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Synthetic Polysaccharides. II. Fractionation of Polyglucose[1]

BY PETER T. MORA, JOHN W. WOOD, PRISCILLA MAURY AND BOBBY G. YOUNG

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Polyglucoses, prepared from glucose by acid-catalyzed polycondensation using different reaction conditions, were fractionated with alcohol in order to study the influence of these conditions on the configurational and macromolecular properties of the highly branched polymers. Three different polymerization methods gave highly polydisperse products, which were fractionated with respect to viscosity, optical rotatory power and also by degree of branching; but only one of these methods, a "solution melt" process, gave polymers which could be fractionated according to number average molecular weight, determined by reducing end-group method. Branching was studied by periodate oxidation. The polymerization of glucose presents a model system in which fractionation by branching can be studied on polycondensation products. Higher polymerization temperatures increase the degree of branching and the higher molecular weight fractions have also higher degree of branching.

The polycondensation of glucose by acid catalysis at elevated temperatures in vacuum was reported in the first communication of this series.[2] Three methods were employed: (1) melt polymerization with infrared heat ("infrared" method); (2) "solution melt" method plasticized with tetramethylene sulfone; (3) "two-stage" process in which the first stage was a melt polymerization with direct heat resulting in a brittle resin which was broken up and re-heated in powdered form in a second stage. For fractionation six polymers were selected, a pair from each method at different polymerization temperatures between 140 and 175°. Details of the polymerization and the properties of the unfractionated polyglucoses were discussed in the previous publication.[2]

There are certain broad considerations which justify detailed study of fractionation of the polyglucoses. We believe that the new synthetic polysaccharides are convenient model systems in two respects. First, the polymerization method is not restricted to glucose, and similar three dimensional (branched) polymers can be prepared from any of the various aldoses with different functionality and with different reactivities of the functional groups. These polysaccharides are easily soluble in water, and therefore accessible to polymer measurements. There are also well-known chemical methods available which can yield detailed information on the structure of polysaccharides. If we find out how to influence the polymerization conditions to bring about changes in macromolecular and structural properties, the polycondensation of sugars can be a useful model system to elicit correlations between solution properties and known configurational properties of branched polymers.

The second field where these synthetic branched polysaccharides can be of use is correlation between macromolecular properties and certain biological properties. Although polyglucoses below about 75,000 molecular weight are biologically inactive and were evaluated as plasma extenders,[3-6] higher molecular weight products have an influence on the blood cell count,[7] have serological activity in cross reaction with antisera elicited by dextrans,[8] and also have serological activity against pneumococcus polysaccharides, which was shown to increase with increasing molecular weight of the polyglucose.[9] It is possible that even higher molecular weight will introduce macromolecular properties suspected to be responsible for a whole group of biological activities, similar to that reported for bacterial endotoxins and for other polysaccharides.[10] By the substitution of the hydroxyls with charged groups, polyelectrolytes can be obtained, which can be studied with regard to their newly acquired biological properties. For example, polyglucose sulfate was found to have anticoagulant activity similar to heparin,[11] as well as other biological effects.[12] Known alterations in macromolecular structure give a set of polymers which we hope will lead to a systematic correlation of these properties with certain biological phenomena, a sort of "macromolecular pharmacology."[13] Also, it may supply models for biological processes to elicit mechanism *per analogiam* in which three-dimensional macromolecules, especially branched polysaccharides, participate, for example, the interaction between synthetic polysaccharides substituted with negative groups and positively charged polypeptides and amino acid polymers.[14]

The objective of the present work was to determine the influence of the different polymerization methods and of polymerization temperature on certain macromolecular and configurational properties of the polyglucoses, by a study of the fractions obtained with ethanol precipitation. The differences between the fractions were investigated

(1) Presented before the Divisions of Carbohydrate and Cellulose Chemistry at a Symposium on Carbohydrate Oxidation, American Chemical Society, 131st National Meeting, Miami, Fla., April, 1957.

(2) P. T. Mora and J. W. Wood, THIS JOURNAL, **80**, 685 (1958).

(3) S. M. Horvath, A. Y. Werner, D. M. MacCanon and D. W. Knapp, *J. Applied Physiol.*, **7**, 49 (1954).

(4) A. L. Gropper, L. G. Raisz and W. H. Amspacher, *Inter. Abstr. Surg., Surg. Gynecol. Obstet.*, **95**, 521 (1952).

(5) There was no immunological activity in humans upon injection; see P. Z. Allen and E. A. Kabat, *J. Exptl. Med.*, **105**, 383 (1957).

(6) For determination of polyglucose in blood and urine see D. D. Van Slyke and F. M. Sinex, *Proc. Soc. Exptl. Biol. Med.*, **79**, 163 (1952).

(7) S. M. Horvath and L. H. Hamilton, *Am. J. Physiol.*, **176**, 319 (1954).

(8) E. A. Kabat and D. Berg, *J. Immunol.*, **70**, 514 (1953).

(9) M. Heidelberger and A. C. Aisenberg, *Proc. Natl. Acad. Sci. U. S.*, **39**, 454 (1953); M. Heidelberger, H. Jahrmätker, B. Björklund and J. Adams, *J. Immunol.*, **78**, 419 (1957); *cf. ibid.*, 427.

(10) M. Landy and M. J. Shear, *J. Exptl. Med.*, **106**, 77 (1957).

