Physicochemical Studies on Starches. Part I. The Characterization of the Starch present in the Seeds of the Rubber Tree, Hevea brasiliensis.

By C. T. Greenwood and J. S. M. Robertson.

[Reprint Order No. 5429.]

The starch present in the endosperm of the seeds of the rubber tree, *Hevea brasiliensis*, is intimately associated with protein. Potentiometric iodine titrations on the purified material indicated that it contained 20% of amylose. Amylopectin (containing only 0·4—0·7% of amylose) and pure amylose fractions were obtained by fractionation of the starch with thymol followed by butan-1-ol. The average length of unit chain in the amylopectin was shown to be 23 ± 1 glucose residues by both methylation and periodate oxidation studies. The latter also indicated that the majority of interchain linkages were of the 1 : 6-type. Osmotic pressure measurements, on the acetylated derivative of the amylopectin in chloroform solution, indicated a molecular weight of about $1{\cdot}8 \times 10^6$ (*ca.* 6000 glucose residues). The acetylated amylose, prepared by a method involving the minimum of degradation, was shown similarly to possess a molecular weight of $4{\cdot}4 \times 10^5$ (*ca.* 1500 glucose residues). Measurements of the limiting viscosity number of both the free and the acetylated components were also carried out.

Starch occurs in most seed materials, and many such sources have recently been examined. In this paper we report an investigation of the starch present in the endosperm of the seeds of the rubber tree, *Hevea brasiliensis*. It was isolated in granular form by a purely mechanical process avoiding reagents likely to cause degradation. It contained a

large amount of protein (41%), which appeared to surround the granules. A modified Sevag technique (Sevag, *Biochem. Z.*, 1934, **273**, 419) reduced this in a portion of the starch to negligible amounts under the desired mild conditions. Portions of non-granular starch were obtained by further extraction of the starch–protein residue with chloral hydrate (Meyer and Bernfeld, *Helv. Chim. Acta*, 1940, **23**, 875) and then with hot water. Protein still contaminated the resultant products and had a marked effect on their interaction with iodine. [This is of general importance in the study of starch-like materials (Anderson and Greenwood, unpublished results).] The purified starch had a maximum binding power of 3·68% of iodine by weight, which under the conditions employed corresponded to an amylose-content of 20%.

A separation of the amylose and amylopectin fractions was carried out. The most satisfactory method for preparing purified amylopectin (0·4—0·7% of amylose) involved the use of thymol as a precipitant (Haworth, Peat, and Sagrott, *Nature*, 1946, **157**, 19),

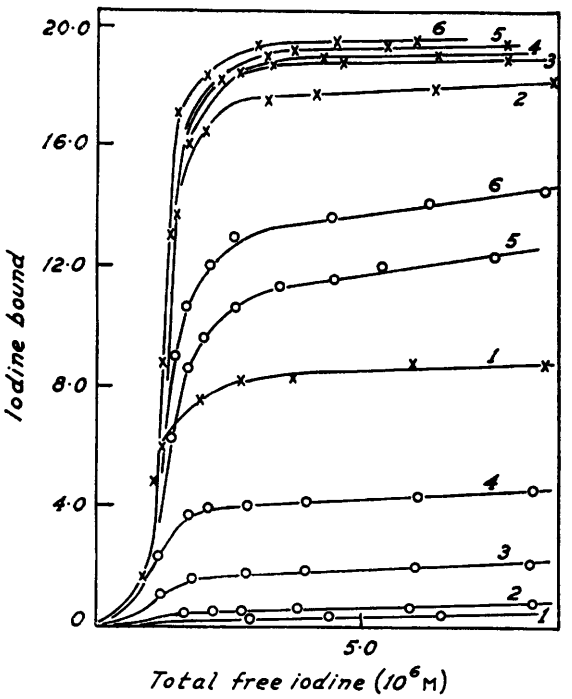

FIG. 1. *Iodine-titration curves of fractionation products.*
—— × —— *Curves for precipitates.*
—— ○ —— *Curves for material in corresponding supernatant liquor.*
1, *Thymol complex.*
2—6, *Butanol complexes* 1—5.

but yielded at the same time an impure amylose fraction (see Table 1). This fraction could then be purified *via* its butanol complex (Higginbotham and Morrison, *Shirley Inst. Mem.*, 1948, **22**, 148). To minimize inadvertent degradation of the linear component at elevated temperatures (Bottle, Gilbert, Greenwood, and Saad, *Chem. and Ind.*, 1953, 541), the fractionation was carried out as far as possible under nitrogen. Table 1 gives the results of potentiometric iodine titrations on, and measurements of the optical densities of the polysaccharide–iodine complexes (Hassid and McCready, *J. Amer. Chem. Soc.*, 1943, **65**, 1154) of, the fractionation products, in particular on material remaining in the supernatant liquors after removal of the various complexes. Amylose binding 19·2% of iodine by weight was obtained after five reprecipitations. Although it has been suggested that all amyloses possess the same maximum iodine-binding power (Higginbotham and Morrison, *loc. cit.*), recent work indicates that this may depend on the source of the amylose and the methods used for fractionation (see, *e.g.*, Schoch in Radley, " Starch and its Derivatives," Chapman & Hall, London, 1953, Vol. I, p. 123). The value will also depend on the potentiometric titration conditions ([iodide ion], [iodine], pH, and temp.). For accurate results, it appears that the only satisfactory method for estimating the percentage of

amylose in a given starch or its fractionation products involves an experimental determination of the maximum iodine-binding power of the *pure* amylose component of that starch and, if possible, the complexing reagent should be varied during this preparation. Percentages of amylose shown in Table 1 have been calculated on this basis. Fig. 1 shows the corresponding iodine titration curves. The weight of material in the supernatant liquor decreased regularly during a successful fractionation (if retrogradation occurred, the amount in the supernatant liquor increased and its properties changed). Amylopectin was obtained from the first butanol supernatant liquor, but the shape of the iodine-titration curves for subsequent supernatant liquors (Fig. 1) and the increase in the wave-length of maximum absorption of the polysaccharide–iodine complex (Table 1) suggested that after one or two re-fractionations actual molecular-weight fractionation of the amylose was occurring and short-chain amyloses were appearing in the supernatant liquors. The

