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Quantitative Determination of Reducing Sugars, Oligosaccharides, and Glycoproteins with [^{3}H]Borohydride

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A very simple and direct radioactive isotope method has been described for the quantitative determination of reducing sugars. The method can be adapted to give any degree of sensitivity desired. The procedure is of general applicability to the determination of a large class of sugars. The stoichiometry of the reaction makes it a comprehensive procedure which greatly facilitates the analysis of sugars, oligosaccharides, and glycoproteins after acid and enzymic hydrolysis. Examples of the versatility of the method are given.

In our studies on glycoproteins, we have constantly searched for analytical procedures by which one sugar could be determined in the presence of others (1). There is a need for a better general analytical method that would be both applicable to all reducing sugars, oligosaccharides, and glycoproteins and, at the same time, be sensitive enough to be used on a microscale.

Borohydride reduces carbonyl compounds including aldoses and ketoses. This reaction is in general stoichiometric and therefore potentially useful as an analytical procedure. Attempts by Skell and Christ (2) and Terenteve (3) employed borohydride in the quantitation of sugars. Their methods depended upon the reduction of sugar with excess borohydride and the determination of the excess unreacted borohydride by the manometric measurement of the hydrogen released upon acidification. The determination of the excess borohydride, by its quantitative reduction of NAD^+ to NADH, has enabled us to develop a spectrophotometric method for the determination of sugars (4).[2]

Since a large excess of borohydride is necessary for the quantitative

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[2] Werner, D. A., Huang, C. C., and Aminoff, David, unpublished observations.

reduction of sugar, the borohydride consumed in reducing the sugar becomes a relatively small portion of the total borohydride present.[2] Hence, both the manometric and spectrophotometric methods for the determination of reducing sugars suffer from the limitation that they determine the unreacted excess borohydride. The use of [^{3}H]borohydride circumvents this problem, since the acid-stable incorporated tritium would be a direct measure of the product formed.

The potential versatility of using tritiated borohydride to reduce sugars has been recognized by a number of investigators (5,6), who used it with specific objectives in mind. In this communication we demonstrate the effectiveness of [^{3}H]BH_4^- as a reagent for the quantitative determination of sugars, oligosaccharides and sugar residues in glycoproteins. A preliminary report of these studies has been presented (7).

MATERIALS AND METHODS

Materials. Tritium-labeled borohydride was obtained from Amersham/Searle Corp. (Arlington Heights, IL) and New England Nuclear Corp. (Boston, MA). Table 1 summarizes the data on the different batches of [^{3}H]BH_4^- used in these studies. The earlier work involved the use of [^{3}H]BH_4^- from Amersham, and, with its high "acid-stable" blank, necessitated the use of charcoal (see below). The New England Nuclear (NEN) material gave a negligible "acid-stable" blank, and many of the problems of using charcoal were thereby eliminated.

The following commercially available sugars were obtained as indicated: *N*-acetyl-D-glucosamine, D(L)-arabinose (C.P.), 2-deoxy-D-ribose, D(L)-galactose (C.P.), D-glucosamine–HCl (C.P.), lactose monohydrate (C.P.), α-melibiose (C.P.), trehalose dihydrate (C.P.), turanose

TABLE 1
Radioactivity and "Acid-Stable" Blank of Various Batches of Tritiated Borohydride Used

Source	Compound	Batch No.	Total dpm × 10^{-6}	Acid stable dpm × 10^{-4}	Acid stable % of Total
Amersham	$NaBT_4$	TRK-45 No. 13	5.5	3.85	0.7
Amersham	KBT_4	TRK-293 No. 9	6.45	3.86	0.75
Amersham	KBT_4	TRK-293 No. 9	57.92	48.34	0.83
Amersham	KBT_4	TRA-293 No. 7	10.56	1.44	0.14
New England Nuclear	$NaBT_4$	NE-T-023 No. 702-008	114.14	0.81	0.007

