260. *The Mechanism of Carbohydrase Action. Part I. The Preparation and Properties of Maltodextrin Substrates.*

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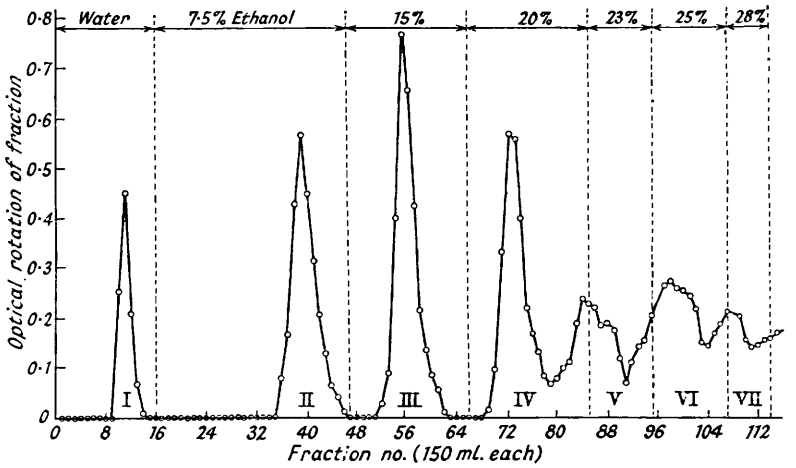
The partial acid hydrolysis of potato amylose, followed by fractionation of the products on a column of charcoal–Celite, leads to the isolation in pure form of a series of linear dextrins of which maltose is the lowest typical member. Certain physical properties of the maltodextrins are described, together with an account of their hydrolysis by β-amylase.

In a preliminary communication (*J.*, 1950, 3692) Bailey, Whelan, and Peat reported the isolation of the lowest members of the series of linear dextrins derived from amylose. The method by which this separation was effected, and some of the properties of the dextrins, are now described in detail.

The basis of the method of fractionation is that described by Whistler and Durso (*J. Amer. Chem. Soc.*, 1950, **72**, 677) who showed that a mixture of mono-, di-, and tri-sac-

Fig. 1. *Chromatographic separation of maltodextrins on charcoal.*



I, Glucose. II, Maltose. III, Maltotriose. IV, Maltotetraose.
V, Maltopentaose. VI, Maltohexaose. VII, Maltoheptaose.

charides can be separated by chromatography on a charcoal–Celite column. Monosaccharides are eluted with water, disaccharides with 5% aqueous ethanol (vol./vol.), and trisaccharides with 15% ethanol. As an example the technique was applied by these authors to the successful resolution of a mixture of glucose, sucrose, and raffinose. It seemed probable that molecules of even greater molecular weight might be resolved by the use of higher alcohol concentrations and if this were so the method would be applicable to the resolution of the dextrins derived by acid hydrolysis from amylose, the linear component of starch. Potato amylose was therefore hydrolysed with sulphuric acid until the degree of apparent conversion into glucose was 32·5%. This particular value was chosen on the basis of a calculation, made by using Kuhn's formula (*Ber.*, 1930, **63**, 1503), which indicated that dextrins containing from three to eight glucose units would be produced at this stage in optimum yield. The amylose hydrolysate was neutralised, absorbed on a charcoal–Celite column, and eluted under suction with water. The apparatus is illustrated in Fig. 5. The optical rotation of each fraction was measured.

Glucose was the only sugar to be eluted by water. Maltose needed 7·5% ethanol, maltotriose 15% ethanol, and further stepwise increments in the alcohol concentration eluted higher saccharides up to maltoheptaose (Fig. 1). Products of even greater molecular weight were later obtained but have not been examined in detail. An absolute separation of the first three sugars was achieved but some mutal contamination occurred

in the elution of maltotetraose and succeeding sugars. In these cases portions of the eluates were rejected after inspection of the elution curves (Fig. 1 and the Table). The fractionation has since been repeated with larger quantities, *viz.*, 20 g., 24 g., and 50 g., of amylose, with similar results.

