction between the iodine and the DMSO which leads to erroneous results if there is a difference in DMSO content between the two half-cells. For this reason, it is essential that the concentration of DMSO in the sample solution and the blank is identical.

(2) Variation in buffer concentration. The effect on the iodine binding capacity of the mature potato starch of varying the concentration of phosphate buffer (pH 5.8) in the half-cells is shown in Table 1.

Table 1. The Iodine Binding Capacity of Potato Starch (var. Pentland Crown) as a Function of Phosphate Buffer Concentration.

<table>
<thead>
<tr>
<th>Phosphate Buffer Concentration</th>
<th>Iodine Binding Capacity (mg I₂ bound/100 mg starch)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.95 X 10⁻⁴ M</td>
<td>4.58</td>
</tr>
<tr>
<td>1.98 X 10⁻³ M</td>
<td>4.54</td>
</tr>
<tr>
<td>4.95 X 10⁻³ M</td>
<td>4.50</td>
</tr>
<tr>
<td>9.90 X 10⁻³ M</td>
<td>4.37</td>
</tr>
</tbody>
</table>

It is obvious that buffer concentration has little effect on the iodine binding capacity — increasing the former 20-fold, decreased the latter by only 4.5%. Hence small differences in concentration between various batches of buffer would not cause any measurable change in the iodine binding capacity. However, it was noted that as the buffer concentration increased, the polysaccharide bound iodine at progressively lower concentrations of free iodine. Kuge and Oto [6] have reported a similar phenomenon, whilst carrying out spectrophotometric measurements on the amylose-iodine complex.

(3) Variation in the procedure for dissolving starch. We have found that the most satisfactory general method of dissolving starch is to use dimethylsulphoxide. In this solvent, starches of high amylose-content give gel-free solutions within 30 min (using hydrous starch granules, and gentle stirring), but longer periods are required for the complete dissolution of waxy starches [9]. The time required is also a function of the type of starch — cereal starches dissolve more easily in DMSO than do tuber starches. We investigated the dependence of the iodine binding capacity of mature potato starch on the period the granules had been in DMSO solution. The results are shown in Table 2.

Table 2. The Iodine Binding Capacity of Potato Starch (var. Pentland Crown) as a Function of the Time the Granules Have Been Treated with Dimethylsulphoxide.

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Iodine Binding Capacity (mg I₂ bound/100 mg starch)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.5</td>
<td>4.38</td>
</tr>
<tr>
<td>6</td>
<td>4.47</td>
</tr>
<tr>
<td>7</td>
<td>4.58</td>
</tr>
<tr>
<td>10.5</td>
<td>4.58</td>
</tr>
<tr>
<td>120</td>
<td>4.52</td>
</tr>
<tr>
<td>240</td>
<td>4.54</td>
</tr>
</tbody>
</table>

From the results, it is obvious that complete solution of the potato starch granule is achieved in 6—7 hrs. Moreover, the starch/DMSO solution appears to be stable over prolonged time intervals. The iodine titration of the potato starch was repeated on the DMSO solution which had been stored for approximately 6 months; the resultant iodine binding capacity was 4.55 mg iodine/100 mg starch.

In the course of this investigation of iodine uptake by mature potato starch, we noted that the results showed a significant, and reproducible, increase (some 5%) in the value of the iodine binding capacity compared to that obtained by our previous technique. In the latter, the starch granules were dissolved in caustic alkali, and the solution neutralised with phosphoric acid prior to titration [5]. We therefore investigated the effect of varying the method of dispersion of the granules on the iodine binding capacity of potato starch. The results are shown in Table 3.


<table>
<thead>
<tr>
<th>Reagent for Dispersion of Granules</th>
<th>Iodine Binding Capacity (mg I₂ bound/100 mg starch)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>4.55</td>
</tr>
<tr>
<td>0.2 M KOH</td>
<td>4.27</td>
</tr>
<tr>
<td>0.5 M KOH</td>
<td>4.41</td>
</tr>
</tbody>
</table>

The highest iodine binding capacity was achieved when DMSO was used to disperse the granules. Moreover, on repeating the experiment, using a number of DMSO/starch solutions, the reproducibility of the results was high (ca ± 1% of the mean iodine binding capacity). The use of caustic alkali as solvent led to a considerable variability (± 3%) in the results obtained. In agreement with the work of Adkins and Greenwood [10] on amylomaize starch, the use of higher concentrations of alkali obviously leads to enhanced iodine binding capacities. Similar results were obtained using maize starch.

Adkins, et al. [9] have reported that to completely destroy the structure of the mature cereal starch granule, it is necessary to precipitate the starch from DMSO solution, and then redissolve it in DMSO. The effect of repeated dissolution of various cereal starches (defatted by the procedure of Schoch [11]) on the measured iodine binding capacity is shown in Table 4.

There is a tendency for the iodine binding capacity to increase as a result of the second precipitation from DMSO. However, the increase is so slight as not to justify the labour involved. Also, in some cases it was found that the second precipitation led to a decrease in the iodine affinity; this decrease was always found to be due to incomplete precipitation of the starch on the second occasion. It was found that the addition of a few drops of saturated sodium chloride solution to the precipitation mixture facilitated flocculation and prevented incomplete precipitation of the starch.

DMSO is also a better solvent than 0.2 m KOH for non-granular starch. (“Non-granular starch” is the term we
The DMSO/ethanol supernatant liquors remaining after the removal of the precipitated starch were distilled under reduced pressure to remove the ethanol. The residual DMSO was then added to the corresponding starch/DMSO solution, and the iodine binding capacities of the mixtures measured. In all cases, the DMSO extract was found to have a markedly depressant effect on the iodine binding capacity. This, of course, confirms that a DMSO/ethanol-soluble material was responsible for the low iodine binding capacities obtained on using untreated starches. This material is almost certainly a fatty-substance.

(4) The defatting procedure. It has long been recognised that fat preferentially complexes with amylose, and thus prevents the binding of iodine to the polysaccharide [11]. The usual method of removing fat is to extract the starch with hydrophilic fat-solvents such as 85% aqueous methanol, or 80% aqueous dioxane [11]. These methods may be efficient, but they are time-consuming, and are difficult to apply to small quantities (<20 mg) of starch.

As we have pointed out elsewhere [12], both DMSO and ethanol are solvents for fats, and hence our dissolution and precipitation technique should provide an efficient (and rapid) method for removing fat from starches. The results in Table 5 show that this is the case.

The DMSO/ethanol supernatant liquors remaining after the removal of the precipitated starch were distilled under reduced pressure to remove the ethanol. The residual DMSO was then added to the corresponding starch/DMSO solution, and the iodine binding capacities of the mixtures measured. In all cases, the DMSO extract was found to have a markedly depressant effect on the iodine binding capacity. This, of course, confirms that a DMSO/ethanol-soluble material was responsible for the low iodine binding capacities obtained on using untreated starches. This material is almost certainly a fatty-substance.

(5) The effect of temperature. In our earlier studies of the effect of temperature on the iodine binding capacity of starch and its components [7], we reported that for normal amylose the change in the amount of bound-iodine was small in comparison to that which occurred for the degraded polysaccharide.

With our improved experimental set-up, we have been able to measure accurately the variation with temperature of the iodine binding capacity of normal amylose and starch. This has enabled us to carry out quantitative calculations.

Table 6 shows the iodine binding capacities of amylose and a number of pea starches measured at 1.5° and 20.5°C.

Table 5. The Effect of Various Defatting Procedures on the Iodine Binding Capacity of Cereal Starches.

