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DETERMINATION OF THE NUMBER-AVERAGE MOLECULAR WEIGHT OF POLYSACCHARIDES BY END-GROUP REDUCTION WITH BOROHYDRIDE-*t*

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ABSTRACT

The reduction with sodium borohydride-*t* of a series of maltose oligosaccharides yields products whose residual radioactivity (after deduction of a blank) has a linear dependence on degree of polymerisation. The procedure has been extended to measure the average degree of polymerisation (or alternatively M_n) of hydrolysed amyloses. For this purpose, it is necessary to use a washing procedure to remove a radioactive impurity in the sodium borohydride. The method should be applicable to measurement of number-average molecular weights of any polysaccharide which has a reducing end-group.

INTRODUCTION

All of the physical methods for molecular weight determination of polymers can be applied to polysaccharides. In the investigation of enzymic synthesis and degradation of polysaccharides such as starch and glycogen, however, it is frequently necessary to determine the number-average molecular weight and usually the method of choice is a chemical end-group analysis. All such methods used to date have some disadvantage, usually associated with unwanted chemical side-reactions. Thus, for example, the periodate method[1] is subject to over-oxidation, and the use of alkaline copper-reducing-agents is non-stoichiometric and requires rigidly controlled experimental conditions. The rate of such reactions may also vary with the nature of the linkage, which makes such standardisation of conditions rather difficult[2]. The reduction with sodium borohydride of reducing sugars, however, apparently proceeds quantitatively and without any side-reactions. Furthermore, the use of tritium-labelled borohydride promised the possibility of introducing radioactivity at one end-group for each polysaccharide molecule. Determination of the activity of the modified polysaccharide would give high sensitivity and hence the potential for measuring higher molecular weights than is normally possible by chemical end-group methods.

The method is similar in principle to that of Isbell and co-workers[3] who were

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able to introduce ^{14}C into the reducing end-group of polysaccharides by chemical methods. The tritiation method, however, appears to be much simpler experimentally.

Amylose has been used as a model polysaccharide in our studies because it is readily hydrolysed to produce fragments with a range of molecular weights, and its chemical structure is well characterised. A glycogen macrodextrin was also used in certain experiments.

MATERIALS

The following substances were used as received from the sources indicated. Maltose monohydrate and anhydrous D-glucose were from Nutritional Biochemicals Inc. Sodium borohydride was from Sigma Chemicals and sodium borohydride-*t* from New England Nuclear Corp., reported to have 200 mCi/mmole. The latter material was pink in colour.

Maltotriose, maltopentaose, and maltohexaose were provided by Dr. N. Palmer and were shown by t.l.c. to contain less than 1% of any impurity of different R_F value.

Partially hydrolysed amylose samples were obtained as follows*. Potato amylose (2.3 g, Koch–Light Laboratories Ltd.) was added with stirring to 9M hydrochloric acid (20 ml) at 0°. After 5 min, the solution was rapidly brought to 30° and kept at that temperature for 5 min before again cooling to 0°. Solid sodium acetate (8 g) and then 40% (w/v) aqueous sodium hydroxide (10 ml) were added, and the resultant suspension was dialysed and freeze-dried. The product is designated as amylose *A* (yield, 1.76 g), and amyloses *B* (yield, 1.01 g) and *C* (yield, 0.33 g) were similarly obtained by similar hydrolysis at 30° for 10 and 20 min, respectively.

METHODS AND RESULTS

Drying procedures. — D-Glucose was dried at 60°/0.1 mmHg/3 h over phosphoric oxide, and maltose monohydrate was kept overnight over anhydrous calcium chloride before weighing. All polysaccharides were kept in an air-dry state in sealed containers and moisture contents were determined by drying samples as for D-glucose.

Counting method. — The method described by Thomas *et al.*[4] for ^{14}C-counting was found to be equally applicable for tritium labelling. A 50-μl sample of the solution to be counted was used, and counting was carried out on the ^{3}H mode of a Packard Tri-carb liquid scintillation spectrometer.