With the exception of maltose, all the dextrin fractions were refractionated on an acid-washed charcoal. This treatment achieved a dual purpose; first, the purity was increased by rejection of extreme fractions, and, secondly, the ash present in dextrins eluted from untreated charcoal was largely removed. There still remained, however, an interfering substance of unknown constitution. It was reported earlier (*J.*, 1950, 3692) that hypoiodite oxidation of the dextrins gave erratic results whereas estimates of chain-length based on copper-reducing power were satisfactory. It was subsequently discovered, however, that interference with the Somogyi copper reagent (*J. Biol. Chem.*, 1945, **160**, 61) became manifest when the dextrins were hydrolysed to glucose with acid for the purpose of determining their concentration. The cause remains unexplained but it was avoided when the Somogyi reagent was replaced by that of Shaffer and Hartmann (*J. Biol. Chem.*, 1921, **45**, 377). For this reason the values of optical rotation reported in our earlier communication had to be corrected; the corrected values are given in the Table. The final products are yellowish-white amorphous solids; crystallisation has not been attempted.

Properties of the maltodextrins.

Sugar	Fractions combined *	Yield, g. †	Molecular weight ‡ M_1	M_2	Calc.	$[\alpha]_D^{15}$, in H_2O	R_F values at 15° § A	B	C
Glucose	10—13	0·81	—	—	180	52·6°	0·39	0·45	0·12
Maltose	36—44	0·91	—	—	342	136·0	0·36	0·41	0·050
Maltotriose	54—62	0·76	500	509	504	160·0	0·305	0·38	0·032
Maltotetraose ...	70—76	0·57	665	666	666	177·0	0·25	0·35	0·018
Maltopentaose...	82—90	0·45	832	843	828	180·3	0·20	0·31	0·012
Maltohexaose ...	94—100	0·33	992	1045	990	184·7	0·18	0·27	—
Maltoheptaose...	104—111	0·22	1147	1150	1152	186·4	0·135	0·23	—

* See Fig. 1. † From 11·2 g. of amylose.

‡ M_1, determined colorimetrically; M_2 determined titrimetrically.

§ Solvents: *A*, phenol–water; *B*, pyridine–fusel oil–water (1 : 1 : 1); *C*, butanol–acetic acid–water (4 : 1 : 5).

Properties of the Maltodextrins.—Although the purified dextrins still contained small amounts (<0·5%) of inorganic matter, the determination of various physical properties indicated that the fractionation procedure had effected absolute mutual separation. Thus, after refractionation, each dextrin gave only a single spot on a paper chromatogram. If it is assumed that acidic fragmentation of amylose effects no change in the configuration of the glucosidic linkages in the fragments then the identity of each dextrin can be determined from a knowledge of the number of glucose units it contains. Indication as to chain-length, available from the order in which the dextrins are eluted from the charcoal column, is supported by evidence detailed below.

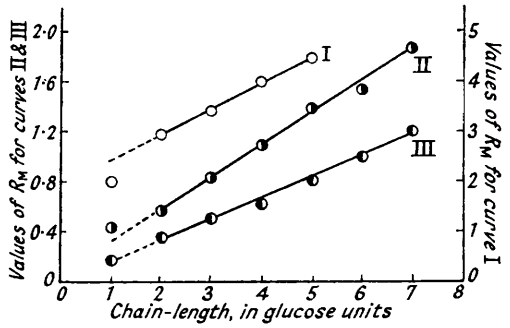
(a) *Paper chromatography.* Paper chromatograms of the maltodextrins obtained with three different solvent systems revealed the expected direct relation between R_M value and molecular weight (Fig. 2) (see Martin, *Biochem. Soc. Symposium* No. 3, 1950, 4; Bate-Smith and Westall, *Biochem. Biophys. Acta*, 1950, **4**, 428). It is noteworthy that glucose itself does not conform to the relationship exhibited by the maltodextrins. Reference will later be made to this question.