<table>
<thead>
<tr>
<th>Procedure</th>
<th>I</th>
<th>II</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Procedure</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I = Defatted starch dissolved in DMSO, precipitated with ethanol, dried and re-dissolved in DMSO;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>II = Defatted starch dissolved in DMSO, precipitated with ethanol, dissolved in DMSO, precipitated with ethanol, dried and re-dissolved in DMSO.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 6. The Iodine Binding Capacity of Amylose and Various Pea Starches Measured at Two Temperatures, and the Calculated Amylose Contents of the Starches.

<table>
<thead>
<tr>
<th>Sample</th>
<th>1.5°C</th>
<th>20.5°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>I.B.C.</td>
<td>%Amyl</td>
<td>%Amyl</td>
</tr>
<tr>
<td>Amylose</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pea (var. Early Onward)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pea (var. Little Marvel)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pea (var. Superb)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pea (var. Dwarf Defiance)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 I.B.C. = Iodine binding capacity (mg iodine bound/100 mg polysaccharide).

The amylose contents of the starches have been calculated at both temperatures from:

\[
\%\text{amylose} = \frac{\text{iodine binding capacity of starch at } T\,^\circ\text{C}}{\text{iodine binding capacity of amylose at } T\,^\circ\text{C} \times 100}
\]

The iodine binding capacity of amylose increases with decreasing temperature; but so also do the iodine binding capacities of the various starches. Thus, when these values are used to calculate the percentage of amylose, using the relevant value for the iodine binding capacity of amylose, there is excellent agreement at both temperatures.

However, amylomaize starch is again an exception for its amylose content apparently increases with decreasing temperatures. By analogy with studies carried out on synthetic, amylose oligomers [13], we have suggested [13] that this type of behaviour provides a simple diagnostic test for the presence of short-chain material (30> chain-length <150 glucose residues) in starch, or its fractions.

(6) Potential oxidizing action of iodine. The reversibility and extent of the iodine binding would be altered if the iodine were oxidizing or degrading the polysaccharide. It is thought that the experimental conditions preclude this action for the titration of an equivalent weight of glucose directly, or the addition of glucose to an equilibrium amylose-iodine system, did not cause any change in observed potential.

Conclusions

Our new technique enables reproducible and highly accurate results to be obtained from the semi-micro determina-
tion of the iodine binding capacity of starch and its components. Indeed, in the case of amylopectin, experience has shown that amyllose-impurities of the order of 0.5 % can be readily detected. We believe that no other method of measuring iodine binding capacity is capable of such a high degree of accuracy.

It has been shown that the most important feature of the experimental technique is the proper dispersion of the starch granules prior to titration.

The experimental procedure described above results in values for the iodine binding capacity of whole starches which are significantly higher (some 5 - 15 %) than those we have previously reported [14]. This effect is attributed to:

1. the DMSO-treatment more-effectively defats the granule,
2. the DMSO-treatment results in the complete disrupting of crystalline structures, and
3. the enzymic method for determining polysaccharide concentration is more accurate than gravimetric methods used previously [5, 14].

We would suggest that these higher values are the more representative of the "true" iodine binding capacities of the starches.

Acknowledgements

This work was supported in part by a Grant from the U.S. Department of Agriculture under P. L. 480. One of us (D. D. Muir) also thanks the Science Research Council for a maintenance grant. Miss E. M. Sheddan is thanked for her assistance with the preliminary experimentation.

Zusammenfassung


Das neue Verfahren führt zu Werten für das Jodbindungsvermögen von Stärken, die um 5 – 15 % höher liegen als bei früheren Bestimmungen. Die Signifikanz dieser Befunde wird diskutiert.

Résumé

Caractérisation de l'amidon et de ses constituants. 3. Une semi-micro méthode différentielle et potentiométrique de titration à l'iode et les facteurs qui influent sur ce dosage. Un appareil amélioré pour une semi-micro méthode différentielle et potentiométrique de titration à l'iode de l'amidon et de ses constituants est décrit. Les manipulations expérimentales nécessaires sont données en détail. Les facteurs qui influent sur la mesure — quantité de diméthylsulfoxyde présente, concentration du tampon, méthode de dispersion de l'amidon, température au cours de la mesure, — sont examinés de façon critique.

La nouvelle technique donne des valeurs de capacité de fixation de l'iode qui sont de 5 à 15 % plus élevées que les mesures précédentes. La signification de ces résultats est discutée.

References


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(Received: November 20, 1970)
Studies on Starches of High Amylose-Content

Part 14*. The Fractionation of Amylomaize Starch by Aqueous Leaching

By W. Banks, C. T. Greenwood, and D. D. Muir, Edinburgh (Scotland)

Amylomaize starch is known to contain an anomalous material which does not conform to the classical definitions of either amylose or amylopectin. A detailed study of this polysaccharide has not yet been carried out, principally because of the difficulty of obtaining it in sufficient quantity. This paper presents a rapid and simple method by which the isolation may be achieved.

(Zusammenfassung siehe Seite 200; Résumé à la page 200)

The fractionation of amylomaize is complicated by the inherent instability of some of the starch-material present in the granule, and we have found that a specialized fractionation scheme has to be rigorously followed if massive retrogradation is to be avoided [1]. The end-result of this fractionation is an "amylopectin", which is heterogeneous, and consists of two polysaccharides, one of which is predominantly amylopectin in character, whereas the other is more akin to amylose. Some degree of separation of the two may be achieved by ultracentrifugation [2], or by complexing with iodine [1], but both methods are laborious.
and inefficient. Indeed, it is the difficulty of obtaining this fraction free from amylpectin which has prevented an extensive study being made of its characteristic properties.

During investigations of the relation between the gelatinization temperature as measured on the hot-stage microscope, and that obtained from viscosity measurements, we noted that at 90 °C the former technique showed the granules to have undergone an irreversible swelling and to have lost their birefringence (i.e. to have gelatinized), whereas temperatures of around 130 °C were necessary to produce the maximum viscosity potential. The changes which occur on heating amylomaize starches in boiling water, are reported here.

Figure 1. Leaching of amylomaize starches in boiling water - Material solubilized (%) as a function of time: (1) "Amylon 50"; (2) "Amylon 70".

Figure 1 shows the percentage solubilization of two amylomaize starches of different apparent amylose-contents (50 and 70 %, respectively) as a function of time of heating in boiling water. In both cases, there is an initial fairly rapid release of soluble material, followed by a much slower secondary process. The starch solubilized is a function of the apparent amylose-content, a larger amount being extracted from the starch of lower amylose-content.

In a separate experiment, the two starches were extracted in boiling water for 1 h, cooled rapidly to room temperature, and the residual gelatinized starch granules removed by centrifugation. Butan-1-ol (sufficient to saturate) was added to both supernatant liquors, and after standing for 2 h the resultant polysaccharide/butan-1-ol complex was removed. Potentiometric iodine titration and enzymic assay showed that this material was amylose. Aliquots of the residual supernatant liquors were taken for estimation of the apparent amylose-content, a larger amount being extracted from the starch of lower amylose-content.

The apparent amylose-content, a larger amount being extracted from the starch of lower amylose-content.

Figure 2 shows that the non-complexable fraction obtained from "Amylon 50": (1) material complexed with butan-1-ol; (2) material not complexed with butan-1-ol.

The non-complexable fraction was also unstable in aqueous solution - visible retrogradation occurred in a 0.1 % aqueous solution, stored at room temperature, in 4 days. This behaviour is a marked feature of the anomalous fraction found in amylomaize [1].