(11) E. London, R. L. Theobald and G. D. Twigg, *Chemistry & Industry*, 1060 (1955); we had observed this activity independently.

(12) Unpublished experiments in collaboration with the Children's Cancer Research Foundation, Boston, and this Laboratory.

(13) *Cf.* P. T. Mora and M. J. Shear, Abstracts of papers presented at the 132nd National Meeting of the American Chemical Society, New York, N. Y., September, 1957, 16-T.

(14) P. T. Mora and B. G. Young, in preparation.

TABLE I

NUMBER AVERAGE MOLECULAR WEIGHT ($\bar{M}_n$) OF POLYGLUCOSE FRACTIONS (CALCULATED FROM REDUCING END GROUP)

No.	Fraction EtOH limits, Vol. %	"Infrared" 140°	"Infrared" 150°	"Two-stage" second stage 150°	"Two-stage" second stage 170°	EtOH limits Vol. %	"Solution melt" 155°	"Solution melt" 175°
..	Unfractionated	7,300	15,150	8,100	13,200		10,850	28,800
0	0-54	....	20,700	....	....			
I	54(0)-58	(15,000)	22,800	(17,500)	(18,200)	0-60	....	33,400
II	58-62	15,050	21,800	16,600	16,500	60-65	16,200	32,800
III	62-66	14,050	22,800	15,400	18,200	65-70	14,700	30,600
IV	66-70	13,500	22,200	18,500	19,300	70-75	10,950	24,500
V	70-75	16,800	21,200	15,100	19,700	75-80	8,250	19,700
VI	75-80 + ether	9,350	20,600	10,700	13,500	80-85 + ether	7,850	13,800
(VII)	Supernatant	Not recovered		Not recovered		Supernatant	Not recovered	

by reducing end-group determination, by viscosity and optical rotation measurements, and by periodate oxidation which reveals differences in branching. There was indication that fractionation occurs not only by molecular weight but also by degree of branching[15] and the data we report here support this conclusion.

Experimental and Results

Fractionation.—Polymerization was carried out as reported in the first communication of this series.[2] Table I and the first column of Table II list the method and average polymerization temperature of the six polymers chosen for this work. A 2% solution of polymer (80–150 g.) was made using 0.1 N sodium bicarbonate which served to neutralize the phosphorous acid catalyst. In addition sodium chloride was added (0.5% w./v.) to aid in the fractionation. The "infrared" 150° polymer had 43% insoluble gel, the "two-stage" (II) 170° product 3.4%, but the rest of the solutions were clear. The solutions were centrifuged to separate the gel.

TABLE II

NUMBER AVERAGE MOLECULAR WEIGHT ($\bar{M}_n$),[a] INTRINSIC VISCOSITY AND OPTICAL ROTATION OF POLYGLUCOSE FRACTIONS

Polymerization method	Temp., °C.	Fraction	$\bar{M}_n$	$[\eta]$, dl./g.	$[\alpha]^{25}D$ in 1 N HCl
"Infrared"	140	I	15,000	0.06	+71.1°
"Infrared"	140	V	16,800	.04	73.3
"Infrared"	150	I	22,750	.08	79.0
"Infrared"	150	V	21,250	.05	82.0
"Two-stage" (II)	150	I	17,500	.07	63.6
"Two-stage" (II)	150	V	15,100	.04	65.7
"Two-stage" (II)	170	I	18,200	.09	67.8
"Two-stage" (II)	170	V	20,000	.04	68.6
"Solution melt"	155	II	16,200	.03	71.2
"Solution melt"	155	V	8,250	.01	69.7
"Solution melt"	175	II	32,800	.03	85.2
"Solution melt"	175	V	20,000	.01	82.8

[a] $\bar{M}_n$ calculated from reducing end group.

To the clear solutions at 40° absolute ethanol was added, in a thin stream from a capillary, with continuous stirring until a sirupy emulsion caused slight turbidity. The turbidity increased when the emulsion was allowed to cool gradually (16 hr.) to room temperature (21°). Then the emulsion was placed in a 14.5° water-bath and left to settle for 72 hr., during which time a sirupy sediment separated. The clear supernatant was decanted carefully and further increments of alcohol were added to separate further fractions similarly. The increments of ethanol necessary to obtain five or six fractions with nearly equal yields were estimated from preliminary precipitation experiments.[2] The sirups were dissolved in water and were freeze-dried to a voluminous white powder. The yields of the different fractions are plotted against the alcohol concentration in Fig. 1. Solid lines correspond to yields of lower temperature polymers, broken lines to the higher temperature products. Roman numerals designate the fractions and Table I gives the ethyl alcohol limits (vol. %). Similar alcohol limits were employed in fractionating the "infrared" and "two-stage" products, except that an "O" fraction was obtained from the 150° "infrared" polymer. The "solution melt" products were more soluble and required somewhat greater increments of alcohol to bring out fractions with comparable yield.

Number Average Molecular Weight ($\bar{M}_n$) by Reducing End-group Determination.—For this and for the following measurements the freeze-dried fractions were dried further to constant weight at 60° in vacuum. A modified Scales reducing method was employed, as reported previously.[2] Results are given in Table I and in Fig. 2. Calculation of $\bar{M}_n$ was on the basis of standardization with gentiobiose, assuming that equimolar quantities of polyglucose and gentiobiose have approximately the same reducing power.