TABLE 1. *Analyses of the fractionation products.*

Product		Wt. (mg.)	$\lambda_{max.}$ (Å)	Iodine affinity *	Amylose (%)
(a) *Precipitates.*					
Thymol complex		—	6000	8·3	43
Butanol complex :	1	—	—	17·4	91
	2	—	—	18·6	97
	3	—	—	18·8	98
	4	—	—	19·0	99
	5	227 †	6550	19·2 ‡	100
(b) *Material in supernatant liquors from* :					
Thymol complex		855	5500	0·14	0·7
Butanol complex :	1	230	5700	0·35	1·8
	2	30	6000	3·65	19
	3	19	—	1·70	9
	4	32	6250	10·6	55
	5	14	—	13·0	68

* Expressed as the maximum iodine binding power (%).
† A further 65 mg. were used in analyses of butanol complexes 1—4.
‡ Average of 6 determinations.

apparent molecular-weight distribution curve of the final amylose and the corresponding iodine affinity (cf. Kerr, Cleveland, and Katzbeck, *J. Amer. Chem. Soc.*, 1951, **73**, 3916) may thus depend on the method of fractionation. In this instance, 7% of the total weight of starch was present in the supernatant liquors. (This is increased to 20% if that from the first butanol complex is included.) Fractionation involving the use of thymol followed by butanol as complexing agents appears to be a general method for preparation of both pure amylose and amylopectin (Anderson and Greenwood, unpublished observations).

Methylation of the amylopectin fraction was carried out by suspending the polysaccharide in liquid ammonia and treating the suspension with sodium and methyl iodide under the conditions described by Hodge, Karjala, and Hilbert (*ibid.*, p. 3312). This method was claimed to give completely methylated starch products without severe degradation. The trimethyl derivative was treated with methanolic hydrogen chloride, and the methylated methyl glucosides obtained were hydrolysed with aqueous hydrochloric acid. Examination of the mixture of reducing sugars both on paper chromatograms and on a column of powdered cellulose showed the presence of 2 : 3 : 4 : 6-tetra-*O*-methyl-, 2 : 3 : 6-tri-*O*-methyl-, and 2 : 3-di-*O*-methyl-glucose, and a mixture of 2 : 6- and 3 : 6-di-*O*-methylglucose, which probably arose from incomplete methylation or some demethylation during hydrolysis. Only traces of mono-*O*-methylglucoses and glucose were detected. The yield of tetra-*O*-methylglucose corresponded to the presence of one non-reducing terminal group for every twenty-three glucose residues in the molecule. The value was confirmed by periodate oxidation of both the amylopectin fraction and the whole starch. It follows that the average length of unit chain in the amylopectin is 23 glucose residues, a value similar to that found in other starches (cf. Brown, Halsall, Hirst, and Jones, *J.*, 1948, 27). Further, the isolation of only 0·75% of glucose from the hydrolysis products

of the periodate-oxidized amylopectin showed that the majority (80%) of the interchain linkages were of the 1 : 6-type.

As a preliminary to determining the size of the amylopectin, the tri-*O*-acetyl derivative was prepared. Difficulty was found in carrying out this esterification with a pyridine–acetic anhydride mixture even on freeze-dried material, although various authors have claimed this to be successful (Higginbotham and Morrison, *loc. cit.*; Meyer, Bernfeld, and Hohenemser, *Helv. Chim. Acta*, 1940, **23**, 885). Potter and Hassid's method (*J. Amer. Chem. Soc.*, 1948, **70**, 3774) involving a prior dispersion in formamide was the most satisfactory. The limiting viscosity number $[\eta]$ (I.U.P.A.C. nomenclature, *J. Polymer Sci.*, 1952, **8**, 257) of the triacetate in chloroform solution was 2·33. Measurements of the osmotic pressure of the acetate in chloroform solution indicated that the number-average molecular weight ($\bar{M}_n$) was approximately 1,800,000. The π/c *versus* c curve (Fig. 2) appeared to be linear for the range of concentrations investigated (cf. Kerr, Cleveland, and Katzbeck, *J. Amer. Chem. Soc.*, 1951, **73**, 111). The molecular-weight data, in conjunction with the average length of unit chain of 23 glucose residues, showed that the amylopectin had a highly branched structure (*ca.* 260 branches per molecule). The amylopectin was also