We conclude, therefore, that aqueous leaching of amylomaize starch provides the first simple, and rapid, method for obtaining the anomalous fraction in amylomaize free from amylpectin. The properties of this material are now being studied in detail.

Acknowledgement

This work was supported in part by a Grant from the United States Department of Agriculture under P. L. 480.

Zusammenfassung


Résumé

Etudes sur les amidons riches en amylose. 14. Le fractionnement de l’amidon d’amyloïdes. Il est connu que l’amidon de l’ amyloïdes contient un matériau anormal qui ne correspond ni à la définition classique de l’amylopectine ni à celle de l’amyllose. Aucune étude détaillée au sujet de ce polysaccharide a été entreprise jusqu’à présent en raison de la difficulté que présente l’obtention d’une quantité suffisante de ce matériau. Ce travail montre une méthode simple et rapide à partir de laquelle l’isolement peut être effectué.

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References


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(Received: March 12, 1971).
Pollen Starch from Amylomaize

Pollen Starch from Amylomaize*


Starch has been extracted from the pollen of band 5 and band 7 amylomaizes which are homozygous for the amylose extender (ae) gene. Microscopic examination showed the starch granules to be small, prolate ellipsoids, having minor diameters of approximately 0.5 μ and major diameters of 1–2 μ. Potentiometric iodine titration of the starches showed the apparent amylose content to be high, and to be grossly dependent on the temperature of measurement. This behavioral pattern is similar to that observed for the endosperm starches of amylomaize. It is therefore concluded that the starches of the gametophytic and sporophytic generations of amylomaize are similar in type.

(Zusammenfassung siehe Seite 382; Résumé à la page 382)

Introduction

One of the earliest characters of the gametophytic generation of higher plants to be investigated was the starch of the pollen of cereals. In 1921, Parnell [1] showed that the pollen of rice plants homozygous for the glutinous or waxy gene (wx), situated in linkage group 1, stained red with iodine, as did the endosperm starch. Similarly, normal (Wx) rice plants gave rise to pollen and endosperm starch which stained blue with iodine. The heterozygous F1 offspring yielded pollen which exhibited chemical dimorphism due to segregation at the waxy locus: pollen grains of F1 plants occurred in approximately equal numbers in two classes, which stained either red or blue with iodine. Parnell concluded that the waxy gene (wx) was a simple recessive to the normal gene (Wx) giving starchy endosperm and pollen.

In fact, the genetic situation is not quite as simple as this. Chandraratna [2] points out: “although numerous workers including Van der Stok (1910) . . . have shown that the glutinous character of the endosperm is determined by a single allele . . . most workers have noted a serious deficiency in the number of glutinous segregants in F2”. No satisfactory explanation for this phenomenon is available, but the meiotic divisions were normal, and no lethality operated at the gametic level in Parnell’s material. Back mutation of wx to Wx occurs at too low a rate to account for the segregation upsets, whilst evidence suggesting differential pollen germination is not substantiated.

Demerec [3], in 1924, carried out similar experiments on waxy maize and normal maize involving the wx locus on chromosome 9. His conclusions were identical with those of Parnell [1] in that his results provided direct evidence for 1:1 segregation, and wx was recessive. An analogous instance occurs in a third cereal, sorghum. Karper [4] demonstrated convincingly a 1:1 waxy : starchy ratio in the pollen of a heterozygote by means of iodine staining. Thus in the case of the starches from rice, maize and sorghum, we may conclude that the characters of the gametophytic generation, controlled by the haploid number of genes, are similar to those of the homozygous diploid, sporophytic generation.

It is surprising, therefore, to note the assertion of Zuber et al. [5] that there is no correlation between the amylose contents of the starches from the endosperm and pollen of amylomaize plants. Such a conclusion, implying as it does that, in one very important property, the gametophytic generation of amylomaize possesses quite different characteristics to those of the sporophytic generation, must have a profound influence on the genetic study of amylomaize. If it is correct, amylomaize, homozygous for the amylose extender gene (ae), must produce in the pollen a starch similar to that in the pollen from normal maize having the homozygous dominant starchy gene (Ae).

Two amylomaizes, band 5 and band 7, were grown in a controlled, glasshouse environment. Both genotypes are homozygous for the amylose extender gene, but their amylose content is influenced by other, modifying gene loci. Pollen was collected from bagged tassels, and the excess, after self-fertilisation was accomplished, was subjected to chemical analysis. The pollens were extracted, the starches isolated and their characteristics determined.

Experimental Methods

Extraction of Starch from Pollen

Immediately after collection, the excess pollen was immersed in HgCl₂ solution (0.01 M). It was then extracted for 2 min in a high-speed mechanical blender, in the presence of HgCl₂. The resultant slurry was screened (75 μ), and the residue re-extracted in the same manner. After a total of eight such extractions, the residue no longer gave a blue stain with iodine. The combined filtrates were allowed to sediment for 24 hrs at room temperature. Gentle suction was then applied to remove the supernatant from the sedimented material. The latter was suspended in dilute aqueous saline, and shaken with toluene (one-eighth volume) overnight. The starch was allowed to sediment, the toluene layer removed, added to water (10 volumes) and air bubbled vigorously through the mixture to release starch granules from the toluene. After standing for 3 hrs, the toluene and the aqueous layer were removed from the sediment and rejected. The starch sediments were combined, suspended in aqueous saline and the extraction with toluene repeated. A total of 15 such extractions was necessary in order to achieve a clear toluene layer, signifying the remov-
al of all contaminating protein. Finally, differential sedimentation was employed to remove a small amount of macerated pollen tissue which had survived the screening procedure. The product-starch was white in colour.

Dissolution of the Starch

The starch (ca. 50 mg) was dissolved in dimethylsulphoxide (DMSO) with gentle mechanical stirring to give a 2.5% (w/v) solution. Ethanol (3 vols.) was then added to precipitate the polysaccharide, which was washed repeatedly with ethanol, and finally dried in vacuo (overnight, 65 °C). The non-granular starch was dissolved in DMSO (2 ml), and an aliquot (0.5 ml) taken for measurement of iodine binding capacity.

Determination of the Amylose Content

The amylose-contents of the pollen starches were determined from measurements of their iodine binding capacity. The experimental technique has been described in detail [6].

Results and Discussion

(i) Microscopic Appearance of the Starches from Amylomaize Pollens

The starches from the pollen of both the band 5 and band 7 amylomaizes were fairly similar on microscopic examination. The granules were small, and, at the magnification used (×900), birefringence could not be detected. They were predominantly prolate ellipsoids, having a minor axis of ca. 0.5 μ, the major axis being ca. 1–2 μ in length. Values from the starch from the band 7 amylomaize pollen were found to be predominantly at the upper end of this scale, whereas those from the band 5 pollen were mainly near the lower limit of 1 μ.

In the presence of iodine/potassium iodide solution, the granules stained deep blue. Whilst staining with iodine is a diagnostic test for waxy granules when it gives a red colouration, it cannot differentiate when it yields a blue colour between starches containing, for example, 30% and 50% of the linear component.

(ii) Iodine Binding Capacity

Figure 1 shows a graph of the bound iodine (mg %) as a function of the concentration of free iodine at (a) 20.5 °C, and (b) 2.5 °C. The extrapolations to zero free iodine concentration to give the iodine binding capacities of the starches are also shown in Figure 1, and the results are summarised in Table 1. Also shown in Table 1 are the apparent amylose-contents of the two starches, calculated on the basis that the iodine binding capacity of amylose is 19.5 mg%/o at 20.4 °C, and 22.4 mg%/o at 1.5 °C [7]. We have found that the difference between amylose contents measured at two temperatures by this technique is significant only when material of comparatively short chain-length (C₅₅₅₅) is present, i.e. when 30 < C₅₅₅₅ < 150. This material, in fact, is found in appreciable amounts only in starches of high amylose-content.