Counting efficiency. — Since no suitable, independent, water-soluble, toluene-insoluble ^{3}H-standard was available, the efficiency was determined by reference to the quoted activity of the borohydride-*t* as follows. To 0.5 ml of an aqueous solution of maltose monohydrate (4.95 mg) at 30° was added 0.1 ml of a solution containing

*This procedure was designed by D. French and J. F. Robyt, Department of Biochemistry and Biophysics, Iowa State University.

sodium borohydride (10.6 mg·ml^{-1}) and sodium borohydride-*t* (0.396 mg.ml^{-1}). After 2 h, 0.1 ml of 3M acetic acid was added and then a 50-μl sample was transferred to paper to give a count of 8.220×10^3 counts.min^{-1}. A blank experiment, excluding only the maltose, gave a count of 1.465×10^3 counts.min^{-1}. The counting efficiency derived from these figures is 5.4%.

Procedure for oligosaccharides. — An aqueous solution (1 ml) containing oligosaccharide (3 mmoles), sodium borohydride-*t* (0.041 mg), and sodium borohydride (1.16 mg) was kept at 30° for 1 h, 3M acetic acid (0.1 ml) was then added, and counting was effected as described above. The observed count was typically in the region 4×10^4 counts.min^{-1}, while the blank experiments in which the oligosaccharides were omitted gave 1.050×10^3 counts.min^{-1}. The results are shown in Fig. 1.

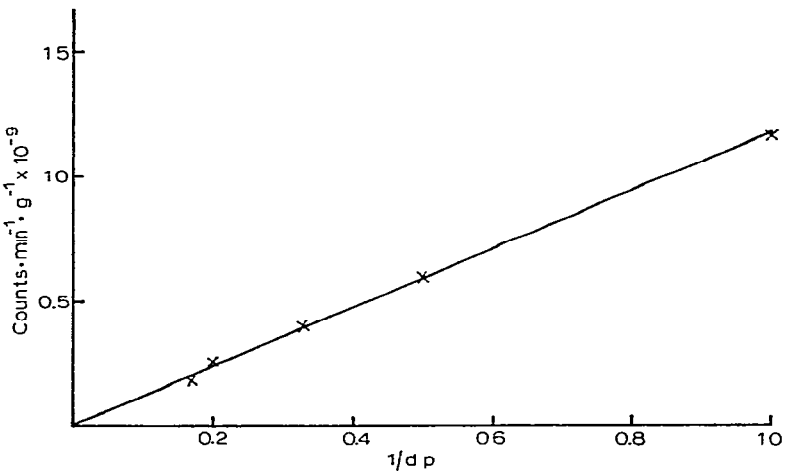

Fig. 1. Relationship of tritium activity to degree of polymerisation of oligosaccharides.

Procedure for polysaccharides. — To 0.5 ml of a solution of the polysaccharide (at least 0.1mM, *e.g.* 800 μg.ml^{-1} for M_n 8000) in either water or 0.25M sodium hydroxide at 30° was added 0.1 ml of a solution containing sodium borohydride (10.6 mg.ml^{-1}) and sodium borohydride-*t* (0.396 mg.ml^{-1}). After 2 h, 0.1 ml (or 0.2 ml when the polysaccharide was dissolved in alkali) of 3M acetic acid was added and 0.05 ml of the solution transferred to paper. The paper was thoroughly dried and then immersed for 10 min at room temperature in a mixture of ethanol–water (19:1) (10 ml) and 10M hydrochloric acid (0.1 ml). After subsequent immersion for a further 10 min in ethanol–water (19:1), the paper was dried and counted in the usual way. Typically, a count of 700 counts.min^{-1} was observed and a blank (without polysaccharide) of 200 counts.min^{-1}. From such figures, a value of 2.0×10^7 counts.min^{-1}.g^{-1} may be calculated (for a concentration of 700 μg.ml^{-1} of a degraded glycogen). This value may be related to the corresponding value for maltose determined by the preceding method, using the same reductant solution (6.25×10^8 counts.min^{-1}.g^{-1}) to derive the d.p. 62 or M_n 10,000.

INVESTIGATION OF VARIABLES

Dependence of observed activity on paper loading. — A maltose solution, which had been reduced with borohydride-*t* as described above, was applied in various volumes to a series of papers and the resultant counts are shown in Table I.