(Pfanstiehl Laboratories, Inc., Waukegan, IL); D(L)-fructose (Dawe's Laboratories, Chicago, IL); sucrose analytical reagent (J. T. Baker, Phillipsburg, NJ); *N*-acetylneuraminic acid (Koch-Light Laboratories, Ltd., Colenbrook Buckinghamshire, England); D-ribose (Mann Research Laboratories, New York, NY); D(L)-mannose (Eastman Kodak Co., Rochester, NY); D-glucose-1-phosphate (Nutritional Biochemicals Corporation, Cleveland, Ohio); *N*-acetylgalactosamine, *N*-acetylmannosamine monohydrate, galactosamine–HCl, and D(L)-xylose (Sigma Chemical Co., St. Louis, MO); L-fucose (Calbiochem, Los Angeles, CA); and D-glucose (Mallinckrodt, St. Louis, MO). All the sugars used for these experiments were tested for purity by paper chromatography in at least two solvent systems (see below) and, with the exception of galactose, only the pure sugars were used. They were dried over P_2O_5 to constant weight before use in the preparation of the standard solutions for analysis.

The charcoal, Darco G-60, used for the removal of "acid-stable" blank was obtained from Atlas Chemical Industries, DE. The Celite used as a filter aid in admixture with the charcoal was obtained from Johns-Manville Products Corp., New York, NY. The charcoal–Celite columns were prepared as follows: 40 g of charcoal was stirred overnight with 400 ml of concd HCl, filtered through Whatman No. 542 filter paper, and washed with water until the effluent pH was the same as the water used for washing. Forty grams of Celite was then added in 500 ml of water and stirred for 1 hr on a magnetic stirrer. The mixture was filtered and washed with absolute ethanol and then stirred with 20% ethanol–water mixture. The washed product was suspended in 200–300 ml of 20% ethanol. Small columns, 1 ml, of the uniformly-mixed suspension of charcoal–Celite were made in Pasteur pipettes.

The oligosaccharides found in milk and used for these studies were obtained from Dr. Adeline Gauhe. Preparation No. TC 150A represents the neutral oligosaccharide fraction obtained from human milk by defatting, deproteinizing, absorbing on charcoal (batchwise), and eluting with 30% ethanol after removing lactose with 10% ethanol. This preparation still contained salts and acidic oligosaccharides, and was further purified by treatment with Ba and Ag acetates. After filtration, the solution was passed through cation and anion exchange columns, and the effluent was freeze dried. This preparation was further purified in our laboratory by preparative paper chromatography in solvent A to remove the excess lactose.

The hog submaxillary glycoprotein with blood group A activity was prepared as already described (8).

A mixture of glycosidases was obtained from an extract of *Clostridium perfringens* and had been partially fractionated with ammonium sulfate

(50–52% saturation) (9). This was dialyzed against 0.9% saline to remove excess ammonium sulfate prior to use.

Methods. The material to be counted was dissolved in water, and 25 μl of the solution added to 10 ml of a toluene/Triton X-100 scintillation mixture (10). It was counted in the Nuclear Chicago Liquid Scintillation Counter (720 series, Des Plaines, IL).

Chromatography of sugars and oligosaccharides was performed on Whatman No. 1 paper by the descending technique and with the following solvent systems: *A*, 1-butanol–pyridine–water (6:4:3, v/v); *B*, phenol saturated with water.

The composition of the oligosaccharides and glycoproteins was also determined by the following colorimetric procedures: galactose and fucose by the anthrone procedure (11), total fucose after a 10-min heating period (12), total sialic acid by a modification (13) of the Svennerholm procedure (14), total hexosamine by the Rondle and Morgan modification (15) of the Elson-Morgan (16) reaction.

STANDARD ASSAY PROCEDURE

Reagents. (a) 0.2 M [^{3}H]KBH_4 (sp act 2.5 mCi/mmole) in 0.1 N KOH; (b) 0.01 M aqueous solution of sugar to be analyzed, e.g., glucose; (c) 1 N HCl.