The point of immediate interest to emerge from these studies is that the amylose-contents of the starches from amylomaize pollen are much greater than those associated with the endosperm starches of normal maize (ca. 28% amylose), or reported previously [5] for the pollen starches of various maize genotypes. The high amylose-contents of our pollen starches, together with the apparent increase in this parameter on conducting the iodine titration at low temperature, strongly suggests that the starch characters of the gametophytic and sporophytic generations are similar, in contrast to the conclusions of Zuber et al. [5]. This earlier work fails to specify whether or not the starches were isolated and purified prior to the determination of amylose content: if they were not so treated, the reaction with iodine may have been partly suppressed by interference from proteins or fats.

However, there are anomalies in the results presented in Figure 1a and Table 1. In particular, for the measurements carried out at room temperature, (a) the apparent amylose-contents of the pollen starches are in the reverse order to those of the endosperm starches, and (b) the shapes of the titration curves are not those expected of amylomaize starches—a linear extrapolation is not usually possible [8]. On the other hand, the slopes of the linear portions of the curves, particularly that for the pollen from band 7 amylomaize, are greater than those encountered in the titration of normal maize starches. These anomalies are to some extent resolved in Figure 1b, in which the iodine binding capacities of the two pollen starches are very similar, and, more importantly, at any finite concentration of free iodine the starch from the pollen of band 7 amylomaize binds more iodine than that from the band 5 type. We have concluded from recent, detailed studies [9] on iodine titrations.

Table 1. The Iodine Binding Capacities, and Apparent Amylose Contents, of two Amylomaize Pollen Starches, measured at 20.4 °C and 1.5 °C.

<table>
<thead>
<tr>
<th>Type</th>
<th>20.4°C</th>
<th>1.5°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Band 5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% of amylose</td>
<td>48</td>
<td>12.3</td>
</tr>
<tr>
<td>% of amylose</td>
<td>55</td>
<td>56</td>
</tr>
<tr>
<td>Band 7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% of amylose</td>
<td>42</td>
<td>12.4</td>
</tr>
<tr>
<td>% of amylose</td>
<td>56</td>
<td></td>
</tr>
</tbody>
</table>

1 I. B. C. = iodine binding capacity (mg %).
2 = % of amylose calculated from 100 × I. B. C./19.5 (see text).
3 = % of amylose calculated from 100 × I. B. C./22.4 (see text).

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tion that the extrapolation necessary to yield the iodine binding capacity is so arbitrary in the case of an amylo-
maize starch as to be meaningless. Accordingly, the appar-
ent amylose contents shown in Table 1 should be inter-
preted only as showing that the starches under examination
are not those typical of normal maize.

We conclude here, then, despite the fact that a much wider
range of genotypes and determinations would be needed to
determine a statistical correlation, that the starches from
the pollen of amylo maize differ from the endosperm
starches in degree, rather than in kind. The differences
between the iodine titrations recorded in Figure 1, and
those recorded for the endosperm starches, probably reflect
a slight variation in the chain-length of the abnormal mate-
rial present in the two cases. Thus, we suggest that the
pollen starches contain a short-chain material which is in-
capable of binding iodine at room temperature, but which
does bind it at low temperature. The endosperm starches
would then contain short-chain material which does bind
some iodine at room temperature, and this binding is con-
siderably enhanced at lower temperatures. We have shown
[7, 10] that such apparently profound differences in iodine-
bounding behaviour can arise between chains which differ in
length only by a few units.

Acknowledgements

The authors are grateful to Dr. R. P. Bear of the Bear Hybrid
Corn Co., Decatur, Illinois, for his gift of the maize seed used in
this work. One of the authors (D. D. Muir) also thanks the
Science Research Council for a maintenance grant.

Zusammenfassung

Pollenstärke von Amylomais. Die Stärke der Pollen der Amylo-
mais-Genotypen Linie 5 und Linie 7, die beide im Gen „Amylose
extender“ (ae) homozygot sind, wurde extrahiert. Im mikrosko-
pischen Bild geben sich die Stärkekörnchen als klein und von ge-
streckt-elliptischer Form zu erkennen; sie messen in der kürzeren
Achse etwa 0,5 μ, in der längeren Achse 1–2 μ. Der potentiom-
etrischen Jodtitration der Stärke zufolge dürfte der scheinbare
Amylose-Gehalt hoch sein und in starkem Maße von der Tempe-
ratur der Messung abhängen. Diese Verhaltensweise ist ähnlich
der der Endo- und Pollenstärke des Amylomais. Es ist daher der Schluß
tzu ziehen, daß die Stärken der gametophytischen und der sporop-
phytischen Generationen des Amylomais von ähnlichem Typ
sind.

Résumé

L’amidon de pollen de l’amylomais. L’amidon a été extrait du
pollen des bandes 5 et 7 de l’amylomais qui sont homozygotes
pour le gène (ae) de l’amylase extender. L’examen microscopique
a montré que les grains d’amidon sont de petits ellipsoides pro-
laits ayant un diamètre minimal d’environ 0,5 μ et un diamètre
maximal de 1–2 μ. La titration potentiométrique à l’iode indique
que la teneur apparente en amylose est élevée et qu’elle dépend
grossièrement de la température de mesure. Ce comportement est
similaire à celui observé chez les amidons d’albumen de l’amyl-
omais. On en conclut que les amidons du gamétophyte et du sporo-
phyte de l’amylomais sont d’un type similaire.

References

(1964) (Longmans), 162.
(1971), 199.
hydrate Res. 17 (1971), 25.
(1966), 152.
published.
(1970), 292.

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(Received: May 24, 1971)
The location of alpha-amylase in developing cereal grains

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When considering quantitative changes occurring in enzymic and other constituents during the development of cereal grains, the change in the relative proportions of the two principal types of tissue present is frequently overlooked. At maturity the endosperm constitutes 82 per cent of the total dry matter, whereas it is not until 16 days after anthesis that this tissue accounts for more than 50 per cent of the total. Before this stage the principal constituent is the pericarp, a tissue of maternal origin and hence of different genetic constitution from the endosperm and embryo. Failure to appreciate the importance of the pericarp during the early stages of development may lead to the planning of experiments on unsound premises and incorrect deductions being drawn from published data.

Attempts to relate enzyme activities, in the whole grain, during the early stages of development, to those of maturity have been made. However, they are of limited value unless the site of the enzyme within the grain is known to remain the same throughout development. Activity of alpha-amylase during grain development in barley reaches a peak shortly after anthesis, it subsequently declines and stabilises 10-15 days after anthesis. The low level is maintained until maturity, when it rises if germination occurs, the rate of increase varying from one cultivar to another. A similar pattern is found in many wheat cultivars.

The site of alpha-amylase activity during germination is well known to be the endosperm. Its location in the very young grain, however, has not previously been demonstrated. Recent unpublished work by one of us (A. D. Evers) has shown that starch in the pericarp is continually synthesised and digested during the early stages of grain development. It seemed likely therefore that the alpha-amylase activity in whole kernels should be associated with starch digestion in the pericarp rather than synthesis in the endosperm. The location of alpha-amylase in kernels between 8 and 14 days after anthesis was therefore investigated.

