UNIVERSITY OF EDINBURGH.

STUDIES ON STARCHES, WITH PARTICULAR REFERENCE TO THE CHARACTERIZATION OF THEIR AMYLOSE AND AMYLOPECTIN COMPONENTS; AND ANALYSES OF SOME STARCH-CONTAINING MATERIALS.

- by -

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THESIS

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PREFACE.

Only the results of my own experimental work are reported in this thesis; certain parts of it have already been published in conjunction with my supervisors, Professor E.L. Hirst, F.R.S., and Dr. C.T. Greenwood, and reprints of these papers have been inserted at the end of the text.

I wish to thank Dr. G.O. Aspinall, Dr. D.J. Bell, Professor E.J. Bourne, Dr. D.J. Manners, Dr. R.M. McCready, Mr. M.J. Nash, Dr. R.L. Whistler, and Dr. W.J. Whelan for kindly providing samples; Mr. J.K. Fleming A.R.I.C. (Messrs. A. and R. Scott, Ltd.,) for milling oat samples; Mr. C.H.C. Matthews for helpful discussions on electronics; and Miss E.M. McAlpine, M.A., for typing this thesis.

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Lastly, I should not like to miss this opportunity of stating my indebtedness to Dr. C.C. Miller, Dr. N. Campbell, Dr. M. Ritchie, Dr. T.R. Bolam, and to all those who have made the years spent in this department - as student and subsequently as a junior colleague - so worthwhile and so very pleasant.

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GENERAL INTRODUCTION.

Starch is one of the most important natural products. It finds many industrial uses and applications, and, in different forms, is used throughout the world as a staple item of the human diet.

Consequently, the structure and properties of starch have deservedly formed the subject of extensive investigations both by chemists and bio-chemists. Because of the inherent difficulties in the many problems which arose, and the continual need for development of new techniques, progress prior to 1925 was slow and uncertain. However, the subsequent elucidation of the precise structural configurations of the simpler sugars e.g. glucose, maltose and cellobiose, gave a firm foundation for future work, and steady progress ensued. One of the most important advances was the realisation of the apparent inhomogeneity of starch; this was followed in 1941 by the first quantitative fractionation into simpler components.

As a result, the highly polymeric nature and main structural features of the molecule are now well established. In general, starches can be separated into two distinct entities, both of which are high polymers of α-D-glucopyranose units joined by α-1:4-glycosidic linkages: (a) Amylose - a mixture of very long, essentially unbranched, chains of these units. (b) Amylopectin - a mixture of much shorter, highly branched chains of these units, the inter-chain, or branching, linkages being

essentially α-1:6.

Yet many problems still remain. Some are concerned with the clarification of controversial issues on which the present experimental evidence, as reported by various workers, is either at variance or considered to be inconclusive; the majority, however, are concerned with the fundamentally important details of fine structure and the molecular size and shape of the starch components. It is now accepted that there is little prospect of progress on these particular problems unless full use is made by the carbohydrate chemist of physical and enzymic methods to supplement purely chemical approaches.

When the researches reported in this thesis were begun, it was apparent that, before the established physical techniques used in polymer chemistry for the determination of molecular size and shape could be profitably applied, work on the following topics was an essential preliminary in order that the materials to be studied could be adequately and accurately characterized:-

(1) The development of a differential potentiometric iodine titration technique for the determination of the % amylose in starches and their fractionated components, and for the possible characterization of other α-1:4-glucosans. It was desirable that the apparatus should be considerably more sensitive and accurate than any of those previously reported, yet also be capable of simple operation as a routine analytical method.

(2) A study of the oxidation of starches by the

periodate ion in view of the low quoted percentage accuracy of the technique, and the then existing lack of agreement in the literature on the following points: (a) conditions for the quantitative estimation of the formic acid released, (b) the length of time required for complete oxidation, and the possibility of over-oxidation, (c) the presence of intact glucose residues after oxidation, suggesting that inter-chain linkages other than 1:6 existed in amylopectin.

(3) A study of the effect of the presence of contaminating protein on results obtained by use of the potentiometric iodine titration and periodate oxidation techniques.

(4) The preparation, and purification, of starches from several botanical sources which had not previously been studied, so that the use at any stage of industrial samples of unknown origin, treatment, and history could be avoided. By preparation of the starches under the mildest possible conditions, so as to minimise degradation, these same samples could later be used for physico-chemical studies of molecular size and shape. The opportunity was taken, after the extraction of each starch, to apply a graded series of extractions to the residual plant material; the fractions isolated were analysed for protein, ash, and uronic acid content, and the polysaccharide content was investigated by chromatographic analyses of the sugars liberated on hydrolysis.

PART I.

Preparation of starches

and analyses of the starch-containing materials.

INTRODUCTION.

Starch is stored as the main reserve metabolic carbohydrate material in the seeds, bulbs, roots, or tubers of most plants. The Compositae group, whose roots or tubers contain inulin, form a notable exception.

In plant storage organs, the starch occurs as granules which are usually surrounded by a thin protein layer. The size and shape of the granules is characteristic of the botanical source (1, 2). The mode of synthesis and deposition of the starch in the granules is not known, although the granules generally appear to be built in layers partially or completely encircling the hilum, which can be seen under polarised light as the centre of a black cross. The exact nature of these layers has not been established, but the "layering" effect is caused by discontinuities in refractive index of the deposited material (3).

Baker and Whelan (4) regard the granule as being constructed of alternate layers of amylose and amylopectin, the thickness of the amylose layers decreasing outwards from the centre of the granule. This hypothesis has, however, been criticised recently by Badenhuizen (5).

Meyer (6, 7) attributed the birefringent properties of the granule to the presence of spherulites, which consist of small crystalline regions held together by secondary valence forces, the crystallinity being primarily due to the amylopectin component. Meyer also considered that the outer concentric shells in the granule contained

90% of amylopectin (8), with which high molecular weight amylose existed in the form of mixed crystals, the inner layer being composed of well-crystallised amylose of low molecular weight. Both layers were believed to contain radially arranged spherulites, formed either by a number of branches of the ramified amylopectin molecule grouping into radially oriented, crystalline, bundles, or by the outer branches of several molecules aligning to form a "fringe-micelle". In amorphous regions, secondary valence forces act tangentially to hold the molecule together. This structure for the granule is generally consistent with the available X-ray diffraction data (68, 69).

Fatty acids (40), phosphorous (generally as phosphate) (41) and protein (42) are usually associated with native starch. It is essential that the starch be freed from these contaminants before structural studies are undertaken, since they interfere in fractionation, in the interaction of starch with iodine (see part II) and in the oxidation of starch by periodate (see part III). Moreover, these contaminants must be removed without causing degradation of the starch if fundamental investigations involving fine structure and molecular size and shape are later to be made. The labile nature of the starch components demands that the extraction of the crude starch, its purification, and subsequent treatment e.g. fractionation, must <u>all</u> be carried out under the mildest possible conditions (9).

In some of the earlier structural investigations it appears that, although the dangers of degradation during

the fractionation process were appreciated, insufficient care was taken in the prior treatment of the starch, e.g. the action of a Waring Blendor has been shown to cause "mechanical degradation" of amylopectin in buffered solutions (59). It has also been found, in the preparation of "waxy" starches, that when the period of contact with sulphur dioxide-water was decreased, an equally pure starch, having considerably higher viscosity, was obtained (95). Table I shows the deviation in results obtained for the molecular weight of samples of amylose and amylopectin prepared from maize, tapioca and potato.

TABLE I.

Starch	Amylose		Amylopectin		
Source	M.Wt.*	D.P.	M.Wt.*	D.P.	Ref.
Maize	230,000	800			16
"	75,000	260			53
"	134,000	465			26
Potato	260,000	930			16
"	1,100,000	3,800			54
Maize			8,000,000	28,000	16
"			420,000	1,450	22
Tapioca			370,000	1,300	22
"			6,000,000	21,000	16

* M.Wt. in all cases measured by the Osmotic Pressure method on the acetate derivative in chloroform as solvent.

The results in Table I may be compared since all the values were obtained by the Osmotic Pressure method, using the same derivative in the same solvent. Either significant degradation has been allowed to occur in some cases, or different varieties of the same botanical source must yield products of greatly differing molecular size. Very few investigations have been made (55-58) of (a) possible variation during growth of the proportions, and molecular size, of the two components in the granule; (b) varation in the starches obtained from different varieties of the same botanical species; and (c) variation in starches from the same variety depending on differing growth conditions from season to season. Further information is required on these possibilities, and pure strains of known varieties should be used for the preparation of all starch samples. It is of interest that Preece and Hobkirk (70) found that the % Nitrogen in thirty-one samples of barley, comprising four different varieties, varied from 1.26-1.56%. This represents a 20% variation in protein content, and it is possible that a similar investigation on such an extensive scale would reveal some variation in starches.

Successful fractionation requires the complete dissolution of the starch granules. For some varieties of starch this is more difficult to achieve than for others, without causing either retrogradation or hydrolytic degradation. Much depends on the natural structure of the granule, which may inadvertantly be modified during the processes of extraction and purification. Starches

which have been dried with the aid of organic solvents are more difficultly soluble than either freeze-dried samples or samples which have never been dried i.e. have been stored under aseptic conditions at 0°C under methanol.

Avoidance of degradation during fractionation involves several technical difficulties (51, 52). In particular, the practice of autoclaving is undesirable (59, 60), and heating under reflux in the absence of oxygen has been suggested as an alternative (9, 52). Some starches e.g. from peas, cannot however be dissolved by refluxing or even by autoclaving, and it has been found necessary to cause gelatinisation of the granules by pre-treatment with liquid ammonia (46, 61) or with sodium hydroxide (36, 60). Since hydroxyl and hydrogen ions both catalyse the degradation of α-1:4 bonds, it appears that the enforced use of such chemical pre-treatment must cause some degradation.

Treatment of maize starch with acid was found by Meyer and Menzi to cause degradation of both starch components equally, but Kerr (114) found preferential degradation of the amylopectin, and this has been confirmed (115). The degradation caused both in neutral (52) and in alkaline solution (52, 64) has also been investigated. Whistler and Johnson (65) found that very little degradation occurred in alkaline solution under nitrogen at 0°C, whilst Rist et al (66) found that a nitrogen atmosphere was not necessary at that temperature. Recently, however, Baum and Gilbert (67) have suggested

that potato amylose contains "oxygen-sensitive" bonds. It therefore appears that all stages in the isolation, purification, and fractionation of a starch should preferably be carried out in an oxygen-free atmosphere, and at temperatures as near 0°C as possible.

Since 1937, starches from more than twenty different sources have been studied, as shown in Table II below.

TABLE II.

Starch Source	Reference
Acorn	43
Apple	44
Arrowroot	45
Banana	27, 46
Barley	47, 48
Canna	49
Easter lily	50
H. brasiliensis seed	9
Horse chestnut	10
Maize	14, 16, 21-26, 28, 100, 112.
Passion fruit	11
Pea (smooth)	12, 36
Pea (wrinkled)	12, 13, 36, 46
Pearl manioc	45
Potato	14-22
Rice	10, 45
Sago	16, 23
Sweet potato	45, 96
Tapioca	16, 22, 45
"Waxy" maize, sorghum, etc.	29, 33, 34, 45, 95
Wheat	10, 16, 35, 45
Wood starches	30-32.

The most widely studied starches have been those from maize (in the U.S.A.) and potato. Starch is easily extracted in good yield from both these sources; in addition, the crude starches are not difficult to purify. Maize is the preferred industrial source of starch from cereals because of its ease of extraction. Clendenning

and Wright (71) have shown that the presence of hydrophilic pentosans - of which maize has the lowest percentage in the cereal family - reduce the ease of extraction of starch. Maize is more characteristic of the majority of starch sources than potato, the crude starch being contaminated with significant quantities of protein and fatty acids. The phosphorous content can be removed, together with the fatty acids, by treatment with water-miscible solvents such as methanol or dioxan (37-39). Potato contains very little protein or fatty acids, and at least part of the phosphorous present is bound as a 6 - phosphate (41), which is not removable by solvent extraction. The behaviour of potato starch in solution may therefore be anomalous.

In seeking suitable new sources of starch, the main criteria appear to be not only the percentage of starch in the source, but also whether it can be extracted in good yield and subsequently freed from contaminating protein by purely physical processes (95). The removal of protein from some crude starches is much more difficult (9, 72, 95) than for others, and may involve a tedious purification process if degradation is to be avoided.

Earlier workers regarded the small percentages of protein associated with purified starches as unimportant so far as structural studies were concerned (42). Only very few of the starches studied have been obtained entirely free of protein (33), about 0.3% generally remaining. This residual protein may be chemically bound to the starch, and in the preparation of the new starches

reported here - from oats, parsnip roots, and iris germanica rhizomes - particular attention was paid to purification from protein in an attempt to obtain further information on this question.

EXPERIMENTAL

I. Notes on reagents and analytical procedures.

Reagents of analytical grade were used throughout, and all analyses were made in duplicate.

Drying procedure: Before analysis, samples were dried at 80°C *in vacuo* for several hours.

Determination of % moisture: This was calculated from the loss in weight on oven-drying at 100°C for five hr.

Determination of % ash: Samples (ca. 200 mg.) were heated to constant weight without sulphating.

Determination of % Protein: This was calculated from %N x 6.25, determinations of % Nitrogen being made by the semi-micro Kjeldahl method.

Determination of % Uronic Acid Anhydride: The method of McCready, Swenson and Maclay (73) was used, modified by the inclusion of an aniline trap to remove furfural before estimation of the carbon dioxide evolved (74).

Hydrolysis conditions: (a) Heating with 1% oxalic acid (w/v) for one hr. on a boiling water-bath was used for sucrose and fructosans. (This applies only to the fractions obtained by cold-water extraction.) This procedure gives complete hydrolysis of fructosans, with insignificant decomposition of fructose (80). (b) Hydrolysis of all other fractions was by 2% sulphuric acid (2 ml. per 20-30 mg. of polysaccharide) in a

sealed tube on a boiling water-bath for 6-8 hr. No decomposition of galactose, glucose, xylose or arabinose results from this treatment (80). (c) method (b) gave incomplete hydrolysis of the residual cellulosic fractions, and Monier-Williams' method (82) using 72% sulphuric acid (w/w) was applied.

Determination of "Acid Lignin": This was made by Bamford and Campbell's method (83) after hydrolysis with 72% sulphuric acid. The "acid lignin" was retained on a sintered glass filter (grade 3), washed, and dried to constant weight.

Paper chromatography: After hydrolysis, solutions were neutralised with barium carbonate, filtered, and the solutions concentrated at 36-40°C under reduced pressure.

The descending method of elution was employed (99), using 3 MM paper. When the quantities of sugars present was very small, Whatman No. 1 paper was used. The solvent system was butan-1-ol-benzene-pyridine-water (5:1:3:3, top layer) in all except the following two cases, in which ethyl acetate-acetic acid-water (3:1:3, top layer) was used (75):- (a) analysis of cold water extracts for free sugars, and (b), separation of mannose from arabinose. Development for 48-60 hr. at 20-21°C on strips 27" long was allowed. Good separation of the sugars present was given in all cases except the separation of glucose from galactose in I. germanica fractions, when development for seventy-two hr. was

required. No reliance was placed on R_F values. Control solutions of standard sugars were run on every chromatogram, and all sugars detected were identified in this manner.

Aldopentoses and aldohexoses were located by spraying side-strips (76) with aniline oxalate and heating at 120-125°C, at which temperature the difference in colour given by hexoses and pentoses is most marked. For ketoses, urea oxalate spray was used with heating at 130°C.

Quantitative estimations of sugars: A known weight of ribose was added as a reference sugar in quantitative hydrolyses, having first been shown absent in all fractions isolated from oats and parsnip roots. Ribose was present, however, in two fractions from Iris germanica; in one fraction xylose was absent and so was introduced as reference sugar; in the other case, rhamnose was used. Sugars were eluted from their located sites by suspending the paper strips over boiling water under reflux for ninety minutes (80). Estimations were made using the Somogyi iodometric method (77), which was found to give very reproducible results. Estimations with alkaline hypoiodite (78) at pH 11.4 (79) were found to be reliable for estimations of glucose only; the method was used for estimation of the % glucose given by starches on hydrolysis. Estimations of fructose and fructosan were made by Arni and Percivals' colorimetric method (81).

Determination of % amylose: The differential potentiometric titration method, using the conditions and apparatus described in Part II, was used.

Determination of the ratio of non-terminal to terminal glucose residues (i.e. $\overline{R}$): The amount of formic acid liberated and the amount of periodate consumed on oxidation of the starch by potassium meta-periodate were determined as described in Part III.

II(a). The preparation and purification of oat starch, and analyses of fractions obtained from oats, Avena sativa Linn.

The sample of oats was grown from a pure strain of the variety "Sun II" in 1952 at Boghall Experimental Farm, Midlothian. The oats were specially milled by Messrs. A. & R. Scott, Ltd., Colinton Mills, Edinburgh, so that minimal mechanical damage to the kernels (groats) resulted. The milled oats were then sorted by hand to ensure freedom from contaminating hulls and damaged, blackened, or diseased kernels.

The groats were ground to a coarse flour in a "Raymond" Laboratory Mill, care being taken to ensure that no significent rise in temperature occurred. To minimise enzymic hydrolysis [cf. (80)], and extract fats, the flour was immediately immersed in a boiling benzene-methanol mixture (2:1, v/v, 1.5 l. per 200 gm. flour); and exhaustively extracted by heating under reflux, with five changes of solvent, for a total of thirty-six hr.

Analyses on the coarse flour:

Found - Moisture ..... = 11.8%
Ash ..... = 2.22%
Protein ..... = 14.0%
Ether extractives = 7.2%

Ratio of sugars obtained on hydrolysis:-

Galactose/glucose/arabinose/xylose = 1/93/3/3

The defatted flour (100 gm.) was then successively extracted with cold and hot water, followed by cold and hot alkali as described below. The yields obtained for each fraction are shown in Table III (a) (p.22), and results of their subsequent analyses in Tables III (a)

and (b) (p.23).

Cold water extractions: The defatted flour (100 gm.) was shaken vigorously with distilled water (1 l., 5 treatments, each of twelve hr.). After each extraction the mixture was filtered through a double layer of muslin. The starchy extracts obtained were combined and centrifuged, giving a proteinaceous starch deposit (fraction F1) which was not dried, and a supernatant solution containing the cold water extractives. This solution was filtered twice through Whatman No. 1 paper to ensure complete absence of any starchy sediment, then reduced in volume and freeze-dried, giving fraction F2.

Hot-water extractions: The combined residues from the cold-water treatments were stirred for three hr. with 1 l. of water at 90°C. The extract, which appeared to contain starch, was treated with thymol (2 gm.) and gave, after storage at 25°C for three days, a characteristic amylose-thymol complex [F3 (a)]. The supernatant liquid from the thymol separation gave F3 (b) when freeze-dried. The residue from the first hot-water extraction was then treated a further four times with water at 90°C; the combined extracts gave F3 (c) after reduction in volume and freeze-drying.

Alkaline extractions: The residue from the hot-water extractions was stirred with sodium hydroxide solution (5% w/v, 500 ml.; 5 treatments, each of two hr.) at room temperature under nitrogen. The supernatants obtained on centrifugation were brought to pH7 by addition of glacial acetic acid, and the resultant precipitate collected,

giving fraction F4. Material in the neutralised solution was precipitated, giving F6, by the addition of ethanol (2 vols.)

A similar procedure using 5% NaOH at 90°C in a nitrogen atmosphere gave F5 and F7 respectively.

The residual material was washed free from alkali, then dried, giving F8.

Analysis of fraction F2 for free sugars: Chromatographic analysis showed that oligosaccharides (fructosans), raffinose, sucrose, glucose and fructose were the only free sugars present. They were present in the ratio of 10:15:65:5:5, and together comprised about 3% by weight of the fraction, i.e. approx. 0.2 % by weight of the defatted groats.

Separation of starch from protein in F1 (crude starch I): A sample of F1 was dried and found to contain 45.6% protein. The crude starch was dispersed in 1M NaCl so as to give a suspension of sp. gr. 1.07, to which was added toluene ($^1/_{10}$ vol.). The mixture was shaken vigorously for at least one hr., then centrifuged (M.S.E. "major" centrifuge) for five mins. at 750 r.p.m., after which the speed was slowly increased to 1200 r.p.m. over a further period of five mins. The brown precipitate at the toluene-water interface was removed and discarded. The deposited material consisted of two well-defined layers; the upper (proteinaceous) layer was loosely packed and easily removed with a spatula, followed by gentle washing with a jet of water. The lower layer was much whiter and densely packed. These two layers were

then separately re-suspended in 1M NaCl, and the extraction procedure repeated six times, finally yielding three fractions. When washed free from salt, these were refluxed with 85% methanol (3 treatments, each of two hr.) to ensure complete removal of fatty materials. The fractions were designated F1 (a), (b), and (c), and had protein contents of 0.3, 7.9 and 28.4% respectively.

Analyses of starch I (a):

% ash ...	=	0.03%
% protein	=	0.3%
% amylose	=	26.0%
$\overline{R}$	=	27.4 glucose units.

Preparation of starch II: Attempts to determine values for $\overline{R}$ and the % amylose in starches I (b) and I (c) indicated that interference was caused by the contaminating protein present. In order to study these effects in greater detail, it was decided to prepare a second sample of impure starch on a larger scale, and obtain a wider range of protein-contaminated samples from the purification process. By using a sample of oats of the same variety, grown on the same farm one year later, it was hoped that a comparison of the purified starches would also yield indications of any seasonal variation.

Crude starch II was extracted and purified exactly as described for starch I. The weight of defatted flour taken was 500 gm., which gave an estimated weight of 300 gm. crude starch. From the purification process Starches II (a), (b), (c), (d), and (e) were obtained, having protein contents of 0.19, 0.45, 2.94, 22.7 and 45.6% respectively. The yield of starch II (a) was

estimated at 48 gm., the bulk of this being stored under methanol at 0°C.

Attempted further purifications: When an aqueous suspension of starches II (b) and II (c) was passed through a column (10 x 1.5 cm.) of Zeokarb-215 resin, the nitrogen content decreased from 0.07 and 0.47% to 0.05 and 0.34% respectively, there being 75% recovery of material. No decrease, however, was found in the case of starch II (a), and further treatments of starches II (b) and II (c) with resin, and also with hot 80% ethanol, did not further reduce these percentages of nitrogen.

Analyses of starch II (a): The white powder consisted of small, bi-refringent granules, polygonal in shape and tending to unite into aggregates. (See plate I, ~~facing~~ p.26). A clear paste, staining blue with iodine, was formed in hot water.

Found:-

Ash ..... = 0.024% (sulphated)

Protein . = 0.19%

$[\alpha]_D^{18}$ ... = + 163° (c., 0.5% in 1N sodium hydroxide).

$[\alpha]_D^{18}$ ... = + 194° (c., 0.34% in 30% perchloric acid).

% Amylose = 26.0% [see Fig. I and Table III (c) (p.24)]

$\bar{R}$ ..... = 27.4 glucose units [see Table III (d) (p.25)]

On hydrolysis, conversion to glucose = 99.1% (alkaline hypoiodite determination at pH 11.4). There was no residue after hydrolysis, and no other sugar could be detected by chromatography.

Analyses of starches II (b) - II (e): An investigation of the effect of presence of protein in these samples

on the determination of (a) the % amylose and (b) value of $\bar{R}$ was made, the results being discussed fully in Parts II and III respectively.

TABLE III (a)

Analyses of oat fractions

isolated by the graded extraction procedure.

Fraction	% of original material /	% protein	% ash	% carbo-hydrate //	% of material not hydrolysed ///	Reaction with iodine
F1a	45	0.3	0.03	99.7	-	+
F1b	9	7.9	0.12	92.0	-	+
F1c	14	28.4	0.31	71.3	-	+
F2	7	20.9	1.08	78.0	1.23	-
F3a	3	5.1	2.13	92.8	-	+
F3b	7	6.9	0.90	92.2	1.14	+
F3c	6	11.5	1.37	87.1	1.58	+
F4	1	68.8	4.28	26.9	18.3	-
F5	Negligible	-	-	-	-	-
F6	3	9.8	6.12	84.1	15.3	-
F7	2	1.4	39.4	59.2	77.3	-
F8	3	1.6	7.65	90.7	71.6	-

Footnotes:-

/ based on dried, defatted material.

// by difference

/// hydrolysis by 2% sulphuric acid at 100°C.

TABLE III (b)

Sugars obtained on hydrolysis/ of oat fractions//.

Fraction	Saccharides/// Oligo- Pentose	Oligo- Hexose	Tri- Pentose	Tri- Hexose	Gal	G ////	F	A	X
F1a	-	-	-	-	-	98	-	-	-
F1b	-	-	-	-	-	90	-	-	-
F1c	-	-	-	-	-	68	-	-	-
F2	1	1	-	-	6	79	5	4	4
F3a	-	-	-	-	3	89	-	4	4
F3b	1	-	1	-	1	88	-	5	4
F3c	1	-	-	-	2	87	-	5	5
F4	-	-	-	-	-	28	-	34	38
F6	-	-	3	-	3	26	-	32	36
F7	-	-	-	-	15	30	-	25	30
F8	-	-	-	-	4	21	-	39	36

Footnotes:-

/ Using 2% sulphuric acid at 100°C.

// Expressed as percentages of hydrolysable carbohydrate.

/// Incompletely hydrolysed material.

//// Gal = galactose; G = glucose; F = fructose; A = arabinose; X = xylose.

TABLE III (c)

Determination of % amylose in Oat starch II (a).

Wt. of sample taken = 14.90 mg.; dissolved in 0.2M KOH (10 ml.) by heating at 98°C for three mins., then shaking vigorously for twelve hr. The solution was neutralised to pH 5.85 by addition of a predetermined volume of phosphoric acid.

Titration conditions:

[I] = 0.01N; pH = 5.85 (phosphate buffer)
Temp. = 20°C; iodine = 0.00992N.

Under these conditions, pure amylose binds 19.2% of its own weight of iodine (9).

(a)	(b)	(c)[x-axis]	(d)	(e)	(f)[y-axis]
5.00	3.50	3.50	1.50	1.50	0.25
5.00	3.00	6.50	2.00	3.50	0.59
5.00	0.75	7.25	4.25	7.75	1.31
5.00	1.50	8.75	3.50	11.25	1.90
5.00	1.00	9.75	4.00	15.25	2.58
5.00	1.30	11.05	3.70	18.95	3.20
5.00	1.20	12.25	3.80	22.75	3.85
5.00	1.35	13.60	3.65	26.40	4.46
5.00	2.15	15.75	2.85	29.25	4.95
5.00	3.95	19.70	1.05	30.30	5.12
5.00	4.20	23.90	0.80	31.10	5.26
10.00	8.90	32.80	1.10	32.20	5.45
10.00	9.00	41.80	1.00	33.20	5.61
15.00	13.70	55.50	1.30	34.50	5.77
15.00	13.80	69.30	1.20	35.70	6.04
20.00	18.65	87.95	1.35	37.05	6.27

Key to table:

(a) = Additions of iodine in Agla units [50 units = 1 ml.] to starch half-cell.
(b) = Additions of iodine in Agla units to blank half-cell.
(c) = Σ(b) : (d) = (a)-(b) : (e) = Σ(d)
(f) = (e)x 0.169, the factor converting iodine uptake in Agla units of 0.00992N iodine per 14.90 mg. starch into mg. of iodine bound per 100 mg. starch.

Result from graph: (see Fig. I).

$$\% \text{ amylose} = \frac{5.0}{19.2} \times \frac{100}{1} = 26.0\%$$

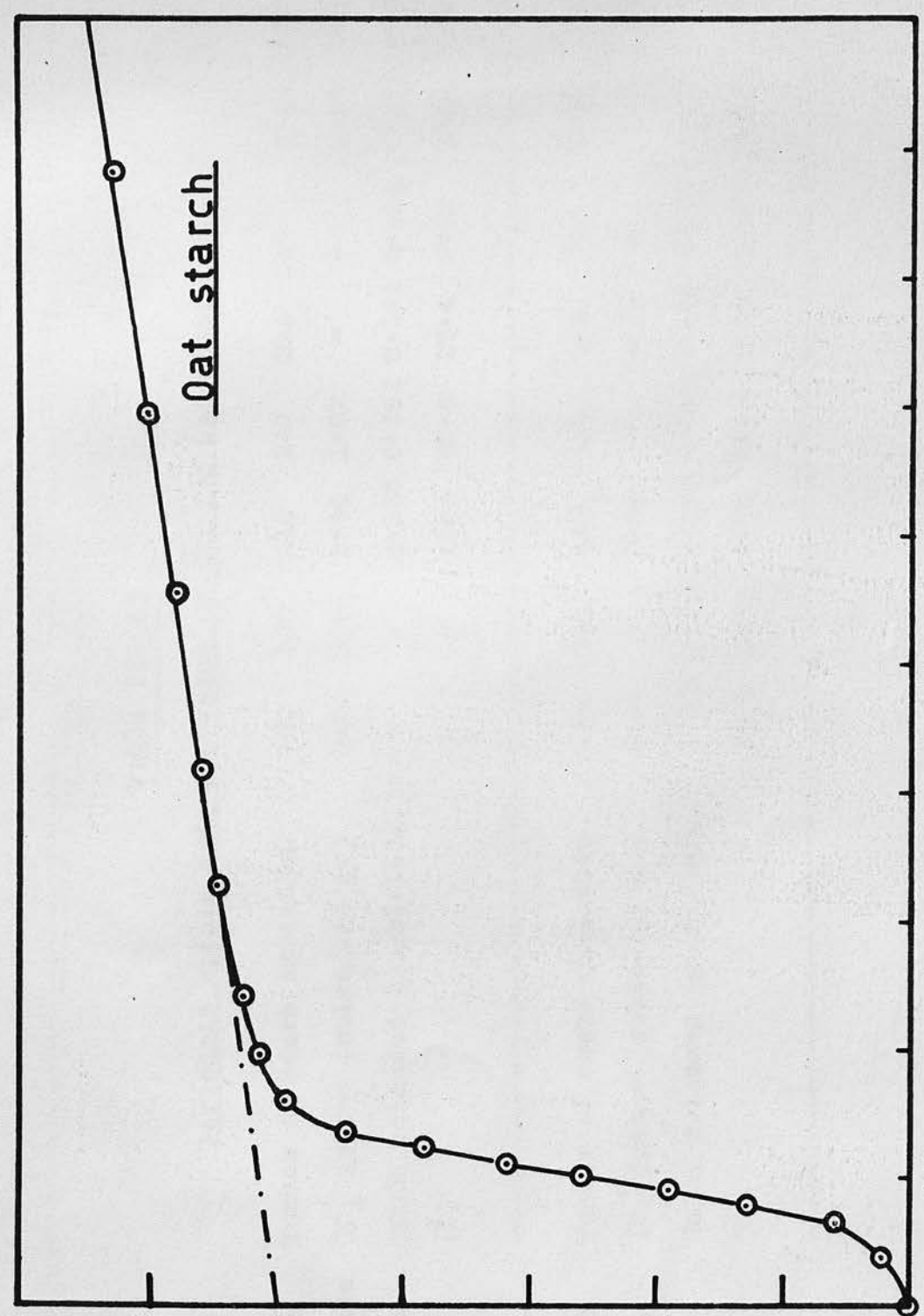
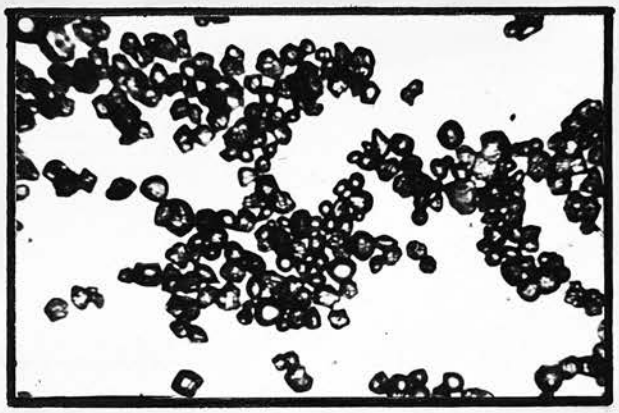

Oat starch
Iodine bound (mg./100 mg. starch).
Total free iodine (units, see Table III(c)).
1
2
3
4
5
6
7
10
50
90

FIG. I

Table III(d)

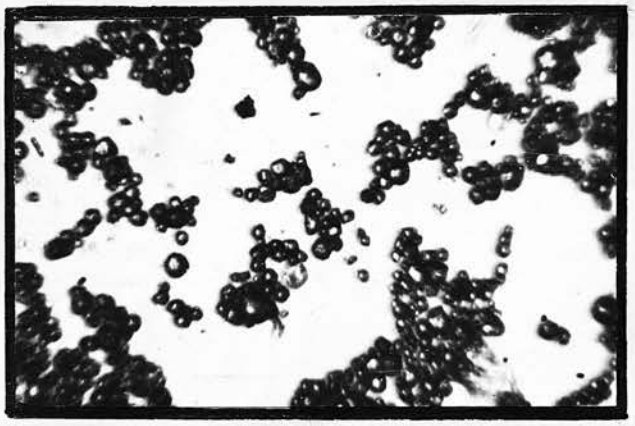
Periodate uptake data and value of $(\bar{R})$ for Oat Starch IIa.

Oxidation temperature									
	Number of hours oxidation	120	160	212	240	284	318	336	402
	IO_4^- uptake (moles/162 g.)	0·80	0·86	0·96	1·02	-	-	1·05	1·06
15-16°C	HCOOH released (g.mol/162g.)	-	-	0·353	0·362	0·365	0·366	0·370	0·388
	$(\bar{R})$	-	-	28·3	27·6	27·4	27·3	27·0	25·8
	Number of hours oxidation	120	140	160	180	212	240	260	284
	IO_4^- uptake (moles/162 g.)	0·97	-	1·02	-	1·03	-	-	1·08
20-21°C	HCOOH released (g.mol/162g.)	0·335	0·357	0·362	0·365	0·370	0·372	0·384	0·413
	$(\bar{R})$	29·9	28·0	27·6	27·4	27·0	26·9	26·0	24·2

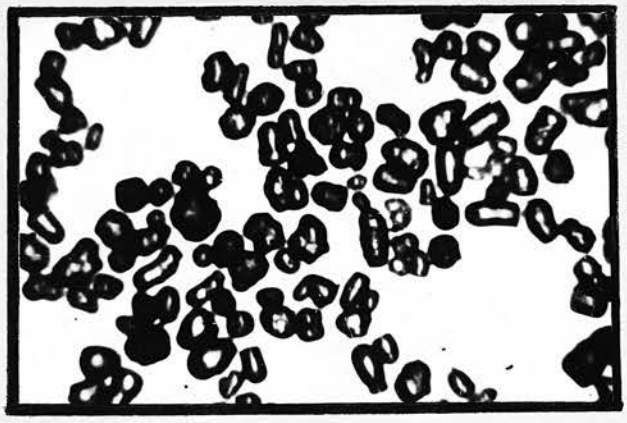


Oat starch.
× 400

Scale: ⌣ = 10μ.



Parsnip starch.
× 400



I. germanica starc
×400

PLATE I

II(b) DISCUSSION.