Procedure. In the general assay procedure for glucose, aliquots of sugar solution were made up to a final volume of 100 μl with water and 50 μl of [^{3}H]KBH_4. The solutions were set aside at 4°C for 24 hr. Unreacted [^{3}H]KBH_4 was decomposed completely by the addition of excess HCl (150 μl). The samples were then carefully taken to dryness *in vacuo* in a desiccator to avoid splattering. The dried samples were then reconstituted with 200 μl of water and redried. This step was repeated once more with a further 200 μl of water, and the final product was reconstituted in 100 μl of water, of which 25 μl were taken for counting in the liquid scintillation counter. Figure 1 shows a typical calibration curve.

The acid-stable blank obtained with some batches of [^{3}H]BH_4^- was fairly high and represented a large percentage of the total counts. This was effectively eliminated by passage through a charcoal–Celite column, as follows: Reaction mixtures were set up, and aliquots of 25 μl were counted directly as before. A second aliquot of 25 μl was then applied to a 1-ml column of charcoal–Celite mixture and eluted with 1 ml of 20% ethanol. The eluate, containing the reduced sugar but not the acid-stable reagent blank, was then dried in the scintillation vial *in vacuo*. The resulting material was reconstituted with 200 μl of water and 10 ml of the scintillation fluid and counted. Figure 2 shows the effectiveness of the procedure for the removal of this acid-stable blank.

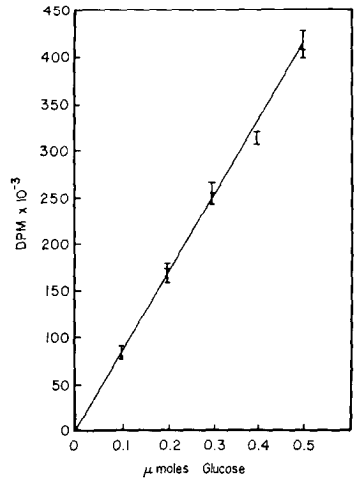

FIG. 1. Calibration curve for glucose. Glucose (0–0.5 μmole) contained in a final volume of 50 μl was incubated at 4°C for 24 hr with 50 μl of 0.2 M [^{3}H]KBH_4 (New England Nuclear Corp.) in 0.1 N KOH. See text for experimental conditions.

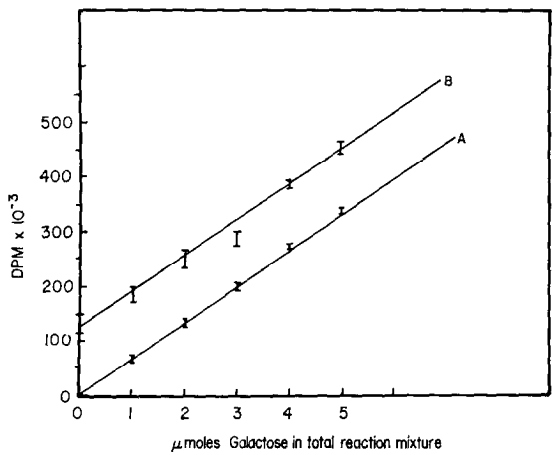

FIG. 2. Effectiveness of charcoal-Celite chromatography in the removal of the acid-stable contaminant from [^{3}H]BH_4^-. The reaction mixture contained 1–5 μmoles of galactose and 100 μmoles of [^{3}H]KBH_4, batch TRK 293 No. 9, in 0.1 N KOH and in a final volume of 100 μl. The solutions were incubated for 24 hr at 4°C, and then 25 μl counted directly after treatment in the *Standard Assay Procedure*. A second 25-μl aliquot from each incubation was applied to a charcoal–Celite column and treated as described in the text. The appropriate corrections for quenching and efficiency of counting were applied to the two sets of readings. (A) After charcoal treatment; (B) before charcoal treatment.