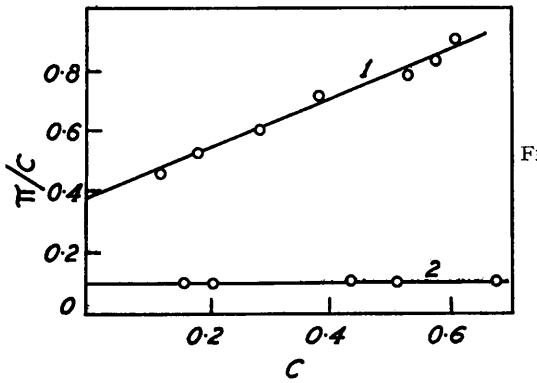

FIG. 2. *Graph of* π/c versus c *for the acetylated starch components in chloroform solution.*
1, *Amylose acetate.*
2, *Amylopectin acetate.*

characterized by measuring the limiting viscosity number of the unsubstituted component in M-potassium hydroxide (Lansky, Kooi, and Schoch, *ibid.*, 1949, **71**, 4066). For comparison, the limiting viscosity number of a sample of rabbit-liver glycogen (kindly provided by Dr. D. J. Manners) was measured in this solvent.

Methylation of the amylose was not attempted. Although other workers have isolated a small quantity of tetra-*O*-methylglucose from the hydrolysed tri-*O*-methyl derivative (see, *e.g.*, Mayer, Wertheim, and Bernfeld, *Helv. Chim. Acta*, 1940, **23**, 865; Bourne, Fantes, and Peat, *J.*, 1949, 1109; MacWilliam and Percival, *J.*, 1951, 2259), the accuracy of such an estimation is not high with limited quantities of material. In view of this, and the labile nature of the linkages in the linear molecule (Bottle, Gilbert, Greenwood, and Saad, *loc. cit.*), it appeared most satisfactory to estimate the chain length of the amylose from the molecular size of the tri-*O*-acetyl derivative, although this procedure cannot indicate whether the molecule is entirely linear (cf. Kerr and Cleveland, *J. Amer. Chem. Soc.*, 1952, **74**, 4036; Potter and Hassid, *loc. cit.*). An estimate of the degradation accompanying acetylation of amylose was obtained by measuring the limiting viscosity numbers of products isolated under different reaction conditions (see Table 3), and the method involving minimum degradation was then used for esterification. The limiting viscosity number of the product was 3·35, whilst the number-average molecular weight obtained from osmotic-pressure measurements in chloroform solution (Fig. 2) was 440,000 (*ca.* 1500 glucose residues). In M-potassium hydroxide, the limiting viscosity number for the unsubstituted amylose was 2·15.

Treatment of the amylose with crystalline sweet potato β-amylase gave 79% conversion into maltose (Dr. D. J. Manners, personal communication). The residual polysaccharide was rapidly degraded by salivary amylase and was therefore composed of α-1 : 4-linked

glucose residues. The nature of the barrier to β-amylolysis is being investigated (cf. Hopkins and Bird, *Nature*, 1953, **172**, 492; Peat and Whelan, *ibid.*, p. 494).

TABLE 2. *Molecular-weight data.*

	Amylose	Amylopectin	Glycogen
Limiting viscosity no. in M-KOH	2·15	1·02	0·10
Limiting viscosity no. of acetate	3·35	2·33	—
Molecular weight of acetate	$4{\cdot}4 \times 10^5$	$1{\cdot}8 \times 10^6$	$4{\cdot}8 \times 10^6$ *
Average degree of polymerization	1,500	6,000	30,000
Average length of unit-chain	—	23	12
Approx. number of branches per molecule	—	260	2,500

* From sedimentation measurements on unsubstituted glycogen (Greenwood and Manners, unpublished results).

The molecular-weight data are summarized in Table 2. The values are of the same order as those reported for the components from other starches (see, *e.g.*, Potter and Hassid, *loc. cit.*; Potter, Hassid, and Joslyn, *J. Amer. Chem. Soc.*, 1949, **71**, 4075; Higginbotham, *Shirley Inst. Mem.*, 1950, **24**, 221).

Although kinetic-energy corrections have been considered in obtaining the limiting viscosity numbers quoted, no correction was applied for shear effects (cf. Krigbaum and Flory, *J. Polymer Sci.*, 1953, **11**, 37). These may be considerably more important in the case of the elongated amylose component than for the branched products. Experiments are now in progress to investigate this. The large difference between the limiting viscosity numbers of the amylopectin and the glycogen samples is significant, and must be related to a fundamental difference in the molecular structure of these branched α-1 : 4-glucosans (cf. Hirst and Young, *J.*, 1939, 1471), as a result of which the amylopectin molecule must possess a more extended shape in solution than glycogen. It has been suggested recently by Hirst and Manners (*Chem. and Ind.*, 1954, 224) that this is due to the two polysaccharides' having different degrees of multiple branching.

EXPERIMENTAL

Preliminary Extraction of Oil from the Rubber Seed Endosperm.—Crushed endosperm was exhaustively extracted with benzene–methanol (1 : 1, v/v). The product was filtered, dried *in vacuo* over calcium chloride, and then ground in a hammer-mill to yield a fine buff-coloured powder (47% of the original weight of endosperm).

Isolation of Starch.—Defatted endosperm (600 g.) was extracted several times (*ca.* 24 hr. periods) by shaking it with cold water under toluene (6 l.), and the crude starch was isolated by filtering the aqueous suspension through muslin. The filtrate yielded a light brown sediment, which was washed by decantation with water and then refluxed three times with 80% aqueous methanol (30 ml./1 g.; 3 hr. periods) to ensure complete removal of fats. The yield was *ca.* 60 g. [Found : N (semi-micro Kjeldahl), 6·5%; *i.e.*, protein, 40·6%].