Because of their importance, the common cereals wheat, barley and maize have attracted considerable study, and the polysaccharide content of these and other types of seed material has recently been examined (9). Investigations into the isolation of wheat starch from contaminating gluten have been made (71, 72), and the cold-water extract from this cereal has been examined by several workers (63, 84-87). The structure of wheat flour pentosan has also been studied (88). A full chemical investigation of barley starch has been made (47), and the cold-water extract from barley partially separated into its components (89-91). The differences between barley and malted barley starches have been investigated (48).

No previous work on oat starch has been reported, although Morris (92) has claimed to have isolated lichenin and araban from the cold-water extract. Analyses of the free sugars present in the fraction obtained by extraction at 40°C have been carried out (70), (94); the same free sugars have been found in very similar quantities to those previously reported, although in this investigation the cold-water extractions were made at room temperature. On hydrolysis, the cold water extract, (fraction F2) also gave sugars in quantities very similar to those described by Preece and Mackenzie if allowance is made for the fact that these authors combined galactose with glucose, and fructose with arabinose. The whole of the fructosan

content of the groats was found in the cold water extract, and no other fraction contained free sugars. The only other sugars found on hydrolysis were galactose, glucose, arabinose and xylose, and these were also present in the hydrolysis product of almost all the other fractions.

Water-soluble pentosans and glucosans other than starch are present. Because of the difficulty of obtaining quantitative extractions by the different solvents used, complete separation of the polysaccharide material into fractions of varying solubility is difficult. As a result, the differentiation between the water-soluble and hemi-cellulosic materials is not sharp. In particular, a large percentage of the glucose present in the hot-water extracts was probably present as starch, which cannot be completely extracted using cold water. The large amount of unhydrolysable material in F7 is accounted for by the high ash content of this fraction; no dialysis was carried out (to avoid loss of carbohydrate) before isolation of the fractions by freeze-drying.

All the fractions obtained from the extraction scheme were proteinaceous; no attempt was made to remove this except in the case of the starch product, where a physical method of purification was successful. The method - a modification of the Sevag technique (93) - depended on treatment of the proteinaceous material with salt and toluene (vigorous shaking for at least one hr. at each treatment was found to be essential) followed by a differential centrifugation separation of the starch from protein. For crude barley starch, McWilliam and

Percival (47) found that treatment with salt and toluene was not required, separation being possible when a suspension of sp. gr. 1.03 and a centrifuge speed of 1,500 r.p.m. was used. In the case of oat starch no purification was given under these conditions. Even after shaking with salt and toluene, the use of this specific gravity and speed gave no separation. The successful combination of sp. gr. 1.07 at 750 r.p.m. (for deposition of starch) followed by 1200 r.p.m. (for loose deposition of protein) had to be found by experiment.

In the case of starch I, it was found that 75% of the starch present in the defatted groats could be purified to a protein content of 0.3% under the mild conditions necessary to minimise degradation. A more prolonged purification process during the treatment of starch II, however, gave starch of protein content = 0.19%, the yield being only 47 gm. from 500 gm. defatted groats. This value (0.19%) could not be further reduced, and it would be difficult to determine positively whether this residual protein is chemically bound to the starch, although such is apparently the case.

The percentage of amylose present (26.0%), and the ratio of non-terminal to terminal glucose units (i.e. $\overline{R}$) found (27.4/1) for starch II (a), was identical to those found in the previous year for starch I (a). It is therefore apparent that there is no seasonal variation in these values for oat starch of the variety studied. The percentage of amylose present in oat starch is higher than for barley (22.0%) and for wheat (25.0%)

(see part II). From the values found for $\overline{R}$ and the percentage of amylose it can be calculated that the average length of unit-chain of the amylopectin component is 20.3 glucose units, a value similar to that found for many other starches (see part III).

III(a). The preparation and purification of parsnip starch, and analyses of fractions from the roots of the parsnip, Pastinaca sativa.

The parsnip roots (var. "Hollow Crown"), grown at King's Buildings, Edinburgh, were harvested in late September, 1954. The roots were immediately washed with cold water then immersed in alcohol (cf. 80)

Found: Moisture, 74%; ether extractives, 9%. After being peeled, and minced to a fine pulp (all under alcohol), the material was exhaustively defatted by refluxing in succession with methanol, a methanol-benzene mixture (2:1, v/v), ether, and finally methanol (90%) [three treatments (each of three hr.) with each solvent; 1 l. per 100 gm. material]. The final treatments with 90% methanol were to remove all traces of benzene and ether, so that no emulsification would occur in the cold water extractions.

Analyses of defatted material:

Found - Ash ..... = 3.02%
Protein ..... = 16.2%
Uronic acid anhydride = 22.4%

Ratio of sugars obtained on hydrolysis:-

Galactose	glucose	mannose	arabinose	xylose	rhamnose
12	57	1	24	4	2

Cold Water Extraction: The defatted material (dry weight estimated at 55 gm. by drying an aliquot) was extracted with cold water (300 ml., eight treatments, each of three mins.) in an "Atomix" blender. This method of extraction was necessary, as vigorous shaking was found to be ineffective because of the fibrous nature of the minced

material. During the short periods of extraction no significant rise in temperature occurred. Each extract was filtered through several layers of muslin and gave on centrifugation a proteinaceous starch (fraction P1) and supernatant liquors. The latter were combined, filtered twice through Whatman No.1 paper to ensure complete freedom from starchy sediment, then reduced in volume and freeze-dried, giving the cold-water soluble material (fraction P2).

Hot-water extractions: The combined residues from the cold-water treatments were extracted by stirring with water (300 ml.) at 98°C (five treatments, each of three hr.). The combined extracts were reduced in volume, and gave fraction P3 on freeze-drying.

Alkaline extractions: The residue was vigorously stirred with sodium hydroxide solution (5% w/v, 200 ml., eight treatments, each of one hr.) under nitrogen at room temperature, and then at 98°C. In both cases the alkaline extract, after centrifugation, was brought to pH7 with glacial acetic acid; no precipitate appeared on neutralisation. The volume was then reduced to 700 ml., salts were removed by dialysis against cold water for ninety-six hr., and the fractions (P4 and P5 respectively) isolated by freeze-drying.

The final residue was washed free from alkali then dried, giving fraction P6.

Yields of fractions obtained: These are shown in Table IV (a) (p.37) the overall recovery being 91% of the estimated weight of original material. The results of

analyses of the various fractions are shown in Tables IV (a) and IV (b) (p.38).

Analysis of fraction P2 for free sugars: Chromatographic analysis showed that raffinose, sucrose, glucose and fructose were the only free sugars present, together comprising 2% by weight of the fraction. No polyfructosans were present.

Identification of Rhamnose: Hydrolysis of some fractions liberated small quantities of a sugar behaving chromatographically as rhamnose. Rhamnose may, however, be readily confused with D(+) apiose under these conditions; since the latter has been shown to exist in other members of the Umbelliferae, and is extremely reactive to alkali (97), the following procedure was used:- The acid hydrolysates of fractions P1, 3, 4 and 5 were examined chromatographically without prior neutralisation (98). This made no apparent difference to the chromatograms or separations obtained. When duplicate chromatograms were run with (a) butan-1-ol saturated with water, and (b) butan-1-ol saturated with aqueous boric acid, the R_F value of the suspected rhamnose was the same as for an authentic sample. The R_F value of authentic D(+) apiose was 0.26 in (a), but only 0.04 in (b); the sugar present was therefore rhamnose.

Analysis of fraction P6: This fraction was only hydrolysed to the extent of 18% by 2% sulphuric acid. Hydrolysis of the residue with 72% sulphuric acid gave 95% conversion to glucose, together with traces of xylose; 2% of "acid lignin" remained. This fraction is therefore

largely cellulosic in nature.

Separation of starch from protein in fraction P1 (crude starch I): A sample was dried and found to contain 37.1% protein, the dry weight of the fraction being estimated at 15.5 gm. i.e. 28% of the dry weight of defatted material. Purification was attempted by centrifuging an aqueous suspension of the starch at different speeds in the range 400-2000 r.p.m. No separation of starch from protein resulted, and it was decided that the quantity of crude starch available was too small to permit development of a successful purification process.

After starch II had been prepared, and a purification procedure perfected (see below), the method was applied to crude starch I, when the following fractions were obtained:-

Starch I (a)	Yield = 1.0 gm.	Protein = 3.7%.
Starch I (b)	Yield = 0.3 gm.	Protein = 12.1%.
Starch I (c)	Yield = 0.2 gm.	Protein = 26.6%.
Starch I (c)	Yield = 1.5 gm.	Protein = 54.5%.

Analyses of starch I (a):

% amylose = 11.1%
$\overline{R}$ ..... = 23.0 glucose units.

Hence the amylopectin component has a calculated average length of unit-chain of 20.4 glucose units.

Preparation of crude starch II: This sample was prepared as described for crude starch I. The estimated weight of defatted material extracted was 500 gm.; this gave 60 gm. crude starch. The parsnip roots were harvested in September, 1955, being of the same variety, and grown on the same site, as those used the previous year for preparation of crude starch I.

Analyses of crude starch II:

Found - Ash ..... = 0.2%
Protein ..... = 6.8%
Uronic acid anhydride = 8.1%

Purification of crude starch II: As found for starch I, no separation of starch from protein was possible by simple differential centrifugation.

The crude starch was dispersed in 1M NaCl so as to form a slurry having sp. gr. = 1.10 (by hydrometer). Toluene ($^{1}/_{10}$ vol.) was added, and the mixture shaken vigorously for forty-eight hr., after which it was stored at 0°C for twelve hr. to allow sedimentation of the starch. A significant degree of purification was obtained. The top (toluene) layer, which was coloured dark brown, was carefully removed, giving (C). More toluene was added to the residual starchy sediment (A) and supernatant salt solution, and the whole process was repeated a further three times.

The combined toluene layers (C) were repeatedly extracted with cold water in a separating-funnel until all salt had been removed. Addition of several volumes of ethanol then gave a clear toluene layer on top of a starch/ethanol slurry, from which the proteinaceous material was obtained by centrifugation, washed free of toluene with ethanol, and dried, giving starch II (c).

The white starchy sediment (A) was washed free from salt. Centrifugation of an aqueous suspension of sp. gr. = 1.10 at 1500 r.p.m. for three mins., followed by three mins. at 2,650 r.p.m., (M.S.E. "Minor" centrifuge) then gave a densely packed layer of pure white starch covered with a thin layer of loosely-packed proteinaceous material

(B) which was a pale brown in colour. This top layer was removed by spatula, followed by washing with a jet of water, and the differential centrifugation process repeated a further twice, after which no off-white top layer formed. The product, after further washing with cold water, gave starch II (a) which was stored at 0°C under methanol. The combined top-layers (B) were washed with water and methanol, giving starch II (b).

Yields and analyses of starches II (a), (b) and (c):-

Starch II (a)	Yield=18.0gm.	% Ash= 0	% Protein= 0.06
Starch II (b)	Yield= 4.7gm.	% Ash=0.19	% Protein= 0.3
Starch II (c)	Yield= 7.5gm.	% Ash=3.2	% Protein=36.8

Further analyses on starch II (a):

Found:-

$[\alpha]_D^{18}$ ... = + 197° (c., 0.30% in 30% perchloric acid)

% Amylose = 11.1% [see Fig. II (p.39a) and Table IV (c) (p.39)]

$\overline{R}$ ..... = 23.0 glucose units.

Periodate oxidation data: At 15-16°C., the periodate uptake, in moles per anhydroglucose unit, reached 1.02 after 268 hr., and 1.05 after 360 hr. During this period the amount of formic acid released gave a value of $\overline{R}$ of 23.0 glucose units.

On hydrolysis, conversion to glucose = 98.7%. There was no residue after hydrolysis, and no other sugar could be detected by chromatography.

Appearance of granules: The granules are spherical in shape, of size estimated at 2-7μ. See plate I (p.26).

TABLE IV (a).

Yields and analyses of fractions from parsnip roots.[/]

Fraction	Yield	% Ash	% Protein	% U.A.A. [//]	% non-acidic hydrolysable polysaccharide [///]	Reaction with iodine
P1	28	0.4	37.1	8.8	53.7	+
P2	20	17.4	24.2	7.4	51.0	-
P3	13	6.6	6.2	41.5	45.7	+
P4	14	7.5	13.4	23.4	55.7	+
P5	5	7.4	5.5	45.3	40.8	+
P6	11	3.1	0	2.2	12.7	-

Footnotes:-

/ based on % of dried defatted material.

// Uronic acid anhydride.

/// hydrolysable by 2% sulphuric acid at 98°C.

TABLE IV (b).

Sugars obtained on hydrolysis of parsnip fractions.[/]

Fraction	% non-acidic hydrolysable polysaccharide[//]	Gal[///]	G	M	A	X	Rh	F
P1	53.7	2.5	86	0	9	1.5	1	0
P2	51.0	22	48	0	25	3	0	2
P3	45.7	13	39	0	37	3	8	0
P4	55.7	24	9	6	47	10	4	0
P5	40.8	23	8	7	57	4	1	0
P6	12.7	6	71	tr.	7	16	0	0

Footnotes:-

/ Expressed as percentages of the non-acidic hydrolysable polysaccharide content.

// Hydrolysable by 2% sulphuric acid at 98°C.

/// Gal = galactose; G = glucose; M = mannose; A = arabinose; X = xylose; Rh = rhamnose; F = fructose.

TABLE IV (c).

Determination of % amylose in Parsnip starch II (a).

Wt. of sample taken = 17.27 mg. Dissolution of sample, and all other conditions as for titration of oat starch II (a) [see Table III (c) p.24] except that iodine solution = 0.01002N.

(a)	(b)	(c)[x-axis]	(d)	(e)	(f)[y-axis]
5.00	4.00	4.00	1.00	1.00	0.147
5.00	2.55	6.55	2.45	3.45	0.51
5.00	2.10	8.65	2.90	6.35	0.93
5.00	1.70	10.35	3.30	9.65	1.42
5.00	1.80	12.15	3.20	12.85	1.88
5.00	3.40	15.55	1.60	14.45	2.14
5.00	4.35	19.90	0.65	15.10	2.22
5.00	4.60	24.50	0.40	15.50	2.27
10.00	9.30	33.80	0.70	16.20	2.38
15.00	14.20	48.00	0.80	17.00	2.50
20.00	19.05	67.05	0.95	17.95	2.64

Factor for conversion of (e) to (f) = 0.147.

Result from graph (fig. II):

$$\% \text{ amylose} = \frac{2.12}{19.2} \times \frac{100}{I} = \underline{\underline{11.1\%}}$$

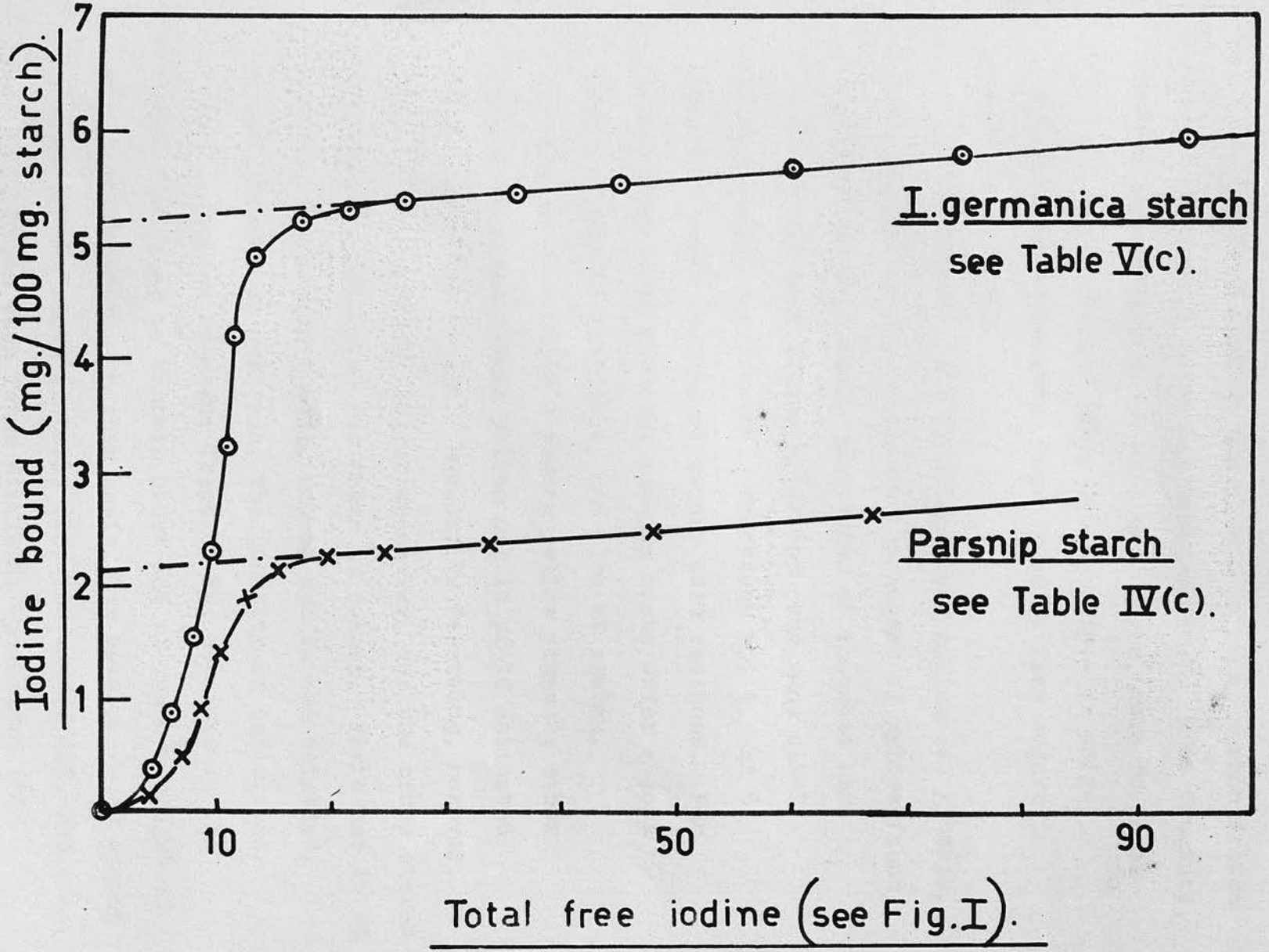

Iodine bound (mg./100 mg. starch).
7
6
5
4
3
2
1
10
50
90
Total free iodine (see Fig.I).
I. germanica starch
see Table V(c).
Parsnip starch
see Table IV(c).

FIG.II

III(b) DISCUSSION.

No previous work on the starch or other carbohydrates present in parsnip roots has been reported. Free fructose, sucrose and raffinose, as well as glucose, were found in the cold water extract (P2), but no oligo- or poly-fructosans were present. There were no free sugars in any other fraction.

On hydrolysis, all fractions gave galactose, glucose, arabinose and xylose, although the amount of xylose found was always small. Small quantities of rhamnose were identified in four of the fractions, and some alkali-soluble mannan was present. Fractions P3, 4, and 5 contained large amounts of uronic acid residues. For future structural studies, parsnip roots offer a good source of pectic materials, and also of araban.

The roots contain a strong yellow pigment; ether extraction gives a dark yellow oil in yield estimated at 9% of the wet weight. Exhaustive defatting, however, removed all traces of colouring matter, and the crude starch subsequently extracted was white in colour. There was 16.2% protein, 22.4% uronic acid, and 3% ash in the defatted, dried, roots. In addition, the quantity of cellulosic material present is high; fraction P6, obtained in 11% yield, was found to contain about 80% of its own weight of cellulosic matter. On this basis, the percentage of starch present in the roots probably does not exceed 20%, and has in addition been found difficult to extract with cold water.

Fractions P1-6 were extracted exhaustively, in order

that the differentiation between fractions should be as sharp as possible. Consequently, the crude starch I obtained (fraction P1) was highly impure, containing only 53% of non-acidic carbohydrate. Starch II was prepared on a much larger scale, and less complete extraction with cold water was made in an attempt to obtain a more easily purified crude starch. From 500 gm. of defatted roots, about 60 gm. of crude starch, containing 80% by weight of starch was extracted. Although eventually starch containing only 0.06% protein was obtained, modification of the protein, and its subsequent separation from the starch, was more difficult to achieve than in the case of oat starch. The conditions for successful differential centrifugation varied widely from those found earlier for barley and oat starches, much higher speeds being required; this is obviously a direct consequence of the unusually small size of the granules.

A further unusual feature of parsnip starch is the low percentage of amylose present (11.1%). To date, no starch having a percentage of amylose intermediate between that of the "waxy" varieties and Pearl manioc (16%) has been reported. At first it was considered that the low apparent values must be due to the presence of residual traces of fatty acids, but further exhaustive defatting did not result in any increase in apparent amylose content. From the value found for the ratio of non-terminal to terminal glucose residues (23:1) it follows that the average length of unit chain of the amylopectin component is 20 glucose units.

Starches I (a) and II (a) were found to have identical values for the percentage of amylose, and also for $\overline{R}$. There therefore appears to be no seasonal variation in these values for parsnip starch of the variety studied.

IIII(a) The preparation and purification of iris starch, and analyses of fractions obtained from Iris germanica rhizomes.

Plants of the order Iridaceae are unusual, since the different members - and even different varieties of the same member - do not all have the same main reserve carbohydrate. In particular, the many varieties of the Iris family show remarkable differences in carbohydrate content.

The endosperm of the ripe seeds of the varieties I. pseudacorus, I. germanica and I. foetidissima were reported (101, 102) to contain a manno-araban consisting of 82% mannose and 18% arabinose. Recent work on the varieties I. ochroleuca and I. siberica, however, revealed the presence of only traces of arabinose (103). No manno-araban could be detected, although a glucomannan was isolated, containing equal quantities of glucose and mannose together with 3% of galactose.

For some species, e.g. I. germanica, I. pallida and I. florentina, the reserve polysaccharide in the rhizomes is solely starch, whilst in I. pseudacorus it is a fructosan, irisin; in yet other varieties e.g. I. foetidissima, both irisin and starch are present in quantity (104, 105). Irisin was first prepared by Wallach (106), and structural studies were later made (107) on a sample, obtained in 7% yield from I. pseudacorus rhizomes, and purified by 50 re-precipitations. Irisin is insoluble in cold, but soluble in hot, water; irisin-containing varieties usually contain, however, other cold-water soluble fructosans of variable composition. It was

believed that the irisin from different varieties also varied in composition, but recently it has been shown (108) that earlier structural work was inaccurate, the presence of tri-methyl fructoses having been missed. The sample studied (108) had a molecular weight of 22,500, there being 8-9 unit chains, each of 17 fructose residues, in the molecule.

Early work (102) showed that, for several varieties, the percentages of reserve carbohydrate were constant, except when the plants were in flower. Kabay (109) made analyses, at intervals from March to November, of the percentages of starch and cold water extractives in I. germanica rhizomes grown in Hungary; the starch content was found to differ greatly from that reported for I. germanica rhizomes grown in other countries. The optimum growth conditions for I. germanica have been investigated (110).

Although the Iris family has attracted a considerable number of investigations, much more information is required, and little work has been carried out using modern techniques. In particular, no structural investigations of any variety of iris starch have been reported.

Rhizomes of the common iris, I. germanica, were taken from the ground in October, 1955, washed free of soil with cold water, and immersed in alcohol.

Analyses on the rhizomes (based on wet weight):-

Found - Ether extractives ..... = 0.92%
Methanol-benzene extractives = 9.3 %
Moisture ..... = 73.5 %

After the rhizomes were peeled and finely minced

(all under alcohol), the material was exhaustively defatted by refluxing with 90% methanol (two treatments), a methanol-benzene mixture (2:1, v/v; ten treatments), ether (two treatments) and finally with 90% methanol. All treatments were for 2-3 hr., using 500 ml. solvent per 100 gm. dry weight of material.

Analyses of defatted rhizomes:

Found - Ash ..... = 4.9%
Protein ..... = 12.8%
Uronic acid anhydride = 9.3%

Ratio of sugars on hydrolysis:-

Galactose/glucose/arabinose/xylose = 23:70:4:3.

Cold water extractions: The defatted material (dry weight estimated at 90 gm.) was extracted with cold water (400 ml.; five treatments, each of three minutes) in an "Atomix" blender, followed by five treatments in which the material was vigorously shaken for a total of seventy-two hr. During the extractions by "Atomix", which were made necessary by the fibrous nature of the minced material, no significant rise in temperature of the extracts occurred. The combined extracts were filtered through several layers of muslin, then centrifuged at 2,800 r.p.m. for five minutes, yielding a proteinaceous starch (fraction IG2S) and supernatant liquors. These were twice filtered through Whatman No. 1 paper to ensure freedom from starchy sediment, then stored at 0°C for one week. During this time a white sediment appeared, which was removed in a "Sharples" supercentrifuge, giving fraction IG2P. An aliquot of the clear extracts was then freeze-dried without prior reduction in volume,

so that no possibility of destruction or inter-conversion of the free sugars should exist. This gave fraction IG1.

Hot-water extractions: The residue from the cold-water treatments was extracted with boiling water (300 ml.) under reflux in a nitrogen atmosphere (five treatments, for four, four, eight, twelve and twenty-four hr. respectively). The volume of the combined extracts was reduced at 36°C under reduced pressure to 700 ml.; freeze-drying gave fraction IG3.

Alkaline extractions: The residue was vigorously stirred with sodium hydroxide solution (5% w/v, 200 ml., five treatments, of four, four, eight, twelve and twenty-four hr. respectively) under nitrogen at room temperature, and then at 100°C. In both cases the alkaline extract, after centrifugation, was brought to pH7 by addition of glacial acetic acid; no precipitate appeared on neutralisation. The volume was then reduced to 700 ml., salts were removed by dialysis against cold water for ninety-six hr., and the fractions (IG4 and IG5 respectively) isolated by freeze-drying.

The final residue was washed free from alkali then dried, giving fraction IG6.

Yields of fractions obtained: These are shown in Table V (a) (p.49) the overall recovery being 90% of the estimated weight of original material. Results of analyses of the fractions are shown in Tables V (a) and (b) (p.50).

Analysis of fraction IG1 for free sugars: (a) Qualitative chromatographic analysis showed that no free mono- or

di-saccharides were present, but that tri- and oligo-saccharides containing fructose, hexoses and pentoses were present in quantity. (b) quantitative analysis of the fructosan and oligosaccharide contents was made, using the method described fully by Wylam (80). The colorimetric method estimated the percentage of fructose residues present at 4% by weight of the fraction. This value compared favourably with the value of 4.4% obtained from the estimation after hydrolysis of total reducing power, in conjunction with the estimated ratio of the constituent sugars.

Analysis of fraction IG6: This fraction was only hydrolysed to the extent of 22.7% by 2% sulphuric acid. On hydrolysis with 72% sulphuric acid, the residue ("acid lignin") amounted to 6.3%; glucose and xylose (in the ratio of 91:9) were the only sugars liberated.

Analysis of fraction IG2P: This fraction was found to contain 95.6% by weight of protein. On hydrolysis, traces only of glucose, galactose and ribose were detected, these sugars being present in the ratio of 7:2:1.

Separation of starch from protein in fraction IG2S (crude starch): By drying an aliquot, the dry weight of the fraction was estimated at 36 gms. i.e. 40% by weight of the dried defatted material. The crude starch contained 8.1% protein. Purification by simple differential centrifugation was found to be possible without prior salt/toluene treatment.

The crude starch was washed free of methanol (under which it had been stored at 0°C). An aqueous slurry was

made, the specific gravity being adjusted to 1.06 by hydrometer. Centrifugation at 800-1,000 r.p.m. for five minutes, followed by three minutes at 2,600 r.p.m. (M.S.E. "minor centrifuge") gave an excellent separation of starch (bottom layer) from proteinaceous matter. The latter formed a pale brown top layer, and was carefully removed. After six such treatments, no further traces of brown material could be seen on top of the starch. Two further treatments were given, however, after each of which the top of the starch deposit was washed with a jet of water.

This purification process gave three fractions, designated IG starch (a), (b) and (c), which analysed as follows:-

	(estimated)		
IG starch (a)	Yield=21. gm.	%Protein= 0.25	%Ash=0.87
IG starch (b)	Yield= 6.4gm.	%Protein= 5.0	%Ash=0.57
IG starch (c)	Yield= 6.2gm.	%Protein=25.6	%Ash=0.68

Further analyses of IG starch (a): Found:-

$[\alpha]_D^{18}$ ... = + 191° (c., 0.34% in 30% perchloric acid).

% Amylose = 27.0 [see Fig. II (p.39a) and Table V (c)(p.51)]

$\bar{R}$ ..... = 28.0 glucose units.

Periodate oxidation data: At 15-16°C, periodate uptake reached 1.02-1.05 moles per anhydroglucose unit after oxidation for 262-308 hr. The formic acid released after this period gave a value of 28.0 glucose units for $\bar{R}$.

On hydrolysis, conversion to glucose was 98.2%. There was no residue after hydrolysis, and no other sugar could be detected by chromatography.

Appearance of granules: The granules tended to be rectangular in shape, of average size 10 x 14μ. See plate I (p.26).

TABLE V (a).

Yields and analyses of fractions from I. germanica rhizomes†.

Fraction	% Yield	% Ash	% Protein	% U.A.A.††	% non-acidic hydrolysable carbohydrate†††	Reaction with iodine
IG1	6.5	26.2	18.0	16.5	37.3	-
IG2S	45.0	3.2	8.1	2.8	85.9	+
IG2P	2.3	0.93	95.6	3.1	0.4	-
IG3	17.5	3.6	4.0	16.4	76.0	+
IG4	11.0	6.5	19.8	9.2	64.5	+
IG5	13.2	6.4	1.4	13.9	78.3	+
IG6	4.5	14.4	0	6.3	2.0	sl.+

Footnotes:-

† as % of dried defatted material.

†† Uronic acid anhydride.

††† Hydrolysable by 2% sulphuric acid.

TABLE V (b).

Sugars obtained on hydrolysis of I. germanica fractions.[/]

Fraction	% non-acidic hydrolysable polysaccharide[//]	Gal[///]	G	A	F	X	Ri.
IG1	37.3	47	27	15	4	3	4
IG2S	85.9	7	93	0	0	0	0
IG2P	0.4	(20)	(70)	0	0	0	(10)
IG3	76.0	46	46	8	0	0	0
IG4	64.5	18	69	3	0	10	0
IG5	78.3	25	65	2	0	8	0
IG6	2.0	28	40	9	0	23	0

Footnotes:-

[/] Expressed as percentages of the non-acidic hydrolysable polysaccharide content.

[//] Hydrolysable by 2% sulphuric acid at 98°C.

[///] Gal = galactose; G = glucose; A = arabinose; F = fructose; X = xylose; Ri = ribose.

TABLE V (c).

Determination of % amylose in Iris germanica starch (a).

Wt. of sample taken = 11.05 mg. Dissolution of sample, and all other conditions as detailed in Table III(c).

(a)	(b)	(c)[x-axis]	(d)	(e)	(f)[y-axis]
6.00	4.20	4.20	1.80	1.80	0.41
4.00	1.80	6.00	2.20	4.00	0.91
5.00	2.20	8.20	2.80	6.80	1.55
5.00	1.65	9.85	3.35	10.15	2.32
5.00	1.00	10.85	4.00	14.15	3.24
5.00	0.65	11.50	4.35	18.50	4.22
5.00	2.00	13.50	3.00	21.50	4.90
5.00	4.05	17.55	0.95	22.45	5.22
5.00	4.15	21.70	0.85	23.30	5.30
5.00	4.75	26.45	0.25	23.55	5.40
10.00	9.65	36.10	0.35	23.90	5.45
10.00	9.65	45.75	0.35	24.25	5.55
15.00	14.45	60.20	0.55	24.80	5.70
15.00	14.45	74.65	0.55	25.35	5.80
20.00	19.25	93.90	0.75	26.10	5.95

Factor for conversion of (e) to (f) = 0.228

Result from graph (fig. II):

$$\% \text{ Amylose} = \frac{5.2}{19.2} \times \frac{100}{1} = 27.0\%$$

IV(b) DISCUSSION.

The dried, defatted rhizomes of I. germanica have been shown to contain about 40% by weight of starch. Fraction IG1 (cold water extractives) was isolated without having been raised above room temperature at any stage; examination for free sugars showed that there were no free mono- or di-saccharides. Water-soluble tri- and oligo-saccharides containing fructose residues were present, however, the total percentage of fructose residues being estimated, by two different methods, at 4% by weight of fraction IG1, i.e. approx. 0.2% by weight of the dried defatted rhizomes. No free sugars were present in any other fraction, and there was no water-insoluble fructosan. I. germanica therefore contains no irisin, the main reserve carbohydrate being starch. This is in agreement with the work of previous investigators (104).

The carbohydrate content is largely composed of glucose residues (70%), but the galactose content is high (23%) and very little arabinose and xylose are present (4% and 3% respectively). Fractions IG1 and IG3 contain high percentages of galactan. The small percentages of ribose found in the hydrolysates of fractions IG1 and IG2P probably arose from nucleo-protein or similar sources (111).

The rhizomes contained no significant colouring matter, and the percentage of ether extractives was unusually low (0.92%). This may not represent the true content of fatty

materials, however, since benzene-methanol extracted 9.3%, although this figure will include some water. The ether extract was colourless, the benzene-methanol extract pale yellow. The ash content of the defatted rhizomes was unusually high (4.9%). Consequently, the cold-water extracts (fraction IG1) had a high ash content (26.2%), but it was surprising to find a high ash content (3.2%) in the crude starch. Although much of this was eliminated in the purification process, the ash content of the three starch fractions obtained was still relatively high (0.87, 0.57 and 0.68% respectively).

Uronic acid residues and protein were present in all fractions. The isolation of a protein-rich fraction (IG2P) containing 95.6% protein was of interest, no such fraction being obtained from either oat or parsnip.

Despite attempts to achieve exhaustive fractionation at each stage, it is remarkable that some starch resisted extraction even by cold and hot alkali, as shown by the slight positive reaction with iodine given by the residual cellulosic fraction IG6. The percentage of "acid lignin" in this fraction was fairly high, and an appreciable quantity of cellulosic material was present. This, however, did not prevent cold-water extraction of the starch, as was found in the case of parsnip roots; the treatments by "Atomix" blender were necessary as a result of the very hard structure of the rhizomes, even when finely minced.

Although containing a significant quantity of protein (8.1%), the crude starch was easily purified. In contrast to the cases of oat and parsnip starch, prior treatment

of the protein by shaking with salt and toluene was not required before separation from the starch by differential centrifugation could be achieved. However, despite the relative ease of purification, the protein content could not be reduced below 0.25%. This is of the same order as obtained for oat starch, but is much higher than for parsnip starch.

From the values found for the amylose content (27.0%), and for the ratio of non-terminal to terminal glucose units (28:1), it can be calculated that the average length of unit-chain in the amylopectin component is 20 glucose residues. These values were identical to those found in 1954 for a sample of I. germanica starch, which, in addition, was not prepared (113) by the method reported in this thesis.

SUMMARY AND GENERAL DISCUSSION.