(b) *Molecular weight.* In all quantitative measurements, the concentrations of the dextrin solution were determined by acid hydrolysis and by measurement of liberated glucose (Pirt and Whelan's method, *J. Sci. Food Agric.*, 1951, **2**, 224). The determination of molecular weight by hypoiodite oxidation being unsatisfactory (see above), resort was made to the determination of the copper-reducing power of the dextrin. Two reagents were used, both developed by Somogyi; the first is a colorimetric reagent (*J. Biol. Chem.*, 1952, **195**, 19) requiring 0·2—0·5 mg. of dextrin, and the second is the titrimetric reagent mentioned earlier (*J. Biol. Chem.*, 1945, **160**, 61) which requires 2—3 mg. of dextrin. The times of heating necessary to develop full reducing power were first determined, the time

increasing with the molecular weight of the dextrin. Secondly, these maximal reducing powers were compared on a molar basis with that of maltose, and not with the reducing powers of glucose and maltose (Bailey, Whelan, and Peat, *J.*, 1950, 3692). The colorimetric method yielded, on this basis, values for the molecular weights which were in excellent agreement with the theoretical values (see Table) although agreement was not quite so close with the titrimetric reagent. This relation does not hold if glucose is used as the standard of comparison. For example, with the titrimetric reagent the ratio R_{Cu} for maltose : R_{Cu} for glucose (molar basis) is 1 : 0·927, whereas R_{Cu} for maltose : R_{Cu} for maltotriose is 1. This stresses an important fact sometimes overlooked, namely, that in such a polymeric series of sugars the lowest normal member is not the monosaccharide but the disaccharide, *i.e.*, the smallest molecule containing the glycosidic linkage. It is well known that the copper-reducing power of a sugar is not stoicheiometric with respect to the aldehydic function and is appreciably influenced by the spatial configuration of the hydroxyl groups. Glucose would not, therefore, be expected to conform to the pattern of behaviour of the maltodextrins and should not be regarded as a standard for comparison.

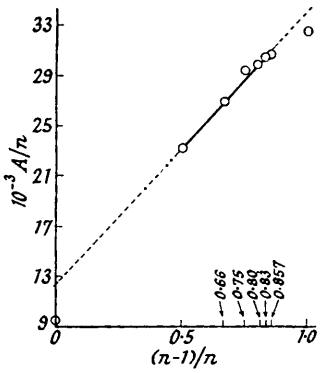
(c) *Optical rotation.* The optical rotations of the sugars (see Table) are plotted in Fig. 3, Freudenberg and Blomquist's relationship (*Ber.*, 1935, **68**, 2070) being used, in which A/n

FIG. 2.



Curve I. Butanol–acetic acid–water.
Curve II. Phenol–water.
Curve III. Pyridine–fusel oil–water.

FIG. 3. *Application of the Freudenberg–Blomquist relationship to the maltodextrins.*

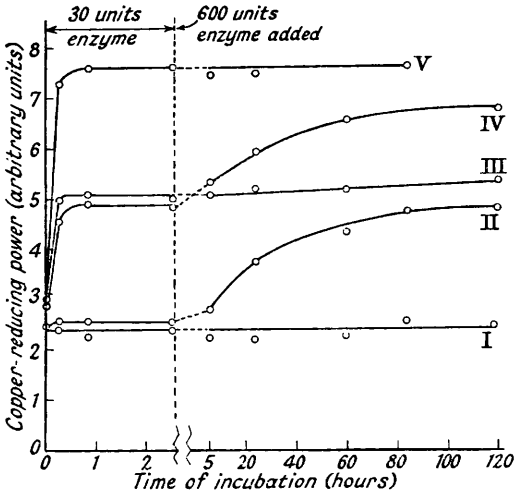


is plotted against $(n-1)/n$, where A is the molecular rotation and n the number of glucose residues per molecule. It will be seen that a linear relationship exists between the values for the maltodextrins but does not hold in the case of the monomer glucose. Whistler and Chen-Chuan Tu (*J. Amer. Chem. Soc.*, 1952, **74**, 3609) have recently reported the isolation, by similar methods, of a series of xylodextrins. It is noteworthy that when we plotted the quoted specific rotations of these xylodextrins, using the co-ordinates of Fig. 3, a linear curve was also obtained and the monomer (xylose) point was not on the curve. The optical rotation of amylose (B.V. 1·41) was found to be $[\alpha]_D$ +201° in aqueous sodium chloride. This value is incorporated in Fig. 3 using $A/n = 32{,}562$ and $(n-1)/n = 1$. It is seen that the amylose point lies below the projection of the straight line joining the maltodextrin points, and the magnitude of the deviation is so great that it must have a real significance. One explanation would be that amylose contains certain structural peculiarities absent from, or less prevalent in, the maltodextrins. It would need to be presumed that these " anomalies " are preferentially removed by the acid treatment in the preparation of the dextrins.