Removal of Protein from the Starch.—Microscopic examination indicated that contaminating protein probably formed a surface layer, as little extraneous material was visible and the granules were noticeably larger than in the purified starch (apart from the difference in size, little change in outward appearance was evident). Treatment with water at 50° did not coagulate the protein, but leached material giving a blue stain with iodine from the granule.

(a) *Treatment with toluene and butanol.* A suspension of starch in M-sodium chloride (2 g./200 ml.) was shaken with an equal volume of toluene for 6 hr. to denature the protein, and then set aside for 3 hr. to allow separation of the starch granules from the toluene–protein layer. (This procedure gave more effective separation than centrifugation at low speeds.) The brown precipitate obtained at the toluene–water interface was removed. The sedimented product consisted of two layers, the lower being mainly starch and the brown upper one mainly protein. These two layers were then each resuspended in M-sodium chloride and re-extracted with toluene as above. Combining the lower white layers, and also the upper layers, and continually re-extracting them with toluene, gave three products: (i) white starch which formed no interfacial precipitate, (ii) pale brown impure starch, and (iii) coagulated protein. Each was then suspended in M-sodium chloride (1 vol.) and shaken overnight with butanol (2·5 vols.), and

the above procedure repeated. The products, after removal of salt by repeated decantation with water, consisted of starch A (*ca.* 15 g.) (Found : N, 0·05%) and a protein-contaminated residue. Starch A was never dried and was stored under ethanol at 0°.

(b) *Extraction with chloral hydrate.* Starch in the residue could not be extracted with either (i) 1% sodium sulphite solution or (ii) 0·3% aqueous sulphur dioxide (Hilbert and McMasters, *J. Biol. Chem.*, 1946, **162**, 229) and so was extracted with chloral hydrate (Mayer and Bernfeld, *loc. cit.*), to yield starch B (5·2 g.) (Found : N, 0·41%).

(c) *Hot-water extraction.* The residue was stirred vigorously with water (2 l.) at 95° under nitrogen for 1 hr. The protein residue was removed on the centrifuge (18 g.) (Found : N, 14·4%), and the supernatant liquor yielded on freeze-drying starch C (20 g.) (Found : N, 4·9%).

Properties of Starch A.—This white powder consisted of small spherical birefringent granules (diam. 4·4 μ). In hot water it formed a clear paste which gave a deep blue colour with iodine. On hydrolysis with 2% sulphuric acid, the starch yielded 98% of the theoretical amount of glucose (quantitative chromatographic determination), and no other sugar could be detected on the paper chromatogram. This material had $[\alpha]_D^{16}$ +161° (*c*, 0·763% in N-NaOH), $[\alpha]_D$ +156° (*c*, 1·0% in 30% $HClO_4$) (Found : ash, 0·05; N, 0·05%). The optical density of the colour developed when starch (1 mg.) was stained with iodine (2 mg.) and potassium iodide (20 mg.) in distilled water (100 ml.) was measured at various wave-lengths between 4000 and 7000 Å in cells of 1 cm. length against the same iodine solution (Hassid and McCready, *loc. cit.*) by means of a Unicam spectrophotometer. For comparison with " blue values " (Bourne, Haworth, Macey, and Peat, *J.*, 1948, 924), optical densities in a 4-cm. cell at 6800 Å were calculated by assuming a linear relation between optical density and (*a*) length of cell and (*b*) concentration at this wave-length. For starch A, this density was 0·31 (the corresponding value for starch B was 0·27).

Potentiometric Iodine Titration.—The differential potentiometric titration technique of Gilbert and Marriot (*Trans. Faraday Soc.*, 1948, **44**, 84) was employed, with the electrometer described by Anderson and Greenwood (*Chem. and Ind.*, 1953, 476). The sensitivity of this electrometer enabled potentials of 0·01 mv to be measured. Titration conditions were : [iodide], 0·01M; [starch], *ca.* 1 mg./50 ml.; [amylose], *ca.* 1 mg./200 ml.; [amylopectin], *ca.* 1 mg./30 ml.; pH, 5·85; temp., 20°. The two half-cells were stirred automatically throughout the experiment. With careful mixing of solutions, a negligible potential difference was observed at the start of the experiment. Points on the titration curve were obtained by adding small increments of iodine to the solution cell, and then adding iodine to the control until the concentration of free iodine in each cell was equal after 2—3 min. had been allowed for equilibration. The total free iodine in the starch solution was plotted against mg. of iodine bound per 100 mg. of polysaccharide. Starch A showed a maximum binding power of 3·86%, which under the conditions used corresponded to an amylose content of 20% (see below). Starch B had a binding power of 3·02% (15·7% of amylose), which suggested that protein present interfered considerably with the iodine uptake of the starch.

Starch A was used in all further investigations.

Periodate Oxidation of the Starch.—A sample (0·469 g.) in water (80 ml.) was treated with 0·25M-sodium metaperiodate (40 ml.) and potassium chloride (5 g.) as described by Brown, Halsall, Hirst, and Jones (*J.*, 1948, 27). The yield of formic acid corresponded to 0·034 mole from 162 g. of the starch after 220 hr. (constant value), *i.e.*, 1 mole per 24 glucose residues in the amylopectin fraction. A duplicate experiment yielded 1 mole per 23 glucose residues.