Crude starches from oat kernels, parsnip roots and I. germanica rhizomes have been prepared, and separated from contaminating protein, by methods unlikely to cause degradation to the starch components. Although attempts were made to remove all traces of protein, small percentages remained in each case. It appears that this residual protein is chemically bound to the starch, in agreement with Stacey's hypothesis that synthesising enzyme systems remain in combination with polysaccharides. (42).

The physical purification process used depends on the protein impurity having a sedimentation rate appreciably lower than that of the starch granules. In the case of barley (47) and I. germanica, such a difference apparently exists naturally; for other sources, however, e.g. H. brasiliensis seeds (9), parsnip, and oats, treatment of the crude starch by shaking with salt/toluene is required before differential centrifugation is possible. Much depends on the physical size of the starch granules, and on the nature of the protein itself, although whether the crude starch is prepared from cereals or roots is evidently not critical. Another possible factor is the way in which starch and protein are associated in the plant; the proteinaceous matter may be present as a layer completely surrounding the starch granules (9), whereas microscopic examination of crude oat starch showed that the starch and protein were present as a physical admixture.

Since the proteinaceous material which has to be removed in the purification of a starch is already cold-water insoluble, the term "denaturation" cannot properly be applied to the process of shaking with salt/toluene described above. Rather does the process appear to be one in which the state of aggregation of the protein-aceous matter is decreased. This may result from part of the protein dissolving in the salt solution; should this happen, then in addition true denaturation of the protein could subsequently occur through contact with the toluene present.

It is possibly significant that although the starch-rich fraction obtained from the purification process contains very little protein (0.2%), the protein-rich fraction always contains much starch (30-40%). If this starch is not bound to protein in any way, then the granule size must be much smaller than for that in the quicker sedimenting starch-rich fraction. In the case of parsnip starch, either modification of the protein material is extremely difficult, or the process of modification must be continued further than is sufficient in the other cases studied. The latter explanation is supported by the fact that parsnip starch granules, which are extremely small, themselves require a high centrifugal force for complete sedimentation.

Although the amylose content of parsnip starch is abnormally low (11%), whilst that of oat (26%) and I. germanica (27%) is higher than for most common starches, the values of $\bar{R}$ found for each were such that,

on calculation, the average length of unit chain of the amylopectin component of all three starches was twenty glucose units, a value found in many previous cases. Despite this apparent constancy in the amylopectin component, however, it is now apparent (cf. 100, 112) that the percentage of amylose in starches varies more widely than was earlier believed. It was possibly fortuitous that the first starches studied should have had amylose contents of about 20-25%; investigation of further natural sources may well reveal the existence of starches having values of amylose in the range 2-16%, and higher than 30%, without resort to hybridisation.

No seasonal variation occurred in the values of $\overline{R}$ and of percentage amylose for the starches studied.

Hydrolysis of the fractions obtained from the graded extraction procedure gave, in all cases, a mixture of sugars. The cold-water soluble fractions obtained were non-starchy, but the quantitative extraction of starch is very difficult, particularly when the cellulosic and pectic structure of the plant source is heavier than normal. Several other factors preventing easy extraction of starch are also known (71, 72, 95). Consequently, the hot-water extracts, and both alkaline extracts, in each of the fractionation schemes contained starch, and the differentiation of starchy from hemi-cellulosic fractions was not sharp.

Parsnip roots have been found to contain high percentages of pectic materials and araban, whilst I. germanica rhizomes have unusually high galactan content.

Both these sources may be of interest for future structural studies of such polysaccharides.

PART II.

The interaction of starches and other α-1:4 glucosans, with iodine; and the development of a valve micro-voltmeter for differential potentiometric titrations.

INTRODUCTION.

Determination of the percentage of amylose present in a starch or its fractionated components is one of the most important analytical techniques in modern starch chemistry. The development of such an analytical method was one of the consequences of Schoch's successful quantitative method of starch fractionation (37).

The first partial fractionation of starch was carried out by Maquenne in 1905 (116), by a process now known to have involved the retrogradation of the more unstable amylose from solution; a component was isolated which was completely hydrolysed to maltose by β-amylase. Maquenne called this component "amylose", and suggested that a second component existed (for which he proposed the name amylopectin) which was not completely hydrolysable by β-amylase, giving a limit dextrin. It was fitting that, when Maquenne's results and suggestions were - much later - shown to be correct, the names he had proposed for the starch components should be widely adopted. In 1920, Samec (117, 118) fractionated potato starch, using an electrophoretic method which depended on the polarity imparted to the amylopectin component by the presence of bound phosphate. Ten years later, Baldwin (119) employed a more general method, preferentially leaching the amylose component from the granules with hot water. This method was used by Meyer in 1940 (120) to obtain for study relatively pure samples of amylose and amylopectin.

Schoch's method of fractionation, which was based on

an observation by Alsberg (121), was used by Bates, French and Rundle in 1943 to obtain fractions with which was developed a potentiometric titration method for determination of the relative proportion of amylose in starches (50). The method was based on the greatly differing affinities of amylose and amylopectin for iodine. Measurement of the potential difference between a calomel electrode and a bright platinum electrode in the starch-iodine solution permitted calculation of the equilibrium concentration of free iodine in the latter. When the amount of bound-iodine was plotted against the total free iodine concentration, a sigmoidal-shaped adsorption isotherm was obtained. For a solution of pure amylose, the activity of the free iodine was constant until the amylose had bound about 20% of its own weight of iodine (the actual amount varying with the iodide concentration present), after which there was a slow, linear increase in activity. The point of intersection of the latter (nearly horizontal) part of the curve with the vertical (constant activity) part was taken as a measure of the "iodine affinity" of the sample. The percentage of amylose in a starch was found by comparing its iodine affinity with that of pure amylose under the same titration conditions.

Only since the introduction of this potentiometric titration technique have quantitative data been obtained on the mode of interaction of amylose with iodine. A definite inclusion complex, and not a solid solution, appears to be formed (123).

The presence of the α-1:4-linkages in amylose permits the long chain of glucose residues to coil naturally into a helical configuration. It has been suggested (124-126), in order to explain retrogradation, that although the stable form in solution is helical, equilibria involving linear chains and intermediate forms probably exist. To explain the blue colour of starch/iodine complexes in aqueous solution, Freudenberg (125) suggested that the hydrogen atoms at positions C1 and C4 on each side of the glucosidic bridge formed a hydrocarbon lining to the helix. Measurements of the dichroism of flow of amylose/iodine solutions (127, 128), studies of optical properties of crystalline amylose before and after staining with iodine (129), and x-ray studies of butanol/amylose and iodine/amylose complexes (129, 130) have all provided evidence for the existence of a helical configuration. The x-ray studies also revealed that the iodine atoms were located inside the helix, there being six D-glucose units, and two iodine atoms, per helix turn. Additional evidence for the value of six D-glucose units per turn was obtained from absorption measurements (131).

The great difference in value of the molar extinction coefficient of iodine in starch and in non-polar solvents has been taken to suggest that the starch-iodine interaction is of a dipolar nature (123). On this basis, and assuming a helical configuration in solution, the increased preferential uptake of iodine by amyloses of longer chain lengths (50) can be explained by the increased stability of the complex formed. Dipolar forces have

also been shown capable of accounting for the stabilisation energy of amylose/iodine complexes in the solid state (133), although the resonance effects of a polyiodide chain at high dipolar interaction could provide additional stabilisation.

From potentiometric studies, Gilbert and Marriott (134) have shown the constitution of the iodine complex to be $(3I_2.2I^-)$ i.e. I_8'' for less than 0.5% of bound iodine in 10^{-3} to 10^{-4} M iodide, complex formation being accompanied by the emission of 11.2 k. cals. per mole of iodine. These authors considered that this resonating ion was responsible for the characteristic blue colouration.

The amount of iodine bound decreases significantly with increased iodide concentration; this effect has been explained by the decrease in volume available to iodine inside the helices. The decreased amount of bound iodine results in a decrease in the wavelength of maximum absorption of the complex (131), and this has been related to decreased length of resonating chains of iodine molecules (135, 136). The wavelength of maximum absorption is also related to the chain length of the amylose (131, 137); Swanson has investigated this effect for synthetic, short-chain, linear glucosans (138). Foster and Paschall (139) have suggested that the wavelength of maximum absorption is affected by the degree of crystallinity as well as by the amylose chain-length.

Although evidence for a helical amylose configuration, and for the resonating dipolar form of interaction between amylose and iodine, is extensive, Schlamowitz considered

the interaction to be non-polar (140). Meyer has proposed (141) that the mechanism is one of adsorption of iodine on colloidal micelles, although his experimental data can also be explained by the helical theory.

In contrast to the deep blue amylose/iodine complex, pure amylopectin gives a red colouration, and binds much smaller amounts of iodine under the same conditions (50). Pure amylopectin is difficult to obtain by fractionation, and the true red colour with iodine is masked by the blue given by traces of contaminating amylose. Whistler and Hilbert (142) proposed that complex formation between iodine and amylopectins was due to hydrogen bonding, but Mikus, Hixon and Rundle (143) considered that the low iodine binding-power of branched α-1:4-glucosans could not be explained on this basis. They suggested that the large number of branch-points prevents helix-formation and decreases the dipolar forces responsible for the amylose-complex formation.

Higginbotham (144), as a result of potentiometric and absorption spectra measurements, suggested that in amylopectin the iodine is bound partly by a helix mechanism and partly by adsorption of I_2 and I_3'.

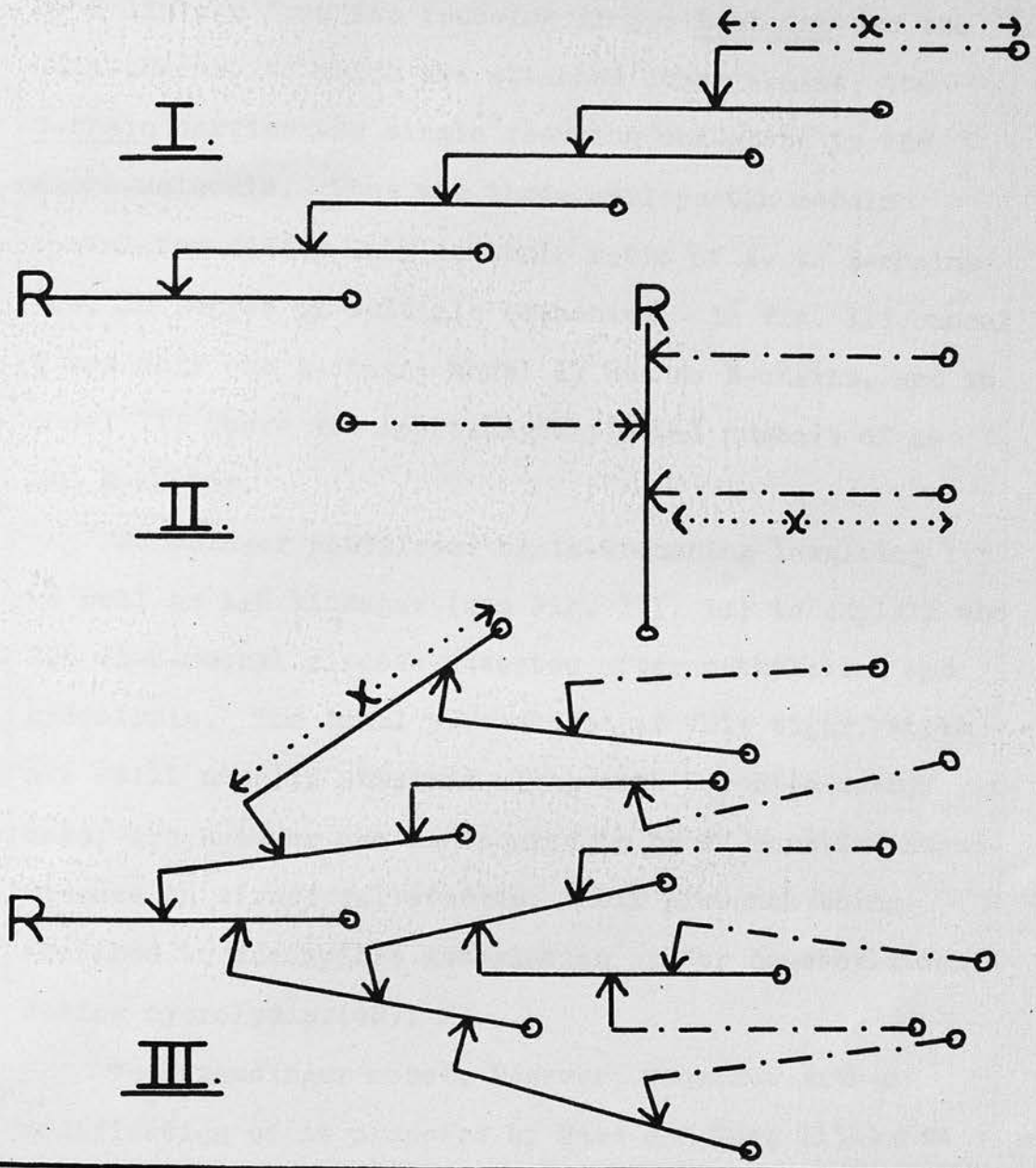
From potentiometric titration studies, Gilbert and Hybart (145) deduced that the iodine was bound as a complex of one or two iodine molecules together with 0, 1, or 2 iodide ions, depending on the iodide concentration present.

Different amylopectins were found (50) to bind different amounts of iodine; differences in absorption

spectra were also observed (131). It was considered that these effects were due to differences in the average length of unit-chain of the amylopectins. Considerable variation in the length of some external branches was considered possible, and Swanson (138) obtained evidence of lengths of up to eighteen glucose units. If free to assume a helical configuration, such long branches would possibly act as short-chain amyloses.

It is now evident that, for amylopectin, the amount of helix formation possible, and hence the iodine-uptake, must be related to fine structure. Various branched structures have been proposed. The "laminated" structure (Fig. III, I) was proposed by Haworth, Hirst and Isherwood (146) on the basis that the non-reducing end group value showed little change for large changes in molecular weight. This structure was the simplest compatible with the data available from methylation studies, but was never intended to be a complete representation of the amylopectin molecule (132). Staudinger suggested a "herring-bone" structure (Fig. III, II) to explain the results of experiments based on the viscosity of starch solutions (147), whilst Meyer (148) proposed the multiple-branched "tree-like" structure (Fig. III, III) to explain enzymic degradation results.

Myrbäck and Sillén (149) showed that these structures all contained the same basic linear chains in different arrangements, and Peat, Whelan and Thomas (34) suggested that these basic chains be termed A-, B-, and C-chains. The A-chain is attached to the rest of the molecule only



Symbols:

↓↓ 1:3 linkage.

↓ 1:6 linkage.

—○ non-reducing end-group.

R— reducing end-group.

·—·—· A-chain

——— B-chain

R—— C-chain

} of α-1:4 linked glucose units. (x ≒ 20).

FIG. III.

by a linkage from its reducing group; B-chains are the main-chains, to which are attached other chains; the C-chain carries the single reducing end-group in the macro-molecule. Thus the three amylopectin models postulated differ only in their ratio of A- to B-chains i.e. in degree of multiple branching. In Fig. III, model I has only one A-chain; model II has no B-chains, and in model III there are approximately equal numbers of A- and B-chains.

Staudinger postulated chain-branching involving 1:3 as well as 1:6 linkages (see Fig. III, II) to explain the 2:6 di-o-methyl glucose detected after methylation and hydrolysis. The small percentages of this sugar, which are still usually obtained along with 3:6 di-o-methyl glucose, are however now considered to be of doubtful significance in structural studies, their presence being ascribed to incomplete methylation and/or demethylation during hydrolysis.(48).

The Staudinger model, however, together with a modification of it proposed by Hess and Lung (151) has been disproved on the basis of studies on the breakdown of amylopectin by hydrolytic enzymes (148, 152); these structures moreover do not explain the results of Hirst and Young's "disaggregation" experiments (10, 153, 154).

Variations in fine structure must exist to explain the difference between the limiting viscosity numbers of solutions of amylopectins and those of the other main type of α-1:4 branched glucosans - the glycogens (122, and cf. 9). In addition, there exist "abnormal" α-1:4

branched glucosans which cannot be classed as amylopectins or as glycogens e.g. those synthesised by plant hybrids (155), by seaweeds [e.g. floridean starch (156)] and by protozoa (157, 180).

Any method capable of giving indications of differences in fine structure is therefore important. Since α-1:4 glucosans are characterised by their interaction with iodine, it appeared that a quantitative method of determining iodine binding-power, which had sufficient accuracy and sensitivity to measure the very small uptake given by highly branched structures, might enable such information to be obtained. An apparatus having the necessary sensitivity would also give very accurate determinations of the purity of starch fractionation products. The construction of such an apparatus was therefore attempted with the proviso that, if possible, it should be capable of use as a simple routine analytical method.

Although colourimetric methods for the estimation of percentages of amylose have been developed (158, 159) which are of great use in routine work and for rapid comparative measurements, the measurements obtained are not absolute, nor capable of the same accuracy as given by the potentiometric method, since the optical absorption characteristics of the iodine complexes of different amyloses and amylopectins may vary considerably. Higginbotham and Morrison (160) observed that the Blue Values of pure amylose fractions from different starches, which bound the same percentage of iodine by potentiometric

titration, ranged from 1.26-1.48; for different amylopectins, the Blue Values (corrected for the presence of small quantities of contaminating amylose) varied from 0.06 to 0.16. Moreover, since the colorimetric method could not detect iodine uptakes of the order of 10% of that of normal amylopectins, it was obvious that a potentiometric titration method had to be devised.

The design was based on the elegant differential method of Gilbert and Marriott (134), which permits very accurate measurements at the necessary low iodine concentrations. Perhaps the only disadvantage of this method is the inherent logarithmic decrease in possible sensitivity as the free iodine concentration in both half-cells increases during a titration. The starch-iodine-iodide solution, and the blank-iodine-iodide solution are arranged as opposing concentration half-cells connected by a salt-bridge; the equilibrium free-iodine concentration can be found directly by a null-deflection measurement. Separate titrations for reagent blanks are not required. As has been mentioned (161), the scope of the differential technique, and the accuracy and reproducability of results obtained by its use depend on the availability of a null-potential indicator of great zero stability combined with high sensitivity.

EXPERIMENTAL.

I. Development of a simple electrometer.

Many electrometer circuits, designed to achieve sensitivity with minimum zero-drift and instability, have been described (162). Complete elimination of zero-drift has been claimed (163) but this was generally attained by use of internal compensation (164), mu-balance (165), continuous charging of batteries (166), or negative feed-back and stages of D.C. amplification (167). Consequently these circuits require some form of tuning, and were not as simple as was desired. In most instances, elaborate precautions with regard to external shielding, thermo-statting and earthing were also necessary (168).

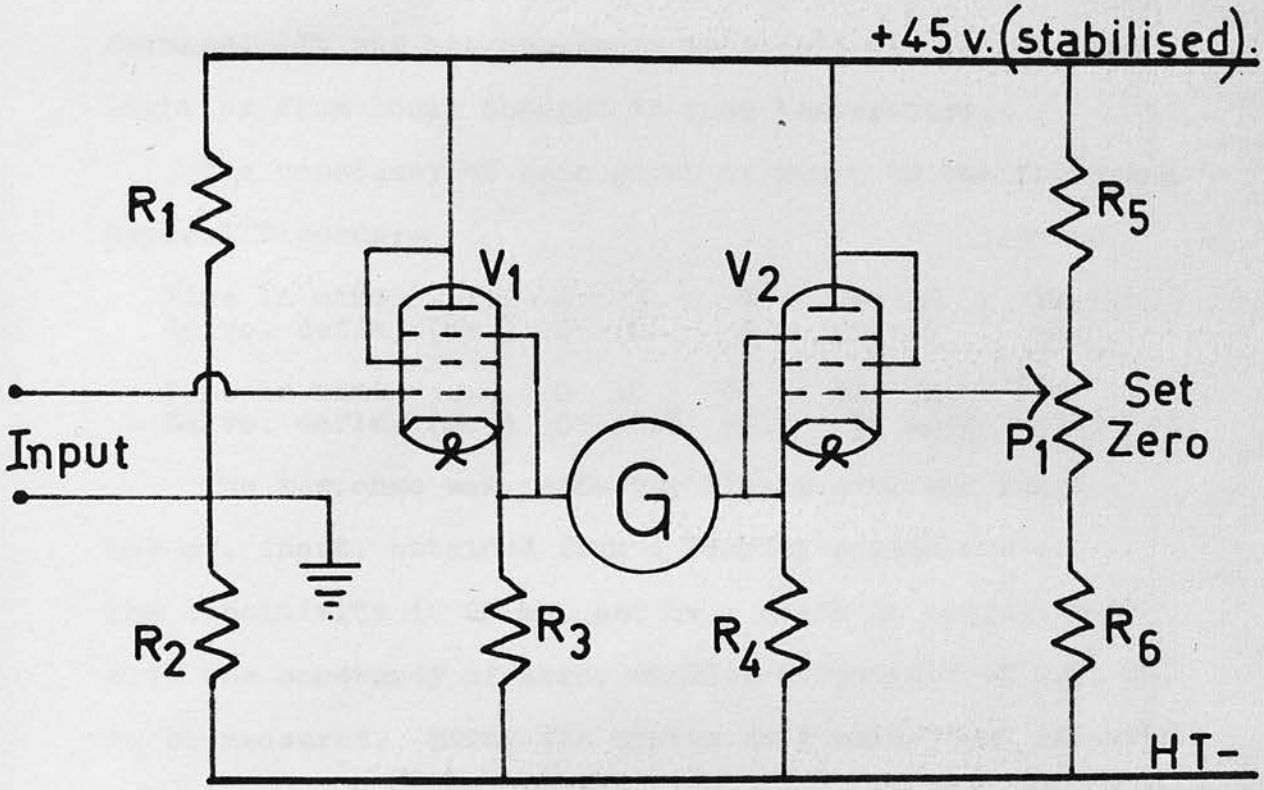
Preliminary investigations were made using an electrometer triode (Type ET1, grid current 10^{-15} amp.) as one of the arms in a Wheatstone-bridge circuit built into an air-tight, vibration-free box, from which light was excluded. Using a spot galvonometer of sensitivity 109 mm. per microamp. and internal resistance 402 ohms, the sensitivity obtained was 6 mm. per millivolt. Zero drift was reduced to an average of 0.5 mm. per minute by using good quality components, allowing adequate wattage dissipation in all resistors, and using aged and well-charged accumulators. (A zero drift of 1-2 mm. per minute had been claimed for circuits of similar sensitivity (168, 169). Neither the sensitivity, nor the rate of zero drift, obtained was considered to meet the standards desired. In addition, the system was so susceptible to

external interference, despite the instalment of a virtual earth system, the provision of an equipotential shield (170), and careful screening, that it was rejected.

The circuit finally adopted employed the well-known principle of using a matched-pair of valves as two of the resistors in a Wheatstone network (171). The other two bridge resistors in this case were made the cathode loads for the valves, as shown in Fig. IV. Since each valve therefore functions as a "cathode follower" the circuit is extremely stable, with a high input impedance (experimentally determined as 700 megohms) and an output to input ratio of 1:11. For a titration cell resistance of two megohms, the grid current was 3×10^{-11} amp. (Although this value is higher than that given by an electrometer triode, no undesirable effects in solution have been observed.)

The valves used (Type VR 116) were a matched pair, selected from ten which were available. This type was chosen for its sturdy filament construction and good heat inertia, which enabled the grid current to be reduced as far as possible by under-running the valves as follows:- (a) The nominal 6.3 volt filaments were run off four volts, supplied by three pairs, arranged in parallel, of 2-volt accumulators, aged and well-charged (172); (b) an H.T. voltage of +45 volts was used, obtained from an electronically stabilised power unit by means of a potential divider system.

The only shielding required was conventional screening of the input coaxial cable to the grid of V1.



Component Values:-

R_1, R_5, 68K.

R_2, 500 ohms.

R_3, R_4, 11K.

R_6, 470 ohms.

P_1, 25 ohms.

V_1, V_2, Type VR 116.

FIG. IV

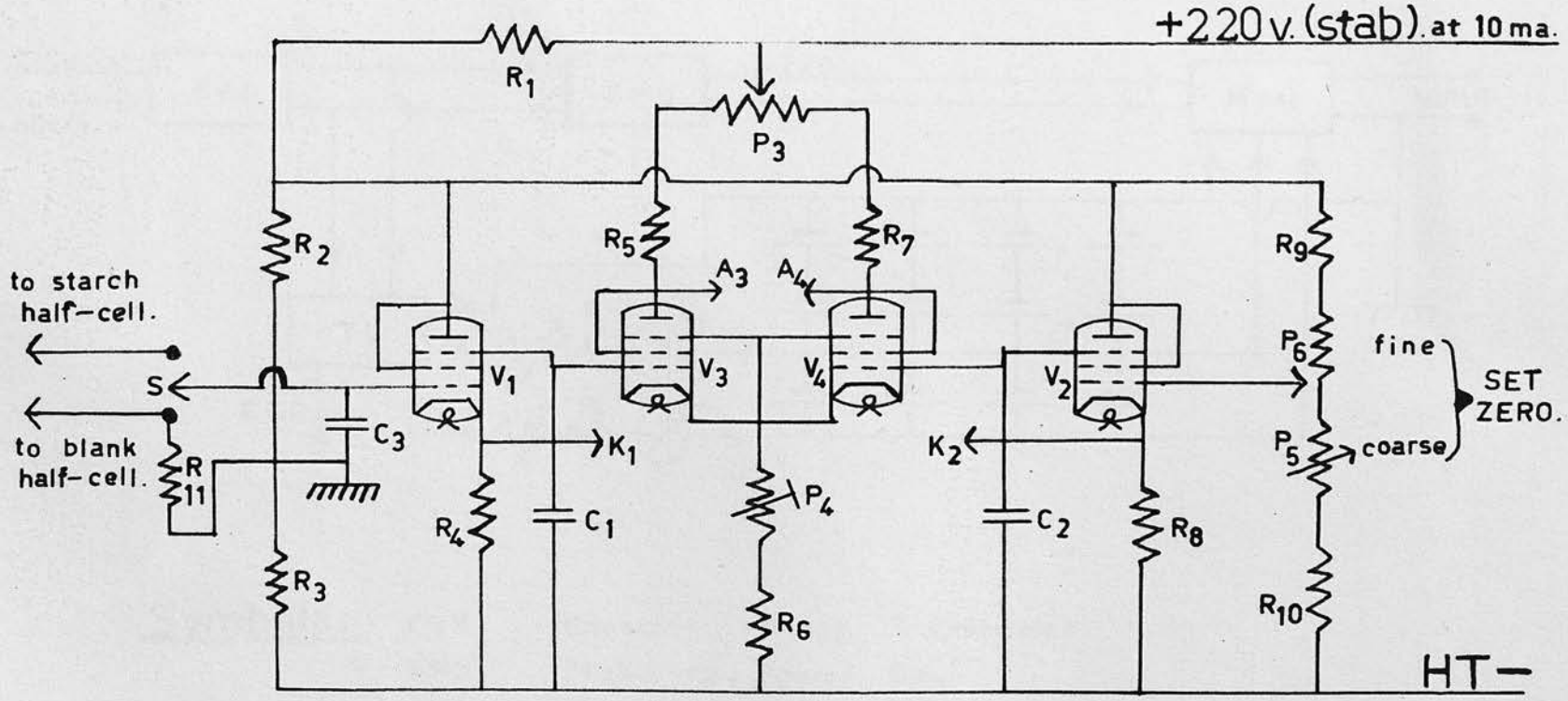
The metallized envelope of the valves was earthed, but screened-type grid top-caps were not required, despite the close proximity of equipment capable of electrical interference. It was not necessary to shield the valves from light or from local changes in room temperature.

The constancy of zero given is shown by the following typical figures:-

Time in mins.	0	3	6	9	12	18
Galvo. defln. (mm.)	0	0	0	0	+1	+1
Time in mins.	0	10	30	50	70	100
Galvo. defln. (mm.)	0	+0.5	+0.5	0	+0.5	+2.5

The response was perfectly linear over the range 0-5 mv. input, obtained from a Tinsley potentiometer. The sensitivity is 28 mm. per mv., which in conjunction with the constancy of zero, enables potentials of 0.01 mv. to be measured. Using the system as a null-point indicator, an uptake of as low as 10^{-5} mg. iodine per mg. polysaccharide can be detected.

This sensitivity gave excellent results for routine analyses of starches and their components, using sample weights of the order of 2 mg. of amylose, 10 mg. of a starch, and 30 mg. of amylopectin. The iodine uptake of highly branched glucosans such as Floridean starch and glycogens could also be studied, although it was found that reliable readings were not possible towards the end of a titration, when the free-iodine concentrations exceeded about 8×10^{-6} M. This unfortunate inherent logarithmic decrease in possible sensitivity in the differential system has already been mentioned. To permit an accurate study of the iodine binding-power of highly



SYMBOLS: C_1, C_2, 0·01 μF, mica; C_3, 0·1 μF, mica. S = two-way switch (wax-block).

P_3, 500 Ω; P_4, 250 Ω; P_5, 10K (all 5 watt). P_6, 10 Ω / 2 watt. (All linear, wire-wound).

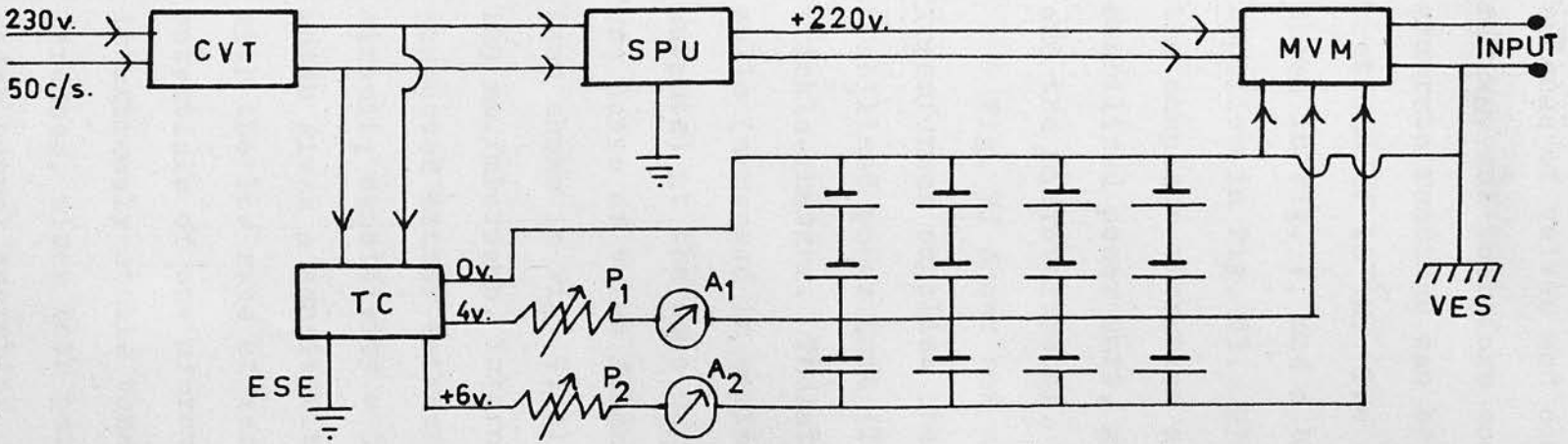
R_1, 100 K; R_2, 68 K; R_3, R_{10}, 500 Ω; R_4, R_8, 10 K; R_5, R_7, 22 K; R_6, 250 Ω; R_9, 65 K. (All 5w., w/w).

R_{11}, 2 MΩ, 1 watt, carbon. ⏚, virtual earth system.

V_1, V_2 = VR 116; heaters, 4V. at 0·88 amp., V_A = 50 v., V_G = 0 v., V_K = 1 v.

V_3, V_4 = VR 65 (SP 61); heaters, 6 v. at 1·25 a., V_A = 110 v., V_G = 1 v., V_K = 2·4 v.

FIG. V.



Symbols:

CVT	Constant Voltage Transformer.
SPU	Stabilised Power Unit.
MVM	Microvoltmeter.
TC	Trickle Charger.
ESE	Electricity Supply Earth.
VES	Virtual Earth System.
A_1	Ammeter set at 0·88 amp.
A_2	Ammeter set at 1·25 amp.
P_1	15 ohms, 5 amp.
P_2	12 ohms, 7 amp.

FIG. VI.

impedance and low grid current of the original circuit, and moreover was still simple, depending on fundamental balance of valves and components rather than on compensating, and therefore complicating, circuitry. Very accurate readings can be made up to free iodine concentrations of 10^{-5}M. The final micro-voltmeter circuit is shown in Fig. V, and a block diagram showing voltage supplies in Fig. VI. Plate II shows a general view of the complete titration apparatus; Plate III shows the stabilised power unit, galvonometer, wax-block switch, and the microvoltmeter.

Fig. VI shows how a harmonic-filtered constant voltage transformer supplies the input voltage to an Ediswan stabilised power unit (Type R1095) and to an accumulator trickle-charger. The latter charges a pile of 12 x 2v cells (arranged in series/parallel to give 4v. and 6v. outputs) at the same currents as are being taken by the two pairs of valve filaments. A highly insulated switch (not shown in Fig. V) allows the galvanometer (sensitivity 109 mm./microamp, internal resistance 402 ohms) to be connected either between K, and K_2 (so giving the original circuit; sensitivity = 30 mm./mv.), or between A_3 and A_4 which gives a sensitivity of 315 mm./mv. In conjunction with the low rate of zero-drift attained, this permits potentials of one microvolt to be measured. The circuit is extremely stable towards external electrical interferences, since both pairs of valves have been selected under actual operating conditions as the best matched pairs obtainable from a large number. The operating conditions

of both pairs differ, and are to some extent interdependent; the choice of individual valves to form pairs, and of optimum values for the resistors, could only be made by continued "refinements". Wire-wound resistors, matched in pairs to within 1%, are used throughout, and all grid leads are of screened-type coaxial cable. For best results, the electrometer must be adequately protected against vibration, mechanical shock, and local changes in room temperature. The value of R6 giving the optimum ratio of sensitivity to stability is 330 ohms; decreasing this value gives increased sensitivity, but the circuit may then tend to oscillate, creating instability. Although this can be minimised by inserting "grid-stopper" resistors (47,000 ohms; 0.5 watt) inside the grid top-caps of V3 and V4, the thermal effects associated with these resistors contributed to zero-drift. The introduction of negative feed-back, either by connecting V3A to V1G and V4A to V2G via 1 megohm resistors, or by cross-connecting V4A to V3Sc and V3A to V4Sc, reduced rather than improved stability, and it was shown that zero-drift is largely due to fluctuations in the L.T.-, and not in the H.T.-supply. Absolute matching of V3 and V4 is achieved by connecting both V3 and V4 grids to V1K (with V1G to V1K via 2 megohm resistor), and, with P4 preset at its optimum value, P3 is adjusted so that the galvanometer deflection when connected across A3/A4 is zero. After returning V4G to V2K, P3 is never altered, and all zeroing adjustments are made using the "set zero" coarse and fine controls for both A3/A4 and K1/K2 systems.

The two-way switch shown in the input circuit to V1

(Fig. V) must be very highly insulated, and must make-before-break so that the grid of V1 is never on open circuit. A satisfactory switch was made from a thick block of paraffin wax containing pools of mercury between which contact was made by a tilting copper-wire framework. See Plate IV. The inter-electrode resistance is approximately two megohms; when checking the zero-reading of the electrometer, the grid of V1 is therefore returned to earth via R11 so that the operating conditions of V1 are changed as little as possible.