Intrinsic Viscosity.—Viscosities were measured in modified Ubbelohde viscometers at 26.5°. Kinetic energy corrections were not applied. Solutions were filtered through sintered glass filters of fine or medium porosity. Results are reported in Table II.

Optical Rotation, Hydrolysis, Reducing Power.—Samples were dissolved in 1 N HCl and the rotation measured within 15 min. There was no observable change in rotation for 1 hr. and the difference from that in aqueous solution is only very slight. Results are reported in Table II. The 1 N HCl solutions were heated in a boiling water-bath for three hours. Aliquots were withdrawn at the times indicated in Fig. 3, frozen immediately in Dry Ice-Cellosolve-bath and thawed only shortly before measurement. For the determination of reducing power as reported above, the aliquots were pipetted into 1 N sodium bicarbonate. Fig. 4 is a representative hydrolysis curve.

Polyglucose Acetate.—One gram each of fraction I of "two-stage" 150° and 170° polymerization products were treated with 9.5 ml. of pyridine and, after swelling, 6.5 ml. of acetic anhydride was added. The mixture was placed in a boiling water-bath for 2.5 hr., another 5 ml. of acetic anhydride was added and the mixture heated for 12 more hr. Some insoluble powder still remained and after 33.5 hr. all went into solution. Aliquots of the supernatant after 12 hr. heating, and the final 33.5 hr. solutions were precipitated by pouring the solutions on crushed ice. The white powdery precipitate was dissolved in glacial acetic acid and reprecipitated, washed with water five times and finally dried at 60° in vacuum over phosphorus pentoxide. Acetyl contents of all the four products were between 41.7–42.0% (theoretical 44.8), m.p. 146–155° dec.

Periodate Oxidation.—The methods employed were similar to the arsenite titration of Jeanes, *et al.*, used on dextrans[16,17] and closely followed a procedure employed at the National Bureau of Standards.[18]

Approximately 1.1 g. of polyglucose was dissolved in water, and 50 ml. of 0.0374 N $NaIO_4$ was added and the solution diluted to 100 ml. (In all the solutions CO_2-free water was used.) The solutions were placed in the dark either at room temperature (21°) or at 4° as indicated, and aliquots were withdrawn for titration at the reported intervals in Tables III and IV and Figs. 5 and 6.

(15) P. T. Mora, *J. Polymer. Sci.*, **23**, 345 (1957).

(16) J. C. Rankin and A. Jeanes, THIS JOURNAL, **76**, 4435 (1954).

(17) A. Jeanes and C. A. Wilham, *ibid.*, **72**, 2655 (1950).

(18) We are grateful to Dr. H. S. Isbell for discussions and for a manuscript entitled: "Methods of Analysis of Clinical Dextrans."

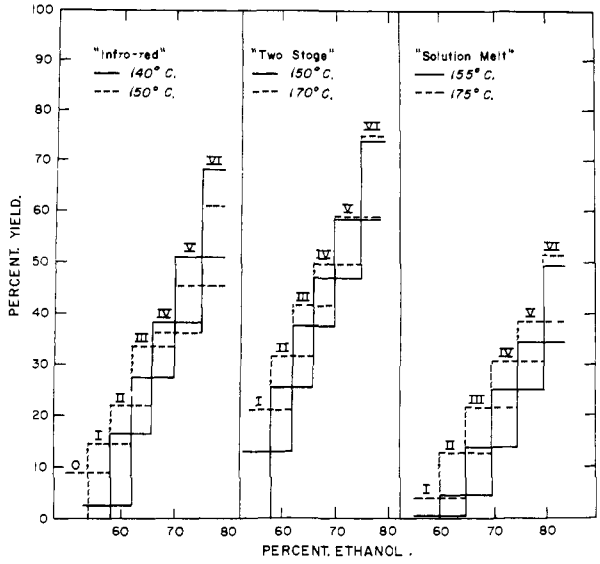

Fig. 1.—Fractionation of polyglucoses.

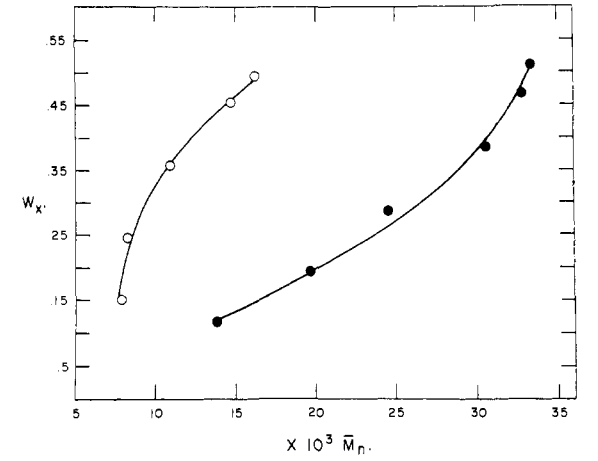

Fig. 2.—Integral distribution curves of polyglucose preparations by "solution melt" method: left, 155°; right, 175° polymer; $\bar{M}_n$ by reducing end group.