TABLE I

EFFECT OF "ACTIVITY LOAD" ON PAPER

Load on paper (ml)	0.02	0.04	0.05	0.06	0.08
Observed activity (counts.min^{-1}.g^{-1})	8.91	8.62	8.73	8.74	8.64

Procedures for washing papers before counting. — The reduction procedure described above for polysaccharides was applied to a partially hydrolysed amylose (M_n 2070) and to the high molecular weight fraction of the alpha-amylase macrodextrin from shellfish glycogen[5] (M_n 22,500). After acidification, each solution was loaded on to a series of papers which were dried and then washed with acidified ethanol for various periods. A blank experiment, omitting only the polysaccharide, was also carried out and the results are shown in Table II.

TABLE II

EFFECT OF ACID WASH (95% ETHANOL, 0.1M HCl)[a]

Time in acid (min)	0	10	20	30
Blank count (c.p.m.)	4500	299	190	150
Amylose count-blank (c.p.m.)	8000	3454	3423	3726
Glycogen count-blank (c.p.m.)	4900	355	362	414

[a]All acid washes followed by washing for 10 min in 95% ethanol.

TABLE III

RATE OF REDUCTION OF MALTOSE AND HYDROLYSED AMYLOSES (*A*–*C*) AT 30°

Maltose			
Time (h)	1.0	3.0	5.5
Activity (c.p.m./g × 10^{-7})[a]	8.1	7.9	8.0
[b]	8.0	7.9	8.1
Amylose			
Time (h)	2.0	4.5	9.5
Activity (c.p.m./g × 10^{-7}) *C*[a]	10.3	10.2	10.4
C[b]	9.5	10.3	10.6
B[b]	6.1	6.1	6.1
A[b]	4.4	4.4	4.4

[a]In water. [b]In 0.25M sodium hydroxide.

Rate of reduction. — The method described above for oligosaccharides was applied to maltose, and samples were withdrawn from the reduction solution at intervals up to 5.5 h. The experiment was also repeated for solutions in 0.25M sodium hydroxide, and the results are shown in Table III. A similar experiment was carried out with solutions of amyloses *A*, *B*, and *C* in 0.25M sodium hydroxide and also, in the latter case, in water. The results are shown in Table III.

TABLE IV

MOLECULAR WEIGHT DETERMINATION ($\overline{M}_n$) OF HYDROLYSED AMYLOSE FRACTIONS BY TRITIATION AND BY THE NELSON METHOD

Amylose sample	*Tritium method*[a] *(standard deviation)*	*Nelson method*
A	4340 (97)[b]	4430
B	3180 (73)[b]	3080
C	1880 (97)[b]	1960
C	1860 (24)[c]	1960

[a]Four analyses on each sample. [b]In 0.25M sodium hydroxide solution. [c]In water.

Comparison with Nelson method for determining the M_n *of amylose.* — The rates of reaction of amylose *B* and maltose with the Nelson reagents were compared as follows. A solution of amylose *B* in 0.5M sodium hydroxide was neutralised with 0.5M acetic acid immediately before sampling and analysis as described by Nelson[6]. In a series of experiments, the time of heating in the boiling-water bath was varied from 10–120 min and the results are shown in Fig. 2. In all subsequent Nelson-determinations, a 30-min heating period was used. Amylose samples *A*, *B*, and *C*

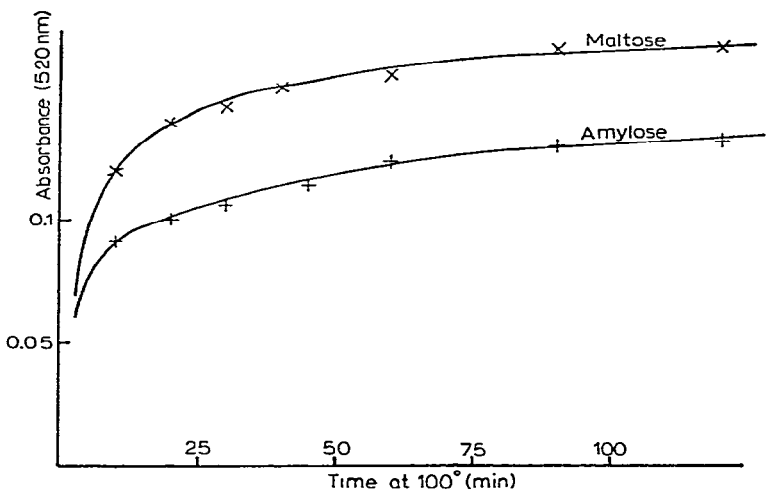

Fig. 2. Effect of time of heating on colour development on Nelson assay of maltose and amylose. Maltose, ×——×; amylose, +——+.