Details of Titration Cells:- The titration cells (1 l. Pyrex flasks) and salt bridge were similar to Gilbert and Marriott's (134), except that stirring was automatic and continuous; See plate IV. Additions of iodine were made via additional necks in each flask. All four necks were fitted with ground-glass joints enabling the apparatus to be completely sealed, stirring being made through Quickfit stirrer glands. [No loss of iodine occurred through volatilization in the time taken for titration (i.e. 40 mins.)] The electrodes consisted of platinum foil (2 x 2 cm.), fused to platinum wire. This was sealed through glass tubing in which was placed mercury. By careful strain-free construction and thorough cleaning, it was possible to obtain a pair of electrodes between which no potential difference existed when placed in the same solution of electrolyte.

Preparation of Starch Solutions:- All samples, regardless of previous treatment, were exhaustively extracted with boiling methanol to remove traces of fatty materials.

This procedure is essential. For example, an undefatted sample of commercial rice starch apparently contained 11.8% of amylose, whilst after being refluxed with methanol (five treatments; each of three hr.) a constant value of 18.5% of amylose was given. (7.3% of the original weight of starch was extracted by the methanol). After defatting, samples were dried in vacuo at 80°C. for several hours before being weighed, by means of a stoppered weighing-stick, into a standard flask. Suitable weights for titration were: starch, 10mg.; amylopectin and glycogen, 30 mg. Solution was achieved by shaking overnight at room temperature with 0.2M-potassium hydroxide (10 ml.), after moistening with ethanol (two drops). In certain cases it was necessary to heat the mixture at 95°C. for three mins. before shaking. [The effect of pretreatment of whole starch with alkali was investigated, since the amylose component degrades in this solvent (52). Moreover, it has been shown that, for very degraded amyloses, the iodine uptake curves approximate to those of amylopectins, being indistinguishable in cases where the amylose chain-length was less than fifty glucose units (150). Ageing at room temperature had no effect, and heating a starch in 0.2M- and also in 1M-potassium hydroxide for thirty mins. at 95°C. had a negligible effect on the iodine affinity of the sample. Thus any degradation caused by this pre-treatment must be so slight as to be insignificant as far as determination of iodine uptake is concerned.] Immediately before addition to the titration half-cell, the alkaline polysaccharide solutions

were brought to pH 5.85 by the addition of a predetermined volume of 0.4N-phosphoric acid. A blank solution containing no starch was similarly prepared.

Titration conditions and procedure:- Titration conditions were: (iodide), 0.01M; pH, 5.85; temp., 20°C. [0.01M-iodide was chosen so that the addition of 0.01M-iodine-potassium iodide did not alter the iodide concentration in the half-cells during a titration, and thus corrections such as applied by Mould (161) were avoided]. The electrolyte solution (2000 ml.) contained 0.1M-potassium iodide (210 ml.) and M/15-phosphate buffer (15 ml.; pH, 5.85). 800 ml. of this solution was placed in each half-cell, and stirred for thirty mins. in the thermostat to allow for temperature equilibrium. The neutralized polysaccharide and blank-solutions were then added to their respective half-cells and the standard flasks carefully rinsed, giving a total volume of 840 ml. [i.e. (iodide), 0.01M]. With careful preparation of all solutions, and with temperature equilibration, no significant off-balance potentials existed at the start of titrations, and the "depolarizing" procedure described by Gilbert and Hybart (177) was not found to be necessary. Points on the titration curve were obtained by adding small increments of 0.01M-iodine-potassium iodide by means of an "Agla" micrometer syringe to the solution cell, then adding the same iodine solution to the control until the concentration of free iodine in each was identical, after two to five mins. (or longer for branched glucosans) had been allowed for equilibration. The difference between

the volume of iodine added to the solution cell and that added to the control gave the amount of iodine bound by the starch, the iodide concentrations in each cell being identical. The total free iodine in the starch solution (i.e. $I_2 + I_3'$) was plotted against mg. iodine bound per 100 mg. polysaccharide.

Reproducibility of Technique:- Results were independent of the length of time taken to complete a titration curve (provided true equilibration had been achieved at each free iodine concentration), and also of variation in the sample weight. The reproducibility of the technique has been found to be within $\pm 2\%$ of the iodine affinity for an unfractionated starch (i.e. for a starch having an iodine affinity of 5.00%, the results of six determinations lay between 4.90 and 5.10%).

As described by Gilbert and Hybart (177), addition of excess thiosulphate enabled the titration curve for any starch sample to be repeated. When the titration was repeated at twenty-four or forty-eight hour intervals for fourteen days, the starch solution being left in contact with iodine throughout, the observed changes in iodine affinity could be attributed to retrogradation of the amylose component. For waxy maize starch, the titration curve was unaltered after the sample had been in contact with iodine for seventeen days. Similarly, for rabbit liver glycogen, the curve was unaltered after contact with iodine for twenty-one and thirty-one days.

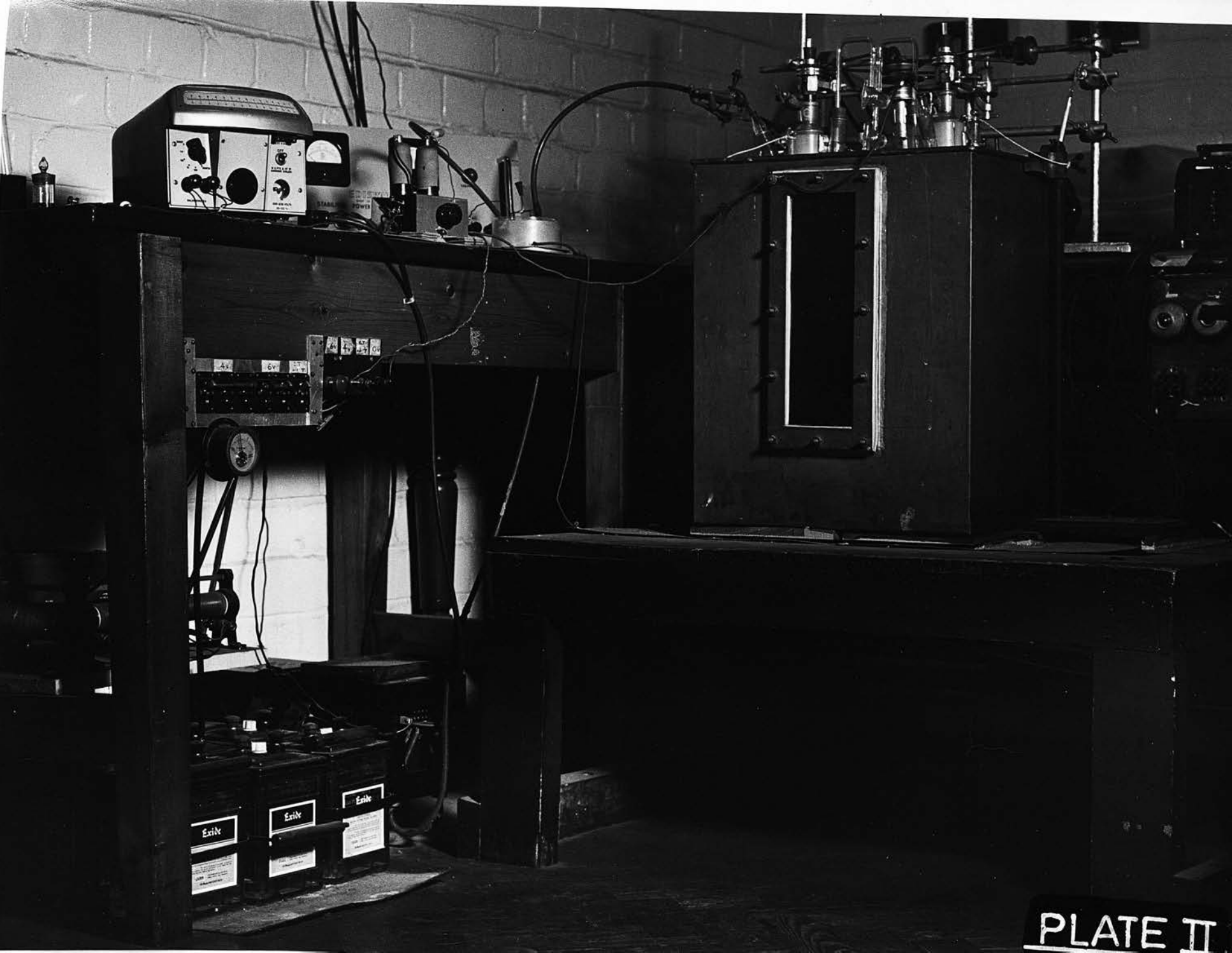


PLATE II

PLATE III

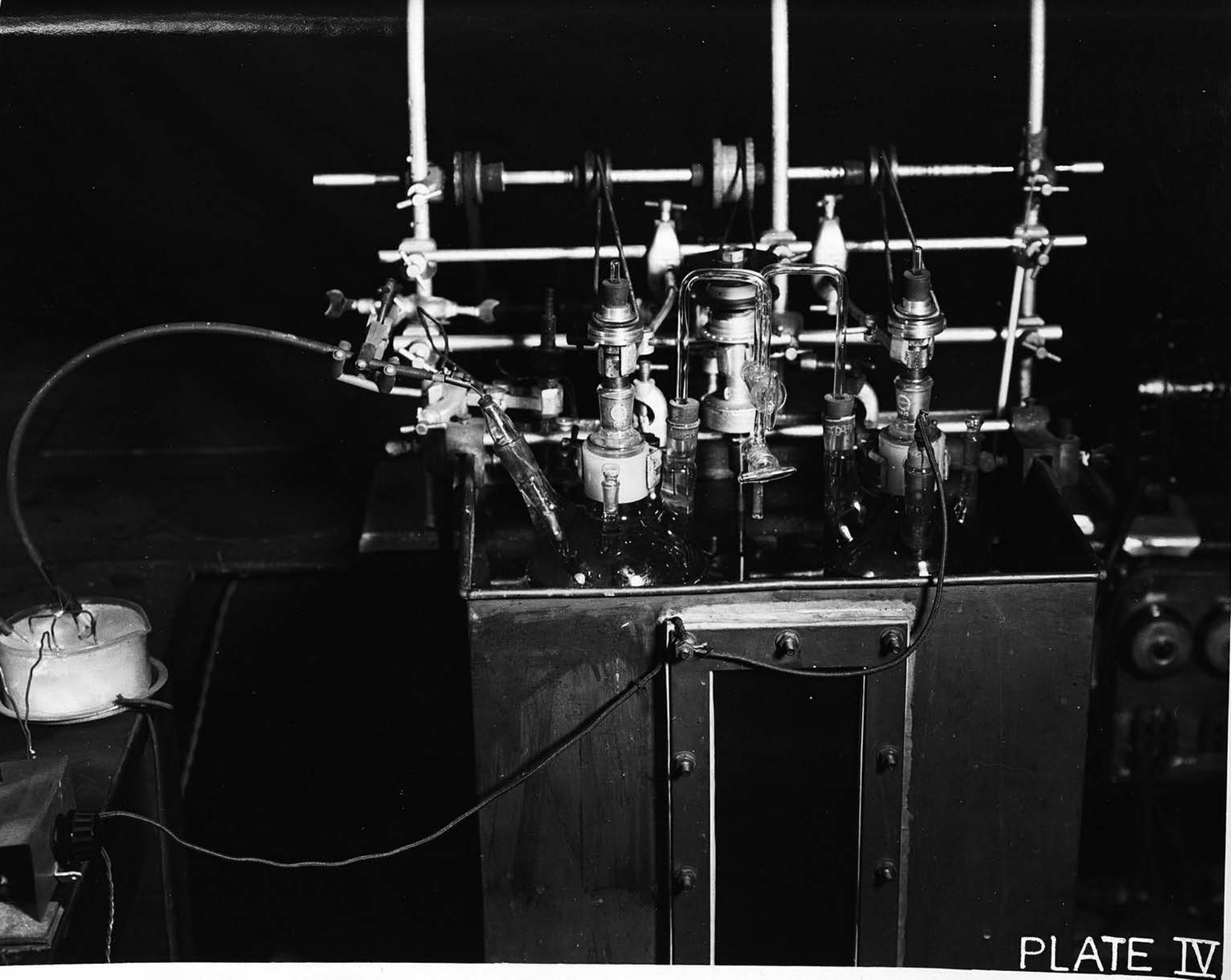


PLATE IV

TABLE VI.

The iodine binding-power of starches.

Source of Starch /	No. of Detns.	Iodine affinity //	% Amylose ///	Slope of linear portion of curve ////
Acorn	2	4.80	25.0	0.08
Arrowroot	4	3.94	20.5	0.13
Banana	3	3.23	16.8	0.10
Barley I	2	4.22	22.0	0.12
Barley II	2	4.22	22.0	0.12
H. brasiliensis seed	3	3.86	20.0	0.10
Iris germanica	3	5.18	27.0	0.09
Maize	3	4.61	24.0	0.11
Oat I	2	5.00	26.0	0.13
Oat II	4	5.00	26.0	0.13
Parsnip	4	2.13	11.1	0.08
Pearl manioc ..	2	3.02	15.7	0.07
Potato I	4	3.94	20.4	0.28
Potato II	2	4.03	21.0	0.24
Potato III	2	4.03	21.0	0.23
Rice	4	3.55	18.5	0.09
Sago	4	5.00	26.0	0.08
Sweet potato ..	2	3.42	17.8	0.27
Tapioca	4	3.21	16.7	0.07
Waxy maize	4	0.27	1.4	0.06
Wheat	4	4.80	25.0	0.05

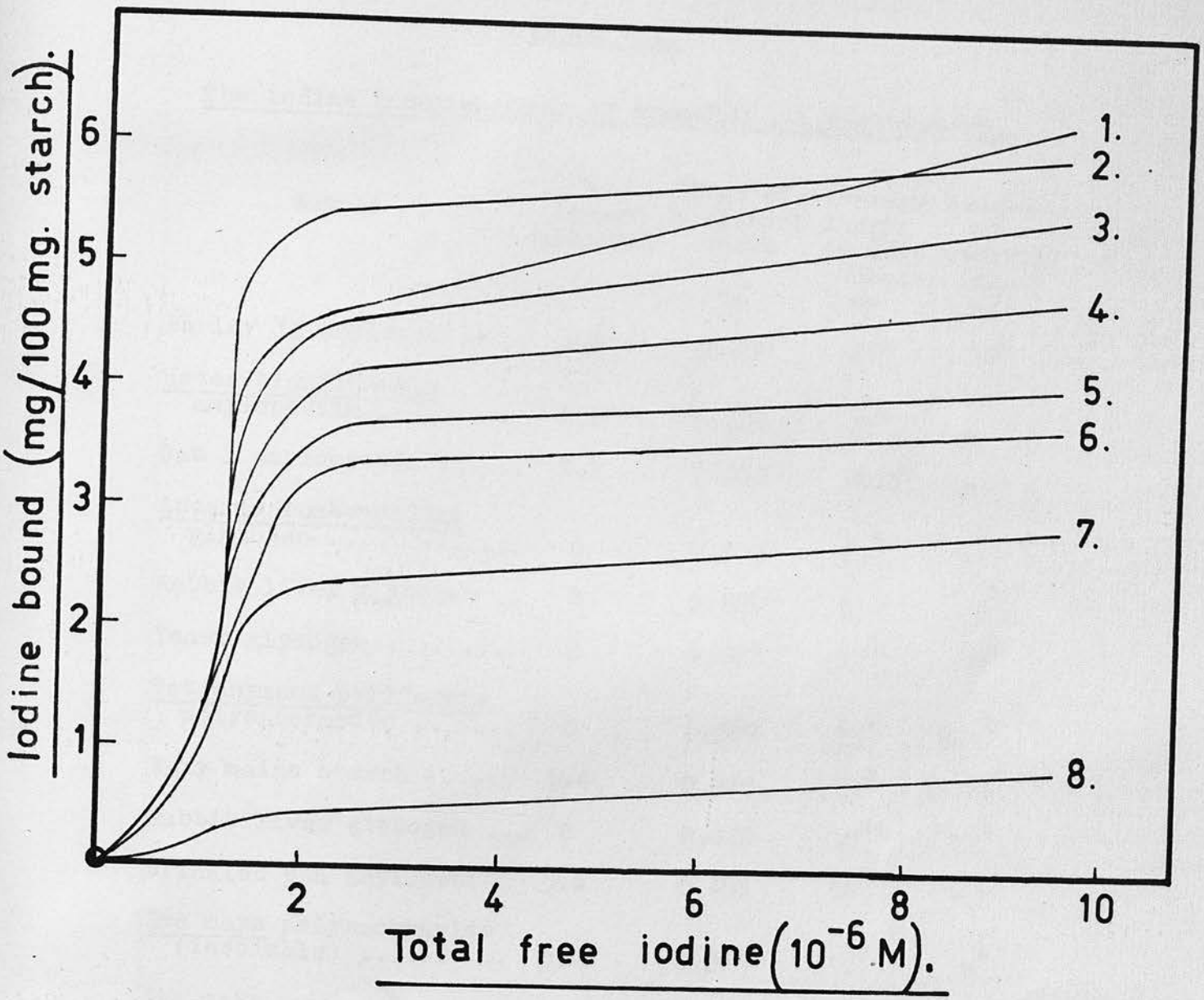
Footnotes:-

/ Origin of samples: See (45) for all except acorn (43); barley I (47); barley II (48); H. brasiliensis seed (9); I. germanica, Oat I and II, Parsnip, all part I, this thesis; Potato II (var. "golden wonder"), Potato III (var. "Redskin") (185).

// Expressed as mg. iodine bound per 100 mg. starch.

/// Calculated as iodine affinity ÷ 19.2.

//// Expressed as % iodine bound per total free iodine concentration x 10^{6}M [in the range of total free iodine (2-10) x 10^{-6}M].



Starch source.

Curve 1: Potato (var. "Golden Wonder").
2: Iris germanica.
3: Barley II.
4: Hevea brasiliensis seed.
5: Rice.
6: Tapioca.
7: Parsnip (var. "Hollow Crown").
8: Waxy maize.

FIG. VII

TABLE VII.

The iodine binding-power of branched α-1:4-glucosans.

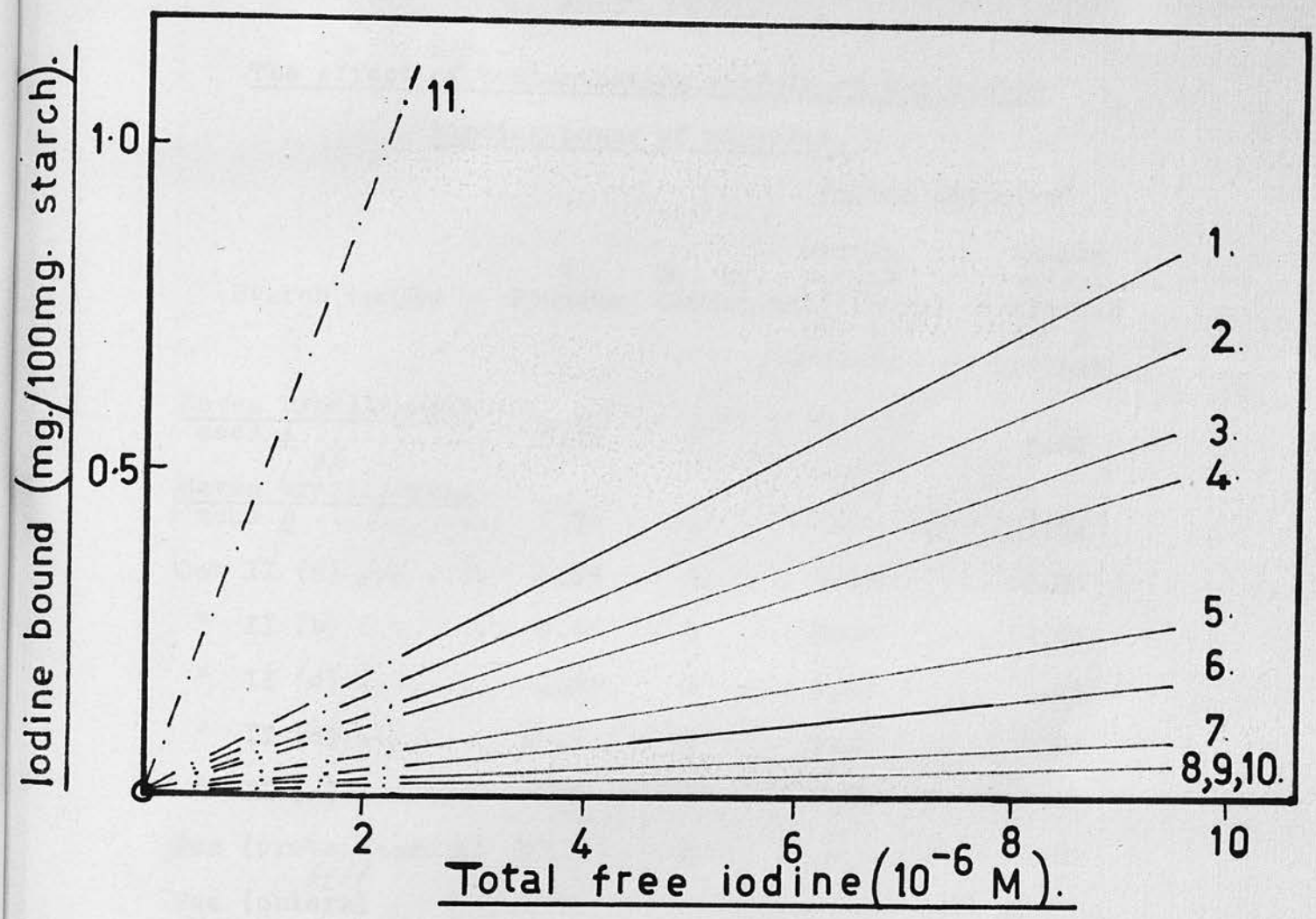
Sample	% linear material	Slope of titration curve /	Average length of unit -chain //	Length of external chain ///
Barley II amylopectin ..	2.6	0.090	23[a]	16[g]
Hevea brasiliensis amylopectin	0.8	0.074	23[a]	-
Oat I amylopectin	3.2	0.052	20.3[a]	-
Ascaris lumbricoides glycogen	0	0.009	12[b]	-
Rabbit liver glycogen ..	0	0.006	13[b]	8[b]
Yeast glycogen	0	0.007	13[h]	8[h]
Tetrahymena pyriformis polysaccharide	0	0.007	13[b]	8-9[b]
Waxy maize starch	1.4	0.060	20[a]	15-16[b]
Rabbit liver glycogen ..	0	0.028	18[c]	12[b]
Wrinkled pea amylopectin	3.4	0.485	36[d]	-
Zea mays polysaccharide (insoluble)	0.26	0.019	12[e]	8[e]
Zea mays " (insoluble)	0.6	0.017	13[f]	9[f]
Zea mays "(soluble)	0.6	0.007	11[f]	7[f]

Footnotes:-

/ Expressed as for Table VI.

// See: [a] as for Table VI; [b] (179); [c] (146); [d] (36); [e] (155); [f] Dr. W.J. Whelan, personal communication.

/// No. of glucose units removed on β-amylolysis + 2.5; [g] (48); [h] (191).



Curve 1: Barley II amylopectin.
2: H. brasiliensis seed amylopectin.
3: Waxy maize starch.
4: Oat I amylopectin.
5: Glycogen ($\overline{C.L.} = 18$ glucose units).
6: Zea mays polysaccharides.
7: Tetrahymena pyriformis polysaccharide.
8: Glycogen (rabbit-liver).
9: " (yeast).
10: " (Ascaris lumbricoides).
11: Wrinkled pea "amylopectin".

FIG. VIII.

TABLE VIII.

The effect of contaminating protein on the iodine binding-power of starches.

Starch sample	% Protein	No. of Detns.	Iodine affinity† sample weight uncorrected for % protein	Iodine affinity† sample weight corrected for % protein
Hevea brasiliensis seed A ††	0.31	3	-	3.86
Hevea brasiliensis seed B	2.56	2	-	3.01
Oat II (a) †††	0.19	4	5.00	5.00
" II (b)	0.45	3	5.44	5.48
" II (c)	2.94	3	5.97	6.14
" II (d)	22.7	3	5.27	6.80
" II (e)	45.6	2	4.78	8.80
Pea (proteinaceous) ††††	37.5	2	-	9.20
Pea (chloral hydrate extracted)	4.93	1	14.6	15.4
Potato I	0	4	-	3.94
" + edestin	2.0	1	-	3.78
" "	10.0	1	-	3.05
" + egg albumin	2.5	1	-	3.66
" "	16.5	1	-	2.93
" + tyrosine	11.0	1	-	3.45

Footnotes:-

† Expressed as mg. iodine bound per 100 mg. starch.

†† See (9).

††† See part I, this thesis.

†††† Kindly provided by Professor E.J. Bourne; see (13).

RESULTS AND DISCUSSION.

Fig. VII shows some typical titration curves for starch samples obtained by plotting the amount of bound-iodine against the total free iodine concentration. Each starch was characterized by its "iodine affinity", which is a measure of the preferential uptake of iodine by the linear amylose component. At the free iodine concentration saturating the amylose, i.e. at approximately 2×10^{-6} M, the amount of iodine bound by the amylopectin is not negligible (see Figs. VIII and IX). An estimate of the amount of iodine bound by the amylose component was therefore obtained by extrapolating the linear portion of the titration curve to zero free iodine concentration. All iodine affinities quoted here were calculated on this basis. The percentage of amylose in a starch can be calculated from this value when the corresponding value for pure amylose is known (50). As has been mentioned (9), the only accurate method of doing this involves the experimental determination of the maximum iodine-binding power of the pure amylose component of the starch under examination. Hence, when it is not desired to fractionate any starch exhaustively, it is more satisfactory to quote its iodine affinity rather than an arbitrary percentage of amylose[cf. (51)]. For comparison with other workers' results, however, percentages of amylose have been calculated, using 19.2% of bound iodine as the maximum iodine-binding power of pure amylose under the experimental conditions used [cf. (9)].

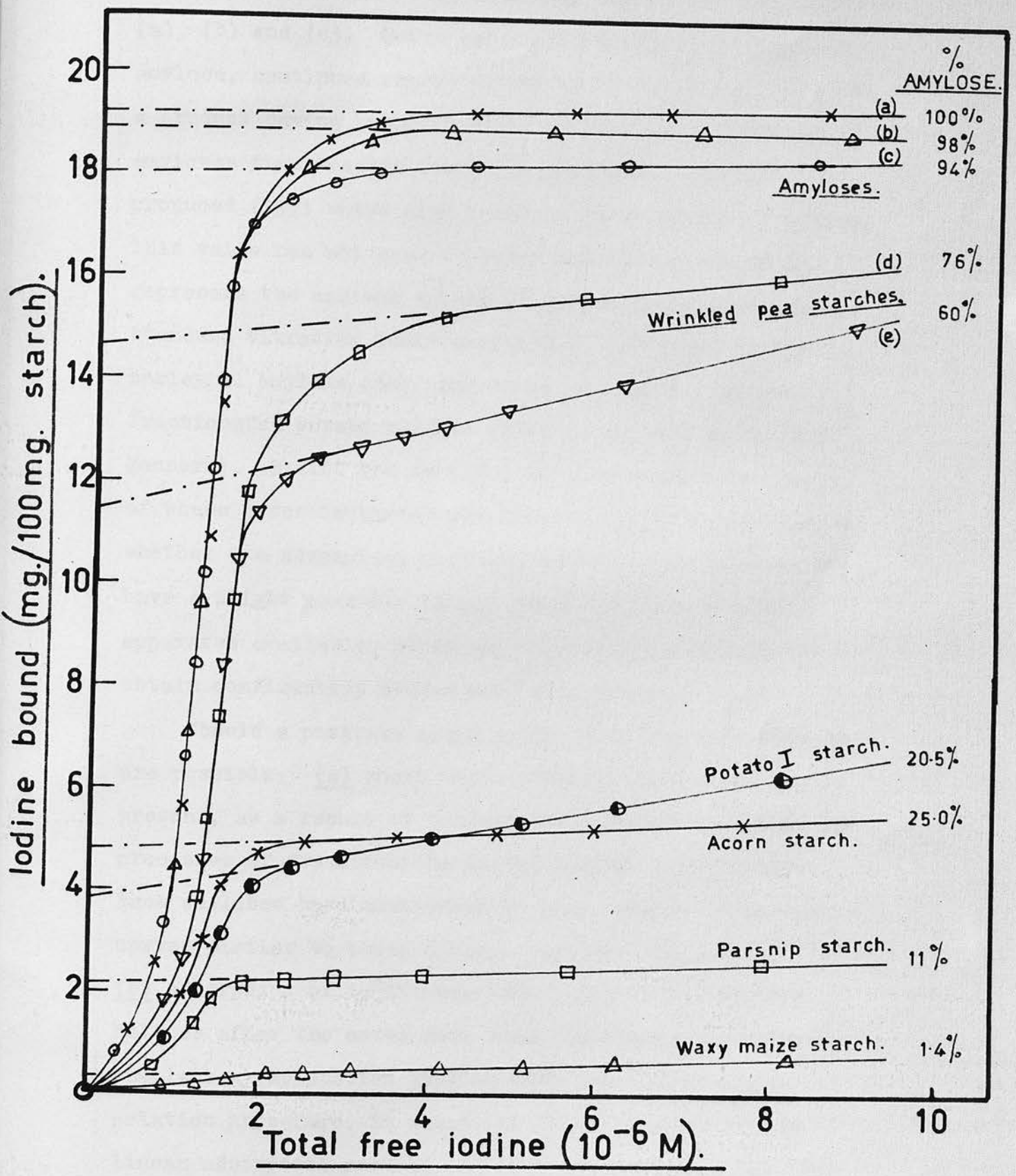

% AMYLOSE.
(a) 100%
(b) 98%
(c) 94%
Amyloses.
(d) 76%
Wrinkled pea starches.
60%
(e)
Potato I starch. 20·5%
25·0%
Acorn starch.
Parsnip starch. 11%
Waxy maize starch. 1·4%
Iodine bound (mg./100 mg. starch).
Total free iodine (10^{-6} M).
0
2
4
6
8
10
12
14
16
18
20

FIG. IX.

In Fig. IX are shown iodine-uptake curves for amyloses (a), (b) and (c). Curve (a) is for H. brasiliensis seed amylose, continued reprecipitations of which did not give a product having higher iodine affinity (9). Although amyloses from several other sources have since been produced (193) which also bound 19.2% by weight of iodine, this value has not been exceeded and is considered to represent the maximum uptake of pure amylose under the standard titration conditions used. Curve (b) is for barley II amylose (48), and curve (c) for a pyridine-fractionated potato amylose (kindly supplied by Dr. D.J. Manners). Whilst the vertical "constant activity" parts of these three isotherms are identical, it is not certain whether the adsorption portions are truly horizontial or have a slight positive slope. Even with the sensitive apparatus available, it is experimentally difficult to obtain confirmatory evidence on this point.

Should a positive slope exist, then two explanations are possible: (a) short chain amyloses (D.P. < 50) may be present, as a result of inadvertant degradation during the processes of fractionation and subsequent purification. Such amyloses have been shown to give linear iodine-uptake curves similar to those of amylopectins (150).
(b) adsorption of iodine may take place on the amylose helices after the cores have been saturated. If correct, the latter explanation implies that the simple extra-polation procedure, in which the positive slope of the linear adsorption part of the isotherm is wholly ascribed to the amylopectin component, will not give accurate

values for the iodine affinity of the amylose component. Further investigations into this matter are necessary. In particular, a study of the molecular-weight distribution of pure amylose fractions to discover if extensive sub-fractionation of this component does occur during fractionation would be of value. It is of interest that, for the samples studied, the slope of the linear portion of the uptake curve for a whole starch is greater than that for the corresponding isolated amylopectin.

Wrinkled pea starches contain high percentages of amylose. Curve (d) is for chloral hydrate precipitated material (% protein = 4.9) obtained from proteinaceous wrinkled pea starch (13); this sample was kindly provided by Professor E.J. Bourne. Curve (e) is for a sample of chloral hydrate precipitated wrinkled pea starch prepared by Fleming and Manners (192).

In Fig. IX, acorn starch (43), potato I, parsnip and waxy maize starches (all as in Table VI) are shown for comparative purposes. As the % amylose increases from 1 to 100%, preferential uptake by the amylose occurs at decreasing free iodine concentrations, although the free iodine concentration required to saturate the amylose component completely is remarkably constant (approximately 2.5×10^{-6} M).

It has been reported that amyloses of increasing chain-length bind iodine before shorter ones, and that the affinity for iodine in amylose complexes is a function of molecular weight (50). Now amyloses (a), (b) and (c) were all obtained by fractionation of starches which have

iodine-uptake curves similar in shape to potato I and acorn. Moreover, fractionation cannot result in increased amylose chain-length. The effect, shown in Fig. IX, of increased preferential uptake at decreased free iodine concentration for increase in amylose content therefore implies that, in the presence of amylopectin in solution, the amylose is prevented from forming the maximum lengths of helices possible by virtue of its chain-length. Whether this effect is due to actual chemical combination of the amylose and amylopectin components in a starch before fractionation could be shown by a carefully controlled series of experiments, in which the iodine-uptake curves of a series of mixtures of amylopectin with different percentages of pure amylose were determined.

Table VI summarizes the results for starch samples. It is of interest that starches from different varieties of the same botanical source may show variation in the apparent percentage of amylose; this is in agreement with Doremur, Creshaw, and Thurbers' results (178). The slope of the linear portion of the titration curve for all the potato starches studied was considerably greater than for other starches. (See Figs. VII and IX for potato starches II and I respectively.)

Contaminants affect the amount of iodine bound by a starch. Interference by fatty acids is well-known. A dipolar mechanism similar to that operative in the case of iodine-binding has been proposed (143); the amylose helix diameter is slightly greater than that of a fatty acid molecule extended along the helix axis. It is

therefore essential that all traces of fatty materials be removed before a titration. The number of treatments with methanol under reflux necessary to achieve this should not be under-estimated. Other contaminants known to interfere are tannins, gallo-tannins, and lignin, particularly in wood-starches (30).

In addition, protein has now been found to have considerable effect (see Table VIII), and its presence causes distortion of the titration curve. For oat starch, the effect is to increase the apparent binding-power; the protein removes free iodine from solution, and estimates of the true iodine affinity are best obtained by not correcting for the percentage of protein present. However, for H. brasiliensis seed and pea starches, and for synthetic mixtures of protein with potato starch I (see Table VIII) the protein apparently suppresses starch-iodine complexing. Interference has also been found during the study of protozoal starches. It is therefore essential to remove contaminating protein before titrations are attempted.

The difference in iodine-binding power of normal amylopectins and glycogens has been found to be sufficiently large to characterise these two structure-types. (See Fig. VIII). The iodine binding-power of these materials must be fundamentally related to differences in (1) the average length of unit-chain, and (2) the degree of multiple-branching. For a group of polysaccharides having a similar degree of branching, it is also probable that variations in the amount of iodine bound are related

to the length of external chain available for helix formation. (cf. 131, 138).