(d) *Action of β-amylase.* Further evidence of the essential purity of the dextrins was obtained from a study of their behaviour towards certain enzymes. The actions of phosphorylase and of α-amylase are reported in forthcoming communications (to be published; Part II, following paper); in the present study the crystalline sweet-potato β-amylase was used. The enzyme was first allowed to act in low concentration on malto-triose, -tetraose, -pentaose, and -hexaose. The results (Fig. 4) show that little hydrolysis of maltotriose took place, but there was rapid hydrolysis of the other three dextrins. The increases in reduc-

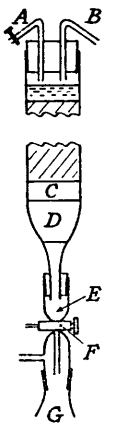
ing power corresponded with the conversion of (i) maltotetraose into two molecular proportions of maltose, (ii) maltopentaose into equimolar proportions of maltose and maltotriose, and (iii) maltohexaose into three molecular proportions of maltose. The enzyme concentration was then increased twentyfold with the result that a slow conversion of maltotriose and more extensive hydrolysis of maltopentaose ensued, occupying several days (Fig. 4). The action of the concentrated enzyme on the previously hydrolysed maltotetraose and -hexaose was slight. The subsequent slow hydrolysis came to an end when maltotriose had been converted into an equimolar mixture of maltose and glucose, and maltopentaose into two molecules of maltose and one of glucose, as determined by reducing power (Fig. 4). It was confirmed by paper chromatography that the deductions as to the nature of these ultimate products of hydrolysis were correct. The maltose fraction eluted from the charcoal column was not attacked by β-amylase. The possibility that the action of the concentrated β-amylase on maltotriose was due to an enzymic impurity cannot be discounted but the lack of action on maltose proves that maltase cannot be that impurity.

FIG. 4. *Action of β-amylase on maltodextrins.*



I, Maltose. IV, Maltopentaose.
II, Maltotriose. V, Maltohexaose.
III, Maltotetraose.

FIG. 5. *Glass column used in charcoal chromatography.*



Dr. Dexter French (personal communication to Professor Peat) reports that a sample of soya-bean β-amylase was without action on maltotriose. It seems possible that a specific maltotriase is involved. It is of interest to compare this observation with that of Roberts and Whelan (*Biochem. J.*, 1952, **51**, xviii) that salivary α-amylase does not attack maltotriose whereas the crystalline enzyme preparation obtained by Meyer *et al.* (*Helv. Chim. Acta*, 1948, **31**, 2158) hydrolysed a sample of our maltotriose (personal communication from the late Professor Kurt Meyer).

The maltodextrins have since been used as substrates in an investigation of the action patterns of α-amylase (Roberts and Whelan, *loc. cit.*) and β-amylase. Their use as primers in amylose synthesis by potato phosphorylase has definitely established that the action of this enzyme is to cause the *simultaneous* apposition of glucose residues (from glucose-1 phosphate) to all primer molecules (Bailey and Whelan, *Biochem. J.*, 1952, **51**, xxiii). In this way the dextrins have been used to prepare linear synthetic polysaccharides, which are homogeneous within narrow limits and of known chain-length. Such synthetic polysaccharides have proved of value in investigations of the mode of action of Q-enzyme (Bailey, Peat, and Whelan, *Biochem. J.*, 1952, **51**, xxiv) and of β-amylase, and also of the relationship of chain-length to iodine stain. Details will be published later. Mould and Synge (forthcoming publication) have used the same synthetic polysaccharides in investigating the fractionation of large molecules.