were analysed by this method and also by the tritiation method described above, and the resultant molecular weights, calculated by reference to the maltose standard, are shown in Table IV.

DISCUSSION

Ideally, in the type of procedure which we have used, in a blank experiment in which an aqueous solution of sodium borohydride-*t* is acidified with acetic acid and dried on paper, the paper should show no residual radioactivity. In every case, however, we have found relatively high blank-values by this procedure and must assume that a non-volatile ^{3}H-impurity is present in the borohydride sample. This blank value is, however, reproducible and, in cases where the product of the analysis has a high relative activity (*i.e.* a low molecular weight), it is possible to accept, and correct for, the blank. This situation pertains in the case of the oligosaccharides and the validity of the method is demonstrated by Fig. 1. The simple procedure for oligosaccharides therefore may be strongly recommended for d.p. determination and is readily applicable on the sub-milligram scale.

In the case of polysaccharides, however, the end-group content is very much lower than with oligosaccharides and hence the ratio of the introduced activity to the blank activity is much smaller. In such cases, the blank activity must be lowered and this has been effected by washing the paper carrying the reduced sample with acidified ethanol before counting. Control experiments demonstrated the need for presence of acid in the wash liquor and Table II shows that most of the anomalous activity is very rapidly lost. This Table also gives strong, presumptive evidence that no active polysaccharide is lost during the washing procedure. By inclusion of the washing procedure and by reference to the relative activity known to be introduced by a given reductant solution with a standard oligosaccharide such as maltose, the method becomes applicable to the accurate determination of molecular weights on quite small amounts of polysaccharide, *e.g.* 500 μg of M_n 10,000. Since this work was completed, a similar reagent-impurity problem has been reported[7] in the micro-analytical determination of sugars with sodium borohydride-*t*. In this case, the active impurity was removed with charcoal.

As a means of verifying the above method, a completely different method for chemical analysis of reducing end-groups has been considered. This method involves the reduction of the end-group by an alkaline copper reagent designed by Nelson[6]. Such reagents induce very complex mixtures of reactions with reducing sugars, but under standard conditions, and when there is only one type of linkage present in the polysaccharide (*cf.* ref. 2), the amount of reagent reduced is usually directly proportional to the amount of reducing end-group present. We were concerned that there might be a significant difference in the rate of such reactions between oligo- and poly-saccharides, but the results in Fig. 2 indicate no significant difference after treatment for 30 min. This period of heating was therefore used in all Nelson determinations, and the $\bar{M}_n$ values for three different hydrolysed samples of amylose were

determined by reference to maltose. Table IV indicates the reproducibility of the tritiation method and shows that this method gives results which agree very closely with the Nelson method. This Table also demonstrates the fact that the tritiation reaction can be carried out on solutions in sodium hydroxide, thus widening its scope to many types of polysaccharide which are insoluble in water, but soluble in alkali.

The time required for completion of the tritiation of both amylose and maltose is considered in Table III, which indicates that the reaction is complete in less than 1h at 30° in both water and sodium hydroxide solution.

Tritiation has two particular types of advantage in molecular weight determination, both dependent on the sensitivity of the method. It is applicable on the microgram scale in the lower range of molecular weight (*e.g.* 50 μg of $\overline{M}_n$ 1000) and it is theoretically applicable to polysaccharides of very high molecular weight. A significant limitation in the latter case, however, is the requirement that each macromolecule must possess a reducing end-group and also that it must contain no other groupings which react with sodium borohydride. Any oxidation of alcohol groups in the polysaccharide to carbonyl groups (*e.g.* during isolation) would thus invalidate the method.

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