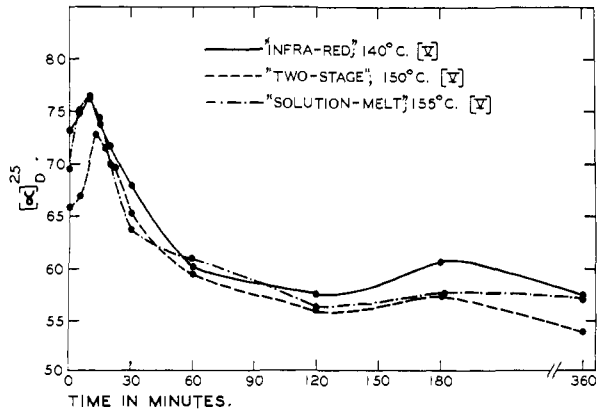

Fig. 3.—Optical rotation of polyglucose fractions during hydrolysis with 1 *N* HCl, 100°.

Formic Acid Produced.—Ten ml. of the oxidation mixture was pipetted into a 125-ml. erlenmeyer flask previously flushed with argon. Ethylene glycol (0.5 ml.) was added and the flask was kept stoppered for 1 hr. in the dark. To the solution ten drops of phenolphthalein indicator was

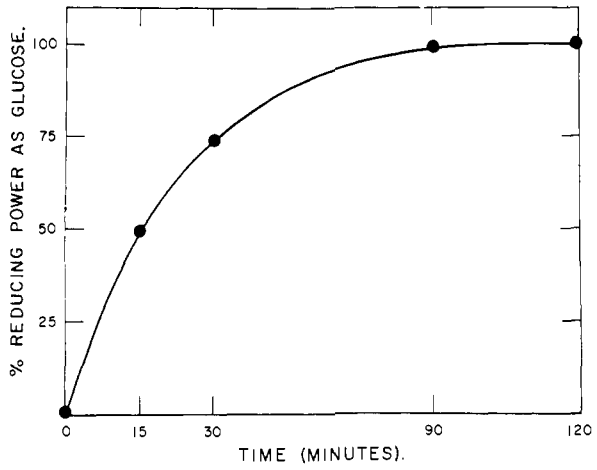

Fig. 4.—Hydrolysis of polyglucose: "solution melt" product, 175°, fraction II, 1 *N* HCl, 100°.

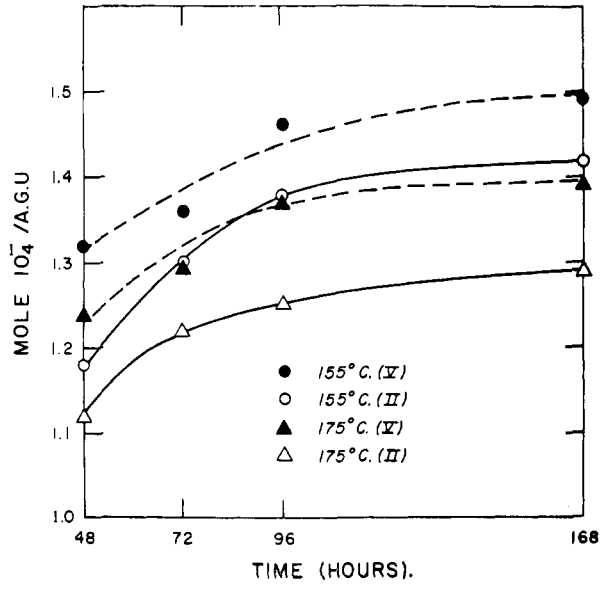

Fig. 5.—Periodate oxidation of polyglucose fractions prepared by "solution melt" method at different temperatures; oxidation at 4°.

added and titration in argon atmosphere with 0.01 *N* $Ba(OH)_2$ was carried out through a small opening to the first detectable pale pink color, compared with a buffered solution at *p*H 8.6. Similar titration was performed on a blank solution containing only $NaIO_4$.

TABLE III

PERIODATE OXIDATION OF "INFRARED" POLYGLUCOSE FRACTIONS

0.0374 *N* $NaIO_4$ room temp., 144 hr.; figures marked by asterisk, 192 hr.

		Mole IO_4^- / A.G.U.		Mole HCOOH / A.G.U.
140°	I	1.35		0.608
140°	V	1.56		.629
150°	0	1.31	1.28*	.545
150°	V	1.13	1.30*	.572

The oxidation product of the reducing end group can be taken as a formate ester (if it is not linked through the second hydroxyl) and this ester can be saponified with 0.01 *N* alkali at room temperature in 30 min. with the production of two additional moles of formic acid when the linkage is at the

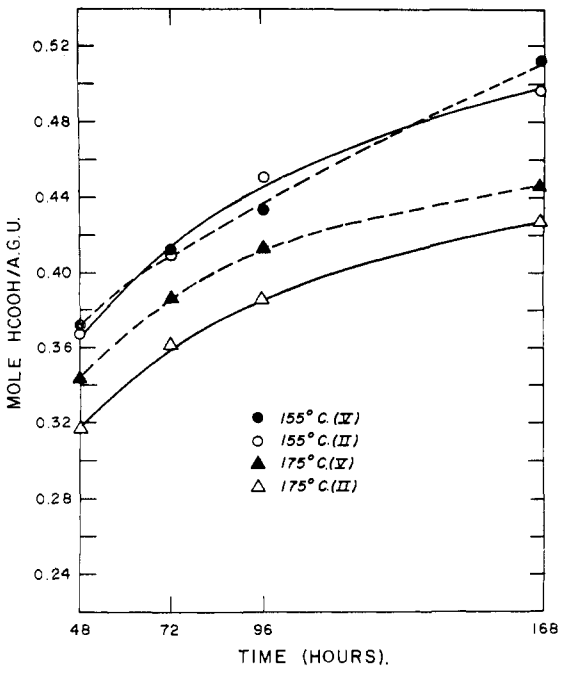

Fig. 6.—Formic acid produced during periodate oxidation of polyglucose fractions prepared by "solution melt" method at different temperatures; oxidation at 4°.

fourth or third hydroxyl, or one mole of formic acid if the reducing end group is linked at the sixth hydroxyl.[19,20] We were careful not to overtitrate to avoid this hydrolysis.