Titrations of different amylopectin samples have always been found to show evidence of preferential uptake of iodine by linear material. To compare iodine binding-powers, therefore, such preferential uptake has been corrected for by extrapolating the titration curve to zero free iodine concentration, with this extrapolated point being taken as the origin for the iodine-binding curve. [This preferential uptake was presumably due to contaminating amylose, which is extremely difficult to remove (cf. 177). The presence of some long branches in the amylopectin cannot be entirely excluded (cf. 138)]. In all the glycogen samples so far examined there was no evidence of any preferential uptake.

Fig. VIII and Table VII show the results for some amylopectins and glycogens. In the range of concentrations employed, the amount of iodine bound is directly proportional, within experimental error, to the total free iodine concentration. This would be expected if the iodine is bound as a co-linear core of iodine and tri-iodide molecules arranged end-to-end in the available helices. One iodine molecule can be accommodated in a helix of about six glucose units (131). Since the maximum length of external chain available for helix formation is only 14-18 glucose units (i.e. about three helices) for amylopectins, and 5-11 glucose residues (i.e. one to two helices) for glycogen (179), the amount of iodine-binding possible is small.

Higginbotham (144) has suggested that adsorption of iodine molecules (or tri-iodide ions) may also occur. However, at the low iodine concentrations used here, adsorption effects would be small, and are indeed unlikely to occur in view of the negligible effect on the titration curves of increased polysaccharide concentrations.

Fig. VIII shows that waxy maize starch behaves as a typical amylopectin, and the polysaccharide from the ciliate Tetrahymena pyriformis (180) as a glycogen. Molecular weight must be of minor importance; e.g. the amylopectin from H. brasiliensis seed starch (D.P., 6000) binds about ten times more iodine than rabbit liver glycogen (D.P., 30,000). [For values of D.P., see (9).]

It was possible to test the hypothesis that fine-structure governed iodine-uptake when samples of "abnormal" branched α-1:4-glucosans became available. Samples of the water-soluble polysaccharides from sweet corn (Zea mays) were kindly provided by Drs. R.L. Whistler and W.J. Whelan. The exact structural nature of these polysaccharides has been in dispute [cf. (28, 155, 181-183)]. Whilst these materials have an average length of unit-chain of 12-13 and 10-11 glucose residues respectively (155, 184), the iodine-uptake was three to four times greater than that for a glycogen of corresponding average chain-length (see Table VII and Fig. VIII), although the molecular weights were of the same order (185). It is therefore suggested that these polysaccharides have a degree of multiple-branching intermediate between glycogen and amylopectin, and are therefore neither in the one class

nor the other. [It is of interest that Wolff, Watson, and Rist (186) reached a similar conclusion from a study of the tricarbanilates of polyglucosans with different linkages.]

An abnormal rabbit liver glycogen [shown by Haworth, Hirst, and Isherwood (146) from methylation studies to have an average length of unit-chain of eighteen glucose residues] bound about five times more iodine than a normal glycogen, and appeared to behave more as an amylopectin-type structure (see Fig. VIII, curve 5). Without additional information, it is not possible to say whether this is due to the increased average length of the external chains (i.e. twelve residues) or to a variation in the degree of branching. [The abnormal character of this glycogen has been confirmed by Professor F. Smith, who found it to possess an abnormal precipitin reaction with concanavalin-A (189).]

The amylopectin from wrinkled pea starch (var. "Perfection") has been shown to possess abnormal iodine-binding power, and an average length of unit-chain of thirty-six glucose units; on the basis of spot-tests it was deduced that no amylose was present (36). A sample of this material, kindly provided by Dr. R.M. McCready, gave however an abnormal titration curve, indicating the presence of some linear material. The iodine-binding power was about six times greater than that for a normal amylopectin (Table VII; Fig. VIII, curve 11).

Although data regarding the average external chain-length of the amylopectins studied are not complete, the

results quoted in Table VII suggest that small variations may exist in the degree of multiple-branching of this group of polysaccharides. Similar effects have been found from a study of a large number of glycogen samples (187). Additional evidence might be obtained from a study of the corresponding limit dextrins (cf. 188).

Hence, potentiometric determinations of the amount of iodine bound by branched α-1:4-glucosans, in conjunction with estimations of chain-length, should enable an estimate of the degree of multiple-branching to be obtained, if the method can be confirmed by enzymic degradative experiments (cf. 190).

SUMMARY.

A sensitive valve micro-voltmeter has been developed to extend the scope and accuracy of differential potentiometric titrations. Accurate values for the iodine affinity of twenty-one different starches have been obtained, and it has been shown that contaminating protein affects the amount of iodine bound. From studies on pure amylose and amylose-rich starches, it has been shown that as the percentage of amylose present increases, preferential uptake of iodine begins at decreased free iodine concentrations. Studies have shown that the iodine-uptake of amylopectins and of glycogens are linear for free iodine concentrations varying between 2×10^{-6} M and 10^{-5} M; in addition, the difference in iodine binding-powers is sufficiently large to characterize these two structure-types. Several "abnormal" α-1:4-glucosans have been investigated and classified as having either glycogen-like, amylopectin-like, or intermediate types of structure. The differences observed in the iodine-binding powers, in conjunction with data for average unit-chain and external chain lengths, suggest that variations exist in fine structure, i.e. in the degree of multiple-branching.

PART III.

The oxidation of starches by potassium meta-periodate.

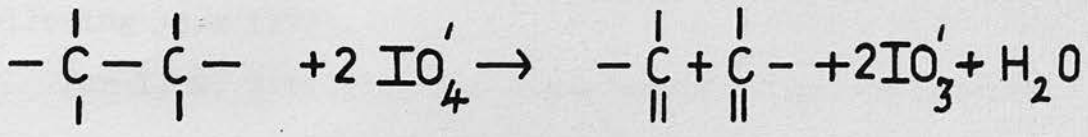
INTRODUCTION.

In starch chemistry, methylation and periodate oxidation have been the main methods used in structural studies.

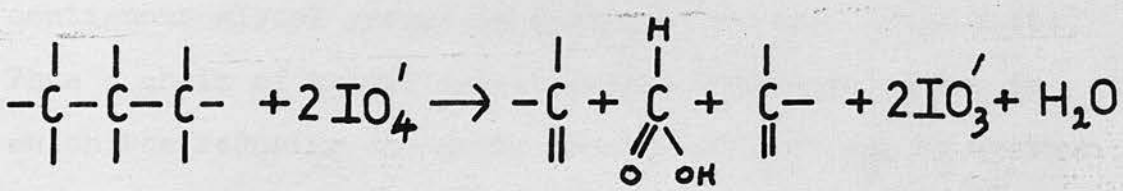
Early work using the classical Haworth technique of exhaustive methylation followed by separation of the methylated sugars obtained on acid hydrolysis (62) was carried out on unfractionated starches. The proportion of 2:3:4:6 tetra-o-methylglucose, originating from the non-reducing end-groups was generally found to be about 4%, corresponding to an average unit-chain length of twenty-five D-glucose units (14). The accuracy of the method was increased by the introduction of chromatography for the separation of the methylated sugars (195), but it is not known whether the small percentages of 2:3-, 2:6-, and 3:6- di-o-methylglucose almost invariably found are structurally significant, since they may result from incomplete methylation or partial demethylation during hydrolysis (48).

The selective oxidative fission of α-glycols by the periodate ion, giving two aldehydic groupings, was discovered by Malaprade (196). The rate of oxidation is greater for cis- than for trans-α-glycols, one mole of periodate being reduced for each carbon-carbon bond broken [see Fig. X (a)]. This possible cleavage of a chain of carbon atoms provided an alternative method of investigating ring-structures, since the use of nitric acid in earlier oxidative degradation studies gave ring-opening at the carbon-oxygen bond. The reaction was first applied

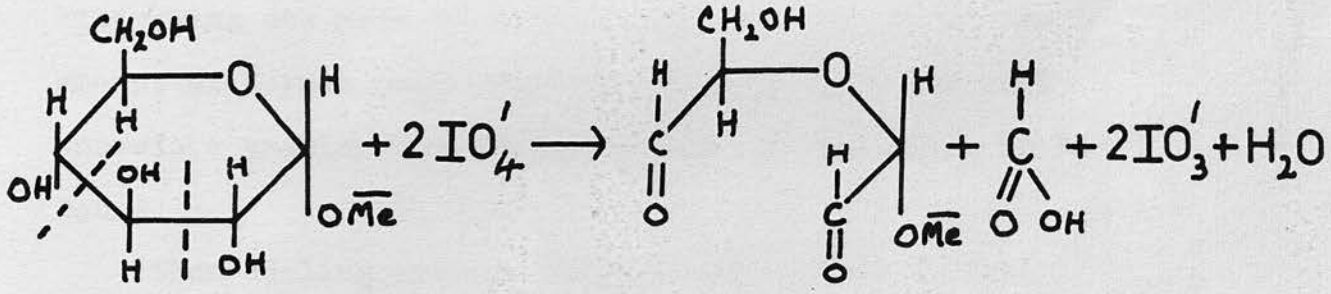
(a)



(b)



(c)



(d)

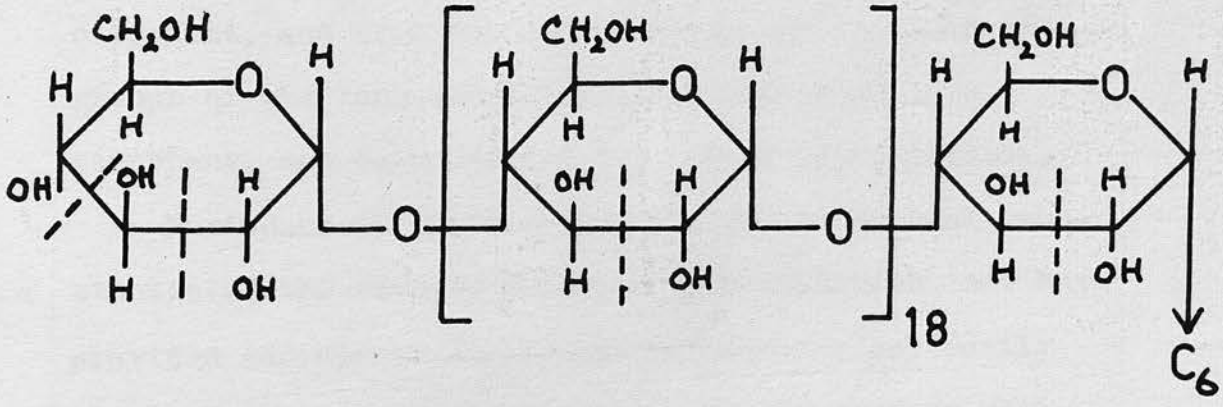


FIG. X

to the study of sugar ring-forms in 1936 by Jackson and Hudson (197) and extended to starch and cellulose in the following year (198).

For 1, 2, 3 triols, the degradative oxidation results in the production of one mole of formic acid and the formation of two aldehydic groupings, two moles of periodate being reduced [Fig. X (b)]. The reaction is also valid for contiguous glycol groups in cyclic structures [Fig. X (c)]. Thus a chain of twenty α-1:4- linked D-glucose units, in which the reducing end-group forms a 1:6 linkage to another similar chain [Fig. X (d)], will consume twenty-one moles of periodate, i.e. 1.05 moles per anhydroglucose unit, since the non-reducing end-group alone consumes two moles, liberating one mole of formic acid. Estimation of the amount of formic acid released per anhydroglucose unit therefore enables the average length of unit-chain to be found.

When dealing with an unfractionated starch, the relatively small amounts of formic acid liberated from the single non-reducing end-group in the amylopectin component, and from the non-reducing and reducing end-groups of the long essentially unbranched amylose component, can be neglected to a first approximation.

Periodate oxidation studies have, in general, substantiated the results obtained by methylation, and have provided additional structural information not easily obtained otherwise. Furthermore, if the average unit-chain length of the amylopectin component as determined by periodate oxidation agrees with that given by

methylation, then there can be no glucose units present which are linked solely through C1 and C6, i.e. containing three unsubstituted hydroxyl groups (45, 194). Close agreement between the results given by the two methods have been found (200); in addition to showing the absence of units linked solely through C_1 and C_6 this has proved that the hemi-acetal type linkage proposed by Pacsu and Hiller (199) does not exist in starch, as has been also indicated by enzymic hydrolysis and dextrin formation.

It is, however, essential that apparent agreement in the values obtained for the average unit-chain length by both methods should not be taken as a criterion that correct conditions for the periodate technique had been established.

Any glucose unit in an α-1:4-glucosan having a cross linkage through either C_2 or C_3 will not be attacked by periodate. If such units exist in starch, then D-glucose will be present in the hydrolysate obtained from the oxidised starch (201). In many such investigations (9, 30, 31, 43, 47, 48, 157), 0.5-1.5% of glucose has been found, indicating that at least 75-90% of the inter-chain linkages must be of the 1:6 type. Several of the authors concerned, however, were undecided whether this small percentage of glucose was of structural significance or due to incomplete oxidation. Gibbons and Boissonas (202) found no glucose after prolonged oxidation, and maintain that <u>all</u> cross linkages are of the 1:6 type. On examination, however, of the reduced oxidation product (i.e. the polyalcohol), Smith and co-workers found 0.5%

of glucose (217). They claimed this to be significant, but it is possible that here also the glucose arose from incomplete oxidation.

In recent years, there has been an increasing tendency for the methylation method of finding average unit-chain lengths to be superceded, in the case of starches, by the periodate oxidation technique, which has the advantages of simplicity and speed, and requires about ten times less material - often a most important consideration. Several difficulties arise, however. Polysaccharides of the α-1:4-glucosan type are easily over-oxidised. To minimise this tendency, Hirst and his co-workers (45) used the sparingly soluble potassium meta-periodate at room temperature, the calculated excess of oxidant, and of formic acid released, being kept low. Oxidation to the theoretical limit was complete after about one week. Potter and Hassid (23) used the sodium salt at 2°C for about twenty-four hr., but these conditions have been found to result in incomplete oxidation (203).

TABLE IX.

Starch source	Reference A	B	C	D
Maize	20	--	25-26	25.2
Potato	24-26	--	27	--
Tapioca	20	22	23	26.6
Wheat	21	--	23	--

Reference: A, (45); B, (215); C, (23); D, (216).

There has also been considerable disagreement in the values obtained by different workers for the average

unit-chain lengths of amylopectins (see Table IX, p. 94). This disagreement probably resulted not only from the use of differing oxidation conditions, but also from differing procedures for determination of the amount of formic acid released. For instance, titration with alkali to the following end-points has been used:- methyl red (204); phenolphthalein (205); phenol red (206, 207); mixed methyl red/methylene blue (pH 6.0) (208, 209); and bromocresol purple (213). Some investigators preferred to use the iodometric method of determining total acidity (210); others used Pirie's steam-distillation method (211), and yet others titrated the formic acid potentiometrically. Of the latter, Kerr and Cleveland (212) titrated to pH 7.1, but other investigations, in which titration to pH 5.5, 6.0, 8.0 and 8.2 was considered to be correct, have been referred to by Morrison, Kuyper and Orten (213).

Even in the determination of residual periodate it was found that (a) the original back-titration method of Fleury and Lange (214), (b) direct titration with arsenite, and (c) direct titration with thiosulphate, all gave differing results for actual reaction mixtures, although agreement was given by the three methods in analyses of pure periodate solutions (205).

It was therefore evident that critical surveys of the conditions necessary for complete periodate oxidation without overoxidation, and for quantitative estimation of the amount of formic acid released were essential. It was hoped that, in consequence, the quoted accuracy ($\pm$ 10%)(45) of values of average unit-chain lengths found by this method would be greatly improved.

EXPERIMENTAL.

All starch samples were dried *in vacuo* at 80°C, for several hours. Reagents were of A. R. quality, or were purified as described by Halsall, Hirst and Jones (45). The nitrogen and sodium hydroxide used during potentiometric titrations were free from carbon dioxide. The distilled water used throughout was always obtained from the same still, and made CO_2- free before use.

Periodate Oxidations: Starch (250-400 mg.) was suspended in 0.56M potassium chloride (60 ml.) to which was added 0.2M sodium *meta*-periodate (20 ml.). Within these limits, the rate of oxidation was found to be independent of the weight of starch; the periodate concentration is about 50% in excess of that theoretically required. Reaction flasks were shaken continuously in darkness in a constant-temperature room.

Potentiometric Titrations: Samples (10 ml.) were withdrawn by pipette from the reaction mixtures at the required time intervals. Ethylene glycol (1 ml.) was added and the mixture shaken in darkness for at least ten minutes, the time found necessary for complete reaction between the glycol and the suspension of potassium periodate. (All excess periodate must be destroyed before the start of a potentiometric titration.) Nitrogen was bubbled through the mixture for ten minutes before titration with 0.01M sodium hydroxide (semi-micro burette). The passage of nitrogen was continued throughout the titration, which was followed by means of a glass electrode and Pye mains-operated pH-meter. Blank determinations showed that

generally no corrections were required for the acidity of starch samples or of other reagents.

Homogeneous samples with respect to formic acid content could be obtained by withdrawal of samples by pipette whilst the reaction flask was shaken gently. Each of seven 10 ml. portions withdrawn consecutively from a reaction mixture gave identical titres with sodium hydroxide, and furthermore this value was the same as that obtained from titrations of the entire contents of each of three individual reaction mixtures (10 ml. each) after the same oxidation time. This procedure did not, however, give consistent results for determinations of excess periodate; the more rapid sedimentation of the excess potassium meta-periodate made impossible the withdrawal of samples homogeneous with respect to this component.

Volatility of formic acid: It was shown that no loss of acid occurred when nitrogen was passed through a solution of formic acid (0.0015M) for one and a half hr. i.e. about five times as long as is taken for a potentiometric titration. Under the conditions used here, periodate oxidation mixtures are approximately 0.0015N with respect to formic acid when oxidation is complete.

Oxidation of Formic Acid by Potassium meta-Periodate: No loss of formic acid nor consumption of periodate occurred when formic acid (0.0015M) was shaken with a saturated solution of potassium meta-periodate for twenty-eight days in darkness at 15-16°. At 20-21°, however, the concentrations of formic acid, and of periodate, decreased by 3% after fifteen days, and by 6% after twenty-one days.

Distribution of Formic Acid in Reaction Mixtures: After oxidation for 240 hr., a starch-potassium periodate reaction mixture (80 ml.) was divided into two. The first half was centrifuged, and portions (10 ml.) of the clear supernatant were titrated (after destruction of excess periodate) against sodium hydroxide (0.01024M) to pH 6.25. The average titre was 1.40 ml. From the second half of the reaction mixture, two heterogeneous 10 ml. portions were withdrawn, treated with glycol, and titrated to pH 6.25. The average titre was 1.43 ml. The remaining two 10 ml. portions were treated separately with glycol then centrifuged, and the oxidized granules washed three times with distilled water followed by centrifugation. The combined supernatant liquids were then titrated to pH 6.25, when the average titre was 1.43 ml.

Determination of Periodate Uptake: Residual periodate concentration was determined by the indirect method of Fleury and Lange (214). The method gave satisfactory results in the presence of all reactants and products when the reaction mixture, to which had been added excess bicarbonate, arsenite and iodide, was shaken in darkness for fifteen min. before back-titrating with iodine. Periodate uptake was determined by the analysis of a series of individual reaction mixtures made up on $^{1}/_{10}$th of the scale described under "periodate oxidations" above. It was found necessary to lubricate the stoppers of the conical reaction flasks with a little silicone grease to prevent losses through "salting-up" effects at the ground glass joints.

Interaction of Formic Acid with Starches and their Oxidation Products: The following mixtures were shaken for 240 hr. in darkness at 15-16°:- (a) control solution of formic acid (10 ml.); (b) formic acid (10 ml.) and oat starch (64.35 mg.); (c) formic acid (10 ml.) and periodate-oxidized oat starch (65.18 mg.); (d) formic acid (10 ml.) and waxy maize starch (60.08 mg.); (e) formic acid (10 ml.) and periodate-oxidized waxy maize starch (55.60 mg.). The contents of each reaction flask were then titrated to pH 6.25 against sodium hydroxide (0.00901M), the titres obtained being:- (a) 16.15 ml., (b) 16.05 ml., (c) 16.15 ml., (d) 15.85 ml., (e) 16.15 ml.

Examination of Polyaldehydic Oxidation Products: No colour reaction occurred with (a) iodine/potassium iodide, (b) dilute sulphuric acid/potassium iodide or (c) sulphuric acid/diphenylamine; Fehling's solution and Schiff's reagent were reduced. The release of acidic decomposition products from the oxidized-starch in the presence of alkali was shown in the following two experiments: (a) oxidized oat starch (50 mg.) was added to water (5 ml.) which had been boiled, and then cooled in the presence of nitrogen. Sodium hydroxide (1 ml., 0.01024M) was added, and the mixture shaken for fifteen hr. The pH was then 6.25, and did not decrease further over twenty-four hr. The further addition of sodium hydroxide (1 ml. 0.01024M) gave an initial value of pH 10.5, which decreased overnight to pH 6.25 and did not decrease further. (b) Oxidized waxy maize starch (35.92 mg.) was added to sodium hydroxide (20 ml., 0.00901M) and shaken for seventeen hr. Titration

of the excess alkali to pH 6.25 required 1.70 ml. formic acid (0.0147M). Hence 232 g. oxidized starch would liberate 1 l. of 1N acid. (A duplicate experiment gave 273 g. as the apparent neutralization equivalent.)

RESULTS AND DISCUSSION.

The ease of oxidation of polysaccharides depends on their structural type; starch is attacked relatively easily, and as a result over-oxidation can occur. This series of investigations was based on the use as oxidant of the potassium salt of meta-periodic acid, which had been shown (45) to give a suitably slow rate of oxidation. It was suspected, however, that the reaction might still be considerably temperature-dependent, and that the conditions, previously described as "oxidation at room temperature", were not sufficiently well-defined.

Accordingly, two series of oxidations were carried out to investigate the temperature dependance factor, the first being at a constant temperature of 15-16°C., and the second at 20-21°C. The results obtained for some of the starches oxidised are shown in Table X, which shows that oxidation at 20-21°C. is about 30% faster than at 15-16°C., and that this increased rate of oxidation is accompanied by a greater tendency for over-oxidation to occur. At 15-16°C., the theoretical uptake of periodate was not reached in less than 240 hr., and even after 350 hr. very little over-consumption of periodate - and hence over-oxidation of starch - occurs.

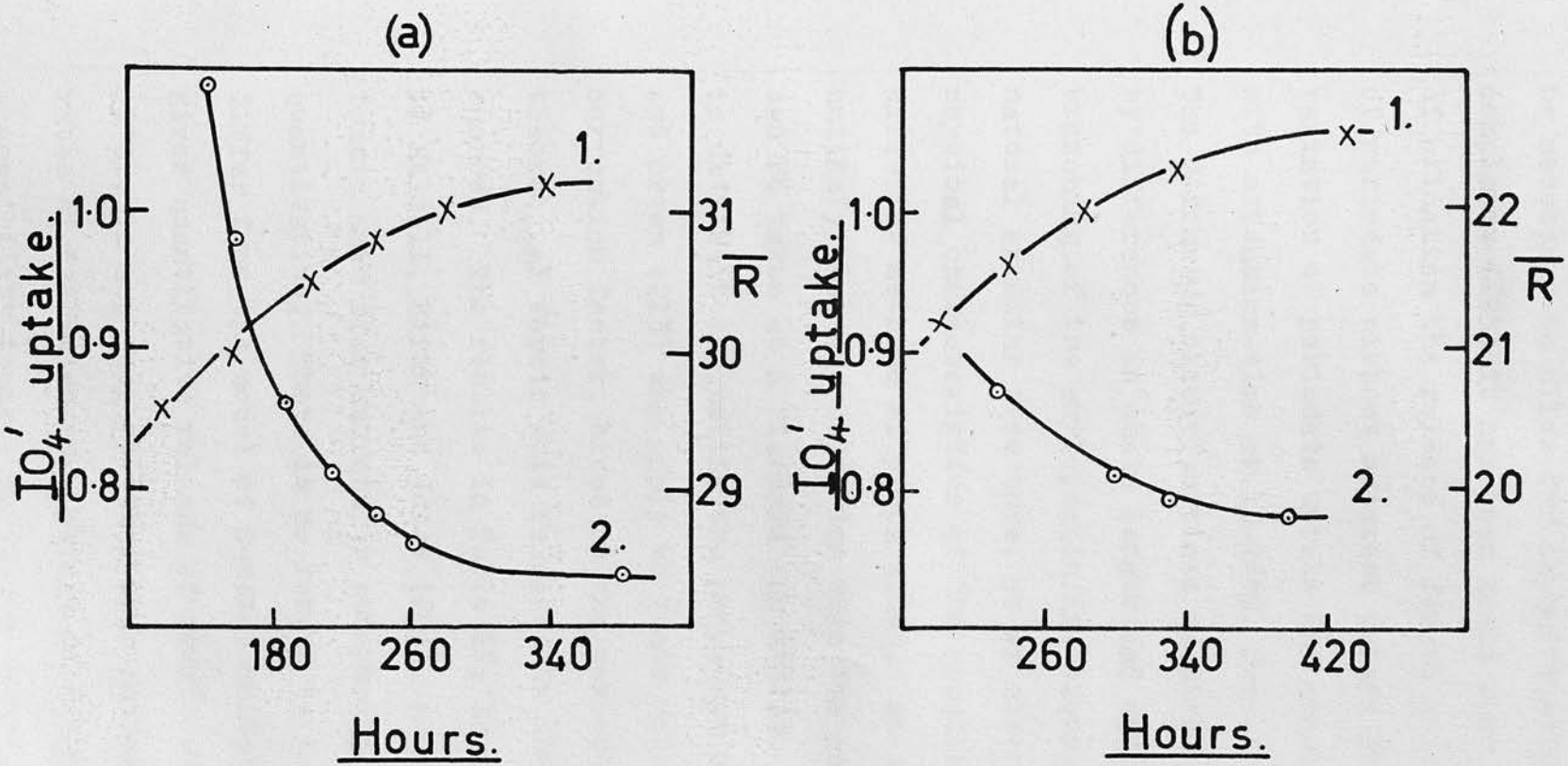
At both temperatures, moreover, the time required for the theoretical uptake of 1.03-1.05 moles of periodate per anhydroglucose unit varied for different starches. It follows that the time required for the periodate-uptake of a starch to reach the theoretical value at constant temperature must be determined.

TABLE X.

Periodate uptake in moles per anhydroglucose unit.

Source of Starch	Temp.	Number of hours oxidation. 72	120	160	200	240	280	336	432
Barley I .	15-16°	-	-	0.91	0.99	1.01	1.04	-	-
Iris germanica	"	-	-	-	0.97	1.02	1.04	1.06	-
Oat II (a)	"	0.77	0.80	0.86	0.96	1.02	-	1.05	-
Parsnip ..	"	-	-	0.90	0.98	1.01	1.03	1.04	-
Potato I .	"	0.81	0.86	0.89	0.95	0.98	1.00	1.02	-
Potato II	"	-	-	0.88	0.94	0.96	-	1.01	1.04
Potato III	"	-	-	0.88	0.94	0.97	-	1.03	1.03
Rice	"	-	-	0.72	0.84	0.93	1.01	1.03	-
Sweet potato ..	"	-	-	-	0.97	1.01	-	-	-
Waxy maize	"	-	-	0.91	0.92	0.96	1.00	1.03	1.05
Oat II (a)	20-21°	-	0.97	1.02	1.03	-	1.08	-	-
Potato II	"	-	0.97	1.03	1.04	1.09	1.13	-	-
Rice	"	-	0.95	1.01	1.03	1.06	1.09	-	-
Waxy maize	"	-	0.98	1.02	-	1.03	1.05	-	-

Footnote: Origin of samples as detailed in Table VI. (p78).



Variation of periodate-uptake and apparent value of $\bar{R}$ with oxidation-time at 15-16°C. for (a) oat starch, (b) waxy maize.

Curve 1 : periodate-uptake (moles/anhydroglucose unit).

Curve 2 : $\bar{R}$ in glucose units.

FIG. XI.

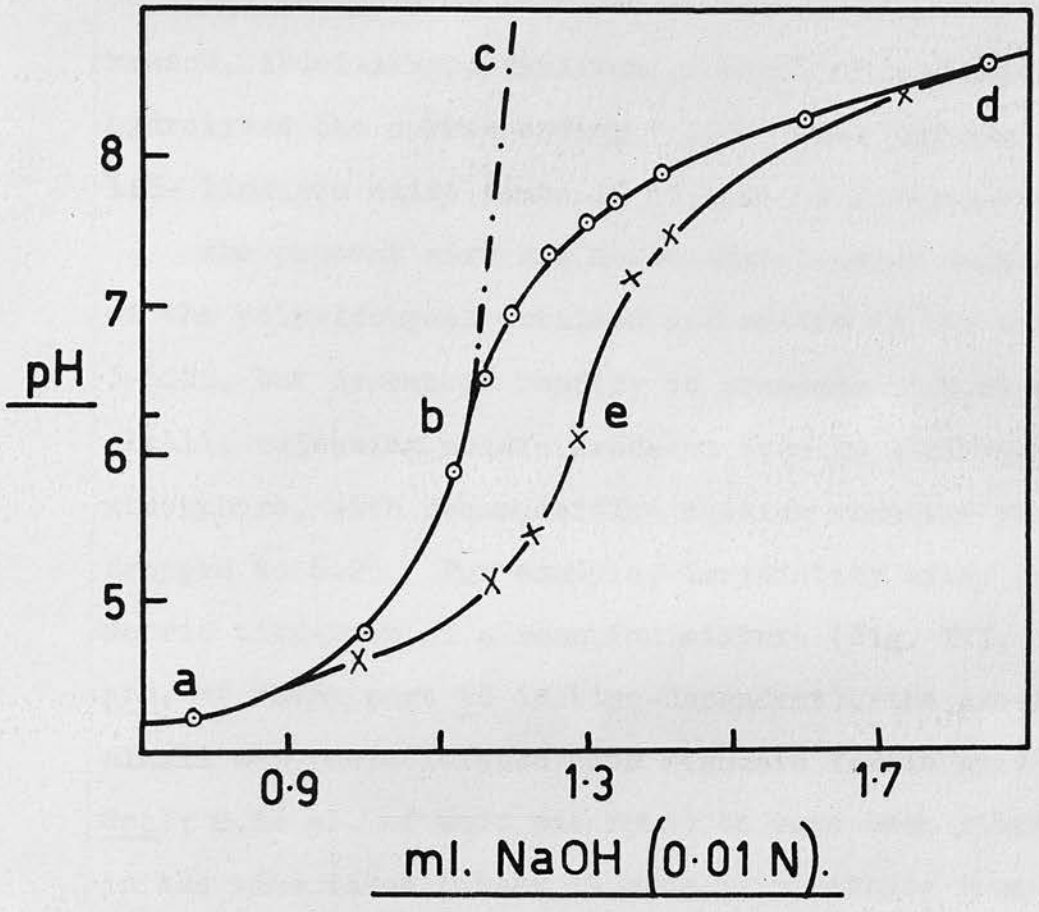
The amount of formic acid liberated in a shorter time cannot be quantitative. Although a longer time may even be necessary to allow for saponification of intermediate complexes (205) it has been found that in the final stages of oxidation the release of formic acid follows consumption of periodate without apparent delay; Fig. XI shows the variation of periodate uptake and apparent value of $\overline{R}$ with oxidation-time at 15-16°C. for oat starch II (a). The differing oxidation times required could be explained by differences in chain length and degree of multiple branching of the amylopectin fractions, by differences in natural granular structure, or by alteration in the physical characteristics of the granules arising from differing methods of preparation. As a result, it appears unlikely that the oxidation time for any simple saccharide can be taken as a standard for starch. In particular, it is difficult to justify the procedure of Morrison, Kuyper and Orten (213) who apply to their results a fixed correction factor, based on the percentage of the theoretical formic acid released in the same time from sucrose. The results in Table XI, in agreement with those of Halsall, Hirst and Jones (204), show that release of formic acid from sucrose is abnormally slow and non-quantitative. There is no reason to suspect that starches differ from the model of β-methylmaltoside (45), which gives quantitative release of formic acid in 150 hr. at 15°C., in any respect other than having variably slower rates of oxidation as a result of having differing accessibility towards periodate attack.

TABLE XI

Periodate uptake and formic acid release (in moles/mole) from sucrose.

	Number of hours oxidation.						
	262	286	300 - 352	408	420	570
Periodate uptake ...	2.98	-	3.12			
Formic acid released	0.87	0.88	-	0.89	0.91	0.92	0.93

After several starches had been oxidised for the time found necessary for the theoretical uptake of periodate to occur, they were examined by the methods described by Hirst, Jones and Roudier (43) for the isolation, hydrolysis of the oxidation products, and the determination of glucose in the hydrolysates. Quantities of glucose between 0.5 and 1% were found. When further samples of the same starches were oxidised for at least 100 hr. longer in each case, traces of glucose, too small for estimation, were only detectable by examination of the chromatograms under ultra-violet light. Recent papers have reported the presence of 1-2% glucose in hydrolysates after oxidation at room temperature for 150 hr. (47) and for 240 hr. (43), but these authors were undecided whether this was of structural significance or due to incomplete oxidation. The latter explanation is supported by the present work, which shows that about 1% of glucose residues in the starches examined are abnormally but not completely resistant to periodate attack. This suggests that 1:2- or 1:3-glucosidic linkages are not present, and is in agreement with the work of Gibbons and Boissonnas (202). However, the polyaldehydic oxidation products are



Titration curves for a reaction mixture.

Curve abc. Control HCOOH v. NaOH.

Curve abd. Reaction mixture v. NaOH.

Curve dea. Back-titration curve for mixture v. HCOOH.

FIG. XII.

very easily hydrolysed, giving solutions containing a brown precipitate, and Jackson and Hudson (218) reported that during hydrolysis some destruction of material occurred and polymer degradation was incomplete. For this reason, Abdel-Akher, Hamilton, Montgomery and Smith (217) hydrolysed the corresponding polyalcohols and claimed that 1:3- linkages exist since 1% of glucose residues were found.

The present work has shown that aqueous suspensions of the polyaldehydes obtained are stable in the range pH 3-6.25, but decompose readily in presence of 0.01-0.001 N alkali, releasing acidic products even in a nitrogen-atmosphere, with decomposition ceasing when the pH has dropped to 6.25. For example, immediately after potentiometric titration of a reaction mixture (Fig. XII, curve abd, of which part bd is time-dependent), the excess alkali was back-titrated with standard formic acid (curve dea); 0.16 ml. of acid was found to have been liberated in the time taken (about 15 mins.) to titrate from b via d to e. Liberation of acid in this manner may explain some anomalous results which have been reported involving over-production of acid during periodate oxidations in alkaline buffered systems (219-221). Similarly, when a calculated excess of potassium hydroxide was added to a series of periodate oxidations of waxy maize starch at 15-16°C., it was found that although the rate of periodate uptake was normal, being only 0.55 moles/162 g. starch after forty hr., the acid liberated reached the theoretical value in only thirty-eight hr., and continued to increase. Small-scale experiments showed that complete decomposition

of about 250 gm. of oxidised starch would yield 1 litre of 1 N acid. By treating starch with periodic acid for five days, Barry (223) obtained oxidation products having neutralisation equivalents of 145, from which he deduced that 2/3 of the aldehydic groups introduced had been further oxidised to carboxyl. Over-oxidation to such an extent, which would lead to a periodate uptake of 1.7 moles/162 gms. starch, could not have occurred in the present cases, where, even after the additional 100 hr. oxidation allowed, the uptake did not exceed 1.1 moles periodate per anhydroglucose unit. Indeed, such oxidation of -CHO groups to -COOH would result in decreased sensitivity to alkali (227). The alkali-sensitivity of some polyaldehydes obtained by periodate oxidation has been investigated by Head (222), and it is considered that the acid formation reported here is due to alkaline hydrolysis of the acetal-linkages accompanied by continued conversion of -CHO to -COOH groups by Cannizzaro-type reactions.

