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Note

A simplified method for determining unsubstituted D-glucose residues in cellulose derivatives*

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Cellulose is generally derivatized by adding the appropriate functional groups to the primary or secondary hydroxyl groups of its D-glucose residues. A nonuniform substitution-pattern usually results, as most such substitution reactions take place under heterogeneous conditions. Klug *et al.*[1] studied the distribution of the substituents in *O*-(2-hydroxyethyl)cellulose, and proposed a procedure for determining the mole percent of unsubstituted D-glucose residues. Briefly, it consists of three stages: (*1*) acid hydrolysis of the cellulose derivative under appropriate conditions, (*2*) neutralization of the acid in the hydrolyzate, and (*3*) determination of the unsubstituted D-glucose in the neutral hydrolyzate by the D-glucose oxidase method[2]. They[1] recommended that, for neutralization of the acid (which must be performed to facilitate application of the D-glucose oxidase method), barium hydroxide or carbonate be used. Although effective, this method is inconvenient, as centrifuging or filtering is necessary in order to remove the barium sulfate; either procedure is time-consuming and tedious. Barium sulfate is a voluminous, white, finely divided precipitate which frequently clogs filters; also, there is the possibility of occlusion of D-glucose by the precipitate if it is allowed to coagulate extensively or is not washed sufficiently after filtration.

It seemed possible that ammonium hydroxide, which upon reaction with sulfuric acid yields a water-soluble salt, might be substituted for the barium hydroxide in the neutralization of the acid in the hydrolyzate, thus eliminating the filtration step. As now shown, this change has no deleterious effect on the subsequent use of the D-glucose oxidase system, or on the final, spectrophotometric measurement.

Nineteen samples of *O*-(2-hydroxyethyl)cellulose were hydrolyzed in duplicate. Two aliquots were then withdrawn from each hydrolyzate; one was made neutral with 0.16M barium hydroxide, and the other, with 10% (v/v) ammonium hydroxide. Each neutral aliquot was analyzed by the D-glucose oxidase procedure, and the resulting values for percent of D-glucose were compared. Values for various samples ranged from 1 to 3% of unsubstituted D-glucose.

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The results from the ammonium method were generally the same as, or slightly higher than, those from the barium method. The precision of the two methods was compared by use of the F test (see Appendix). The value of F_{18}^{18} obtained from the data was 2.09; the value of F_{18}^{20} from an F distribution table is 2.19. The two methods of neutralization are equally precise at the 95% confidence level.

The results from the two methods were compared by using the t test to see if there was a significant difference. This calculation gave $t_{38} = 6.49$. At the 95% confidence level, $t_{40} = 2.02$. Thus, the means for the two methods are significantly different, a result not unexpected. The problems with the filtration of the precipitated barium sulfate, and the possibility of occlusion of D-glucose thereby, may account for the difference in the two sets of results. Experiments in these laboratories have shown that extensive washing will consistently elute all of the D-glucose present[3]. However, less handling is required in the ammonium method.

Neutralization with ammonium hydroxide is the method of choice due to its greater ease and the smaller likelihood of occlusion or other interference. Furthermore, interference by the ammonium sulfate in the solution upon the absorbance measurement or the enzyme reagents used to develop color was found to be negligible. One calibration curve, obtained by using various aliquots of a standard D-glucose solution, was compared with a calibration curve for a solution to which 5.4 mg of ammonium sulfate per aliquot had been added. The amount of ammonium sulfate needed was calculated from the amount of sulfuric acid known to be in the solution, taking the aliquot factor into account. The absorbance was measured at 525 nm (see Table I). Inspection of the data revealed no appreciable difference between the

TABLE I

COMPARISON OF CALIBRATION POINTS WITH AND WITHOUT ADDED AMMONIUM SULFATE

D-Glucose (mg/25 ml)	*Ammonium sulfate added*	*Absorbance*
25	no	0.060
25	yes	0.053
50	no	0.133
50	yes	0.140
75	no	0.230
75	yes	0.220
100	no	0.321
100	yes	0.322
150	no	0.495
150	yes	0.500

calibration curves. No bias of the data was seen. Thus, the presence of ammonium sulfate at the levels normally encountered in the procedure described herein does not appear to have any effect.

EXPERIMENTAL

Hydrolysis of samples. — To a solution of *O*-(2-hydroxyethyl)cellulose (0.30–0.35 g) in distilled water (84 ml) is added 72% sulfuric acid (3 ml), and the solution is boiled under reflux for 7 h, cooled, transferred to a 100-ml volumetric flask, and diluted to the mark with distilled water.

Neutralization of hydrolyzed samples (barium method). — A 10-ml aliquot of the hydrolyzate is withdrawn. A pH electrode is placed in it and 0.16M barium hydroxide is added until the pH is 4–5. The sample is then filtered through Whatman No. 42 filter paper into a 100-ml volumetric flask (if the solution is cloudy, it is refiltered). The precipitate is washed with distilled water until the bulb of the volumetric flask is full, and the funnel is removed. The solution is then diluted to the mark with distilled water.

Neutralization of hydrolyzed samples (ammonium method). — A 10-ml aliquot of hydrolyzate is withdrawn, distilled water (20 ml) is added, and a pH electrode is placed in the solution. Ammonium hydroxide [10% (v/v)] is added until the pH is 4–5. The solution is quantitatively transferred to a 100-ml volumetric flask, and diluted to the mark with distilled water.

Calibration curve. — Into each of four clean, 25-ml, volumetric flasks is respectively pipetted 0.25, 0.50, 1.00, and 1.50 ml of a standard solution of D-glucose (0.10 mg/ml). Enough distilled water is added to each flask to give a total volume of 2 ml. A flask reserved for a blank experiment is also prepared. D-Glucose oxidase reagent (Worthington Biochemical, Freehold, N.J., catalog no. 7923; 8 ml) is pipetted into each flask, and the flasks are incubated for 1 h at 37°. Into each flask is pipetted 5M sulfuric acid (12.5 ml), and the solution is made to the mark with distilled water. The absorbance at 525 nm *vs.* that of the blank is measured with a Beckman Model B spectrophotometer, and a calibration curve is plotted.

D-Glucose oxidase method. — A 4-ml aliquot of each sample of hydrolyzate is pipetted into a 25-ml, volumetric flask. An aliquot (8 ml) of D-glucose oxidase reagent is added, and the samples are incubated, treated with sulfuric acid, and measured as in the preparation of the calibration curve. The percentage of unsubstituted D-glucose is calculated by use of the data obtained, taking the aliquot factor into account.

APPENDIX[4]

$F = S_1^2/S_2^2$, where $S_1^2 > S_2^2$, S_1^2 is the variance of the barium hydroxide neutralization, and S^2 is that of the ammonium hydroxide neutralization

$S^2 = [\Sigma xi^2 - (\Sigma xi)^2/N]/(N-1)$, where xi is the result in the ith trial, and N = the number of trials

$t = [\bar{d}/S_d]/N_d$, where $\bar{d} = [d_1 + d_2 + \ldots d_i]/N_d$, d = the difference between the values given by the two methods, and $S_d = d^2/N - (\Sigma d)^2/N^2$.

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