Determination of Glucose Residues in the Starch linked through $C_{(1)}$, $C_{(4)}$, *and* $C_{(6)}$.—After completion of the above oxidation the solutions were freed from periodate and hydrolysed, and the percentage of glucose remaining was quantitatively estimated by MacWilliam and Percival's method (*J.*, 1951, 2259). The amount (1·2%) of glucose found indicated that 98·8% of the residues in the starch were linked through $C_{(1)}$–$C_{(4)}$ or $C_{(1)}$–$C_{(6)}$, and that, as the chain length was 24 glucose units, about 70% of the interchain linkages were of the 1 : 6-type.

Fractionation of the Starch.—The starch was defatted by refluxing it several times with methanol, and then portions were fractionated with thymol as a complexing agent (Haworth, Peat, and Sagrott, *loc. cit.*), followed by butanol (Higginbotham and Morrison, *loc. cit.*). Fractionation was carried out under nitrogen to minimise degradation of amylose (Bottle, Gilbert, Greenwood, and Saad, *loc. cit.*). The course of the fractionation was followed by potentiometric and optical measurements on the polysaccharide–iodine complexes of the precipitated material, and in addition on material in the supernatant liquors.

(a) *Fractionation I.* This will not be reported in detail. Starch (8 g.) yielded amylose I

[*ca.* 1·70 g.; $O.D._{4\ cm.}^{6800}$ (*i.e.* optical density as above) 1·00; $\lambda_{max.}$ 6250 Å; max. iodine-binding power, 15·4%], amylopectin I (4·3 g.; $O.D._{4\ cm.}^{6800}$ 0·08; $\lambda_{max.}$ 5250 Å; max. iodine-binding power, 0·07%; amylose, 0·4%), and supernatant material (1·60 g.). Improvements in methods were incorporated in fractionation II.

(b) *Fractionation II.* A suspension of starch (*ca.* 2 g.) in water (40 ml.) was added to vigorously stirred boiling water (260 ml.) under nitrogen, and boiling continued for 20 min. The temperature was then allowed to fall to 60°, powdered thymol (0·5 g.) was added, and the mixture stirred at 60° for 30 min., then kept at room temperature (15—17°) for 3 days to allow the thymol complex to be precipitated. The suspension was then passed three times through a Sharples supercentrifuge to remove the amylose complex as completely as possible. The clear supernatant liquor was freeze-dried, refluxed with methanol to remove thymol, redissolved, and freeze-dried again, to yield amylopectin II (1·38 g.; $O.D._{4\ cm.}^{6800}$ 0·08; max. iodine-binding power, 0·13%; amylose, 0·7%). The thymol–amylose complex was directly dispersed in boiling water (200 ml.; under nitrogen), and the amylose reprecipitated by saturating the solution with butanol of analytical quality (20 ml.; redistilled over sodium hydroxide), stirring at 95° for ½ hr., and then allowing the solution to cool slowly to room temperature (in a Dewar flask, 24 hr.). The resultant butanol complex was removed on the supercentrifuge, and then redispersed and reprecipitated with butanol. After four butanol re-fractionations the amylose obtained (II*a*) was stored as its butanol complex (*ca.* 240 mg.; $O.D._{4\ cm.}^{6800}$ 1·21; max. iodine-binding power, 18·7%). 250 mg. of material were obtained when the clear supernatant liquors from each reprecipitation were freeze-dried. Total recovery was approximately 90%, losses being purely mechanical.

To check the value of the limiting iodine-binding power of the amylose, the whole of the above fractionation procedure was repeated with 800 ml. of water to disperse the starch (*ca.* 1·5 g.), and re-fractionation from 400 ml. of water saturated with butanol. Analyses of the fractionation products are given in Table 1. Amylose II*b* was isolated by stirring the butanol complex several times with butanol and then drying it *in vacuo* at 75° (Schoch, *loc. cit.*).

The amylopectin fractions were used without further purification.

Examination of the amylopectin.

Determination of Non-reducing End-group by Periodate Oxidation.—The method described above was used. 0·048 mole of formic acid liberated from 162 g. of amylopectin corresponded to one non-reducing terminal end-group per 23 glucose residues.

Determination of Glucose Residues linked through $C_{(1)}$, $C_{(4)}$, *and* $C_{(6)}$.—0·75% of glucose isolated as above from the periodate-oxidized amylopectin indicated that 82% of the interchain linkages were of the 1 : 6-type.

Methylation of the Amylopectin.—The modification by Hodge, Karjala, and Hilbert (*loc. cit.*) of the liquid-ammonia method of Freudenberg, Boppel, and Meyer-Delius (*Naturwiss.*, 1938, **26**, 123) was used. Dry liquid ammonia, and a reaction temperature of −30°, were essential. After every four additions of reagents the product was isolated, dialysed, and freeze-dried before continuing the treatment. The sample (3 g.) was treated with seventeen additions of reagents, after which the methylated product (2·7 g.) was isolated (Found: OMe, 43·5%), having $[\alpha]_D^{16}$ +204° (*c*, 0·5 in $CHCl_3$). Further methylation was carried out by Purdie and Irvine's method (*J.*, 1903, 1021). The product (2·1 g.) was purified by precipitation from chloroform solution with light petroleum (b. p. 40—60°) (Found: OMe, 43·8. Calc. for tri-*O*-methylamylopectin: OMe, 45·6%).