Periodate Reduced.—Twenty ml. of saturated solution of sodium bicarbonate was transferred to a 125-ml. erlenmeyer flask, which was previously flushed with argon. Ten ml. of the oxidation mixture was added immediately followed by the addition of 10 ml. of 0.1 *N* sodium arsenite solution and 2 ml. of 20% KI solution. The flask was immersed in an ice-water-bath and stirred occasionally for 15 minutes. The excess sodium arsenite was titrated with 0.1 *N* iodine (standardized against arsenic trioxide), keeping the solution at approximately 4° during the titration. The same titration was performed with a blank solution and the moles of periodate consumed per mole anhydro-glucose unit (A.G.U) was calculated.

TABLE IV

PERIODATE OXIDATION OF "SOLUTION MELT" AND "TWO-STAGE" POLYGLUCOSE FRACTIONS

0.0374 *N* $NaIO_4$, room temp.

		Mole IO_4^-/A.G.U. 72 hr.	Mole IO_4^-/A.G.U. 96 hr.	Mole HCOOH/A.G.U. 72 hr.	Mole HCOOH/A.G.U. 96 hr.
		"Solution melt"			
155°	I	1.43	1.47	0.598	0.631
155°	V	1.45	1.55	.605	.678
175°	II	1.22	1.32	.448	.546
175°	V	1.37	1.49	.556	...
		"Two-stage" (II)			
150°	I	1.41	1.51	0.573	0.639
150°	V	1.43	1.59	.607	.668
170°	I	1.33	1.51	.553	.634
170°	V	1.40	1.52	.586	.658

Detection of Glucose in the Hydrolysate of Periodate Oxidized Polyglucoses.—Fractions I and V of the two "solution melt" products and of the 150° and 170° "two-stage" polymers were oxidized with 0.0374 *N* $NaIO_4$ at room temperature in the dark for 76 days. The solutions were passed through Amberlite IR 45 ion exchange resin, were freeze-dried and finally dried to constant weight at 60° in vacuum. The samples were then hydrolyzed with 1 *N* HCl for 3 hr. in a boiling water-bath during which time the solution turned yellow. The hydrolyzed solutions were again passed through Amberlite IR 45 resin, freeze-dried and dried to constant weight. Aliquots of these samples in duplicate were chromatographed parallel to glucose with a mixture of butyl alcohol, ethyl alcohol and water (4:1:5). Parallel strips were sprayed with aniline–hydrogen phthalate to indicate the area where glucose was expected. These areas were cut out, eluted with water and the amount of glucose was determined by anthrone reagent by comparing the absorptions at 625 mμ to the absorption of glucose. The amount of glucose equivalent to the hydrolysate was between 1 and 6%. Similar chromatographic results by qualitative estimation of intensity of spots indicated on the average the presence of about 2% glucose in the hydrolysate.

Discussion

The alcohol fractionation indicates that there are differences in the solubility of the polyglucoses prepared by the different methods. The less steep rise of the yields of fractions from the "solution melt" process shows (Fig. 1) that these polymers have somewhat different solubility behavior, because of different molecular weight distribution, or different structure (branching), or both. The lower yields at higher alcohol concentrations of polyglucose fractions from the 150° "infrared" process was due to formation of large amounts of gel (43%).

There was no fractionation by number average molecular weights calculated on the basis of reducing end-group determination on the "infrared" and "two-stage" products, but fractionation of "solution melt" polyglucoses was evident by these values (Table I). This, together with the agreement between osmotic and end-group number average molecular weights,[15] indicates that in the "solution melt" product there is one reducing end group per molecule, and that the previously presented requirement of polycondensation mechanism is fulfilled.[2] The shapes of the integral distribution curves in Fig. 2 show that the lower temperature (155°) "solution melt" polyglucose has a greater concentration of polymer in the lower molecular weight regions (below 15,000 $\overline{M}_n$), while in the 175° product the distribution is shifted toward the higher molecular weights. The weight fraction distribution predicted for branched polycondensation products is very broad[21–23] and the efficiency of our fractionation must be considered low, due to the high concentration of solute (2%). Under these conditions, number average molecular weights may represent only a small portion of the polymer near the low molecular weight end. Actually, the unfractionated polymers contain 70–80% of non-dialyzable polyglucose which is above about 40,000 molecular weight.[15]

The assumption that there is one reducing end group (unreacted glucosidic hydroxyl) in each *n*-mer, is based on the restrictions set up in the treatment of mechanism of polyfunctional condensation where the $A-R-B_{f-1}$ monomer (A is the glucosidic hydroxyl, B the four non-glucosidic hydroxyls) can condense only by A to B or *vice versa*, and intramo-

(19) I. A. Wolff, B. T. Hofreiter, P. R. Watson, W. L. Deatherage and M. M. MacMasters, THIS JOURNAL, **77**, 1654 (1955).