Spring wheat plants (cv. Kolibri) grown in pots under glass, were kindly made available by Mr A. C. Kendall of Rothamsted Experimental Station, Harpenden, Hertfordshire. The basal grain of the central spikelet on the main culm was removed and dissected into pericarp-testa and endosperm-germ fractions. Samples were collected, 8, 10, 12 and 14 days after anthesis. Each freshly dissected fraction was homogenised in a tissue grinder with 2 cm3 0.2M acetate buffer pH 5.5 containing 10-3M CaCl2. After centrifugation the supernatant was assayed for alpha-amylase activity. Results are given in the Table.

<table>
<thead>
<tr>
<th>Days after anthesis</th>
<th>Pericarp-testa</th>
<th>Endosperm-germ</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>10</td>
<td>&lt;0.3</td>
</tr>
<tr>
<td>10</td>
<td>16</td>
<td>&lt;0.3</td>
</tr>
<tr>
<td>12</td>
<td>15</td>
<td>&lt;0.3</td>
</tr>
<tr>
<td>14</td>
<td>17</td>
<td>&lt;0.3</td>
</tr>
</tbody>
</table>

Clearly the alpha-amylase present in the initial growth stage is associated primarily with the pericarp-testa fraction of the grain and the amount in the endosperm-germ is negligible. This fact has to be taken into consideration when considering the possible implication of alpha-amylase in biosynthesis.

Received 11 April 1972

References
A review of the chemistry of hydroxyethyl starch, with reference to its use as a blood plasma volume expander

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The uses of hydroxyethyl starch have been recently extended by the proposals for its employment as a blood plasma volume expander and cryoprotective agent. These potential pharmacological uses for the derivative demand that its chemistry and biochemistry be thoroughly understood. This review examines the methods of preparing hydroxyethyl starch, the chemical composition of the product, its physical properties and the factors governing its resistance to starch-degrading enzymes.

Introduction

Hydroxyethyl starch (HES), prepared by the reaction of starch with ethylene oxide, is readily water-soluble and, in contrast to native starch, aqueous solutions are stable over prolonged periods. These properties, together with the low cost of manufacture have led to its extensive use as an industrial chemical.

In the last decade, however, considerable interest has been shown in the pharmacological properties of HES, first as a blood plasma volume expander, and latterly as a cryoprotective agent for erythrocytes. The reason for the choice of HES as an artificial colloidal agent was that the branched component of starch, amylopectin, is very similar in structure to glycogen, the reserve polysaccharide of animals, and therefore was liable to be compatible with the body tissue. Attempts to use native starch as a plasma expander were not successful because of the presence of amylases in the blood. These enzymes rapidly degraded the starch molecules, and hence this colloidal agent did not survive sufficiently long in the intravascular system to be satisfactory as a blood plasma volume expander. It has long been known, however, that the partial derivatization of starch makes it much more resistant to the highly-specific enzyme systems which attack it. Consequently, Wiedersheim [1] studied HES as a possible plasma expander, and found it to be relatively non-toxic.

This work was subsequently confirmed, and considerably expanded by Walton et al. [2—7]. Indeed, it is now generally accepted that HES produces no more deleterious effects in experimental animals than does the widely-used bacterial polysaccharide, dextran, and that HES has superior storage properties. However, one report has appeared [8] which claims that dextran is decidedly less toxic than HES, but the applicability of this work has been questioned [9].

This Review deals with the chemistry, and physical chemistry of HES-subjects which have not been considered in detail before.

The Chemical Structure of HES

The most commonly accepted model for HES is one in which the substituent hydroxyethyl group is found on carbon atom 6 of the glucose ring [2, 7, 10]. This model was given by Schoch in 1963 [11] — but this author did stress the random nature of the hydroxyethylation process during the discussion of one of his papers [12, 13]. We believe that the molecular structure of HES is of paramount importance with regard to the pharmacological uses of HES, and therefore this aspect is dealt with in some detail.

In the first instance, a distinction has to be made between molar substitution and degree of substitution, and for the purposes of this Review they will be defined as follows:

1. Molar substitution (M.S.) is the mole fraction of hydroxyethyl groups per anhydroglucose residue, and it may be obtained from:

$$\text{M.S.} = \frac{\text{wt.} \% \text{ hydroxyethyl group}}{100 - \text{wt.} \% \text{ hydroxyethyl group}} \times \frac{162}{44}$$

$$\text{(1)}$$

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As each glucose residue in starch contains three functional groups which may react with ethylene oxide, the M.S. of a totally-substituted starch polymer is three.

2. **Degree of substitution (D.S.)** is the fraction of glucose residues substituted (at any of the three reactive hydroxyl groups). In order to obtain this parameter, it is necessary to measure both the M.S., and also the proportions of unsubstituted anhydroglucose residues. The D.S. may then be obtained from:

\[
\text{D.S.} = 1 - \left( \frac{\text{wt. of anhydroglucose} \times [(1.0 - \text{M.S.}) \times 162 + 206 \times \text{M.S.}]}{162 \times \text{wt. of polymer}} \right)
\]

These two parameters — M.S. and D.S. — are quite distinct, and one very important reason for this difference arises because of a type of reaction which has rarely been given the attention it deserves, namely the formation in HES of poly(ethylene oxide) side-chains.

Ethylene oxide reacts with the hydroxyl groups to give an ether linkage, and concurrently generates a new hydroxyl group. Thus, for the reaction at carbon atom 6, for example:

\[
\text{CH}_2\text{OH} + \text{CH}_2\text{CH}_2\text{H} \rightarrow \text{CH}_2\text{O} - \text{CH}_2\text{CH}_2\text{OH}
\]

This newly-introduced hydroxyl group is also able to react with ethylene oxide as follows:

\[
\text{CH}_2\text{O} - \text{CH}_2\text{CH}_2\text{OH} + \text{CH}_2\text{CH}_2\text{H} \rightarrow \text{CH}_2\text{O} - \text{CH}_2\text{CH}_2\text{O} - \text{CH}_2\text{CH}_2\text{OH}
\]

and so on. The end result of this series of reactions is a side-chain of poly(ethylene oxide) having the structure:

\[
\text{CH}_2\text{O} - (\text{CH}_2\text{CH}_2\text{O})_n - \text{H}
\]

Following the arguments of Spurlin [14], and assuming (a) that the introduction of a substituent at any one of the three hydroxyl groups in the glucose residue does not affect the reactivities of the remaining two hydroxyl groups and (b) the substitution of each of the three functionalities is kinetically of the first order we may write:

\[
S_2 = 1 - \exp (-k_2t), \\
S_3 = 1 - \exp (-k_3t), \\
S_6 = 1 - \exp (-k_6t),
\]

where \(S_2\), \(S_3\) and \(S_6\) are the fraction of hydroxyl groups substituted at positions C2, C3 and C6 at time \(t\), and \(k_2\), \(k_3\) and \(k_6\) are the corresponding rate constants. The rate of formation of polymeric side-chains \((S_p)\) is given by

\[
dS_p = k_6 (S_2 + S_3 + S_6) \, dt,
\]

where \(k_6\) is the rate constant of the polymerization reaction. The substitution pattern will be decided solely by the relative values of the various rate constants, \(k_2\), \(k_3\), \(k_6\) and \(k_p\). If, for example, \(k_6 \gg k_2\), \(k_3\) or \(k_p\), then the resultant HES will consist (to a first approximation) of a series of anhydroglucose units substituted only at carbon atom 6. Similarly, if \(k_p \gg k_2\), \(k_3\) or \(k_6\), the HES would have relatively few glucose units substituted but each one that was would carry a long side-chain or poly(ethylene oxide).