Experimental

Preparation and Use of Charcoal–Celite Column.—" Activated charcoal for decolorising purposes " (British Drug Houses) was used in the initial fractionations, but for refractionation B.D.H. " Acid-washed charcoal " was preferred for reasons stated earlier. Both brands of charcoal contain an iron compound, probably organic, which contaminates the trisaccharide and higher fractions, being first extracted by the 15% ethanol. Incidentally, this substance is a powerful amylase inhibitor. It may be removed by washing the packed column with an aqueous 0·2M-citrate buffer (pH 7·0) until the yellow iron–citrate complex is completely eluted; about 30 ml. of buffer per 100 g. of charcoal–Celite are required. Celite 535 (Johns-Manville Co., London) is preferred to Hyflo-Supercel since the latter contains appreciable amounts of water-soluble inorganic matter. Equal parts by weight of charcoal and Celite are intimately mixed and washed with distilled water (*ca.* 3 l./100 g. of mixture). The mixture is thoroughly dried at 80° and stored until required. A glass column is used with pads of glass wool (*D*) and cotton wool (*C*) at the bottom (Fig. 5). The mixture is added as a thick slurry in water and is allowed to settle before suction is applied; this precaution is necessary for the speedy elution of the column. The liquid level is never allowed to fall below the level of the charcoal–Celite. When operating the column the concentrated aqueous solution of the mixture to be fractionated is added and suction applied from a water-pump attached to the adapter *E*. When the solution has been absorbed a liquid head is provided by suction through tube *A*, tube *B* being connected to a reservior of eluant. Tube *A* is then closed, and the head of liquid is automatically maintained. Fractions are collected in a graduated receiver (*G*) and when a fraction is removed, air is admitted through the three-way tap (*F*) in order not to interrupt the vacuum applied to the column. Before measurement of the optical rotation, each fraction is clarified by filtration through a modified Seitz filter (H. A. Jones, Beaumaris).

Preparation of Maltodextrins.—Potato amylose (11·2 g.; air-dried; B.V., 1·45; *J.*, 1951, 801) was wetted with ethanol (40 ml.). 6N-Sodium hydroxide (40 ml.) and water (800 ml.) were added with vigorous shaking; the amylose dissolved at room temperature within a few minutes. The solution was neutralised (phenolphthalein) with 6N-sulphuric acid and a further 56 ml. of acid, and water to 1 l. were added, to give a final acid normality of 0·33. The solution was heated in a boiling-water bath, and portions (2 ml.) were removed at intervals for determination of copper-reducing power (as glucose) (Pirt and Whelan, *J. Sci. Food Agric.*, 1951, **2**, 224). After 80 and 130 minutes the %-conversions (as glucose) were 11·1 and 22·6, respectively. The solution was cooled after 165 minutes and neutralised (phenolphthalein); the conversion was then 32·5%. The original amylose solution was slightly opalescent but during the hydrolysis a precipitate suddenly formed and the supernatant became water-clear. Later examination of this precipitate (*ca.* 500 mg.) suggested that it was a partly degraded amylose. The neutralised solution was concentrated to 200 ml. under diminished pressure at 40°, and was dialysed against distilled water (4 × 1 l.). The combined dialysates were concentrated to 20 ml. and the syrup was added to a charcoal–Celite column (55 × 4·8 cm.) which was irrigated as described above. Fractions of 150 ml. were collected, and the optical rotations measured by using a 4-dm. tube. The eluant was changed as indicated in Fig. 1. At first the column delivered 300 ml. of eluate/hour but the rate gradually decreased to 100 ml./hour. The sugar-containing fractions were combined as shown in the Table and evaporated to dryness under diminished pressure. In later work it was found to be advantageous to redissolve the solid residue by warming it with ethanol and adding water until solution of the sugar was complete; a white powdery residue derived from the adsorbents remained undissolved and was removed by filtration. Refractionation of the dextrins was carried out in the same way and it was usual to reject the first and the last of the fractions which exhibited an appreciable optical rotation. The criterion of purity of the dextrins taken was presence of a single zone on the paper chromatogram. The charcoal column might possibly be used for de-ionising; in the above experiment (see Fig. 1) the sodium sulphate was largely eluted between fractions 6 and 9; glucose was first detected in fraction 10.