(20) *Cf.* J. M. Bobbitt, *Adv. Carbohydrate Chem.*, 11, 1 (1956), pp. 18–19.

(21) P. J. Flory, THIS JOURNAL, **74**, 2718 (1952).

(22) P. J. Flory, "Principles of Polymer Chemistry," Cornell Univ. Press, Ithaca, N. Y., 1953, pp. 361–378.

(23) S. Erlander and D. French, *J. Polymer Sci.*, **20**, 7 (1956).

lecular rings are not formed. Figure 7 shows one possible configuration of a low n-mer, where the branching is due to the addition of a monomer through its A group to one B group in the polymer. The one reducing glucosidic hydroxyl in the polymer (A*) is marked with an asterisk. The average number of unreacted non-glucosidic hydroxyls (B*) per monomer is given by

$$N_B^* = \frac{3n+1}{n}$$

where n is the degree of polymerization. In higher polymers N_B^* is substantially 3. By acetylation only about 94% of the three free hydroxyls were substituted.[24] This might be the consequence of incomplete substitution, due to insufficient reaction conditions or to steric hindrance, or might be the sign of inner ring formation through B*–B* condensation (changes in N_B^* due to one A*–B* reaction would be negligible at higher n); finally, this might be caused by decomposition of the monomer. This later possibility was shown not to occur above 0.5% by paper chromatography of the unfractionated products.[2]

The intrinsic viscosity data in Table II show that fractionation by molecular weight takes place in all polyglucose preparations and also the higher temperature polymerizations produce polymers with higher intrinsic viscosity. The absence of difference in the molecular weights calculated from reducing end-group data in the "infrared" and "two-stage" fractions has to be taken therefore as a sign of deviation from the theoretical structure. The optical rotation of the "infrared" and "two-stage" fractions shows further that there is some configurational difference in these products as contrasted with the "solution melt" polymers. Only the latter follows the empirical observation on unfractionated polymers[2] that higher temperatures produce polyglucose fractions with higher molecular weight, higher intrinsic viscosity and higher optical rotation. Therefore, only in the "solution melt" fractions there is no deviation from the postulated structure based on reaction mechanism as indicated by these methods.[25] The reason for this lies probably in the increased mobility of the monomers and n-mers during polymerization. This allows the reactants to come into contact suitably through the A* groups, which are the most reactive and which thereby reduces the chance of other reactions.[2] The absence of A*–A* link at higher degrees of polymerization was explained by the lowering of the fraction A*/B* in an n-mer as the reaction proceeds.[2]

The changes in optical rotation during hydrolysis are alike in the three polymers (Fig. 3). The temporary increase in the specific rotation during the first few minutes of hydrolysis is similar to that observed in cyclohexanes, which was attributed to conformation changes.[26] When hydrolysis proceeds further, the optical rotation decreases and the value approaches that of glucose. When the extent of hydrolysis is followed by measurement of reducing power the curve is monotonic and the reducing power of glucose is recovered quantitatively (Fig. 4).

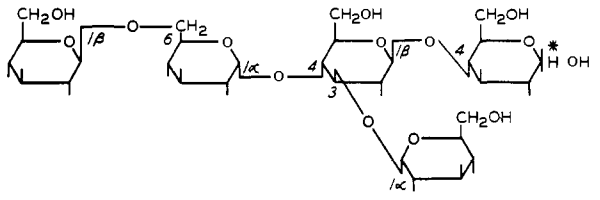

Fig. 7.—Schematic structure of a possible reversion product of glucose.

Complete structural analysis of these heterogeneous polyglucose fractions by customary methylation methods would be a staggering task, since the structure probably would contain all the variations of linkages and branching up to the functionality of glucose in a random distribution.[27] Only the relative frequencies of the linkages will be controlled by the relative reactivities of the hydroxyls under the employed polymerization conditions, but even these will change if we introduce the slightest alteration in any of the numerous parameters controlling the polymerization.[2]

Periodate oxidation is a relatively simple way to study certain aspects of over-all configurational difference, such as degree of branching. Under controlled conditions two neighboring hydroxyls on the glucose monomers will be oxidized by one mole of periodate and three vicinal hydroxyls will consume two moles of periodate with the production of one mole of formic acid from the group in the middle. In conforming to previous treatments of periodate oxidation on glucose polymers, and to make the considerations simple, we assume that all the monomer is in the glucopyranose structure. One mole of formic acid will be produced by the consumption of two moles of periodate from each of the following monomeric units: non-reducing end groups (E), 1–6 linear chain members linked at 1- and 6-position, and reducing end group linked through the 4th carbon. The reducing end group linked through the 6th carbon will supply two moles of formic acid with the consumption of three moles of periodate, but the contribution of reducing end groups is insignificant at higher degrees of polymerization and can be neglected.

The amount of formic acid produced comes substantially from the linear chain members linked at 1- and 6-position and from non-reducing end groups (E). The number of branches in each polymeric species is E-1, since each new branching will introduce one new E. Since it was found by methylation[27] that about one-third of the B groups are branching points, another one-third have to be end groups and only one-third can be linear. About

(24) Methylation can be carried out also only to 42% methoxyl content (theoretical 45.6%). Private communication, Professor E. Pacsu, Princeton University.

(25) *Cf.* the agreement of number average molecular weights by reducing end group and by osmotic methods in similar polyglucoses (ref. 15).