Using these extreme examples, it can be seen that two HES starches with radically different chemical structures could have identical values of M.S., although these differences would be reflected in their different values of D.S.

From the viewpoint of persistence in the blood, the product in which monosubstitution is dominant is to be greatly preferred because the modification of the individual glucose residues of the starch polymer will prevent \(\alpha\)-amylolysis attack, whereas the presence of a few long side-chains of poly(ethylene oxide) in the starch will have virtually no effect in slowing down \(\alpha\)-amylolysis but there may be possible toxicological effects (see later).

It had to be stressed that there is, at present, no a priori method by which values may be ascribed to \(k_2\), \(k_3\), \(k_6\) and \(k_p\). Rather, it is necessary to determine experimentally the substitution pattern at a given M.S., and, from the derived values of the various rate constants, postulate the expected structure at a different M.S.; this postulate has to be tested then by experiment.

**Preparation of HES**

In commercial practice, HES is prepared by the reaction of ethylene oxide with starch [15, 16], often in the presence of catalysts such as inorganic salts [16], inorganic alkalis [16, 17] and certain organic bases [18, 19]. Reaction systems have been designed to produce an essentially granular product by inhibition of gelation, by limiting the amount of water present in the reaction system [18, 20], by the addition of inorganic salts, or by carrying out the reaction in certain alcoholic media [21, 22]. HES has also been prepared by the reaction of ethylene chlorohydrin with pregelled starch, in pyridine [23], and with the starch alkoxide [24], again in pyridine. Starch/poly(ethylene oxide) graft co-polymers have been prepared [25].

With such a large variety of possible methods of preparation of HES, it would be most surprising if the relative values of the four rate constants \(k_2\), \(k_3\), \(k_6\) and \(k_p\) were unchanging. Thus it is possible that samples prepared in different laboratories could differ fundamentally in their chemical structures, and this may consequently affect their pharmacological uses.

**Measurement of M.S.**

Before discussing the various reports on the substitution pattern in HES, it is necessary to consider the methods by which the M.S. — the most fundamental parameter in HES — may be determined. Basically, the methods involve the cleavage of the hydroxyethyl ether group with hydroiodic acid under a variety of conditions, and subsequent estimation of the reaction products derived from the hydroxyethyl group. Using constant boiling hydroiodic acid at atmospheric pressure, cleavage of the hydroxyethyl ether linkage yields a mixture of ethyl iodide and ethylene. The method of Morgan [26], and the subsequent modification of Lortz [27], utilizes this reaction, the ethyl iodide and ethylene being estimated volumetrically after reaction with silver.
nitrile, and bromine in acetic acid, respectively. Van der Bij [28] employed 70% hydroiodic acid to cleave the ether group and, by carrying out the reaction under pressure, obtained ethyl iodide as the sole reaction product. This was extracted from the reaction mixture with trichlorethylene and estimated by gas chromatography. Anderson and Zaidi [29] employed conditions similar to those of Morgan [26], but trapped the reaction products and estimated them by quantitative infra-red spectroscopy. Tai et al [30] employed a secondary method of analysis, in which the acetaldehyde produced from the controlled pyrolysis of HES and starch was measured by gas chromatography. Primary calibration of the technique was achieved by means of samples standardized against the method of Lortz [27].

None of the above methods are very accurate because the extreme conditions used to obtain complete cleavage of the ether linkages cause interfering side-reactions. Anderson and Duncan [31] attributed this interference in part to the oxidation of the polymer backbone. In any case, it appears that, under the best possible conditions, an error of ±(3-5) % is to be expected in the determination of the hydroxyethyl ether content, and therefore in the M.S. of HES: this error is certainly higher than one would suppose from the literature.

Distribution of the Hydroxyethyl Groups

The different approaches which have been used to characterize the distribution pattern of hydroxyethyl groups in HES will be next considered. Experiments on the polymer itself have yielded useful, but rather limited information, and the most important method of characterization has been acid hydrolysis, followed by identification of the products. The latter, however, has proved to be an unusually complex task.

The properties of three samples of hydroxyethyl amylose of different molar substitutions (0.3 < M.S. < 1.1) were investigated by Husemann and Kafka [32]. They found that the extent of reaction of sodium metaperiodate with the polysaccharide decreased during the substitution process. The periodate ion oxidizes hydroxyl groups on adjacent carbon atoms, which in the case of amylose means that oxidation occurs at carbon atoms 2 and 3. Hence, the significance of a decrease in periodate consumption with increasing M.S. is that substitution must take place predominantly at carbon atoms 2 or 3, rather than at carbon atom 6. This conclusion was supported when it was found that more tritylation (i.e. reaction to form the trityl ether) occurred with increasing M.S. – it is accepted that this reaction is predominantly associated with primary hydroxyl groups (i.e. -CH₂OH groups). Each substitution introduces a new primary hydroxyl group, and if substitution occurred most readily at carbon atom 6 of the glucose ring (i.e. on the primary hydroxyl group) then the number of primary hydroxyl groups would not change, whereas if the favoured substitution occurred at the hydroxyl groups on carbon atoms 2 and 3 (the secondary hydroxyl groups) the number of primary hydroxyl groups would increase. The latter was, in fact, observed experimentally, as shown by the increasing tritylation. From these results, Husemann and Kafka [32] concluded that substitution at carbon atom 2 is favoured, but that reaction at positions 3 and 6 increased with increasing M.S. In terms of the parameters used in equations (3) – (5), this would imply that \( k_2 \) is greater than either \( k_3 \) or \( k_6 \), although these latter two rate constants do have finite rate constants. The possibility of polysubstitution, i.e. a finite value for the rate constant \( k_p \) of equation (6) was not considered in this work.

Schoch [12] gave — without any experimental details — the following values for the substitution pattern of HES of M.S. ~ 0.9: 60% of the reaction occurred at carbon atom 6, 20% at carbon atom 2, and 10% at carbon atom 3. This would imply that \( k_6 > k_2 > k_3 \), a conclusion radically different from that of Husemann and Kafka [32]. Schoch [12] also added that there was no evidence of any complex substitution having taken place i.e. the value of the rate constant \( k_p \) was apparently zero. On the other hand, it should be noted that his analytical data account for only 90% of the substituent hydroxyethyl groups found to be present, and it is possible that the remainder is to be found in the form of polysubstituted products.

Lott and Brobst [33] characterized a series of samples of hydroxyethyl amylose (HEA) of varying M.S. After acid hydrolysis of the polymer, the products were characterized by gas chromatographic separation of their trimethyl silyl ethers. Although some difficulty was experienced in separating certain of the derivatives from each other (particularly the 3-O-hydroxyethyl-ß-D-glucose from 6-O-hydroxyethyl-α-D-glucose) they found that, for 0.23 < M.S. < 1.03, the substitution patterns corresponded to rate constants in the order \( k_2 > k_3 > k_6 \). They also pointed out that with increasing M.S., increasingly large proportions of the hydroxyethyl content were to be found as complex derivatives. The latter are, in fact, probably attributable to the presence of poly-(ethylene oxide) side chains.