Examination of the Dextrins.—(a) *Paper chromatography.* This was carried out at room temperature, the three mixtures of solvents shown in the Table being used. It was usual to employ a spray of benzidine (0·5 g.), dissolved in glacial acetic acid (10 ml.), 40% trichloroacetic acid (10 ml.), and ethanol (80 ml.) to detect the sugar zones.

(b) *Optical rotation.* All quantitative measurements were made on solutions in which the concentration of dextrin was determined by acid hydrolysis to glucose (Pirt and Whelan, *loc. cit.*) and measurement of the reducing power developed. Varying amounts of this reducing

solution were used in the determination, and the relation between reducing power and volume of solution was plotted. With the Somogyi titrimetric reagent (*J. Biol. Chem.*, 1945, **160**, 61) the usual direct relationship was not found but no such interference took place with the Shaffer–Hartmann reagent (*ibid.*, 1921, **45**, 377) which was therefore used in these determinations. In later work it was found possible to remove the interfering substance by the use of the Somogyi deproteinising agents (*ibid.*, 1945, **160**, 69) before the acid hydrolysis. It should be mentioned that the unhydrolysed sugar solution did not exhibit this interference.

(c) *Molecular weight.* Two methods of determination were used. The first utilises the Somogyi copper reagent (*ibid.*, 1952, **195**, 19) and the Nelson arsenomolybdate reagent (*ibid.*, 1944, **153**, 375), as follows. The sugar solution (2 ml.; containing $\gtrless 0{\cdot}6 \times 10^{-6}$ mole) was placed in a 25-ml. flask and heated in a boiling-water bath with copper reagent (2 ml.). Maltose and maltotriose were heated for 10 minutes and higher dextrins for 25 minutes. After cooling in cold water for $2\frac{1}{2}$ minutes the arsenomolybdate reagent (2 ml.) was added with shaking; after a further minute, water was added to 25 ml. The intensity of colour developed was measured, 5—30 minutes after dilution, against that in a control from which sugar was absent, a Spekker photometer, Ilford 608 filters, and 4-cm. cells being used. With maltose, 0·1 mg. $\equiv 0{\cdot}294 \pm 0{\cdot}003$ (scale reading). The molar concentration, and hence the molecular weight, of a dextrin was obtained by comparing its reducing power with that of maltose. In the second method the Somogyi titrimetric reagent (*loc. cit.*) was used as prescribed by Pirt and Whelan (*loc. cit.*). With maltose a heating time of 20 minutes was necessary for the full development of reducing power. Maltotriose required 30 minutes' heating and 45 minutes was found to be a suitable minimum time for the higher dextrins up to maltoheptaose.

(d) *Action of* β*-amylase.* Digests were prepared containing approximately equimolar amounts ($2{\cdot}1 \times 10^{-5}$ M) of maltose, malto-triose, -tetraose, -pentaose, and -hexaose; 0·2M-acetate buffer (pH 4·8; 1·5 ml.), crystalline β-amylase (30 units; 0·5 ml.), and water to 11·5 ml. were included. Enzyme activity was determined as in *J.*, 1945, 882. The digests were incubated at 35° and portions (1 ml. each) were withdrawn at intervals up to 2·5 hours for measurement of reducing power, the Somogyi 1945 reagent being used (*loc. cit.*). At this stage a portion (7 ml.) of each digest was removed and mixed with the enzyme (600 units). Incubation was then continued under toluene with further measurements of reducing power as indicated in Fig. 4. The reducing power of the digest components other than the dextrins was determined by using a control digest from which sugar was absent.

The authors are grateful for the advice and encouragement given by Professor Stanley Peat, F.R.S. They wish to thank Professor A. K. Balls, for the gift of crystalline sweet-potato β-amylase, and the Department of Scientific and Industrial Research, for the award of maintenance grants (to J. M. B. and P. J. P. R.).

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