Hydrolysis of Methylated Amylopectin.—(*a*) The sample (50 mg.) was hydrolysed in a sealed tube by the method of Hough, Hirst, and Jones (*J.*, 1949, 928). The hydrolysate was examined by paper-strip chromatography with butanol–ethanol–water (4 : 1 : 5, v/v) as the mobile phase, and revealed 2 : 3 : 4 : 6-tetra-*O*-methyl- (R_G 1·0 (4·8%), 2 : 3 : 6-tri-*O*-methyl- (R_G, 0·83) (85·0%), di-*O*-methyl- (R_G, 0·59, 0·51) (9·5%), and a trace of mono-*O*-methyl-glucose and glucose. This result indicated the presence of one non-reducing terminal group per 22 ± 1 glucose residues.

(*b*) The material (1·8 g.) was heated with methanolic hydrogen chloride (110 ml.; 1%) for 5 hr. at 100°, then neutralized with silver carbonate, washed, freed from excess of silver with hydrogen sulphide, and concentrated. The syrupy glycosides obtained were boiled with 2% hydrochloric acid (88 ml.) for 8 hr. The solution was then neutralized with silver carbonate as above, deionized by shaking it with resins, and concentrated to give a clear syrup

(1·75 g., 95%). This mixture of methylated glucoses was separated on a column (64 × 3 cm.) of cellulose (Hough, Jones, and Wadman, *J.*, 1949, 2511; Chanda, Hirst, Jones, and Percival, *J.*, 1950, 1289). Elution with butanol–light petroleum (b. p. 100—120°) (3 : 7, v/v) saturated with water gave fractions (1) 0·242 g., (2) 1·172 g., and (3) 0·139 g. By elution with butanol a further fraction (4) 0·058 g. was obtained, whilst elution with water gave a trace of a fraction, which from examination on a paper chromatogram, was mono-*O*-methylglucose and glucose. The last fraction was not examined further.

Examination of the fractions. Fraction (1). Examination by paper-strip chromatography indicated the presence of a single sugar (R_G, 1·0) corresponding to 2 : 3 : 4 : 6-tetra-*O*-methylglucose. Hydrolysis of a small portion (10 mg.) with 2% sulphuric acid, and re-examination revealed an additional substance (R_G, 0·84). Hypoiodite oxidation indicated that fraction (1) contained only 30% of tetra-*O*-methylglucose. Fraction (1) (210 mg.) was then rehydrolysed at 100° with 1% hydrochloric acid (20 ml.) for 5 hr., and the methylated glucoses were separated on Whatman 3MM papers with benzene–ethanol–water (149 : 45 : 15, v/v) as the mobile phase for 4 hr. Elution gave fractions (1*a*) (59 mg.) and (1*b*) (197 mg.) (94% recovery). Hypoiodite oxidation indicated that fraction (1*a*) contained 99% of tetra-*O*-methylglucose. After being recrystallized twice from light petroleum (b. p. 40—60°), the material (50 mg.) had m. p. 85—87°, which was not depressed on admixture with an authentic specimen of tetra-*O*-methyl-D-glucopyranose; $[\alpha]_D^{16}$ +85° (final) (*c*, 0·4 in H_2O) (Found : OMe, 52·0. Calc. for $C_{10}H_{20}O_6$: OMe, 50·5%). The anilide had m. p. 136—138°, alone or mixed with authentic tetra-*O*-methyl-D-glucopyranosylaniline (Found : N, 4·3; OMe, 39·5. Calc. for $C_{16}H_{25}O_5N$: N, 4·5; OMe, 39·9%). From the above results the amount of 2 : 3 : 4 : 6-tetra-*O*-methylglucose was calculated to be 72·3 mg., corresponding to one non-reducing terminal group per 23 glucose residues.

Fraction (1*b*) was shown to consist of a single substance (R_G 0·84) corresponding to 2 : 3 : 6-tri-*O*-methylglucose. It was crystallized from ether and had m. p. 115—117° (alone or mixed with an authentic specimen of 2 : 3 : 6-tri-*O*-methylglucose), $[\alpha]_D^{17}$ +70° (*c*, 0·4 in H_2O), $[\alpha]_D^{17}$ +65° (initial), −35° (final) [*c*, 0·4 in cold methanolic hydrogen chloride (2% for 10 hr.)] (Found : OMe, 41·3. Calc. for $C_9H_{18}O_6$: OMe, 41·9%).

Fraction (2). Paper chromatography indicated that this fraction consisted of one component (R_G 0·84), corresponding to 2 : 3 : 6-tri-*O*-methylglucose. It had m. p. 115—117° (alone or mixed with authentic 2 : 3 : 6-tri-*O*-methyl-D-glucose), $[\alpha]_D^{17}$ +68° (*c*, 1·0 in H_2O), $[\alpha]_D^{17}$ +67° (initial), −36° (final) [*c*, 1·0 in cold methanolic hydrogen chloride (2%, for 10 hr.)] (Found : OMe, 41·5. Calc. for $C_9H_{18}O_6$: OMe, 41·9%).