The products from the acid hydrolysis of a commercial sample of HES (M.S. = 0.6) were separated by Bollenback et al [34] by paper chromatography, and the relative proportions of each estimated by reaction with hypiodite [35]. These authors concluded that their results were consistent with a kinetic scheme for the hydroxyethylation process in which \( k_2 > k_3 > k_6 \), and added that there was apparently only a low level of disubstitution (ethylene oxide reacting with an already-present hydroxyethyl group to form the dimeric substitution product \(-O-\text{CH}_2\text{-CH}_2\text{-O-}\text{CH}_2\text{-CH}_2\text{OH} \)), i.e. \( k_p < k_3 \). The conclusion regarding the low level of complex derivatives is somewhat suspect, however, because of the experimental technique (paper chromatography) employed to separate the products — Shyluk et al [36] have demonstrated the difficulty in separating mono- and di-hydroxyethyl glucose derivatives.

A commercial sample of HES (M.S. = 0.1) was also used in the work of Srivastava and Ramalingam [37], who oxidized the HES with periodate (noting that the periodate uptake indicated that the main substitution had not taken place on carbon atom 6), reduced the resultant dialdehyde starch with borohydride, and acid-hydrolyzed the product. The hydrolysate was then examined, by means of adsorp-
tion and paper-chromatography. Their results were consistent with a kinetic scheme in which \( k_2 \gg k_q > k_s \). No mention was made of complex derivatives — and indeed at the low M.S. employed in this work, the amount of such products would be too small for detection by the authors' techniques.

Anionic graft co-polymers of poly-(ethylene oxide) and starch over a very wide range of M.S. were prepared by Taban and Zilka [25, 38]. In this instance, no detailed investigation of the relative reactivities of the different hydroxyl groups to grafting was carried out, but again peridode oxidation suggested that the reaction occurred preferentially at the secondary hydroxyl groups (those on carbon atoms 2 and 3).

These literature reports on the distribution of hydroxyethyl groups in HES are confusing, and it would appear unlikely that the view that substitution takes place mainly at the primary hydroxyl group on carbon atom 6 is correct — all the other work suggests that the rate constant with the highest value is \( k_5 \). However, even when it is agreed that \( k_5 \) is numerically the largest, some doubt exists as to whether or not \( k_2 \) or \( k_q \) has the smallest value. Additionally the situation is further complicated by the lack of appreciation of the possibility of complex derivatives being formed, i.e. the relation of \( k_p \) to \( k_2 \), \( k_q \) and \( k_p \).

These different studies show so little agreement that it has to be considered that each set of workers has obtained the correct distribution of hydroxyethyl groups for their particular sample, but that the actual chemical structures differ from sample to sample, because of slight differences in the mode of preparation.

As we have stressed earlier, the ultimate structure of HES is determined solely by the relative values of four rate constants — \( k_2, k_3, k_4 \) and \( k_p \). If the conditions of preparation of HES can be so altered as to change the relative orders of these rate constants, then products of vastly different structure may result.

The Physical Structure of HES

Some of the most important parameters which can be used to characterize a polymer are measurements of the size and shape of the macromolecule. By size, we imply a knowledge of both the molecular weight and also the molecular dimensions of the dissolved material. These considerations are of vital importance in the use of HES as a blood plasma volume expander because ultimately the colloid must be ejected from the body via the kidneys, and the rate at which this occurs will be determined by the size of the macromolecule.

The physical characterization and solution behaviour of hydroxyethyl starch has not been extensively investigated. There have been five investigations of the shape and molecular weight distribution of HES in solution, and one report of the physical properties of a series of hydroxyethyl amylloses.

Information concerning the shape of molecules in solution may be obtained by measuring the molecular weight of the polymer by light-scattering or osmotic pressure measurements [39, 40], and relating this to a parameter such as viscosity or sedimentation coefficient which measures the hydrodynamic volume of the polymer. Extensive reviews have appeared [40, 41] detailing the theory and applications of these measurements to the elucidation of polymer shape, but much information may be obtained from an examination of the experimentally-obtained parameters in the Mark-Houwink relation:

\[
[\eta] = K_a M^a
\]

and the corresponding relation \( S_0 = K_b M^b \)

where \([\eta]\) is the limiting viscosity number, \( S_0 \) is the sedimentation coefficient at infinite dilution, \( M \) is the molecular weight, and \( K_a \) and \( K_b \) are constants for a given polymer-solvent system. The exponents \( a \) and \( b \) have values which depend (i) on the nature of the polymer, i.e. branched or linear, (ii) the shape of the polymer i.e. coil or rigid rod, and (iii) the nature of polymer-solvent interactions.

For viscosity, when polymer-solvent interactions are at a minimum (i.e. a Flory theta-solvent) only the first two effects govern the magnitude of \( a \). In this case, if \( a < 0.5 \) the polymer is branched; if \( a = 0.5 \) the polymer is a flexible Gaussian coil, and values of \( a > 0.5 \) indicate extension of the polymer due to rigidity in the polymer backbone. In cases where measurements have been made in good solvents, and polymer-solvent interactions play a major part in polymer size, various theories have been proposed to allow the short and long range interactions to be separated, but have been shown by Banks and Greenwood [42] to be limited in their application.

Husemann and Resz [43] fractionated three samples of hydroxyethyl amyllose by acetone precipitation and measured the average degree of polymerization (DP) by osmotic pressure measurements and related these to the viscosity.

Recalculating these authors' data gives values of \( a \), which vary considerably with M.S. as shown below:

<table>
<thead>
<tr>
<th>Molar Substitution</th>
<th>( a )</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.31</td>
<td>1.65</td>
</tr>
<tr>
<td>0.60</td>
<td>1.21</td>
</tr>
<tr>
<td>1.08</td>
<td>0.64</td>
</tr>
</tbody>
</table>

Extrapolation of these results to the case where M.S. is zero would appear to give values of \( a \approx 1.0 \). This would correspond to native amylose existing in solution as a totally extended coil, a view contrary to the generally-accepted evidence supporting the model of amylose as a flexible Gaussian coil [41].

In the other physical investigations of the solution properties of HES, the branched fraction of starch, amylopectin, has been used. Little experimental work has been reported on the characteristics of the parent polymer, and present theoretical treatments are somewhat limited for branched polymers in solution.

The physical characteristics of five samples of HES of M.S. in the range 0.83—0.03 were reported by Greenwood and Hourston [44]. It was found that the viscosity potential of these samples was about a fifth of that of dextran, for a comparable molecular weight.
An HES with an M.S. value of 0.85 was fractionated by Cerny et al [45] by acetone and isopropanol precipitation, the number-average molecular weight measured by osmometry and the weight-average molecular weight by light-scattering, and these parameters related to the viscosity. They obtained the equations:

\[ \eta_0 = 5.29 \times 10^{-5} M_w^{0.300} \]

and

\[ \eta = 3.27 \times 10^{-3} M_w^{0.351} \]

Positive values of the virial coefficients were found in both light scattering and osmotic pressure measurements. The equations indicate the branched nature of the polymer, and the disparity between the number average and weight average results would seem to be caused by different molecular weight distributions between samples. These authors also calculated a branching index by comparison of the intrinsic viscosity, radius of gyration, and second virial coefficients with those of hydroxyethyl cellulose and ethylhydroxyethyl cellulose, and found that an estimation of the expansion factor which measures solvent-polymer interaction was not possible using current theories. The comparison of hydroxyethyl cellulose and ethylhydroxyethyl cellulose with HES would seem also to be unjustified in obtaining the branching parameter, since the required analogue is hydroxyethyl amylose and the nature of the glycosidic linkages in the parent amylose and cellulose has been shown [46] to confer quite different properties on the derivatives.