The fact that formic acid release follows periodate uptake very closely (Fig. XI), suggests that there is no preferential initial oxidation at reducing end-groups followed by stepwise oxidation of successive glucose units along the unit-chains. It has been suggested (228) that oxidation of the reducing end-group proceeds via the formation of an intermediate formate ester. It is still possible, however, that over-oxidation to carboxyl of a small percentage of the initially formed aldehydic groups may occur before primary oxidation of every glucose unit

present has occurred. In this event, theoretical uptake of periodate would not be a reliable indication that complete oxidation of the polysaccharide had occurred. Determinations of the carboxyl content of the poly-aldehydic oxidation products could be used to clarify this matter, but unfortunately the analytical methods at present in existence are not sufficiently sensitive to give conclusive evidence of the presence of 1-2% of carboxyl groups.

There was the possibility that esterification of C_6 in the starch may occur through interaction with some of the liberated formic acid (cf. 224, 225). This would account for the release of some acid when the oxidation product was treated with alkali, and would imply that the estimation of formic acid by titration of aliquots centrifuged free from oxidised starch granules would not be quantitative. As esterification might have already occurred during preparation of the polyaldehydes, quantities were shaken for twelve days at 15-16°C. with concentrations of formic acid ten times greater than that normally released during oxidation, so that further esterification could occur. The formic acid was recovered quantitatively: in a similar experiment using pure starches, evidence of 0.2% removal of acid was obtained. Hence no significant quantity of formic acid becomes chemically bound as ester during periodate oxidations lasting twelve days at 15-16°C.

Halsall, Hirst, Jones and Sansome (57) obtained evidence that different samples of the same starch,

derived from plants differing in botanical variety and growth-conditions, contained the same proportions of end-group. As this result has been substantiated for barley, oat and potato starches, (see Table XII), the deviating periodate values in Table IX could possibly be explained by experimental errors arising through incomplete oxidation and by the use of differing procedures for the determination of formic acid. Morrison, Kuyper and Orten (213) showed that choice of end-point in the range pH 5.5-8.0 should have little influence on quantitative titration of pure formic acid. Whilst potentiometric titration studies, supported by iodometric determinations, have shown this to be correct, they have also shown that quantitative titration of formic acid in the presence of all normal oxidation products is complete at pH 6.25, and that over-titration to pH 8.0 would reduce the value obtained for a true value for $\overline{R}$ of 25-30 glucose units by about 20%. In Fig. XIII (a), which shows potentiometric titration results for control solutions, the curve for pure formic acid (curve 1) differs above pH 6.5 from that for the same quantity of acid in the equivalent of a centrifuged reaction mixture (curve 2). A still greater divergence occurs above pH 6.25 when 50 mg. oxidized starch (carefully washed free from acid) is added, giving the equivalent of an uncentrifuged reaction mixture (curve 3). Cori and Larner (226) noted "an apparent buffering action" during titration of periodate reaction mixtures: this is now explained by the alkaline degradation of oxidized starch. From the equivalence point of curve (1) at pH 7.1,

Key to curves in Figure XIII.

Figure XIII (a):

Titration curves for control solutions.

Curve 1. HCOOH v. NaOH.

Curve 2. (HCOOH + KCl + $NaIO_4$ + Ethylene glycol) v. NaOH.

Curve 3. [(2) + oxidized starch] v. NaOH.

Figure XIII (b):

Titration curves for reaction mixtures.

Curve 1. Control HCOOH v. NaOH.

Curve 2. Centrifuged reaction mixture v. NaOH.

Curve 3. Uncentrifuged reaction mixture v. NaOH.

Figure XIII (c):

Titration curves for proteinaceous reaction mixtures.

Curve 1. Control HCOOH v. NaOH.

Curve 2. Centrifuged reaction mixture v. NaOH.

Curve 3. Uncentrifuged reaction mixture v. NaOH.

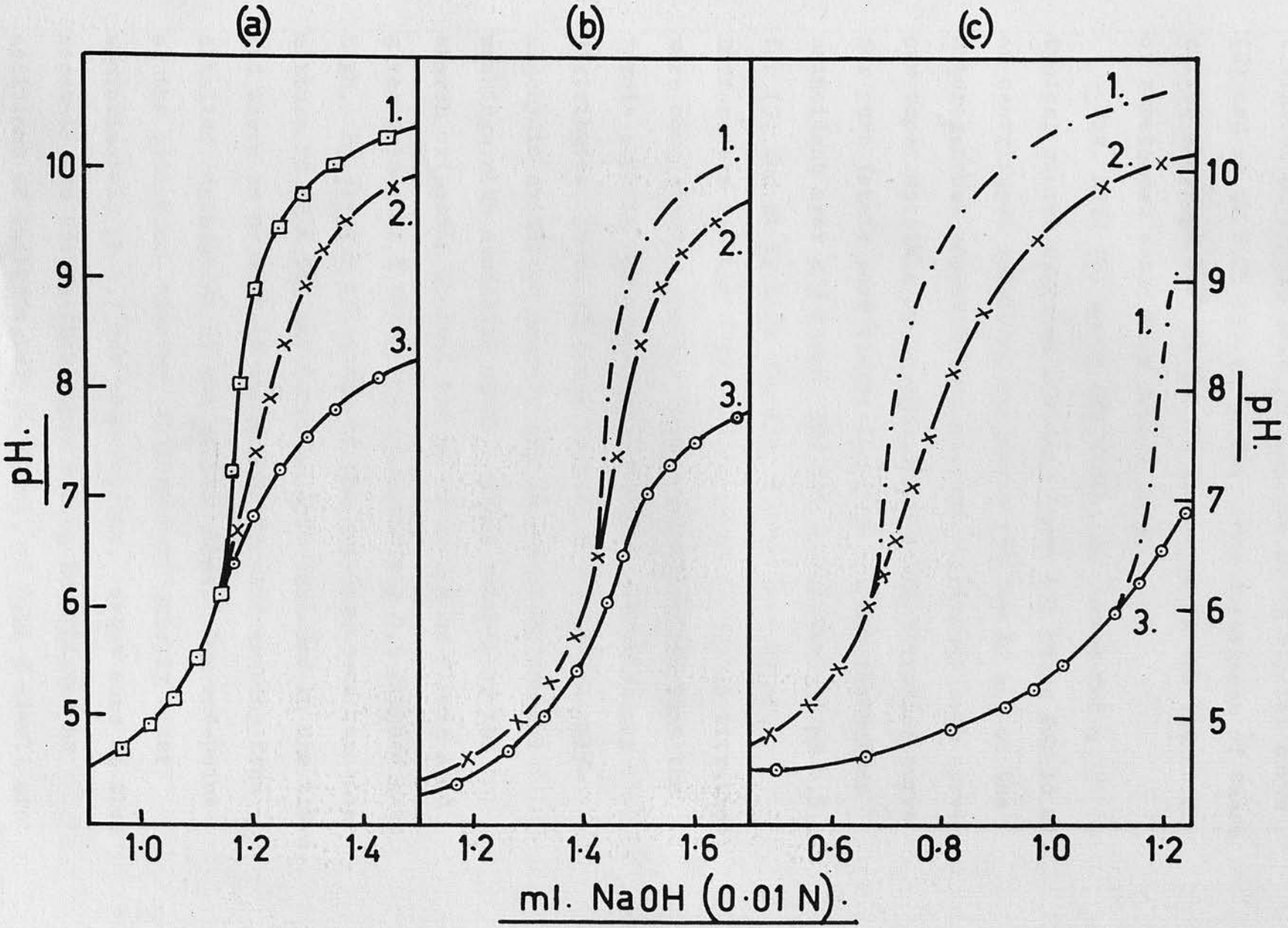


FIG. XIII.

it is seen that, under these experimental conditions, titration of formic acid is complete at pH 6.5 for curve (2) and at pH 6.25 for curve (3). The divergence of these curves above pH 6.25 is due to inclusion in the titration of substances other than formic acid.

Fig. XIII (b) shows the titration curves for a typical starch reaction mixture, curve (2) being for 10 ml. of centrifuged solution, and curve (3) for 10 ml. of the heterogeneous uncentrifuged mixture. Although both curves are superimposible up to pH 6.25 with the titration curve for pure formic acid (curve 1), they are not themselves coincident over any range, and the end-points, at pH 6.5 for (2) and at pH 6.25 for (3), differ by 0.03 ml. Differences of this order (about 2% of the total titration) were consistently found. Investigation showed that the formic acid in the reaction mixture is not uniformly distributed, about 2% being loosely bound to the polyaldehydic oxidation product and removable by several washings with distilled water. Hence removal of the starch oxidation product before titration of formic acid gives values of $\bar{R}$ which are approximately 0.5 glucose unit high. By careful titration of the heterogeneous reaction mixture to pH 6.25, any bound acid is included in the titre, and there is no risk of including any acid arising from alkaline degradation of the polyaldehyde. The end-point of the titration, however, follows very quickly after approximately pH 5.7 has been reached. Great care is then necessary to ensure that rapid mixing is achieved as additions of approximately 0.002 ml. of 0.01 N alkali are

made, so that local concentrations of alkali never exist.

The average values of $\overline{R}$ found for the starches studied are presented in Table XII, the experimental error being within $\pm$ 0.5 glucose unit.

The behaviour of some protein-contaminated oat starches (obtained as intermediates in the purification of the starch as described in Part I, this thesis) on periodate oxidation has been studied, the results being shown in Table XIII (p.112). For protein contents of less than 3%, uptake of periodate was normal, and the correct value of $\overline{R}$ is given when the sample weight is corrected for the percentage of protein present. The differences in titration curves of centrifuged and non-centrifuged samples increases however with increasing protein-content. Fig. XIII (c) shows the curves obtained for oat starch II (d), which contained 23% protein. The end-point for the centrifuged solution (pH 6.5; curve 2) gives a value of $\overline{R}$ of forty glucose units, whilst that for the non-centrifuged solution (pH 6.25; curve 3) gives the correct value of twenty-eight for $\overline{R}$. Thus the presence of protein causes further complexing of formic acid, and it is even more essential than in the case of non-proteinaceous samples that the heterogeneous reaction mixture be titrated. No reliable estimate of $\overline{R}$ could be obtained from the potentiometric titration curves for oat starch II (e) under any conditions.

TABLE XII.

Average values of R found for unfractionated starches, and calculated number of glucose residues per non-reducing end-group in the amylopectin fraction.

Source of Starch	Temp.	No. of Detns.	Oxidatn. Time (hr.)/	Av. value of $\bar{R}$ //	Amylose content (%)///	Calc. value of C.L. for Amylo-pectin
Arrowroot ..	15-16°	3	284-312	27.3	20.5	21.7
Banana	15-16	2	244-336	26.3	16.8	21.9
Barley I ...	15-16	4	260-282)	29.5	22.0	23.0
Barley II ..	15-16	2	260-282)			
I. germanica	15-16	2	262-308	28.0	27.0	20.4
Maize	15-16	2	300-384	26.5	24.0	20.1
Oat I and II	15-16	5	240-318)	27.4	26.0	20.3
	20-21	1	164-240)			
Parsnip	15-16	2	268-360	23.0	11.1	20.4
Pearl manioc	15-16	2	244-312	24.1	15.7	20.3
Potato I ...	15-16	2	291-383	28.3	20.4	22.5
Potato II ..	15-16	2	336-455)	28.3	21.0	22.4
	20-21	1	186)			
Potato III .	15-16	2	335-455	28.3	21.0	22.4
Rice	15-16	3	286-384)	27.5	18.5	22.4
	20-21	1	164-212)			
Sago	15-16	2	244	25.0	26.0	18.5
Sweet potato	15-16	2	266-310	28.2	17.8	23.2
Tapioca	15-16	4	264-300	26.2	16.7	21.8
Waxy maize .	15-16	4	302-400)	20.0	1.4	19.7
	20-21	1	164-284)			
Wheat	15-16	4	260-306	26.2	25.0	19.6

Footnotes:-

/ The minimum time quoted is the number of hr. of oxidation (found by separate experiment - see Table X) for periodate uptake to reach 1.03-1.04 moles/162 g. starch: the range quoted shows the period in which no over-oxidation occurred, the formic acid released being constant within the limits corresponding to $\bar{R} \pm 0.5$ glucose units.

// All values obtained were within $\pm$ 0.5 glucose units from the average.

/// Values for % amylose, and origin of samples, as in Table VI.

TABLE XIII.

Value of $\bar{R}$ found for proteinaceous oat starches *** by periodate oxidation.

Starch Sample	% Protein	Oxidation-time at 15-16°C: 10 Days		11 Days		12 Days		13 Days	
		C*	NC**	C	NC	C	NC	C	NC
Oat IIb	0·45	29·9	29·1	29·0	28·3	28·2	27·5	27·9	27·3
IIc	2·94	31·1	29·7	30·2	28·8	28·4	27·6	28·1	27·1
IId	22·7	48·8	32·8	46·5	28·0	40·5	27·8	38·6	25·3
IIe	45·6	79·6	34·1	66·8	28·4	64·7	26·2	51·8	23·5

* Value obtained by titration of reaction mixture centrifuged free of oxidised granules.

** Value obtained by titration of non-centrifuged reaction mixture.

*** Sample weight corrected for percentage protein present.

The potentiometric titration method has been found to give reproducible and reliable results, under the conditions described, for the determination of the amount of formic acid released on periodate oxidation. Whilst the iodometric method described by Smith (210), which determines the total acidity, was found to give good agreement for pure starches, the potentiometric method has the advantage of showing from the shape of the titration curve whether acids other than formic are being titrated, so indicating the presence of impurity or occurrence of non-Malaprade reactions.

Periodate oxidation, under the experimental conditions described, is therefore a reliable and easy routine method for determining the average unit-chain length in starches, and has in addition an accuracy and reproducability better than that reported for the methylation technique.

SUMMARY.

The oxidation of eighteen different starches by potassium meta-periodate has been studied, and values for the ratio of non-terminal to terminal glucose units present in each are presented, the accuracy claimed being ± 0.5 glucose unit. The rate of oxidation is appreciably temperature-dependent, and, in addition, the time taken for the theoretical uptake of periodate ion varies from starch to starch. Potentiometric titration studies showed that, in the presence of all reaction products, formic acid is quantitatively determined by titration to pH 6.25. Oxidised starches are stable below this pH value, but decompose rapidly in dilute alkali, yielding acidic products in quantity. Oxidised starches bind about 2% of the formic acid released during oxidation; hence complete estimation of this acid can only be made by titrating the heterogeneous reaction mixture to pH 6.25. Results of an investigation of the presence of protein on the periodate oxidation of oat starches are reported.

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APPARATUS AND TECHNIQUE

A SIMPLE ELECTROMETER FOR SMALL-SCALE POTENTIOMETRIC TITRATIONS

By D. M. W. Anderson and C. T. Greenwood

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An electrometer was required as the null-point indicator in the measurement of the uptake of iodine by starch and other polysaccharides using the differential potentiometric titration technique of Gilbert and Marriott.[1] The investigation of samples on a small scale necessitated an instrument of high sensitivity; at the same time a simple design was aimed at so that determinations could be made on a routine basis.

Many electrometer circuits, designed to achieve sensitivity with minimum zero-drift and instability, have been described.[2] Complete elimination of zero-drift has been claimed,[3] but this has generally been attained by use of internal compensation,[4] mu-balance,[5] the continuous charging of batteries,[6] or negative feed-back and stages of D.C. amplification.[7] Consequently, these circuits require some form of tuning, and are not as simple as was desired. In most instances, elaborate precautions with regard to external shielding, thermostatting and earthing were also found to be necessary.[8]

Preliminary investigations were made using an electrometer triode (grid current 10^{-15} amp.) as one of the arms in a Wheatstone bridge circuit built into an air-tight vibration-free box, from which light was excluded. Using a spot galvanometer of sensitivity 109 mm. per micro-amp. and internal resistance 402 ohms, the sensitivity obtained was 6 mm. per mv. Zero drift was reduced to an average of 0·5 mm. per minute by using good quality components, allowing adequate wattage dissipation in all resistors, and using aged and well-charged accumulators. (A zero drift of 1—2 mm. per minute has been commonly claimed for circuits of similar sensitivity).[8, 9] Neither the sensitivity nor the rate of zero drift obtained was considered to meet the standards required. In addition, the system was so susceptible to external interference despite the instalment of a virtual earth system, the provision of an equipotential shield,[10] and careful screening, that it was rejected.

The circuit finally adopted employs the well-known principle of using a matched pair of valves as two of the resistances in a Wheatstone network,[11] with the other two bridge resistors in this case acting as cathode loads for the valves, as shown in Fig. 1.

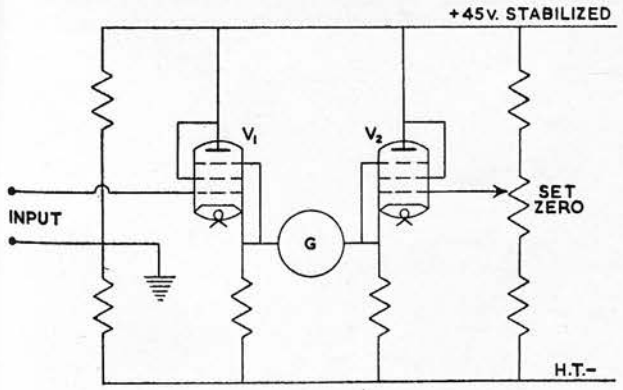

FIG. 1

Since each valve functions as a cathode follower, the circuit is extremely stable, with a high input impedance (experimentally determined as 700 megohms) and an output to input voltage ratio of 1 : 11. For a titration cell resistance of 2 megohms, the grid current is 3×10^{-11} amp. (Although this value is higher than that given by an electrometer triode, no undesirable effects in solution have been observed.)

The valves (Type VR116) are a matched pair, selected from ten which were available. This type was chosen because of its sturdy filament construction and good heat inertia, which enabled the grid current to be reduced as far as possible by underrunning the valves as follows: (*a*) The nominal 6·3 v. filaments are run off 4 volts, supplied by three parallel pairs of 2-volt accumulators, aged and well-charged;[12] (*b*) H.T. voltage is + 45 v., obtained from an electronically stabilized power unit by means of a potential divider chain.

The only shielding employed is conventional screening of the input coaxial cable to the grid of V.1. The metallized envelope of the valves is earthed, but screened-type grid top-caps are not required, despite the presence in the same room of motors and other equipment capable of electrical interference. It has not even been found necessary to shield the valves from light, or from local changes in room temperature.

The constancy of zero attained is shown by the following typical figures obtained in different experiments:

Table I

Time in mins. ..	0	3	6	9	12	18
Galvo. defln. (mm.) ..	0	0	0	0	+1	+1
Time in mins. ..	0	10	30	50	70	100
Galvo. defln. (mm.) ..	0	+0·5	+0·5	0	+0·5	+2·5

Response is perfectly linear over the range 0—5 mv. input, obtained from a Tinsley potentiometer. The sensitivity (using the galvanometer mentioned above) is 28 mm. per mv., which in conjunction with the constancy of zero, enables potentials of 0·01 mv. to be measured.

With this sensitivity, an uptake of 2×10^{-10} moles iodine per mg. polysaccharide can be detected. This has enabled the iodine uptake of lichenin, Floridean starch, amylodextrin and various glycogen samples to be studied, in addition to routine determinations on starch and its components. Sample weights of the order of 2 mg. of amylose, 12 mg. of a starch, and 30 mg. of highly branched polysaccharides are sufficient for characterization. It is hoped to publish the results of this work elsewhere.

In its present form, this circuit is applicable to many potentiometric titrations, having the advantages of inexpensive and simple construction, high sensitivity and accuracy, freedom from external interference, and simplicity in manipulation. While sensitivity can be increased by incorporating a more sensitive galvanometer, this can be achieved, and the use of the circuit extended to measurements with glass electrodes, by using valves of higher mutual conductance.

Acknowledgment

The authors thank Prof. E. L. Hirst, F.R.S., for his interest in this work, and Mr. C. H. C. Matthews for invaluable discussions.

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THE CHARACTERIZATION OF BRANCHED α : 1-4-GLUCOSANS

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During recent potentiometric studies of the iodine-uptake of many branched α : 1-4-glucosans,[1] it has been observed that the difference between the binding-power of normal glycogens and amylopectins is sufficiently large to characterize the two structure-types.

Fig. I shows typical curves obtained: the slopes of the curves for the amylopectins from barley,[2] *Hevea brasiliensis* seed,[3] and oat[4] starches (curves 1, 2 and 3, respectively) are quite distinct from those of *Ascaris lumbricoides* and rabbit liver glycogens (curves 4 and 5). In all samples so far examined, the normal glycogen-type structure shows no evidence of any iodine binding-power due to linear material in the early stages of the titration (i.e. for total I_2-concentration $< 1 \times 10^{-6}$M). The preferential iodine-uptake by traces of such material occurring in all the amylopectin-type curves has been corrected for by extrapolating the curve for the branched material to zero total iodine concentration, and hence only uptake by branched molecules is being compared.

On this basis, waxy maize starch behaves as a typical amylopectin (curve 6), and the polysaccharide from the ciliate *Tetrahymena pyriformis*[5] as a glycogen (curve 7).

This potentiometric method gives unambiguous characterization, and has the advantage of accuracy and reproducibility under standard conditions ([I′] = 0·01M; pH = 5·85; temperature = 20° C.); the titration curve can be repeated on the same 10—20 mg. sample; and the polysaccharide can be recovered if desired.

In the range of concentrations employed, the amount of iodine bound is directly proportional to the free iodine concentration. It is possible that the length of unit-chain alone determines the amount bound by any branched glucosan, molecular size being of secondary importance, but further investigations on " abnormal "-type branched glucosans are necessary.

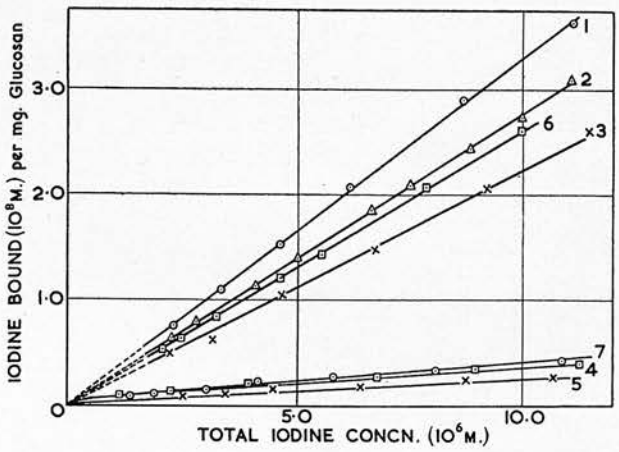

FIG. 1

Comparison of titration curves for branched glucosans

Curve 1. Barley amylopectin (C.L.* = 24—26)
2. *Hevea brasiliensis* seed amylopectin (C.L. = 23)
3. Oat amylopectin
4. *Ascaris lumbricoides* glycogen (C.L. = 12)
5. Rabbit liver glycogen (C.L. = 12)
6. Waxy Maize starch (C.L. = 18—20)
7. *Tetrahymena pyriformis* polysaccharide (C.L. = 12)

* C.L. is the chain-length expressed as the number of glucose units

The authors thank Prof. E. L. Hirst, F.R.S., for his interest in this work, and Dr. D. J. Manners for providing the samples of glycogen investigated.

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AN INVESTIGATION OF THE POLYSACCHARIDE CONTENT OF OATS, *AVENA SATIVA* L.

By D. M. W. ANDERSON and C. T. GREENWOOD

A graded extraction procedure has been applied to the polysaccharides present in the kernels of oats, *Avena sativa* L. Analyses of the sugar, protein and ash content of the fractions obtained are reported. A method for the purification of the starch present in the kernels is given. Oxidation of the unfractionated starch with potassium metaperiodate showed that the ratio of terminal to non-terminal glucose units was 1 to 27·4. Differential potentiometric iodine titrations indicated the presence of 26·0% of amylose in the starch, and hence the average length of unit-chain in the amylopectin component was calculated to be 20·3 glucose units. Examination of the impure intermediate starch products showed that the protein impurity interfered considerably with the interaction of the starch with potassium metaperiodate.

Introduction

The polysaccharide content of many types of seed material has recently been examined Because of their importance, the common cereals, wheat, barley and maize, have attracted considerable study, and work carried out on waxy maize starch has been reviewed recently.[1] Investigations into the isolation of wheat starch from the contaminating gluten have been carried out by McMasters & Hilbert[2, 3] and by Glendenning & Wright,[4] and the cold-water extract from this cereal has been examined by several workers.[5, 6, 7, 8] McWilliam & Percival[9] have carried out a full chemical investigation of the starch from barley, and the corresponding cold-water extract has been partially separated into its components.[10, 11, 12] The cold-water extract from each of the common cereals has been shown to give several sugars on hydrolysis,[12] but the structural significance of these is not yet completely established. In the case of the

cereal, oat, little work on the polysaccharide content has been reported, although Morris[13] has claimed to have isolated lichenin and araban from the cold-water extract.

In the present work, the distribution of all the component polysaccharides in the oat kernel has been investigated, whilst special attention has been devoted to determine whether *pure* starch could be isolated in good yield. Chemical methods were avoided in the early stages of extraction to reduce possible damage to the starch granules. After removal of fats and deactivation of enzymes, oat kernels (*var.* Sun II, 1952 crop) were successively extracted with cold and hot water followed by cold and hot alkali. The fractions obtained were hydrolysed with acid, then examined qualitatively and quantitatively for the liberated component sugars ; in addition, the amount of ash, protein, and unhydrolysable material in each was determined. The starch fraction was purified free from protein, and the percentage of amylose and the ratio of non-terminal to terminal groups determined. The presence of contaminating protein in the starch was found to interfere with both these determinations. In order to study these effects in more detail, a second sample of impure starch from the same variety of oats (1953 crop) was extracted with M-sodium chloride and toluene to provide a wider range of protein-contaminated samples.

Experimental

Before analyses, samples were dried by heating *in vacuo* at 80° for several hours. Solutions were concentrated under reduced pressure at 40°. Percentages of nitrogen were found by duplicate semi-microKjeldahl determinations.

Extraction of fats from the kernels

Oat kernels (*var.* Sun II, 1952 crop) were ground to a coarse flour (Found : moisture, 11·8 ; ash, 2·22 ; protein, 14·0 ; ether-extractable substances, 7·2%), and this was exhaustively extracted with a boiling benzene–methanol mixture (2 : 1, v/v ; 1·5 l./200 g. flour).

Cold-water extraction

Defatted kernels (100 g.) were shaken vigorously with distilled water (1 l.) (five treatments, each of about 12 h.). After each extraction, the turbid extract obtained on filtration was centrifuged to give a proteinaceous starch deposit (fraction F1), and a supernatant liquid containing water-soluble material. Supernatant liquids were combined, reduced in volume and freeze-dried to yield fraction (F2).

Purification of fraction (F1) (Starch I)

This portion was treated by fractional centrifuging using the procedure detailed below for Starch II, to give fractions F1*a*, *b* and *c*.

Hot-water extraction

The residue from the cold-water extraction was stirred for 3 h. with 1 l. of water at 90°. The extract, which appeared to contain starch, was treated with thymol (2 g.) to give, after keeping at 25° for three days, a characteristic amylose–thymol complex (F3*a*). The supernatant liquid from the thymol separation yielded fraction F3*b* after freeze-drying. The residue from the first cold-water extraction was extracted four more times with hot water as before and the extracts combined, giving after freeze-drying, fraction F3*c*.

Alkaline extractions

The residue from the cold- and hot-water extractions was stirred five times with sodium hydroxide (5% w/v ; 500 ml. ; about 2 h. each time) at room temperature under nitrogen. The supernatant liquids obtained on centrifugation were brought to pH 7 with glacial acetic acid, and the resultant precipitate collected (fraction F4). Material in the solution was precipitated (fraction F6) by the addition of ethanol (2 vol.).

A similar procedure using sodium hydroxide (5%, w/v ; 500 ml. ; about 2 h. each time) at 90° in a nitrogen atmosphere gave fractions F5 and F7.

The residual material was washed free from alkali and dried (F8).

Qualitative analysis of the fractions for sugars

Samples (F1–F7) were hydrolysed with 2% sulphuric acid (2 ml./20–30 mg.) at 100° for 8 h. For (F8), the hydrolysis-time was increased to 18 h. The solutions were then neutralized with barium carbonate, the precipitated sulphate removed, and the concentrated solutions examined by partition chromatography. [Whatman No. 1 filter paper; descending method; solvent system: butanol–benzene–pyridine–water (5 : 1 : 3 : 3—top layer).] After development for 48 h., good separation of all sugars was achieved, except for fraction (F2), where the fructose and arabinose were not resolvable. Two chromatograms were examined for each sample, aldoses being located with aniline oxalate spray, and ketoses with urea oxalate. All sugars detected were identified by comparisons with control solutions.

In addition, the unhydrolysed cold-water extract was examined, when maltose, galactose, mannose, arabinose and xylose were shown to be absent.

No ribose was liberated from any fraction on hydrolysis.

Quantitative analysis of fractions for sugars

For quantitative analyses, a known weight of ribose was added as a reference sugar. After hydrolysis (as above) any non-hydrolysable material was removed, and weighed. The sugars were then separated on Whatman 3MM paper using the above solvent system at 21° for 48 h., and eluted with water from their located sites.[14] Estimations of sugar concentration were made on aliquots using Somogyi's reagent.[15] Fructose and fructosans in F2 were estimated using Arni & Percival's colorimetric method.[16]

Purification of oat starch II

Crude oat starch (Starch II, separated from the 1953 sample of oats) was purified by dispersing in 1M-sodium chloride so as to give a suspension of specific gravity 1·07, to which was added toluene ($\frac{1}{10}$ vol.). The mixture was shaken vigorously for at least 1 h., then centrifuged for 5 min. at 750 r.p.m. before the speed was slowly increased to 1200 r.p.m. over a further period of 5 min. (M.S.E. 'Major' centrifuge). The brown precipitate at the toluene–water interface was removed and discarded. The deposited material consisted of two well-defined layers; the upper (proteinaceous) layer was loosely packed and easily removed with a spatula followed by gentle washing with a jet of water. The lower layer was much whiter and very densely packed. These two layers were then separately re-suspended in 1M-sodium chloride, and the extraction procedure repeated six times. The nitrogen content of the final starch (IIa) was only 0·03%, and other fractions of higher nitrogen content were isolated at intermediate stages of the purification process.

After washing free from salt, these products were refluxed with 85% methanol (three treatments, each of 2 h.) to ensure complete removal of fatty materials, finally yielding fractions of which Starches II*a–e* are representative (for protein content see Table II).

When an aqueous suspension of Starches II*b* and *c* was passed through a column (10 × 1·5 cm.) of Zeokarb-215 resin, the nitrogen content decreased from 0·07 and 0·47% to 0·05 and 0·34% respectively (75% recovery of material). No decrease, however, was found in the case of Starch II*a*, and further treatments of II*b* and *c* with resin, and also with hot 80% ethanol, did not further reduce these percentages of nitrogen.

*Analysis of Starch II*a

The white powder consisted of birefringent granules, forming in hot water a clear paste which stained blue with iodine. Hydrolysis with 2% sulphuric acid (2 ml./20 mg.) at 100° for 7 h., yielded 99·1% of the theoretical amount of glucose (alkaline hypoiodite determinations[17] at pH 11·4[18]). There was no residue after hydrolysis, and no other sugar could be detected by chromatography. The starch had $[\alpha]_D^{18}$ + 163° (*c*, 0·5% in N-NaOH), $[\alpha]_D^{18}$ + 194° (*c*, 0·34% in 30% $HClO_4$) (Found: sulphated ash, 0·024; N, 0·03%).

Determination of the percentage of amylose

The differential potentiometric titration technique of Gilbert & Marriott[19] was employed to measure iodine-binding power. The electrometer used and the titration conditions have been previously described.[20]

Determination of the ratio of terminal to non-terminal groups [i.e. $(\overline{R})$] in the starch

The amount of formic acid liberated and the amount of periodate consumed on oxidation of the starch by potassium metaperiodate were determined as described by Anderson, Greenwood & Hirst.[21]

Results

The chromatographic analyses of the unhydrolysed cold-water extract (F2) indicated that oligosaccharides (fructosans), raffinose, sucrose, glucose and fructose (approximately 10, 15, 65, 5 and 5% of the total, respectively) were the only free sugars present. Table I shows the results of analyses of the hydrolysed fractions. The potentiometric iodine titration curve for Starch II*a* (Fig. 1) shows that the starch bound 5·0% of its own weight of iodine. If the maximum iodine binding-power of pure amylose under these experimental conditions is 19·2%,[22] then the starch contains 26·0% of amylose.

Table I

Analyses of the fractions isolated by the graded extraction procedure

Fraction	% of original material (approx.)	% Protein (N×6·25)	% Ash	% residue after hydrolysis	Reaction with iodine	Percentages of sugars obtained on hydrolysis*: Pentosan† Oligosaccharides	Galactose	Glucose	Fructose	Arabinose	Xylose
F1*a*	45	0·3	0·03	—	+	—	—	98	—	—	—
F1*b*	9	7·9	0·12	—	+	—	—	90	—	—	—
F1*c*	14	28·4	0·31	—	+	—	—	68	—	—	—
F2	7	20·9	1·08	1·23	—	2‡	6	79	5	4	4
F3*a*	3	5·1	2·13	—	+	—	3	89	—	4	4
F3*b*	7	6·9	0·90	1·14	+	2	1	88	—	5	4
F3*c*	6	11·5	1·37	1·58	+	1	2	87	—	5	5
F4	1	68·8	4·28	18·3	—	—	—	28	—	34	38
F5	Negligible	—	—	—	—	—	—	—	—	—	—
F6	3	9·8	6·12	15·3	—	3	3	26	—	32	36
F7	2	1·4	39·4	77·3	—	—	15	30	—	25	30
F8	3	1·6	7·65	71·6	—	—	4	21	—	39	36

* Expressed as percentages of hydrolysable carbohydrate
† Incompletely hydrolysed polysaccharides
‡ This fraction also contained some hexosans

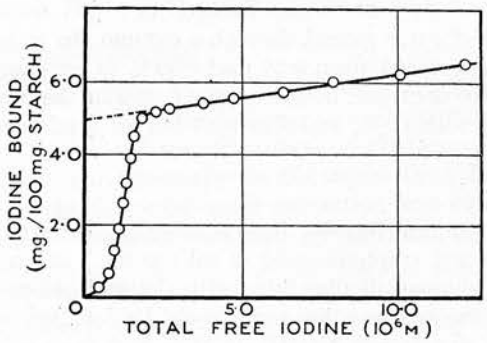

FIG. 1.—*The potentiometric iodine titration curve for Starch II*a

([I′] = 0·01M; pH = 5·85; temp. = 20°)

The results of periodate uptake showed that at 15–16° oxidation was complete in 240–318 h. The amount of formic acid liberated in this time corresponded to a value for $(\overline{R})$ of 27·4 glucose units. The average length of unit chain in the amylopectin component of the starch was hence calculated to be 20·3 glucose units. The effect of protein on the observed value of $(\overline{R})$ is shown in Table II for centrifuged and non-centrifuged samples.