Fraction (3). Chromatographic examination of this fraction revealed the presence of two substances; one (R_G 0·84) corresponding to 2 : 3 : 6-tri-*O*-methylglucose, the other (R_G 0·59) to 2 : 3-di-*O*-methylglucose. When separated on Whatman 3MM papers and extracted with acetone, a portion gave fractions (3*a*) (18·8 mg.) and (3*b*) (75 mg.) (94% recovery). Fraction (3*a*) was shown to be 2 : 3 : 6-tri-*O*-methylglucose as above. Fraction (3*b*) was chromatographically pure and was a pale yellow syrup, which did not crystallize after two weeks in the cold. It had $[\alpha]_D^{17}$ +106° (initial), +68° (final) (*c*, 1·0 in H_2O) (Found : OMe, 29·3. Calc. for $C_8H_{16}O_6$: OMe, 29·7%). The di-*O*-methylglucose was converted into the gluconolactone and then into 2 : 3-di-*O*-methylgluconophenylhydrazide (Evans, Levi, Hawkins, and Hibbert, *Canad. J. Res.*, 1942, **20**, *B*, 175). The product had m. p. 160—162° (Evans *et al.* give 166·5—167°) (Found : OMe, 19·4; N, 9·0. Calc. for $C_{14}H_{22}O_6N_2$: OMe, 19·7; N, 8·9%).

Fraction (4) was shown chromatographically to consist of 2 : 6- or 3 : 6-di-*O*-methylglucose, or a mixture of the two (R_G 0·51). When it was rehydrolysed and re-examined no other sugar was detected. The fraction was a yellow syrup, which failed to crystallize during two weeks in the cold and had $[\alpha]_D^{17}$ +78° (initial), +60° (final) (*c*, 0·5 in H_2O), $[\alpha]_D^{17}$ +60° (initial), −10° (final) [*c*, 0·5 in methanolic hydrogen chloride (2%; 10 hr. const.)] (Found : OMe, 29·2. Calc. for $C_8H_{16}O_6$: OMe, 29·7%). The uptake of periodate (Bell's method, *J.*, 1948, 992) indicated that 64% of the fraction was 2 : 6-di-*O*-methyl-D-glucose. When excess of periodate was destroyed, the solution evaporated to dryness, and the chloroform-soluble material examined chromatographically, 34% of 3 : 6-di-*O*-methylglucose was found.

Acetylation of the Amylopectin.—Amylopectin (245 mg.) was acetylated with formamide as a dispersive agent (Potter and Hassid, *loc. cit.*). Purification by precipitation from chloroform with light petroleum (b. p. 80—100°) gave a fibrous product (Ap 1) (370 mg., 92%) (Found : Ac, 44·2. Calc. for amylopectin tri-*O*-acetate : Ac, 44·8%), [η] 2·33 (in $CHCl_3$).

Examination of the amylose.

Degradative Effect of Acetylation.—Portions of amylose 1 were acetylated by Higginbotham and Morrison's method (*loc. cit.*) at (*a*) room temperature (16°) for 74 and 186 hr. to yield acetates (A 1) and (A 2) and (*b*) 100° for 4 and 8 hr. to yield acetates (A 3) and (A 4) respectively. The products were purified as above. Acetate A 1 (54 mg.) (Found: Ac, 44·3. Calc. for amylose tri-*O*-acetate: Ac, 44·8%) had $[\eta]$ 2·63 in $CHCl_3$. Acetate A 2 (52 mg.) (Found: Ac, 43·9%) had $[\eta]$ 2·45. Acetate A 3 (47 mg.) (Found: Ac, 44·1%) had $[\eta]$ 2·55. Acetate A 4 (42 mg.) (Found: Ac, 44·0%) had $[\eta]$ 2·38. When the limiting viscosity numbers $[\eta]$ were plotted against the time of reaction (t), the rate of breakdown was so small that it was assumed legitimate to extrapolate the $[\eta]$–t curve to zero time to obtain the limiting viscosity number of the undegraded amylose. This enabled the rate of degradation to be calculated and showed that minimum degradation occurred at room temperature in the time required to give complete acetylation.

Molecular weights (M) and corresponding degrees of polymerization (D.P.) were later calculated from the equation $M = 1{\cdot}3 \times 10^5 \times [\eta]$ (where $[\eta]$ is the limiting viscosity number) obtained from the osmotic pressure measurements, as shown in Table 3.

TABLE 3. *Results of viscosity measurements.*

Product	Acetylation time (hr.)	$[\eta]$	$10^{-5}M$	Approx. D.P.	Rate of degradation *
A 1	74	2·63	3·42	1190	0·001
A 2	146	2·45	3·19	1110	0·001
A 3	4	2·55	3·32	1150	0·025
A 4	8	2·38	3·10	1080	0·023
—	0	2·80 †	3·64 †	1260 †	—

* Expressed as the number of bonds broken per initial amylose molecule per hr.
† Extrapolated values.

Acetylation of Amylose 2a.—Amylose–butanol complex (containing *ca.* 240 mg. of amylose) was acetylated as above for three days at room temperature, to yield amylose acetate (A 5) (400 mg.) (Found: OAc, 44·3%), $[\eta]$ 3·35.

Physical measurements.

Measurements of Limiting Viscosity Number.—The limiting viscosity number $[\eta]$ for the acetylated products was determined from the relation $[\eta] = \lim_{c \to 0} (\eta_{sp.}/c)$. Specific viscosities ($\eta_{sp.}$) in chloroform solution were determined with a modified Ubbelohde viscometer (Davis and Elliott, *J. Coll. Sci.*, 1949, **4**, 313) at 22·5° ± 0·001°. Solvent flow time was 192·0 sec. By measuring the viscosity of a series of liquids, the kinetic-energy correction factor B in the equation $\eta = Adt + Bd/t$ (d = density of liquid) for a capillary viscometer was shown to be zero. The viscosity of the most concentrated solution was measured first, and the dilutions were made *in situ* in the viscometer. Final concentrations (c) were determined in duplicate by the method described by Gilbert, Graff-Baker, and Greenwood (*J. Polymer Sci.*, 1951, **6**, 585) and were expressed as g. per 100 ml. of solution. Results were as tabulated.