(26) K. Freudenberg, G. Blomquist, L. Ewald and K. Soff, *Ber.*, **69**, 1258 (1936), K. Freudenberg, *J. Polymer Sci.*, **23**, 791 (1957).

(27) Methylation data on certain polyglucose preparations indicate that about every third glucose unit is branched in one way or another, and multiple branchings occur up to the functionality of glucose (private communication, Professor E. Pacsu; *cf.* also P. T. Mora and E. Pacsu, U. S. Patent 2,719,179 (1955)). Methylation by $C^{14}H_3I$ and separation and estimation of the hydrolysis products by tracer paper chromatography is planned on selected fractions in our laboratory.

one-half of the linear units are expected to be linked through the 1- and 6-positions, because, at least at lower temperatures, the reactivity of the sixth hydroxyl approximately equals the sum of the other three B hydroxyls, which are equal to each other.[14,28] The probability that a new branch will be formed by the addition of monomer at a given monomeric unit which is not an end group, is proportional to the product of the B* groups on the unit with the frequency of the unit, therefore $3 \times {}^1/_2 \times {}^1/_3 = {}^1/_2$ on the 1–6 linear chain members. Addition of monomer at E does not introduce branching, only extends the chain. Therefore, one-half of the decrease of formic acid production by oxidation of polymers prepared by different polymerization conditions is a direct measure of the disappearance of the linear 1–6 units and of the increase in branching.

One mole of periodate is consumed for each of the following units in addition to that which produces formic acid: 1–4 and 1–2 linear monomers, 1–4–6 and 1–2–6 branched monomers and for 2- and 3-linked reducing end groups. The end groups are negligible at a high degree of polymerization. The sums of the frequency of branched units at 6 which consume periodate (1–4–6 and 1–2–6) and the corresponding unbranched units (1–4 and 1–2) are equal, since in each case they represent two-thirds of the frequency of the 1–6 linear monomers, due to the above-mentioned relative reactivities of the B hydroxyls. Therefore, half of the difference in the extra periodate consumed above the two moles necessary for one mole formic acid is due to 1–2 and 1–4 linear units, and the total amount of periodate also can be taken as the relative measure of linearity.

Linear units linked through the third hydroxyl and the branchings where no vicinal hydroxyl is left will not be affected by periodate oxidation. Glucose detected in the hydrolysate of product oxidized for 76 days indicates that these links occur, but the quantity of the glucose found was low because over-oxidation must have occurred.

We do not claim that the periodate oxidation figures represent structural information in a definitive sense. It is very difficult to make any quantitative interpretation not only because of the many possible structural variations but also because the periodate oxidation of polysaccharides is a rather ill-defined continuous process in time where over-oxidations may occur depending sensitively on light and on temperature. The only value of the data reported in Tables III and IV and Figs. 5 and 6 is to compare polyglucoses with regard to relative degree of branching, when the oxidation was carried out under similar conditions. Higher values of moles periodate consumed and formic acid produced per anhydro-glucose unit (A.G.U.) indicates relatively less branching in the corresponding polyglucose and *vice versa*.

Data in Table III on fractions of the "infrared" product show that at higher polymerization temperature higher degree of branching is produced. This is in agreement with the statistical reasoning based on the decrease of difference in relative reactivities between the primary (6) and secondary (2,3,4) hydroxyls at higher temperatures.[2,15] The fractions which precipitate first with alcohol have higher viscosity (Table II) and higher degree of branching (the 144 hr. periodate figure of the 150° "0" fraction is obviously in error and the 192 hr. figure corrects the correlation). Theoretical treatments[29,30] predict that as a result of an increase of number of branching points the intrinsic viscosity would be independent of or even decrease with increasing molecular weight, provided that the degree of branching is constant. Our higher molecular weight fractions have higher intrinsic viscosity which, in accordance with the periodate figures, indicate that we do not have a homologous series and we do achieve fractionation by degree of branching. That there is more branching in the higher molecular weight products might be due to the differences in polycondensation mechanism: *i.e.*, both higher degree of branching and higher molecular weight products will be produced by higher temperatures. It is not known, then, whether we obtain by alcohol precipitation the more branched, higher molecular weight fractions first from the heterologous mixture of polymers because solely of the effect of molecular weight or because of solubility differences caused truly by branching; and this can be decided only if we compare the solubility of two homodisperse fractions with equal molecular weight, but with different branching. In starch[31] and in polyvinyl chloride[32] fractionation the linear fractions are precipitated first and the branched fractions need higher non-solvent concentration, but in these cases the precipitated linear phase is semi-crystalline and the thermodynamic considerations of fractionation cannot be based on the assumption that only size parameters cause differences in chemical potential.[33] There is no published theory on fractionation by branching. One suggestion was that fractionation may occur because every branching point prevents the smooth contact between large parts of the molecular chain and so renders intramolecular coagulation less likely.[32] Our observation on polyglucose fractionation and one paper on dextran[34] are inconsistent with this explanation, and clearly more research is indicated.

Similar periodate oxidation results were obtained on the "solution melt" and "two-stage" preparations (Table IV). Each figure of periodate consumption or formic acid production of the higher temperature polymerization products is lower than of the corresponding fraction of the lower temperature product, indicating a greater degree of branching in the higher temperature polymers. Also, fractionation occurs by degree of branching in each

(28) H. Fraenkel, *Ann.*, **555**, 187 (1944).