Table II

Value of $(\overline{R})$ *found for proteinaceous starches* by periodate exidation*

Sample	% Protein	Oxidation-time at 15–16°							
		10 days		11 days		12 days		13 days	
		C	NC	C	NC	C	NC	C	NC
II*b*	0·45	29·9	29·1	29·0	28·3	28·2	27·5	27·9	27·3
II*c*	2·94	31·1	29·7	30·2	28·8	28·4	27·6	28·1	27·1
II*d*	22·7	48·8	32·8	46·5	28·0	40·5	27·8	38·6	25·3
II*e*	45·6	79·6	34·1	66·8	28·4	64·7	26·2	51·8	23·5

* Sample weight corrected for percentage protein present
C = Value obtained by titration of reaction mixture centrifuged free of oxidized granules
NC = Value obtained by titration of non-centrifuged reaction mixture

Discussion

The samples of groats studied had been *completely* freed from contaminating hulls, although a significant proportion of the original oats were green-tipped. Results similar to those of Macleod & Preece[23] have been obtained for the analysis of the free sugars in the cold-water extract of the defatted kernels. All the polysaccharide fractions obtained from the extraction scheme were contaminated with protein ; no effort was made to remove this except in the case of the starch product, where a physical method of separation was successful. The high ash content of some fractions was due to the fact that no dialysis was carried out (to avoid loss of carbohydrate) before isolation of the fractions by freeze-drying. The whole of the fructosan content of the kernels was found in the cold-water extract F2, whilst galactose, glucose, arabinose and xylose were present in the hydrolysis product of nearly all the fractions.

Complete separation of the polysaccharide material into products of varying solubility is thus difficult. As a result, the differentiation between the water-soluble and hemicellulosic materials is not sharp, but water-soluble pentosans and glucosans other than starch are present. A large percentage of the glucose in the hot-water extracts was probably present as starch (which cannot be completely separated using cold water), whilst that in the alkaline-soluble fractions was probably hemicellulosic in nature. The large amount of unhydrolysed material in F7 is accounted for by the high ash content of this fraction, whilst that for F8 is probably due to the presence of cellulosic material.

On hydrolysis, the cold-water extract F2 was shown to give rise to sugars in quantities very similar to those described by Preece & Mackenzie,[12] bearing in mind that these authors combined galactose with glucose and fructose with arabinose.

About 75% of the starch present in the kernel could be purified to a protein content of 0·3% under the mild conditions necessary to minimize degradation. A prolonged purification procedure produced a quantity of starch having a protein content of 0·19%. This percentage could not be further reduced and it would be difficult to determine whether this residual protein is chemically bound to the starch. The percentage of amylose is higher than for barley starch, but is of the same order as for wheat.[21] Further work is in progress on the isolation of the amylose from the starch fraction. The average length of unit-chain of the amylopectin component appears to be similar to that of many other starches.[21]

Examination of the samples of protein-contaminated starches showed that, with these materials, abnormal results were obtained from both periodate oxidation and potentiometric iodine titration experiments. The effect of this impurity on periodate oxidation is shown in Table II ; the value of $(\overline{R})$ obtained for protein-contaminated starches most closely approximates to the correct value only when estimations are carried out by titrating *uncentrifuged* samples of the reaction mixture to pH 6·25.[21] This technique is suitable for samples containing up to 23% protein : above this value consumption of periodate exceeds the theoretical and no reliable figure for $(\overline{R})$ can be obtained. On the other hand, the presence of even 3% of protein seriously interferes with the observed iodine-uptake. This effect will be described in detail elsewhere. These data show the necessity for *rigorous* removal of protein from polysaccharide samples before structural examinations are attempted.

For Starch I*a*, the values obtained for $(\overline{R})$ and the percentage of amylose were identical with those for Starch II*a*. Since the oats, although of the same variety, were grown in different years, possible variations in growth conditions produced no change in these characteristics of the isolated starches (compare references 21 and 24).

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Physicochemical Studies on Starches. Part II. The Oxidation of Starches by Potassium Metaperiodate.*

By D. M. W. Anderson, C. T. Greenwood, and E. L. Hirst.

[Reprint Order No. 5721.]

The oxidation of 18 different starches by potassium metaperiodate has been studied. At constant temperature, the time taken for the theoretical uptake of periodate ion varies from starch to starch. Only traces of glucose were found in the hydrolysates from oxidised starches when oxidation was continued until over-consumption of periodate had occurred. Potentiometric-titration studies showed that, in the presence of all reaction products, formic acid is quantitatively determined by titration to pH 6·25. Oxidised starches are stable below this pH, but decompose rapidly in dilute alkali, yielding acidic products in quantity. Oxidised starches bind about 2% of the formic acid released during oxidation; hence complete estimation of this acid can only be made by titrating the *heterogeneous* reaction mixture to pH 6·25. The effect of the presence of protein on the periodate oxidation of oat-starch samples has been investigated. Values for the ratio of non-terminal to terminal groups of the starches studied are presented, the accuracy claimed being ±0·5 glucose unit. The average length of unit-chain of the corresponding amylopectin components has been calculated from these values and the results of determinations of the percentages of amylose.

In recent years there has been an increasing tendency for the methylation method of determining the ratio of non-terminal to terminal groups [*i.e.*, $(\bar{R})$] in starches to be superseded by the method involving the estimation of formic acid released from the non-reducing end-groups during periodate oxidation. The periodate method has the advantages of simplicity and speed and requires about ten times less material. Theoretically, it can also provide additional evidence for the presence or absence of inter-chain linkages involving $C_{(2)}$ or $C_{(3)}$ (Halsall, Hirst, Jones, and Roudier, *Nature*, 1947, **160**, 899) and, in conjunction with methylation data, of glucose residues linked solely through $C_{(1)}$ and $C_{(6)}$ (Brown, Halsall, Hirst, and Jones, *J.*, 1948, 27). However, the accuracy of periodate determinations of the value of $(\bar{R})$ for starches has been quoted as ±10% (*idem*, *loc. cit.*). There has also been considerable disagreement in the values obtained for the average lengths of unit-chain of amylopectins by different workers (see Table 1). The studies reported here were undertaken in an effort to reduce the experimental errors

Table 1. *Values quoted from periodate oxidation results for the average length of unit chain of amylopectins.*

Source of starch	Reference A	B	C	D
Maize	20	—	25—26	25·2
Potato	24—26	—	27	—
Tapioca	20	22	23	26·6
Wheat	21	—	23	—

A, Brown, Halsall, Hirst and Jones, *loc. cit.*; B, Meyer and Settele, *Helv. Chim. Acta*, 1953, **36**, 197; C, Potter and Hassid, *J. Amer. Chem. Soc.*, 1948, **70**, 3488; D, *idem*, *ibid.*, 1951, **73**, 997.

involved in periodate oxidations, and so obtain accurate values of $(\bar{R})$ for use in conjunction with the results from differential potentiometric-titration studies of the iodine uptake of starches and their components.

* Part I, *J.*, 1954, 3769.

Oxidations were carried out with potassium metaperiodate (Halsall, Hirst, and Jones, *J.*, 1947, 1399) at 15—16° and at 20—21°. At both temperatures, the time required for the theoretical uptake of 1·03—1·05 moles of periodate per anhydroglucose unit varied for different starches. Some typical results are shown in Table 2. In no case was the theoretical uptake reached in less than 240 hr. at 15—16°, and even after 350 hr. at this

TABLE 2. *Periodate uptake, in moles per anhydroglucose unit.*

		Time of oxidation (hours)							
Source of starch	Temp.	72	120	160	200	240	280	336	432
Barley I [a]	15—16°	—	—	0·91	0·99	1·01	1·04	—	—
Oat [b]	,,	0·77	0·80	0·86	0·96	1·02	—	1·05	—
Potato I [c]	,,	0·81	0·86	0·89	0·95	0·98	1·00	1·02	—
Potato II [d]	,,	—	—	0·88	0·94	0·96	—	1·01	1·04
Potato III [e]	,,	—	—	0·88	0·94	0·97	—	1·03	1·03
Rice [c]	,,	—	—	0·72	0·84	0·93	1·01	1·03	—
Sweet potato [c] ...	,,	—	—	—	0·97	1·01	—	—	—
Waxy maize [c]	,,	—	—	0·91	0·92	0·96	1·00	1·03	1·05
Oat [b]	20—21	—	0·97	1·02	1·03	—	1·08	—	—
Potato II [d]	,,	—	0·97	1·03	1·04	1·09	1·13	—	—
Rice [c]	,,	—	0·95	1·01	1·03	1·06	1·09	—	—
Waxy maize [c]	,,	—	0·98	1·02	—	1·03	1·05	—	—

[a] McWilliam and Percival, *J.*, 1951, 2259; [b] Anderson and Greenwood, unpublished work; [c] Samples described in *J.*, 1948, 27; [d] *Var.* "Golden Wonder"; [e] *Var.* "Redskin" ([d,e] Greenwood, unpublished work).

temperature, very little over-consumption of periodate (and hence very little over-oxidation of starch) occurs. At 20—21°, the oxidation is about 30% faster, but there is a greater tendency for over-oxidation.

It follows that the time required for the periodate-uptake of a starch to reach the theoretical value *at constant temperature* must be determined. The amount of formic acid liberated in a shorter time cannot be quantitative. Although a longer time may even be necessary to allow for hydrolysis of intermediate complexes (Hughes and Nevell, *Trans. Faraday Soc.*, 1948, **44**, 941), it has been found that in the final stages of oxidation the release of formic acid follows consumption of periodate without apparent delay (see Fig. 1). The differing oxidation times required could be explained by differences in chain length and degree of multiple branching of the amylopectin fractions, by differences in natural granular structure, or by alteration in the physical characteristics of the granules arising from differing methods of preparation. As a result, it appears unlikely that the oxidation time for any simple saccharide can be taken as a standard for starch. In particular, it is difficult to justify the procedure of Morrison, Kuyper, and Orten (*J. Amer. Chem. Soc.*, 1953, **75**, 1502), who apply a fixed correction factor, based on the percentage of the theoretical formic acid released in the same time from sucrose; results agreeing with those of Halsall, Hirst, and Jones (*J.*, 1947, 1427) have been obtained here which show that release of formic acid from sucrose is abnormally slow and non-quantitative. There is no reason to suspect that starches differ from the model of methyl β-D-maltoside (Brown, Halsall, Hirst, and Jones, *loc. cit.*) which releases formic acid quantitatively in 150 hr. at 15°, in any respect other than having variably slower rates of oxidation.

After several starches had been oxidised for the time found necessary for the theoretical uptake of periodate, they were examined by the methods described by Hirst, Jones, and Roudier (*J.*, 1948, 1779) for the isolation and hydrolysis of the oxidation products, and for the determination of glucose in the hydrolysates. Quantities of glucose between 0·5 and 1% were found. When further samples of the same starches were oxidised for at least 100 hr. longer in each case, traces of glucose, too small for estimation, were detectable only by examination of the chromatograms under ultra-violet light. Recent papers have reported the presence of 1—2% of glucose in hydrolysates after oxidation at room temperature for 240 hr. (Hirst, Jones, and Roudier, *loc. cit.*) and for 150 hr. (McWilliam and Percival, *loc. cit.*), but these authors were undecided whether this was of structural significance or due to incomplete oxidation. The latter explanation is supported by the present work, which shows that about 1% of glucose residues in the starches examined are

abnormally but not completely resistant to periodate attack. This suggests that 1 : 2- or 1 : 3-glucosidic linkages are not present, and is in agreement with the work of Gibbons and Boissonnas (*Helv. Chim. Acta*, 1950, **33**, 1477). However, the polyaldehydic oxidation products are very easily hydrolysed, giving solutions containing a brown precipitate, and Jackson and Hudson (*J. Amer. Chem. Soc.*, 1938, **60**, 989) reported that during hydrolysis some destruction of material occurred and polymer degradation was incomplete. For this reason, Abdel-Akher, Hamilton, Montgomery, and Smith (*ibid.*, 1952, **74**, 4970) hydrolysed the corresponding polyalcohols and claimed that 1 : 3-linkages exist since 1% of glucose residues was found. However, the period of oxidation used may not have been sufficient for complete oxidation.

The present work has shown that aqueous suspensions of the polyaldehydes obtained are stable in the range pH 3—6·25, but decompose readily in 0·01—0·001N-alkali, releasing acidic products even in a nitrogen atmosphere, with decomposition ceasing when the pH

FIG. 1. FIG. 2.

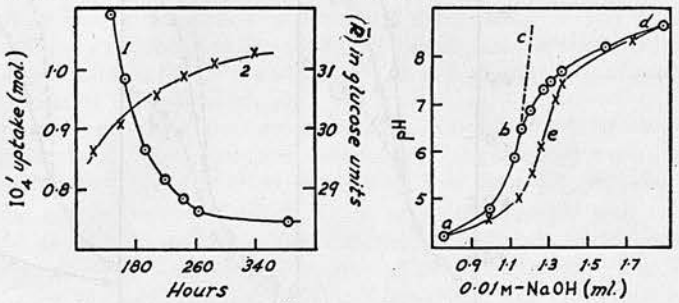

FIG. 1. *Variation of periodate-uptake and apparent value of* $\bar{R}$ *with oxidation time at* 15—16° *for oat starch.*

Curve 1. $(\bar{R})$ *(glucose units).*
Curve 2. IO_4'*-uptake (moles/anhydroglucose unit).*

FIG. 2. *Titration curves for a reaction mixture.*

Curve abc. Control $H{\cdot}CO_2H$–NaOH.
Curve abd. Reaction mixture–NaOH.
Curve dea. Back-titration curve for mixture–$H{\cdot}CO_2H$.

has dropped to 6·25. For example, immediately after potentiometric titration of a reaction mixture (Fig. 2, curve *abd*, of which part *bd* is time-dependent), the excess of alkali was back-titrated with standard formic acid (curve *dea*); 0·16 ml. of acid was found to have been liberated in the time taken (about 15 min.) to titrate from *b via d* to *e*. Liberation of acid in this manner may explain some anomalous results which have been reported involving over-production of acid during periodate oxidations in alkaline-buffered systems. Similarly, when a calculated excess of potassium hydroxide was added to a series of periodate oxidations of waxy maize starch at 15—16°, it was found that, although the rate of periodate uptake was normal (0·55 mole/162 g. of starch after 40 hr.), the acid liberated reached the theoretical value in only 38 hr. and continued to increase. The alkali-sensitivity of some aldehydes obtained by periodate oxidation has been investigated by Head (*J. Text. Inst.*, 1947, **38**, T 389), and it is considered that the acid formation reported here is due to alkaline hydrolysis of the acetal linkages accompanied by continued conversion of $-CHO$ into $-CO_2H$ by Cannizzaro-type reactions and *not* to over-oxidation by periodate, as the uptake did not exceed 1·1 moles/anhydroglucose unit (cf. Barry, *J.*, 1942, 578).

There was the possibility that esterification of $C_{(6)}$ in the starch may occur with some of the liberated formic acid (cf. Gottlieb, Caldwell, and Hixon, *J. Amer. Chem. Soc.*, 1940, **62**, 3342; Tarkov and Stamm, *J. Phys. Chem.*, 1952, **56**, 262). This would account for the release of some acid when the oxidation product is treated with alkali, and would imply that the estimation of formic acid by titration of aliquot portions centrifuged free from oxidised starch granules would not be quantitative. As esterification might have already occurred during preparation of the polyaldehydes, quantities were shaken for twelve days

at 15—16° with concentrations of formic acid ten times greater than that normally released during oxidation, so that further esterification could occur. The formic acid was recovered quantitatively: in a similar experiment with pure starches, evidence of 0·2% removal of acid was obtained. Hence no significant quantity of formic acid becomes chemically bound as ester during periodate oxidations lasting 12 days at 15—16°.

As Halsall, Hirst, Jones, and Sansome (*Biochem. J.*, 1948, **43**, 70) have obtained evidence that different samples of the same starch, derived from plants differing in botanical variety and growth-conditions, contain the same proportions of end-group, and as this result has been substantiated (see Table 3), the deviating periodate values in

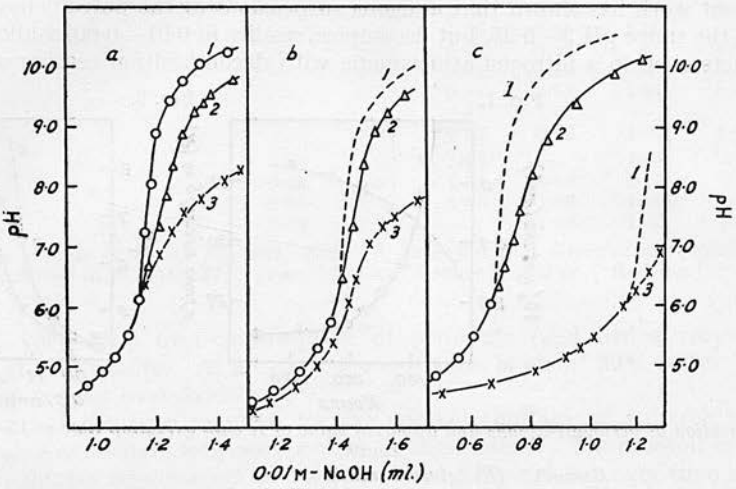

FIG. 3 *a*. *Titration curves for control solutions.*
Curve 1. $H{\cdot}CO_2H$–NaOH.
Curve 2. ($H{\cdot}CO_2H$ + KCl + $NaIO_4$ + *ethylene glycol*)–NaOH.
Curve 3. [*As* (2) + *oxidised starch*]–NaOH.

FIG. 3 *b*. *Titration curves for reaction mixtures.*
Curve 1. *Control* $H{\cdot}CO_2H$–NaOH.
Curve 2. *Centrifuged reaction mixture*–NaOH.
Curve 3. *Uncentrifuged reaction mixture*–NaOH.

FIG. 3 *c*. *Titration curves for a proteinaceous reaction mixture.*
Curve 1. *Control* $H{\cdot}CO_2H$–NaOH.
Curve 2. *Centrifuged reaction mixture*–NaOH.
Curve 3. *Uncentrifuged reaction mixture*–NaOH.

Table 1 could possibly be explained by experimental errors arising through incomplete oxidation and the use of differing procedures for the determination of formic acid. Abdel-Akher and F. Smith (*J. Amer. Chem. Soc.*, 1951, **73**, 994) prefer the iodometric method; Kerr and Cleveland (*ibid.*, 1952, **74**, 4036) titrate to pH 7·1, and papers in which titration to pH 5·5, 6·0, 8·0, and 8·2 was used have been referred to by Morrison, Kuyper, and Orten (*loc. cit.*), who showed that choice of end-point in the range pH 5·5—8·0 should have little influence on quantitative titration of *pure* formic acid. This is correct, but potentiometric titration studies carried out here, supported by iodometric determinations, have shown that quantitative titration of formic acid in the presence of all normal oxidation products is complete at pH 6·25, and that over-titration to pH 8·0 would reduce the value obtained for a true ($\bar{R}$) value of 25—30 by about 20%. In Fig. 3 *a*, which shows typical potentiometric titration results for control solutions, the curve for pure formic acid (curve 1) differs above pH 6·5 from that for the same quantity of acid in the equivalent of a centrifuged reaction mixture (curve 2). A still greater divergence occurs above pH 6·25 when 50 mg. of oxidised starch (carefully washed free from acid) are added, so giving the equivalent of an uncentrifuged reaction mixture (curve 3). Cori and Larner (*J. Biol. Chem.*, 1951, **188**,

17) noted " an apparent buffering action " during titration of periodate reaction mixtures : this is now explained by the alkaline degradation of oxidised starch. From the equivalence point of curve 1 at pH 7·1, it is seen that under these experimental conditions, titration of formic acid is complete by pH 6·5 for curve 2 and pH 6·25 for curve 3. The divergence of these curves above pH 6·25 is due to inclusion in the titration of substances other than formic acid.

Fig. 3 *b* shows the titration curves for a typical reaction mixture, curve 2 being for 10 ml. of centrifuged solution and curve 3 for 10 ml. of the heterogeneous mixture. Although both curves are superimposable with curve 1 for pure formic acid to pH 6·25, they are not themselves coincident over any range, and the end-points at pH 6·5 for (2) and pH 6·25 for 3 differ by 0·03 ml. Differences of this order (about 2% of the total titration) were consistently found. Investigation showed that the formic acid in the reaction mixture is not uniformly distributed, about 2% being loosely bound to the polyaldehydic oxidation product and removable by several washings with distilled water. Hence removal of the starch oxidation product before titration of formic acid gives values of ($\bar{R}$) which are about 0·5 glucose unit high. By careful titration of the heterogeneous reaction mixture to pH 6·25, any bound acid is included in the titre and there is no risk of including any acid arising from alkaline degradation of the polyaldehyde.

The behaviour of some protein-contaminated oat starches [obtained as intermediates in the purification of the pure starch (Anderson and Greenwood, unpublished work)] on periodate oxidation has been studied. For protein contents of less than 3%, uptake of periodate is normal, and the correct value of ($\bar{R}$) is given when the sample weight is corrected for the percentage of protein present. The differences in titration curves of centrifuged and non-centrifuged samples increase with increasing protein-content. Fig. 3 *c* shows the curves obtained for a product containing 23% of protein. The end-point for the centrifuged solution (pH 6·5; curve 2) gives a value of ($\bar{R}$) of 40, whilst that for the non-centrifuged solution (pH 6·25; curve 3) gives the correct value of ($\bar{R}$) of 28. Thus

TABLE 3. *Average values of ($\bar{R}$) found for unfractionated starches, and calculated number of glucose residues per non-reducing end-group in the amylopectin fraction.*

Source of starch	Temp.	No. of determns.	Oxidation time (hr.) *	Av. value of ($\bar{R}$) †	Amylose ‡ content (%)	Calc. chain-length for amylopectin
Arrowroot [c]	15—16°	3	284—312	27·3	20·5	21·7
Banana [c]	15—16	2	244—336	26·3	16·8	21·9
Barley I [a]	15—16	4	260—282	29·5	22·0	23·0
Barley II [f]	15—16	2	260—282			
Iris germanica [g]	15—16	2	262—308	28·0	27·0	20·4
Maize [c]	15—16	2	300—384	26·5	24·0	20·1
Oat I and II [b]	15—16	5	240—318	27·4	26·0	20·3
	20—21	1	164—240			
Parsnip [h]	15—16	2	268—360	23·0	11·1	20·4
Pearl manioc [c]	15—16	2	244—312	24·1	15·7	20·3
Potato I [c]	15—16	2	291—383	28·3	20·4	22·5
Potato II [d]	15—16	2	336—455	28·3	21·0	22·4
	20—21	1	186			
Potato III [e]	15—16	2	335—455	28·3	21·0	22·4
Rice [c]	15—16	3	286—384	27·5	18·5	22·4
	20—21	1	164—212			
Sago [c]	15—16	2	244	25·0	26·0	18·5
Sweet potato [c]	15—16	2	266—310	28·2	17·8	23·2
Tapioca [c]	15—16	4	264—300	26·2	16·7	21·8
Waxy maize [c]	15—16	4	302—400	20·0	<1	20
	20—21	1	164—284			
Wheat [c]	15—16	4	260—306	26·2	25·0	19·6

* The time necessary (found by separate expt.) for periodate uptake to reach 1·03—1·05 moles/162 g. starch : the range quoted shows the period in which no over-oxidation occurred, the formic acid released being constant within the limits corresponding to ($\bar{R}$) ± 0·5 glucose unit.

† All values obtained were within ±0·5 glucose unit from the average.

‡ Values obtained from potentiometric iodine titration curves (Anderson and Greenwood, unpublished work).

[a–e] See Table 1. [f] Aspinall, Hirst, and McArthur. [g] Aspinall and Johnstone. [h] Greenwood (all unpublished work).

the presence of protein causes further complex-formation with formic acid, and the *heterogeneous* reaction mixture must be titrated. For samples containing more than 23% of protein, periodate uptake exceeds the theoretical value and no reliable estimate of ($\bar{R}$) can be obtained from the potentiometric-titration curves.

The potentiometric-titration method has been found to be the simplest and most reproducible for determining the formic acid released on periodate oxidation. Whilst the iodometric method, which determines total acidity, gives good agreement in determinations on pure starches, the potentiometric method has the advantage of showing from the shape of the titration curve whether acids other than formic are being titrated, so indicating the presence of impurity. The steam-distillation method has been found to give less consistent results for the small amounts of formic acid normally released, and it is slower.

The average values of ($\bar{R}$) found for the starches studied are presented in Table 3, the experimental error being within $\pm 0{\cdot}5$ glucose unit. The values deduced for the average length of unit-chain in the corresponding amylopectin components are also shown.

Under the experimental conditions described, periodate oxidation is a reliable and easy routine method for determining values of ($\bar{R}$) for starches, having an accuracy and reproducibility better than that of the methylation technique.

Experimental

All starch samples were dried *in vacuo* at 80° for several hours. Reagents were of analytical grade, or were purified as described by Halsall, Hirst, and Jones (*loc. cit.*). Nitrogen and sodium hydroxide used during potentiometric titrations were free from carbon dioxide.

Periodate Oxidations.—Starch (250—400 mg.) was suspended in 0·56M-potassium chloride (60 ml.) to which was added 0·2M-sodium metaperiodate (20 ml.). Within these limits the rate of oxidation was independent of the weight of starch. Reaction flasks were shaken continuously in the dark in a constant-temperature room.

Potentiometric Titrations.—Samples (10 ml.) were withdrawn by pipette at the required times. Ethylene glycol (1 ml.) was added and the mixture shaken in the dark for at least 10 min., the time found necessary for complete reaction between the glycol and the suspension of potassium periodate. (All excess of periodate *must be destroyed* before the start of a potentiometric titration.) Nitrogen was bubbled through the mixture for 10 min. before titration with 0·01M-sodium hydroxide (semimicro-burette). The passage of nitrogen was continued throughout the titration, which was followed by means of a glass electrode and Pye mains-operated pH-meter. Blank determinations showed that generally no correction was required for the acidity of the starch samples or of other reagents.

Withdrawal of samples by pipette whilst the reaction flask was *shaken gently* introduced no error. Each of seven 10-ml. portions withdrawn consecutively from a reaction mixture gave the same titre with sodium hydroxide, and, further, the value of ($\bar{R}$) deduced was the same as that obtained from titrations of the entire contents of each of three individual reaction mixtures (10 ml. each) after the same oxidation time. This procedure did not, however, give consistent results for determinations of excess of periodate as the more rapid sedimentation of potassium metaperiodate made impossible the withdrawal of samples homogeneous with respect to this component.

It was shown that no loss of acid occurred when nitrogen was passed through 0·0015M-formic acid for 1½ hr.

Oxidation of Formic Acid by Potassium Metaperiodate.—No loss of formic acid or consumption of periodate occurred when 0·0015M-formic acid was shaken with a saturated solution of potassium metaperiodate for 28 days in the dark at 15—16°. At 20—21°, however, the concentrations of formic acid and of periodate decreased by 3% after 15 days, and by 6% after 21 days.

Distribution of Formic Acid in Reaction Mixtures.—After oxidation for 240 hr., a starch–potassium periodate reaction mixture (80 ml.) was divided into two. The first half was centrifuged, and portions (10 ml.) of the clear supernatant liquid were titrated (after destruction of excess of periodate) against 0·01024M-sodium hydroxide to pH 6·25. The average titre was 1·40 ml. From the second half, two *heterogeneous* 10-ml. portions were withdrawn, treated with glycol, and titrated to pH 6·25: the average titre was 1·43 ml. The remaining two 10-ml. portions were treated separately with glycol, then centrifuged, and the oxidised granules were

washed three times with distilled water by centrifugation. The combined supernatant liquids and washings were then titrated to pH 6·25, the average titre being 1·43 ml.

Determination of Periodate Uptake.—Residual periodate concentration was determined by Fleury and Lange's indirect method (*J. Pharm. Chim.*, 1933, **17**, 107). This method gave satisfactory results in the presence of all reactants and products when the reaction mixture, to which had been added excess of bicarbonate, arsenite, and iodide, was *shaken in the dark for* 15 *min.* before back-titration with iodine. Periodate uptake was determined by analysis of a series of individual mixtures (10 ml., containing *ca.* 50 mg. of starch). The stoppers of the conical reaction flasks were lubricated with a little silicone grease.

Periodate Uptake and Formic Acid Release from Sucrose.—Results obtained (expressed in moles/mole of sucrose) were : (*a*) periodate uptake : 2·98 (262 hr.) ; 3·12 (300 hr.) ; (*b*) formic acid release : 0·87 (262 hr.) ; 0·88 (286 hr.) ; 0·89 (352 hr.) ; 0·91 (408 hr.) ; 0·92 (420 hr.) ; 0·93 (570 hr.).

Interaction of Formic Acid with Starches and their Oxidation Products.—The following mixtures were shaken for 240 hr. in the dark at 15—16° : (*a*) control solution of formic acid (10 ml.) ; (*b*) formic acid (10 ml.) and oat starch (64·35 mg.) ; (*c*) formic acid (10 ml.) and periodate-oxidised oat starch (65·18 mg.) ; (*d*) formic acid (10 ml.) and waxy maize starch (60·08 mg.) ; and (*e*) formic acid (10 ml.) and periodate-oxidised waxy maize starch (55·60 mg.). The contents of each reaction flask were then titrated to pH 6·25 against 0·00901M-sodium hydroxide, the titres obtained being (*a*) 16·15, (*b*) 16·05, (*c*) 16·15, (*d*) 15·85, and (*e*) 16·15 ml.

Examination of Polyaldehydic Oxidation Products.—No colour reaction occurred with (*a*) iodine–potassium iodide, (*b*) dilute sulphuric acid–potassium iodide, or (*c*) sulphuric acid–diphenylamine ; Fehling's solution and Schiff's reagent were reduced. The release of acidic decomposition products from the oxidised starch in the presence of alkali was shown in the following experiments : (*a*) Oxidised oat starch (50 mg.) was added to water (5 ml.) which had been boiled, and then cooled, in the presence of nitrogen. 0·01024M-Sodium hydroxide (1 ml.) was added, and the mixture shaken for 15 hr. The pH was then 6·25, and did not decrease further during 24 hr. Further addition of 0·01024M-sodium hydroxide (1 ml.) gave an initial value of pH 10·5, which decreased overnight to pH 6·25 and did not decrease further. (*b*) Oxidised waxy maize starch (35·92 mg.) was shaken with 0·00901M-sodium hydroxide (20 ml.) for 17 hr. Titration of the excess of alkali to pH 6·25 required 1·70 ml. of 0·0147M-formic acid. Hence 232 g. of oxidised starch would liberate 1 l. of N-acid (duplicate experiment gave 273 g. as the apparent neutralisation equivalent).

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Physicochemical Studies on Starches. Part III. The Interaction of Starches and Branched α-1 : 4-Glucosans with Iodine; and a Valve Microvoltmeter for Differential Potentiometric Titrations.*

By D. M. W. ANDERSON and C. T. GREENWOOD.

[Reprint Order No. 6182.]

The uptake of iodine by 20 different starches has been studied by differential potentiometric titration, and iodine affinities for these samples are quoted. Contaminating protein alters significantly the apparent iodine affinity of starches. The iodine binding power of amylopectins, glycogens, and other branched α-1 : 4-glucosans has been studied. The differences observed, in conjunction with data for average unit-chain and external-chain lengths, suggest that variations in fine structure (*i.e.*, in degree of multiple branching) exist. In addition, details are given of a valve microvoltmeter developed to extend the scope and accuracy of differential potentiometric iodine titrations.

A QUANTITATIVE estimate of the amount of iodine bound by starch and its components can be obtained by the potentiometric-titration method introduced by Bates, French, and Rundle (*J. Amer. Chem. Soc.*, 1943, **65**, 142). Colorimetric methods developed subsequently (Hassid and McCready, *ibid.*, p. 1154; Bourne, Haworth, Macey, and Peat, *J.*, 1948, 924) are more arbitrary, although useful for comparative measurements. The optical absorption characteristics of the iodine complexes of different amyloses and amylopectins vary (cf. Baldwin, Bear, and Rundle, *J. Amer. Chem. Soc.*, 1944, **66**, 111; Kerr, Cleveland, and Katzbeck, *ibid.*, 1951, **73**, 3916), and the measurements are not absolute or capable of the same accuracy, particularly for amylopectin (cf. Higginbotham and Morrison, *Shirley Inst. Mem.*, 1948, **22**, 141).

Bates and his co-workers (*loc. cit.*) measured the potential between a bright platinum electrode in the starch–iodine–iodide solution and a standard calomel electrode, and thus were able to calculate the equilibrum concentration of free iodine in the mixture. However, the elegant *differential* method of Gilbert and Marriott (*Trans. Faraday Soc.*, 1948, **44**, 84) is much more satisfactory for accurate work at the necessary low free-iodine concentrations. In this technique, the starch–iodine–iodide solution and the blank–iodine–iodide solution are arranged as opposing half-cells connected by a salt bridge; the equilibrium free-iodine concentration in the starch solution can then be found directly, and separate titrations for reagent blanks are not required. As has recently been mentioned (Mould, *Biochem. J.*, 1954, **58**, 593), the scope of the differential technique and the accuracy and reproducibility of results obtained by its use depend on the availability of a null-potential indicator of great zero stability combined with high sensitivity. Such an electronic device, providing high sensitivity (30 mm./mv) and zero stability has already been described (Anderson and Greenwood, *Chem. and Ind.*, 1953, 476). This electrometer gives excellent results for routine analyses of unfractionated starches, but reliable readings are not possible when the free-iodine concentrations exceed 8×10^{-6}M. The logarithmic decrease in possible sensitivity with increasing free-iodine concentration in both half-cells is an inherent disadvantage of the differential-titration technique. Nevertheless, a ten-fold increase in sensitivity was sought, to permit an accurate study of the iodine-binding power of branched α-1 : 4-glucosans (*e.g.*, the amylopectin component of starch) on which relatively little work has yet been carried out. It appeared possible that such a study could give some details of fine structure.

* Part II, *J.*, 1955, 225.