Granath et al [8] carried out extensive physical characterization on HES of various M.S. For a sample of chemical material of M.S. about 0.6, these authors obtained the relation: \[ [\eta] = 2.91 \times 10^{-3} M_w^{0.45} \]. The low exponent was attributed to the branched nature of the polymer, and they also noted that the viscosity increased with molar substitution, and attributed this fact to hindered rotation about the glycosidic bonds in the substituted polymer. Gel permeation chromatography was shown to provide a convenient method for the fractionation of hydroxyethyl starch for biological purposes. The relation between elution volume and molecular weight was determined, and various models for gel permeation were also examined.

The interaction of HES and dextran with albumin has been examined by means of osmotic pressure measurements [10]. Cerny et al [10] concluded that both were equally effective in plasma expansion. A viscosity study was carried out on HES/erythrocyte mixtures, but due to the heterogeneous nature of the HES, the authors were reluctant to define exactly the ideal size and shape which HES should have for the purpose of plasma expansion.

Tamada et al [47] measured the relationship between molecular weight, determined by the Archibald method, and limiting viscosity number, and obtained the relation:

\[ [\eta] = 4.72 \times 10^{-8} M_w^{0.517} \]

This would indicate a considerable expansion of the highly branched polymer in salt solution and is not in accord with the observations of Cerny et al [45] or Granath et al [8].

These investigations on hydroxyethyl starch show that the derivative exhibits the predominant characteristics of the parent amylopectin i.e. a low viscosity for a given molecular weight. The broad molecular weight distribution is also noteworthy, and would seem to be both a function of the branching and of the hydrolysis involved in preparation of the derivative. The exact nature of the polymer-solvent interaction is unknown, as the results of Husemann and Resz [43] could be interpreted to show that polymer-solvent interaction decreases with increasing substitution, and those of Granath et al [8] to show the diametrically opposite view. Further investigations on the hydroxyethyl derivative of amylose are required to establish the exact nature of the effect of the substituents on the solution behaviour of the native polymers.

The Enzymic Degradation of HES

The resistance of hydroxyethyl starch to \( \alpha \)-amylases has been recognized for a long time, and was used by Ziese [48, 49], to differentiate \( \beta \)-amylase from the endo \( \alpha \)-amylase. These observations were confirmed by Scholander and Myrbäck [50] and later by Greenwood and Hourston [44]. Husemann and Resz [43] carried out a systematic investigation of the action of \( \alpha \)-amylase on hydroxyethyl starches of various M.S., and measured the rate of hydrolysis of glycosidic bonds by \( \alpha \)-amylase from Aspergillus oryzae. They found that as M.S. increased, the rate of hydrolysis decreased rapidly, but was still finite at a M.S. value of 1.03. Since the rate of \( \alpha \)-amylolytic attack will be governed by the number of unsubstituted glucose residues, it seems likely that the substitution pattern described by Lott and Brobst [33], where 20% of the glucose residues are unsubstituted at a M.S. of 1.03, is essentially correct. The parameter governing susceptibility of hydroxyethyl starch to \( \alpha \)-amylolytic attack would appear to be degree of substitution, and in cases where this differs substantially from molar substitution, e.g. in the case of extensive poly-substitution, the value quoted for M.S. is likely to give a misleading impression of susceptibility to \( \alpha \)-amylase action.

General Properties of HES

Tahan and Zilkha [25] report that as the M.S. increased from 0.4 to 1.0, HES formed by graft polymerisation became progressively more soluble in cold water, and at D.S. = 3.0, the products are thermoplastic, and both water and alcohol soluble. These polymers would seem to combine the characteristics of both the starch granule and polyethylene-oxide, and this was mirrored in their reported melting temperatures and alcohol solubility. Staserman [51] reported that as M.S. increased, the ability to bind iodine was sharply diminished, reflecting perhaps the disrupting effect of the substituent groups on the ability of amylose to form a helix. This is also reflected in the stability of hydroxyethyl starch under conditions designed to promote crystallisation [52].

Conclusions

The methods for characterization and preparation of HES are fairly well developed, but agreement has not yet been
The physical methods available for the analysis of HES are also well developed, but again investigations are limited and there is an apparent lack of agreement on the influence of substitution on the hydrodynamic properties of the polymer. This problem can only be settled by detailed and extensive investigation of the properties of model compounds.

Relatively little information on the resistance of HES to enzymic attack is available, and the relation between degree of substitution and resistance to endo-amylase attack would appear to be a profitable field of study.

It would appear that much of the basic chemistry of HES remains to be explored.

Acknowledgments

This work was supported in part by a grant from the United States Department of the Navy, Office of Naval Research.

Zusammenfassung


Résumé

Etudes au sujet de l'amidon hydroxyéthylé. Partie I. Une revue de la chimie de l'amidon hydroxyéthylé, en tenant compte de son utilisation comme excipient de volume du plasma sanguin.

L'utilisation de l'amidon hydroxyéthylé a été récemment étendue en ce sens qu'il est employé comme excipient de volume du plasma sanguin et comme antigel. Ces utilisations potentielles pharmacologiques de ce dérivé exigent une profonde connaissance de sa chimie et de sa biochimie. Dans cet article on examine les méthodes de préparation de l'amidon hydroxyéthylé, la composition chimique du produit, ses propriétés physiques et les facteurs qui déterminent sa résistance vis-à-vis des enzymes dégradant l'amidon.

References


(Received: April 17, 1972)
ABSTRACT OF THESIS

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Date ................................................................. 1st JANUARY, 1973.
Title of Thesis ........................................................ PHYSICOCHEMICAL STUDIES ON STARCHES.

After a brief Introductory Section, general methods of characterization of starch and its fractions are detailed in Section 2 of this work. The methods of chemical and physical investigated employed in later sections are reported, and some modifications of general techniques proposed.

Section 3 presents two special techniques devised to facilitate later experiments. Firstly, a method of estimation of the starch-content of a cereal grain is described. Starch is extracted from previously macerated plant material by treatment with calcium chloride solution (d = 1.3 g/ml) at 130° - 135°C. A complete extraction of all starches, including those of high amylose-content, is achieved, and the extracted starch estimated by a combination of highly-specific enzymes.

Secondly, an extensively-modified technique of semi-micro, differential, potentiometric iodine-titration is proposed. A simplified electronic circuit is described utilizing a digital voltmeter as a null detector. Control experiments are described which have established analytical conditions allowing the estimation of the iodine-binding capacity of starch and its fractions to an accuracy of ± 1.5%.

The next Section describes a number of experiments on amylomaize starch. After a short review outlining the outstanding problems, a simple method of isolating the anomalous material from this starch is described. A partial characterization of this low-molecular weight material was performed and revealed that some of the branched fraction was fundamentally different from normal maize amylopectin. The starch from the pollen of amylomaize was examined in detail and shown to be similar in nature to the parent endosperm starch.

In Section 5, the biogenesis of starch in general and barley starch in particular is investigated. The character of two barley starch genotypes during growth is detailed and this information related to general theories of biosynthesis. The nature of compound starch granules in varieties of pea and potato is investigated, and a hypothesis concerning the formation of starch granules is advanced.

The final Section of this work, reviews the chemistry of the hydroxyethyl derivative of starch with special relation to its use as a blood plasma expander and cryoprotective agent for human erythrocytes. Serious deficiencies in the present knowledge of this polymer are revealed. The rest of this Section presents some experiments performed to clarify these points of dispute, a model of hydroxyethyl starch is proposed, and the relevance to its intended pharmaceutical use discussed.

Use other side if necessary.