(29) B. H. Zimm and W. H. Stockmayer, *J. Chem. Phys.*, **17**, 1301 (1949).

(30) P. J. Flory and T. G. Fox, Jr., THIS JOURNAL, **73**, 1904 (1951); also *cf.* ref. 22.

(31) For review of starch fractionation see T. J. Schoch, *Adv. Carbohydrate Chem.*, **1**, 247 (1945); also in J. A. Radley, "Starch and its Derivatives," John Wiley and Sons, Inc., New York, N. Y., 1954, Chapter 6, pp. 123–200.

(32) A. Peterlin, Symposium on Macromolecular Chemistry, Milan, Sept., 1954; *Ricerca Sci., Supplemento A*, 553 (1955).

(33) *Cf.* Reference 22, pp. 339–345, 512 and 563.

(34) L. H. Arond and H. P. Frank, *J. Phys. Chem.*, **58**, 953 (1954).

polymer, indicated by both the periodate and formic acid data at both 72 and 92 hr. If we compare polymers prepared by different methods (but consider our above reservations) we may conclude that the 155° "solution melt" product has about the same degree of branching as the 150° "two-stage" polymer, but the 175° "solution melt" polymer has a higher degree of branching than the 170° "two-stage" polymer. This would be in line with the lower intrinsic viscosity of the "solution melt" polymers, and can be explained by the more free mobility of the monomers and the lower *n*-mers in the polymerizing mixture, leading to more frequent additions of these species through their A* groups to the B* groups of the same monomeric unit in the polymer.

Figures 5 and 6 indicate the same correlations in relative degrees of branching in "solution melt" polyglucose fractions, showing the periodate oxidation as a time process, except in this experiment the formic acid figures in the lower temperature product are not sufficiently different to illustrate fractionation by branching.

BETHESDA 14, MD.

[CONTRIBUTION FROM THE KETTERING-MEYER LABORATORY,[1] SOUTHERN RESEARCH INSTITUTE]

Synthesis of Potential Anticancer Agents. XIII. Ribosides of 6-Substituted Purines

BY JAMES A. JOHNSON, JR.,[2] H. JEANETTE THOMAS AND HOWARD J. SCHAEFFER

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The syntheses of six 6-substituted-9-β-D-ribofuranosylpurines from 6-chloro-9-β-D-ribofuranosylpurine have been accomplished.

Since it is known that certain unnatural purines are converted into nucleosides by enzymes[3] or into nucleotides by enzymes[4] and biological systems,[5] there is a distinct possibility that purine antagonists, such as 6-mercaptopurine, are ribosidated[6] or ribotidated[4] before they become active biological agents. In order to test this hypothesis, we have prepared several 6-substituted purine ribosides so that a comparison of their anticancer activity with the free purines can be made. An ideal starting material for our synthetic program was 6-chloro-9-β-D-ribofuranosylpurine (I) which was first prepared by Brown and Weliky[7] by the condensation of chloromercuri-6-chloropurine with 2,3,5-tri-O-acetylribofuranosyl chloride. The structure and stereochemistry of I were established by its conversion into adenosine with methanolic ammonia at 100°. We have prepared I by the recently published[8] improved procedure in which 2,3,5-tri-O-benzoylribofuranosyl chloride was condensed with chloromercuri-6-chloropurine.

The usefulness of 6-chloropurine riboside (I) in the synthesis of 6-substituted purine ribosides has already been demonstrated by the preliminary report[9] from this Laboratory of the synthesis of 6-mercapto-9-β-D-ribofuranosylpurine (II) from I. A modification of the method which we employed has been described recently by Kissman and Weiss,[10] who prepared several 6-substituted aminopurine ribosides from 6-chloro-9-β-D-(2′,3′,5′-tri-O-benzoylribofuranosyl)-purine. The present paper gives complete details of the synthesis of II (see the Experimental section) and describes further transformation products of 6-chloropurine riboside (I).

Treatment of 6-chloropurine riboside (I) with two equivalents of sodium methoxide for 30 minutes at 65° gave a good yield of 6-methoxy-9-β-D-ribofuranosylpurine (III). The course of the reaction was followed by examining the ultraviolet spectra of aliquots removed from the reaction mixture at increasing time intervals and observing the shift in the peak from 263 mμ (6-chloro) to 248 mμ (6-methoxy).

The synthesis of 6-methylaminopurine riboside (IV) was accomplished by heating a solution of I with aqueous methylamine at 80° for 16 hours. The replacement of the chloro group[11] proceeded smoothly, and the desired product was isolated in good yield. No attempt has been made to determine whether a shorter reaction time would be sufficient since little decomposition occurred even with these reaction conditions.

Recently, it was demonstrated[12] that 6-benzylmercaptopurine is as effective as 6-mercaptopurine against Adenocarcinoma 755. In order to determine if the corresponding riboside would possess or have enhanced anticancer activity, we undertook the synthesis of 6-benzylmercapto-9-β-D-ribofuranosylpurine (V). The reaction of I with two equivalents of sodium benzylmercaptide at 65°

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(11) Dr. Alexander Hampton of the Sloan-Kettering Division of Cornell University Medical College informed us that he has prepared 6-methylaminopurine riboside (IV) in a 64% yield by the reaction of 6-methylmercaptopurine riboside with methylamine for six hours at 125°. The physical properties of the compounds prepared by the different procedures are in good agreement.

(12) H. E. Skipper and J. R. Thomson, unpublished results.