Mikus, Hixon, and Rundle (*J. Amer. Chem. Soc.*, 1946, **68**, 1115) consider that the low iodine-binding power of branched α-1 : 4-glucosans is inexplicable in terms of hydrogen bonding (cf. Whistler and Hilbert, *ibid.*, 1945, **67**, 1161). They suggested that the large number of branch-points prevents helix formation and decreases the dipolar forces thought to be responsible for complex formation between iodine and the amylose component of starch. [Higginbotham (*Shirley Inst. Mem.*, 1949, **23**, 171) has suggested that, in amylopectin, adsorption of I_2 and I_3^- occurs in addition to complex formation in helices.] The amount of helix formation possible, and hence the iodine uptake, must be related to fine structure. The several model structures proposed for amylopectin [*i.e.*, the " laminated " structure (Haworth, Hirst, and Isherwood, *J.*, 1937, 577; Halsall, Hirst, and Jones, *J.*, 1949, 3200), the " herring-bone " structure (Staudinger and Eilers, *Annalen*, 1937, **527**, 195), and the " ramified " structure (Meyer and Bernfeld, *Helv. Chim. Acta*, 1940, **23**, 857)] all contain different arrangements of the same linear basic chains (Myrbäck and Sillén, *Acta Chem. Scand.*, 1949, **3**, 190), which Peat, Whelan, and Thomas (*J.*, 1952, 4546) have suggested be termed A-, B-, and C-chains. The three models differ, therefore, only in their ratio of A : B chains, *i.e.*, in the *degree of multiple branching*. Similar considerations also apply to other branched α-1 : 4-glucosans. Variations in fine structure must exist to explain the difference in limiting viscosity numbers of the two branched glucosans, amylopectin and glycogen (cf. Greenwood and Robertson, *J.*, 1954, 3769). Any method which can give further indications of differences in fine structure is important.

Experimental

Details of Valve Microvoltmeter.—The few valve millivoltmeters described in recent years (see, *e.g.*, Morton, *Trans. Faraday Soc.*, 1948, **44**, 588; Gray, *Discuss. Faraday Soc.*, 1950, **8**, 331, and personal communication; Scroggie, *Wireless World*, 1952, **14**; Furman, *Analyt. Chem.*, 1954, **26**, 84) were found either to be incapable of modification for our purpose, or, when constructed, had a zero-drift about 100 times greater than required.

Attempts to improve the sensitivity of the original circuit (Anderson and Greenwood, *loc. cit.*) by using miniature valves of high mutual conductance ($g_m = 10$) with 22-v heaters (run from the stabilized high-tension supply) were unsuccessful as the valves would not function under these " under-run " conditions. The desired standard was finally achieved by improving the stability of both high- and low-tension voltage supplies, then amplifying the output by a matched pair of valves functioning as a cathode-coupled amplifier. This design had the advantage of retaining the satisfactory high input impedance and low grid current of the original circuit, and moreover was still simple, depending on fundamental balance of valves and components rather than compensating, and therefore complicating, circuitry. The final circuit is shown in Fig. 1*b*. Very accurate readings can be made up to free-iodine concentrations of 10^{-5}M.

Fig. 1*a* shows how a harmonic-filtered constant-voltage transformer supplies the input voltage to an Ediswan stabilized power unit (Type R1095) and to an accumulator trickle-charger. The latter charges a pile of 12 × 2-v cells (arranged in series/parallel to give 4-v and 6-v outputs) at the same currents as are being taken by the two pairs of valve filaments. A highly insulated switch allows the galvanometer (sensitivity 109 mm./microamp., internal resistance 402 ohm) to be connected either between K_1 and K_2 (so giving the original circuit; sensitivity = 30 mm./mv) or between A_3 and A_4, which gives a sensitivity of 315 mm./mv. In conjunction with the low rate of zero drift attained, this permits potentials of 1 microvolt to be measured. The circuit is extremely stable towards external electrical interferences, since both pairs of valves have been selected *under actual operating conditions* as the best matched pairs obtainable from a large number. The operating conditions of both pairs differ, and are to some extent interdependent; the choice of individual valves to form pairs and of optimum values for the resistors could only be made by continued " refinements." Wire-wound resistors, matched in pairs to within 1%, are used throughout, and all grid leads are of screened-type coaxial cable. For best results, the electrometer must be adequately protected against vibration, mechanical shock, and local changes in room temperature. The value of R_6 giving the optimum ratio of sensitivity to stability is 330 ohms; decreasing this value gives increased sensitivity, but the circuit may then tend to oscillate, creating instability. Although this can be minimized by inserting " grid-stopper " resistors (47,000 ohms; 0·5 w) inside the grid top-caps of V_3 and V_4, the thermal effects associated with these resistors contributed to zero drift. The introduction of negative feed-back, either by connecting V_3A to V_1G, and V_4A to V_2G, *via* 1 megohm

resistors, or by cross-connecting V_4A to V_3Sc and V_3A to V_4Sc, reduced rather than improved stability, and it was shown that zero drift is largely due to fluctuations in the low-tension, and not in the high-tension, supply. Absolute matching of V_3 and V_4 is achieved by connecting both V_3 and V_4 grids to V_1K (with V_1G to V_1K *via* a 2-megohm resistor), and, with P_4 pre-set at its optimum value, P_3 is adjusted so that the galvanometer deflection when connected across A_3/A_4 is zero. After V_4G has been returned to V_2K, P_3 is never altered, and all zeroing adjustments are made by using the " set zero " coarse and fine controls for both A_3/A_4 and K_1/K_2 systems.

The two-way switch shown in the input circuit to V_1 (Fig. 1*b*) must be very highly insulated and must make-before-break so that the grid of V_1 is never on open circuit. A satisfactory switch was made from a thick block of paraffin wax containing pools of mercury, between which contact was made by a tilting copper-wire framework. The inter-electrode resistance is

FIG. 1(*a*) *and* (*b*).

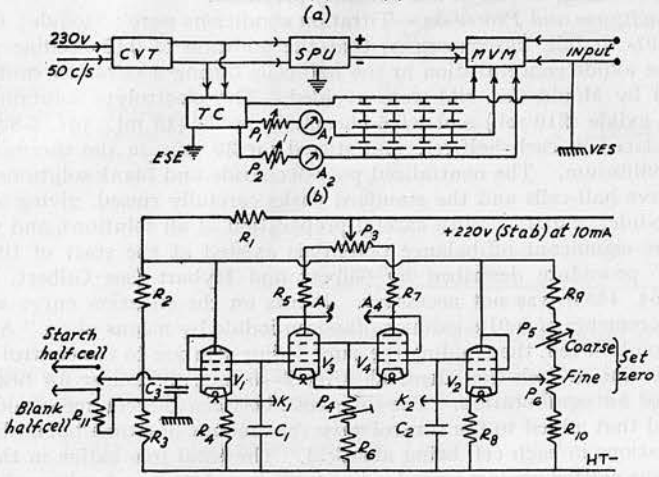

FIG. 1(*a*). *Block diagram of circuit.*

CVT, Constant voltage transformer. SPU, Stabilized power unit. MVM, Microvoltmeter. TC, Trickle-charger. ESE, Electricity supply earth. VES, Virtual earth system. A_1, Ammeter (set at 0·88 amp.). A_2, Ammeter (set at 1·25 amp.). P_1, 15 Ω/5 amp. P_2, 12 Ω/7 amp.

FIG. 1(*b*). *Microvoltmeter circuit.*

C_1, C_2, 0·01 μF, *mica*. C_3, 0·1 μF, *mica*. P_3, 500 Ω; P_4, 250 Ω; P_5, 10 *K* (*all* 5 w). P_6, 10Ω/2 w. (*All linear, wire-wound.*)

R_1, 100 *K*; R_2, 68 *K*; R_3, R_{10}, 500 Ω; R_4, R_8, 10 *K*; R_5, R_7, 22 *K*; R_6, 250 Ω; R_9, 65 *K*. (*All* 5 w, *wire-wound.*)

R_{11}, 2*M*, 1 w, *carbon*.

V_1, V_2 = VR 116; V_3, V_4 = VR 65 (SP61); V_1 *and* V_2, *heaters* 4 v *at* 0·88 *amp.*; V_A, 50 v; V_G, 0 v, V_K, 1 v; V_3 *and* V_4, *heaters* 6 v *at* 1·25 *amp.*; V_A, 110 v; V_G, 1 v; V_K, 2·4 v.

approximately 2 megohms; when the zero-reading of the electrometer is being checked, the grid of V_1 is therefore returned to earth *via* R_{11} so that the operating conditions of V_1 are changed as little as possible.

Details of Titration Cells.—The titration cells (1-l. Pyrex flasks) and salt bridge were similar to Gilbert and Marriott's (*loc. cit.*), except that stirring was automatic and continuous. Additions of iodine were made *via* additional necks in each flask. All four necks were fitted with ground-glass joints, enabling the apparatus to be completely sealed, stirring being made through Quickfit stirrer glands. [No loss of iodine occurred through volatilization in the time taken for titration (*i.e.*, 40 min.).] The electrodes consisted of platinum foil (2 × 2 cm.). By careful strain-free construction and thorough cleaning, it was possible to obtain a pair of electrodes between which no potential difference existed when placed in the same solution of electrolyte.

Reagents.—All reagents were of " AnalaR " grade, used without further purification.

Preparation of Starch Solutions.—All samples were exhaustively extracted with boiling methanol to remove traces of fat. This is essential. For example, an undefatted sample of commercial rice starch apparently contained 11·8% of amylose, but after being refluxed

with methanol (5 treatments; each of 3 hr.) a constant value of 18·5% of amylose was given (7·3% of the original weight of starch was extracted by the methanol). After removal of fat, samples were dried *in vacuo* at 80° for several hours before being weighed by means of a stoppered weighing-stick into a graduated flask. Suitable weights for titration were: starch, 10 mg.; amylopectin and glycogen, 30 mg. Dissolution was achieved by shaking the starch overnight at room temperature with 0·2M-potassium hydroxide (10 ml.) after moistening it with ethanol (2 drops). In certain cases it was necessary to heat the mixture at 95° for 3 min. before shaking. [The effect of pretreatment of whole starch with alkali was investigated as the amylose component degrades in this solvent (Bottle, Gilbert, Greenwood, and Saad, *Chem. and Ind.*, 1953, 541). Ageing at room temperature had no effect, and heating a starch in 0·2M- and 1M-potassium hydroxide for 30 min. at 95° had a negligible effect on the iodine affinity of the sample.] Immediately before addition to the titration half-cell, the alkaline polysaccharide solutions were brought to pH 5·85 by the addition of a predetermined volume of 0·4N-phosphoric acid. A blank solution containing no starch was similarly prepared.

Titration Conditions and Procedure.—Titration conditions were: [iodide], 0·01M; pH, 5·85; temp., 20°. 0·01M-Iodide was chosen so that the addition of 0·01M-iodine–potassium iodide did not alter the iodide concentration in the half-cells during a titration, and thus corrections such as applied by Mould (*loc. cit.*) were avoided. The electrolyte solution (2 l.) contained 0·1M-potassium iodide (210 ml.) and M/15-phosphate buffer (15 ml.; pH, 5·85). This solution (800 ml.) was placed in each half-cell, and stirred for 30 min. in the thermostat to allow for temperature equilibrium. The neutralized polysaccharide and blank solutions were then added to their respective half-cells and the standard flasks carefully rinsed, giving a total volume of 840 ml. (*i.e.*, [iodide], 0·01M). With careful preparation of all solutions, and with temperature equilibration, no significant off-balance potentials existed at the start of titrations, and the "depolarizing" procedure described by Gilbert and Hybart (see Gilbert, Greenwood, and Hybart, *J.*, 1954, 4454) was not necessary. Points on the titration curve were obtained by adding small increments of 0·01M-iodine–potassium iodide by means of an "Agla" micrometer syringe to the solution cell, then adding the same iodine solution to the control until the concentration of free iodine in each was identical, after 2—5 min. (or longer for branched glucosans) had been allowed for equilibration. The difference between the volume of iodine added to the solution cell and that added to the control gave the amount of iodine bound by the starch, the iodide concentrations in each cell being identical. The total free iodine in the starch solution (*i.e.*, $I_2 + I_3^-$) was plotted against mg. of iodine bound per 100 mg. of polysaccharide.

Reproducibility of Technique.—Results were independent of the time taken to complete a titration curve (provided true equilibration had been achieved at each free iodine concentration), and also of the sample weight. The reproducibility is within $\pm 2\%$ of the iodine affinity for an unfractionated starch (*i.e.*, for a starch having an iodine affinity of 5·0%, the results of 6 determinations lay between 4·9 and 5·1%).

As described by Gilbert and Hybart (*loc. cit.*), addition of excess of thiosulphate enabled the titration curve for any starch sample to be repeated. When the titration was repeated at 24- or 48-hr. intervals for 14 days, the starch solution being left in contact with iodine throughout, the observed changes in iodine affinity could be attributed to retrogradation of the amylose component. For waxy maize starch, the titration curve was unaltered after the sample had been in contact with iodine for 17 days. Similarly, for rabbit-liver glycogen, the curve was unaltered after contact with iodine for 21 and 31 days.

Results and Discussion

Fig. 2 shows some typical titration curves for starch samples obtained by plotting the amount of bound iodine against the total free-iodine concentration. Each starch was characterized by its "iodine affinity," which is a measure of the preferential uptake of iodine by the linear amylose component. At the free-iodine concentration saturating the amylose, the amount of iodine bound by the amylopectin is not negligible (see Anderson and Greenwood, *Chem. and Ind.*, 1954, 642, and below). An estimate of the amount of iodine bound by the amylose component was therefore obtained by extrapolating the linear portion of the titration curve to zero free-iodine concentration. All iodine affinities quoted here were calculated on this basis. The percentage of amylose in a starch can be calculated from this value when the corresponding value for pure amylose is known (Bates and his co-workers, *loc. cit.*). However, as previously mentioned (Greenwood and Robert-

son, *loc. cit.*), the only accurate method of doing this involves experimental determination of the maximum iodine-binding power of the *pure* amylose component of the starch under examination. Hence, when it is not desired to fractionate any starch exhaustively, it is more satisfactory to quote its iodine affinity, rather than an arbitrary percentage of amylose (cf. Schoch in Radley, " Starch and its Derivatives," Chapman and Hall, London, 1953, Vol. 1, p. 123). For comparison with other workers' results, however, percentages of amylose have been calculated, using 19·2% of bound iodine as the maximum iodine-binding power of pure amylose under our experimental conditions (Greenwood and Robertson, *loc. cit.*).

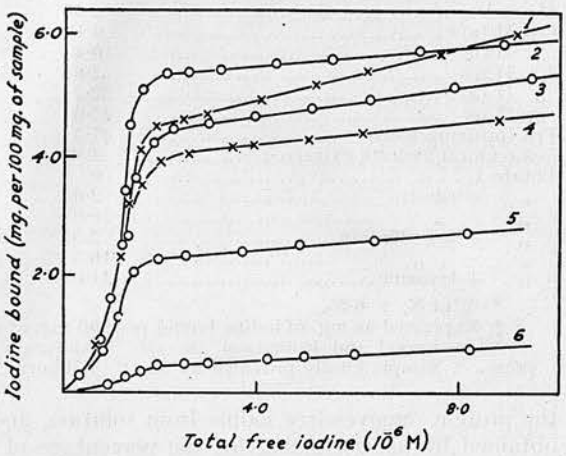

FIG. 2. *Typical iodine-titration curves for starches.*
1, *Potato* (*Golden Wonder*). 2, Iris germanica. 3, *Barley II*. 4, Hevea brasiliensis. 5, *Parsnip*. 6, *Waxy maize*.

Table 1 summarizes the results for starch samples. It is of interest that starches from different varieties of the same botanical source may show variation in the apparent percentage of amylose; this is in agreement with Doremur, Creshaw, and Thurber's results (*Cereal Chem.*, 1951, **28**, 308). The slope of the linear portion of the titration curve for all the potato starches studied was considerably greater than for other starches.

TABLE 1. *The iodine-binding power of starches.*

Source of starch *	No. of detns.	Iodine affinity †	Amylose (%) ‡	Slope of linear portion of curve §
Arrowroot	4	3·94	20·5	0·13
Banana	3	3·23	16·8	0·10
Barley I	2	4·22	22·0	0·12
Barley II	4	4·22	22·0	0·12
Hevea brasiliensis seed	3	3·86	20·0	0·10
Iris germanica	3	5·18	27·0	0·09
Maize	3	4·61	24·0	0·11
Oat I	2	5·00	26·0	0·13
Oat II	7	5·00	26·0	0·13
Parsnip	4	2·13	11·1	0·08
Pearl manioc	2	3·02	15·7	0·07
Potato I	7	3·94	20·4	0·28
Potato II	4	4·03	21·0	0·24
Potato III	2	4·03	21·0	0·23
Rice	6	3·55	18·5	0·09
Sago	4	5·00	26·0	0·08
Sweet potato	2	3·42	17·8	0·27
Tapioca	4	3·21	16·7	0·07
Waxy maize	5	0·27	1·4	0·06
Wheat	6	4·80	25·0	0·05

* Origin of samples as in Part II of this series (*loc. cit.*), except for *Hevea brasiliensis* seed (Greenwood and Robertson, *loc. cit.*).
† Expressed as mg. of iodine bound per 100 mg. of starch.
‡ Calc. as iodine affinity ÷ 19·2.
§ Expressed as % of iodine bound per total free-iodine concn. × 10^6(M) [range of total free iodine (2—10) × 10^{-6}M].

Contaminants affect the amount of iodine bound by a starch. Interference by fatty

acids is well known (cf. Mikus, Hixon, and Rundle, *loc. cit.*). In addition, protein has now been found to have considerable effect (see Table 2), and its presence causes distortion of the titration curve. For oat starch, the effect is to increase the apparent binding power;

TABLE 2. *The effect of contaminating protein on the iodine-binding power of starches.*

Starch sample	Protein (%) *	No. of detns.	Iodine affinity † Sample wt. uncorr.	Iodine affinity † Sample wt. corr. for protein
Hevea brasiliensis seed A [a]	0·31	3	—	3·86
Hevea brasiliensis seed B	2·56	2	—	3·01
Oat II (*a*) [b]	0·19	6	5·00	5·00
,, II (*b*)	0·45	3	5·44	5·48
,, II (*c*)	2·94	3	5·97	6·14
,, II (*d*)	22·7	3	5·27	6·80
,, II (*e*)	45·6	2	4·78	8·80
Pea (proteinaceous) [c]	37·5	2	—	9·20
Pea (chloral hydrate extracted)	4·93	1	—	15·1
Potato I	0	7	—	3·94
,, + edestin	2·0	1	—	3·78
,, ,,	10·0	1	—	3·05
,, + egg albumin	2·5	1	—	3·66
,, ,,	16·5	1	—	2·93
,, + tyrosine	11·0	1	—	3·45

* % of N, × 6·25.
† Expressed as mg. of iodine bound per 100 mg. of starch.
[a] Greenwood and Robertson, *loc. cit.* [b] Anderson and Greenwood, *J. Sci. Food Agric.*, in the press. [c] Sample kindly provided by Dr. E. J. Bourne; see *Nature*, 1948, **161**, 206.

the protein removes free iodine from solution, and estimates of the true affinity are best obtained by *not* correcting for the percentage of protein present. However, for rubber seed and pea starches, and for synthetic mixtures of protein with potato starch, the protein apparently suppresses starch–iodine complex formation. Interference by protein has also been found during the study of protozoal starches (unpublished observations). It is therefore essential to remove contaminating protein before titrations are attempted.

As briefly reported (Anderson and Greenwood, *loc. cit.*), the difference in iodine-binding power of normal amylopectins and glycogens is sufficiently large to characterize these two structure types. The iodine-binding power of these materials must be fundamentally related to differences in the average length of unit chain, degree of multiple branching, and external-chain length. For a group of polysaccharides having a similar degree of branching, it is also probable that variations in the amount of iodine bound are related to the length of *external chain* available for helix formation.

Titrations of different amylopectin samples have always shown evidence of preferential uptake of iodine by linear material. To compare iodine-binding powers, therefore, such preferential uptake has been corrected for by extrapolating the titration curve to zero free-iodine concentration, with this extrapolated point being taken as the origin for the iodine-binding curve. [This preferential uptake was presumably due to contaminating amylose; this is extremely difficult to remove (cf. Gilbert, Greenwood, and Hybart, *loc. cit.*), and the presence of some long branches in the amylopectin cannot be entirely excluded (cf. Swanson, *J. Biol. Chem.*, 1948, **172**, 825).] In all the glycogen samples so far examined there was no evidence of preferential uptake.

Fig. 3 and Table 3 show the results for some amylopectins and glycogens. In the range of concentrations employed, the amount of iodine bound is directly proportional, within experimental error, to the total free-iodine concentration. This would be expected if the iodine is bound as a co-linear core of iodine and tri-iodide molecules arranged end-to-end in the available helices. One iodine molecule can be accommodated in a helix of about six glucose units (Baldwin, Bear, and Rundle, *J. Amer. Chem. Soc.*, 1944, **66**, 111). Since the length of external chain available for helix formation is only 14—18 glucose units (*i.e.*, about three helices) for amylopectins, and 8—11 glucose residues (*i.e.*, 1—2 helices) for glycogen (cf. Manners, *loc. cit.*), the amount of iodine-binding possible is small.

Higginbotham (*loc. cit.*) has suggested that *adsorption* of iodine molecules (or tri-iodide

TABLE 3. *The iodine-binding power of branched α-1 : 4-glucosans.*

Sample	Linear material (%)	Slope of titration curve *	Av. length of unit chain	Length of external chain †
Barley II amylopectin	2·6	0·090	23 [a]	16 [g]
Hevea brasiliensis amylopectin	0·8	0·074	23 [a]	—
Oat I amylopectin	3·2	0·052	20·3 [a]	—
Ascaris lumbricoides glycogen	—	0·009	12 [b]	—
Rabbit-liver glycogen	—	0·006	13 [b]	8 [b]
Tetrahymena pyriformis polysaccharide	—	0·007	13 [b]	8—9 [b]
Waxy maize starch	1·4	0·060	20 [a]	15—16 [b]
Rabbit-liver glycogen	—	0·028	18 [c]	12
Wrinkled-pea amylopectin	3·4	0·485	36 [d]	—
Zea mays polysaccharide (insoluble)	0·26	0·019	12 [e]	8
,, ,, ,,	0·6	0·017	13 [f]	9
,, ,, (soluble)	0·6	0·007	11	7

* Expressed as for Table 2. † No. of glucose units removed on β-amylolysis +2·5.
[a] Part II, *loc. cit.*; [b] Manners, *Ann. Reports*, 1953, **50**, 288; [c] Haworth, Hirst, and Isherwood, *J.*, 1937, 377; [d] Potter, Silveira, McCready, and Owens, *J. Amer. Chem. Soc.*, 1953, **75**, 1335; [e] Dvonch and Whistler, *J. Biol. Chem.*, 1949, **181**, 889; [f] Dr. W. J. Whelan, personal communication; [g] Aspinall, Hirst, and McArthur, *J.*, 1955, in the press.

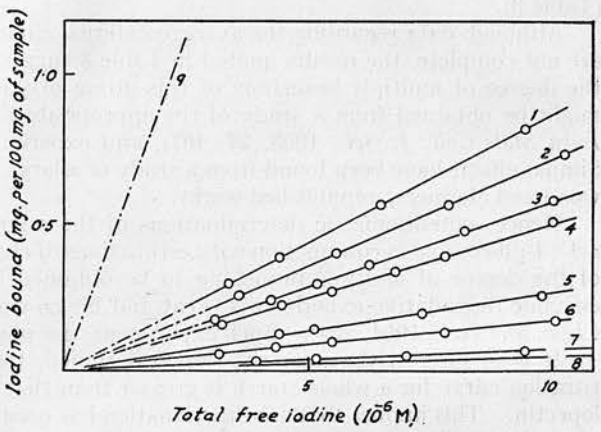

FIG. 3. *The iodine-binding power of branched α-1 : 4-glucosans.*

1, *Barley II amylopectin.* 2, Hevea brasiliensis *seed amylopectin.* 3, *Waxy maize starch.* 4, *Oat I amylopectin.* 5, *Glycogen (chain-length 18 glucose units).* 6, *Zea mays polysaccharides.* 7, Tetrahymena pyriformis *polysaccharide.* 8, *Rabbit-liver glycogen.* 9, *Wrinkled-pea amylopectin.*

ions) may also occur. However, at the low iodine concentrations used here, adsorption effects would be small, and are indeed unlikely to occur in view of the negligible effect on the titration curves of increased polysaccharide concentrations.

Fig. 3 shows that waxy maize starch behaves as a typical amylopectin, and the polysaccharide from the ciliate *Tetrahymena pyriformis* (Manners and Ryley, *Biochem. J.*, 1952, **52**, 480) as a glycogen. Molecular weight must be of minor importance. For example, the amylopectin from rubber-seed starch (D.P. 6000) binds about ten times more iodine than rabbit-liver glycogen (D.P. 30,000). (For values of D.P., see Greenwood and Robertson, *loc. cit.*)

It was possible to test the hypothesis that fine structure governed iodine-uptake when samples of " abnormal " branched α-1 : 4-glucosans became available. Samples of the water-soluble polysaccharides from sweet corn (*Zea mays*) were kindly placed at our disposal by Drs. R. L. Whistler and W. J. Whelan. The exact structural nature of these polysaccharides has been in dispute (cf. Morris and Morris, *J. Biol. Chem.*, 1939, **130**, 535; Hassid and McCready, *J. Amer. Chem Soc.*, 1941, **63**, 1132; Sumner and Summers, *Arch. Biochem.*, 1944, **4**, 7; Cameron, *Genetics*, 1947, **32**, 459; Dvonch and Whistler, *J. Biol. Chem., loc. cit.*). Whilst these materials have an average length of unit chain of 12—13 and 10—11 glucose residues respectively (*idem, loc. cit.*; Whelan, personal communication), the iodine-uptake was three to four times greater than that for a glycogen of corresponding average chain length (see Table 3), although the molecular weights were of the same order (Greenwood, unpublished work). It is, therefore, suggested that these polysaccharides have a degree of multiple branching *intermediate* between those of glycogen and amylo-

pectin, and are therefore neither in the one class nor the other. [It is of interest that Wolff, Watson, and Rist (*J. Amer. Chem. Soc.*, 1953, **75**, 4897) reached a similar conclusion from a study of the tricarbanilates of polyglucosans with different linkages.]

An abnormal rabbit-liver glycogen [shown by Haworth, Hirst, and Isherwood (*loc. cit.*) from methylation studies to have an average unit chain of 18 glucose residues] bound about five times more iodine than a normal glycogen and appeared to behave more as an amylopectin-type structure. Without additional information, it is not possible to say whether this is due to the increased average length of the external chains (*i.e.*, 12 residues) or to a variation in the degree of branching. [The abnormal character of this glycogen has been confirmed by Professor F. Smith (personal communication), who found it to possess an abnormal precipitin reaction with concanavalin-A.]

The amylopectin from wrinkled-pea starch (*var.* Perfection) has been shown to possess abnormal iodine-binding power, and an average unit chain of 36 glucose residues (Potter, Silveira, McCready, and Owens, *loc. cit.*). These authors deduced from spot tests that no amylose was present. A sample of this material, kindly provided by Dr. R. M. McCready, gave an abnormal titration curve indicating the presence of some linear material. The iodine-binding power was about six times greater than that for a normal amylopectin (Table 3).

Although data regarding the average external chain length of the amylopectins studied are not complete, the results quoted in Table 3 suggest that small variations may exist in the degree of multiple branching of this group of polysaccharides. Additional evidence might be obtained from a study of the appropriate limit dextrins (cf. Foster and Smith, *Iowa State Coll. J. Sci.*, 1953, **27**, 467), and experiments on these lines are in progress. Similar effects have been found from a study of a large number of glycogen samples (Greenwood and Manners, unpublished work).

Hence, potentiometric determinations of the amount of iodine bound by branched α-1 : 4-glucosans, in conjunction with estimations of chain length, should enable an estimate of the degree of multiple branching to be obtained if the method can be confirmed by enzymic degradative experiments (Peat and his co-workers, *loc. cit.*; Hirst and Manners, *Chem. and Ind.*, 1954, 224). Such experiments are now in progress in these laboratories.

It is of interest that, for the samples studied, the slope of the linear portion of the titration curve for a whole starch is greater than that for the corresponding isolated amylopectin. This implies that sub-fractionation has occurred, and emphasizes the importance of study of all supernatant and precipitated materials obtained during fractionation (cf. Greenwood and Robertson, *loc. cit.*).

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46. *Carbohydrates of the Roots of the Parsnip*, Pastinaca sativa.

By D. M. W. Anderson and C. T. Greenwood.

During the isolation of starch from parsnips, *Pastinaca sativa*, other polysaccharides present in the roots were separated by successive extractions with cold water, hot water, cold 5% sodium hydroxide, and hot 5% sodium hydroxide. Results of analyses of the fractions obtained are shown in the Table.

Examination of the unhydrolysed cold water extract (P2) by chromatography indicated that raffinose, sucrose, glucose, and fructose were present as free sugars, together comprising about 2% of the fraction. No polyfructosans were detected. No free sugars were present in any of the other fractions. Hydrolysis of fractions P1, P3, P4, and P5 liberated a sugar which behaved chromatographically as rhamnose. Rhamnose may, however, be readily confused with D(+)-apiose under these conditions, and the latter has been shown to exist in other members of the *Umbelliferae*.[1] Comparison with authentic D(+)-apiose (kindly placed at our disposal by Dr. D. J. Bell) established that the sugar was in fact rhamnose. The presence of this sugar in plant materials is not unusual.[2] Fraction (P6) was only 18% hydrolysed under the conditions described, but the residue on treatment with 72% sulphuric acid gave 95% of glucose together with traces of xylose, and was therefore cellulosic material, (2% of lignin remained).

Analyses of fractions isolated from parsnip roots (% of dry weight).

Fraction	Yield	Ash *	Protein †	Uronic acid anhydride	Polysaccharide ‡	Sugars obtained on hydrolysis §						
						Gal	G	M	A	X	R	F
P1 (cold water sediment)	28	0·4	37·1	8·8	53·7	1·3	46·2	0	4·9	0·8	0·6	0
P2 (,, extract)	20	17·4	24·2	7·4	51·0	11·2	24·4	0	12·8	1·6	0	1·0
P3 (hot water ,,)	13	6·6	6·2	41·5	45·7	6·0	17·6	0	17·0	1·4	3·7	0
P4 (cold NaOH ,,)	14	7·5	13·4	23·4	55·7	13·2	5·1	3·4	26·2	5·6	2·2	0
P5 (hot ,, ,,)	5	7·4	5·5	45·3	40·8	9·2	3·2	2·9	23·4	1·6	0·4	0
P6 (residue) [a]	11	3·1	0	2·2	12·7	0·8	9·1	0	0·9	2·0	0	0

* Not sulphated. † %N × 6·25.

‡ Hydrolysable non-acidic polysaccharide (calculated by difference).

§ Hydrolysis conditions: 2% H_2SO_4 in a sealed tube at 98° for 7 hr.

Chromatographic conditions: butan-1-ol–benzene–pyridine–water (5 : 1 : 3 : 3; top layer) solvent phase; 48 hr. development time; aniline oxalate spray for aldoses; urea oxalate for ketoses; estimations by Somogyi's reagent.

Gal = galactose; G = glucose; M = mannose; A = arabinose; X = xylose; R = rhamnose; F = fructose.

[a] This fraction was only 18% hydrolysed under these conditions (see text).

Traces only of alkali-soluble mannan exist, and surprisingly little xylan is present. The uronic acid content of all fractions is high, however, and the roots provide a good source of pectic material and also of araban. The starch (from P1) in unusual in that potentiometric titrations with iodine have shown it to contain only 11·1% of amylose,[3] and this material is being investigated in detail

Experimental.—Before analysis, samples were dried at 80° *in vacuo* for several hours. Solutions were concentrated under reduced pressure at 40°. Percentages of nitrogen were determined by duplicate semi-micro Kjeldahl determinations, whilst estimations of uronic acid anhydride were made by McCready's method.[4]

Extraction of roots. (*a*) Removal of oil. Parsnips were peeled, minced, and then exhaustively extracted by successive treatments with boiling methanol, methanol–benzene (2 : 1 v/v), and ether (Found, on defatted material: ash, 3·02; protein, 16·2; uronic acid anhydride, 22·4%).

(*b*) Extractions with water. Defatted roots (55 g.) were extracted with cold water (8 × 300 ml.; each 3 min.) in an " Atomix " blender. Each extract was filtered through muslin to yield on centrifugation a protein-contaminated starch (P1) and supernatant liquors. The latter when reduced in valume and freeze-dried yielded the cold-water extract (P2). The residual material was similarly extracted with water at 98°, and the polysaccharides isolated by freeze-drying to yield fraction (P3).

(*c*) Extractions with alkali. The residue was vigorously stirred with 5% sodium hydroxide (w/v : 8 × 200 ml.; each 1 hr.) at room temperature, and then at 98°. In each case the alkaline extract after centrifugation was brought to pH7 with acetic acid. The volume was then reduced, salts were removed by dialysis for 96 hr., and the fractions (P4 and P5, respectively) were isolated by freeze-drying. The final residue was washed free from alkali and dried (P6).

The overall yield was 91% of the dry weight of original material.

Analysis of fractions. Qualitative and quantitative estimations of the sugars liberated on hydrolysis were carried out as previously described,[5] with the exception that separation of arabinose from mannose in fractions P4 and P5 was achieved by use of ethyl acetate–acetic acid–water (3 : 1 : 3 v/v; non-aqueous phase) as solvent.[6]

With the exception of fructose (which is about 35% decomposed), the liberated sugars were stable under the hydrolysis conditions used (see Wylam [8]). (Since fructosans were absent, the appearance of fructose in hydrolysed P2 was only supplementary to the results obtained from a study of the unhydrolysed cold-water extract.) A weighed amount of ribose was added to each fraction (preliminary experiments having shown this sugar to be absent from all hydrolysates) to permit estimation of losses during analysis. In each case, the total weight of sugars found was between 75 and 90% of the expected quantities. Values quoted in the table have been corrected, the loss for each sugar being assumed to be proportionate to the actual weight found.

Although uronic acids were present in all hydrolysates (naphtharesorcinol test), no attempt was made to separate them chromatographically.

Confirmation of the presence of rhamnose. The acid hydrolysates of fractions P1, P3, P4, and P5 were examined chromatographically without preliminary neutralization,[7] since apiose is extremely reactive to alkali.[1] The R_F value (0·24) of the suspected rhamnose and of an authentic sample of rhamnose was the same when duplicate chromatograms were run with (*a*) butan-1-ol saturated with water, and (*b*) butan-1-ol saturated with aqueous boric acid. The R_F value of authentic D(+)-apiose was 0·26 for (*a*), but only 0·04 in (*b*). This showed that the sugar present was rhamnose, not apiose.

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[1] Bell, Isherwood, Hardwick, and Cahn, *J.*, 1954, 3702.
[2] Cf. Hirst, *J.*, 1949, 522.
[3] Anderson and Greenwood, *J.*, 1955, 3016.
[4] McCready, Swenson, and Maclay, *Analyt. Chem.*, 1946, **18**, 290.
[5] Anderson and Greenwood, *J. Sci. Food Agric.*, 1955, **6**, 587.
[6] Jermyn and Isherwood, *Biochem. J.*, 1949, **44**, 402.
[7] Gaillard, *Nature*, 1953, **171**, 1160.
[8] Wylam, *J. Sci. Food Agric.*, 1954, **4**, 167.

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