Product	c	$\eta_{sp.}$	$\eta_{sp.}/c$	Product	c	$\eta_{sp.}$	$\eta_{sp.}/c$
A 1	0·201	0·705	3·51	A 2	0·210	0·626	2·98
	0·134	0·436	3·25		0·140	0·399	2·85
	0·100	0·307	3·06		0·105	0·286	2·73
	0	—	2·63 *		0	—	2·45 *
A 3	0·186	0·568	3·05	A 4	0·200	0·536	2·85
	0·124	0·375	3·02		0·133	0·336	2·68
	0·093	0·274	2·95		0·100	0·258	2·58
	0	—	2·55 *		0	—	2·38 *
A 5	0·575	2·376	4·13	Ap 1	0·540	1·568	2·90
	0·384	1·533	3·99		0·360	0·956	2·68
	0·287	1·102	3·84		0·270	0·675	2·50
	0·230	0·839	3·65		0·216	0·547	2·53
	0·192	0·687	3·58		0·180	0·443	2·46
	0·143	0·522	3·65		0·135	0·340	2·52
	0·115	0·463	3·53		0·108	0·263	2·40
	0	—	3·35 *		0	—	2·33 *

* Extrapolated values.

Measurements were also carried out on the unsubstituted components dissolved in M-potassium hydroxide. The viscometer used had a solvent flow time of 227 sec., and no kinetic-energy correction was necessary. Solutions were prepared by shaking polysaccharide (50—100 mg.) in M-potassium hydroxide (15 ml.) vigorously at room temperature for 30 min. Before being placed in the viscometer, each solution was filtered under gravity through sintered glass (G3, then G4). The results, which were reproducible in the short time required for dissolution and measurement, are tabulated. Measurements on a sample of rabbit-liver glycogen in the same solvent are also included.

Product	c	$\eta_{sp.}$	$\eta_{sp.}/c$	Product	c	$\eta_{sp.}$	$\eta_{sp.}/c$
Amylose	0·333	0·960	2·88	Amylopectin ...	0·474	0·766	1·62
	0·222	0·585	2·64		0·316	0·443	1·41
	0·167	0·417	2·50		0·237	0·313	1·32
	0·111	0·265	2·39		0·158	0·193	1·22
	0	—	2·15 *		0	—	1·02 *
Glycogen	0·666	0·073	0·110				
	0·333	0·035	0·105				
	0·267	0·029	0·108				
	0·167	0·017	0·102				
	0	—	0·100 *				

* Extrapolated values.

Measurement of Osmotic Pressure (with W. N. BROATCH).—Osmotic pressures (π) of amylose acetate (A5) in chloroform were determined at 22·5°. The osmometer used was a modified Fuoss–Mead instrument (*J. Phys. Chem.*, 1943, **47**, 59) designed for complete immersion in a thermostat (cf. Masson and Melville, *J. Polymer Sci.*, 1949, **4**, 323). The membrane was of gel-cellophane (No. 600) conditioned to solvent after dehydration with acetone–water mixtures. It was held taut in the osmometer by rings (cf. Gilbert, Graff-Baker, and Greenwood, *loc. cit.*). Observed osmotic pressures (measured to 0·001 cm.) were corrected for the solution density (*idem, loc. cit.*). Density determinations showed that this correction was $-0{\cdot}002\ h_s c$ (h_s = height from the mid-point of the vertical membrane to the solution meniscus; c = concentration), and the results shown in Fig. 2 include this. Concentrations were estimated in duplicate as above.

Measurements on amylopectin acetate (Ap 1) were made in the osmometer previously described (*idem, loc. cit.*), in which for greater ease in manipulation, the solvent capillary diameter was altered to 0·04 cm. The membrane was as above. The density correction was $+0{\cdot}003\ h_s c$, and is included in the results shown in Fig. 2.

In each experiment, the value of the intercept $(\pi/c)_0$ was obtained from the graph of π/c *versus* c and was found to be 0·380 and 0·097 for amylose acetate and amylopectin acetate, respectively. Substitution of these values in van't Hoff's equation gave the number-average molecular weights ($\bar{M}_n$), since $\bar{M}_n = \boldsymbol{R}T(c/\pi)_0/100d_0\boldsymbol{g}$ ($\boldsymbol{R}$ = $8{\cdot}315 \times 10^7$ ergs/degree/mole; $\boldsymbol{g}$ = gravitational constant; and d_0 = density of chloroform at 22·5°). The value of $\bar{M}_n$ was $4{\cdot}4 \times 10^5$ for A 5 and $1{\cdot}8 \times 10^6$ for Ap 1.

The authors are indebted to Professor E. L. Hirst, F.R.S., for his interest and advice, and to the Dunlop Rubber Company, Limited, for generous supplies of the rubber tree seeds. They thank Dr. D. J. Manners for carrying out the enzyme experiments, Dr. G. A. Gilbert for the loan of an osmometer, and Dr. E. J. Bourne for helpful discussion regarding methylation. Thanks are also offered to the Department of Scientific and Industrial Research for a maintenance allowance to one of them (J. S. M. R.).

DEPARTMENT OF CHEMISTRY, UNIVERSITY OF EDINBURGH. [*Received, May 31st*, 1954.]