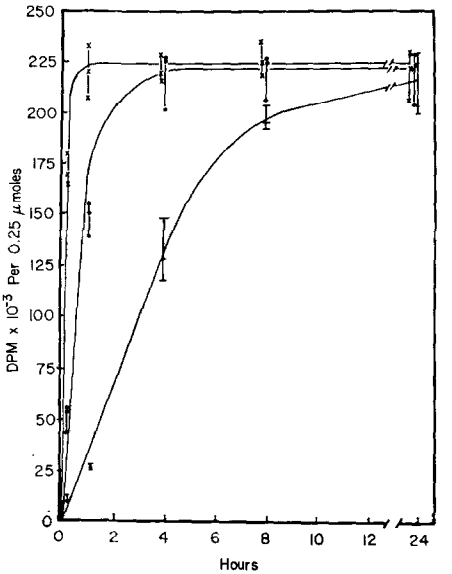

FIG. 3. Effect of temperature on the rate and extent of incorporation of tritium into glucose. Glucose (0.25 μmole) mixed with 10 μmoles of [^{3}H]KBH_4 in 100 μl of solution was incubated for varying periods of time at 4°C (|——|); 25°C (●——●); 37°C (×——×).

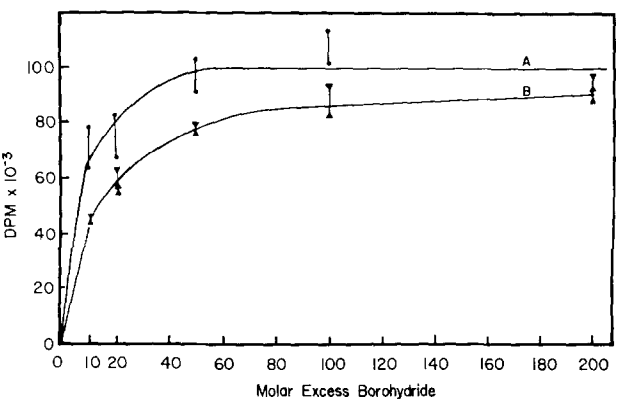

FIG. 4. Effect of excess [^{3}H]KBH_4 on the extent of reduction of glucose at 4°C for 5 hr (B) and for 48 hr (A). The final reaction mixtures contained 0.1 μmole of glucose and varying amounts of [^{3}H]KBH_4 (1, 2, 5, 10, or 20 μmoles) representing a 10-, 20-, 50-, 100-, or 200-fold *molar* excess of the reducing agent over the sugar.

Variables Affecting the Assay

Effect of Temperature. Reaction mixtures were prepared as in the standard procedure with a constant amount of glucose, and the resulting solutions were kept at different temperatures. Figure 3 shows the results obtained.

Effect of Excess [^3H]KBH_4. The effect of excess molar ratio of [^{3}H]KBH_4 to sugar on the extent of reduction of glucose at 4°C for 5 and 48 hr is shown in Fig. 4.

Applications of Assay

Reductive Equivalent of Monosaccharides and Disaccharides. Each reaction mixture contained 0.25 μmoles of the sugar and 10 μmoles of [^{3}H]KBH_4 in a final volume of 100 μl. Reduction of the sugar occurred at 4°C for 24 hr. The reaction was stopped by the addition of 150 μl of 1 N HCl, and the samples treated as in the *Standard Assay Procedure.* Triplicate samples of each sugar were taken, and the extent of reduction was compared to that of glucose. Table 2 shows the results obtained. With sialic acid and the hexosamine hydrochlorides, it was necessary to assure the alkalinity of the reaction mixture by the addition of 2.5 μmoles of 0.1 N KOH before reduction.

Purity of the Galactose Preparation. Five micromoles of galactose was reduced with [^{3}H]KBH_4 in the usual way (4°C, 24 hr), and the sample acidified, washed, and subjected to ion exchange chromatography on Dowex 50 (200–400 mesh, H^+ form) eluted with water, concentrated, and applied to Dowex 1 (200–400 mesh, OH^- form), again eluted and concentrated. Total recovery from the ion exchange resins was monitored by counting. The final concentrated eluate was applied to Whatman No. 1 paper and developed for 60 hr in solvent A. Strips of the chromatogram were stained with periodate and $AgNO_3$, and the spots visualized and compared to nonradioactive markers, galactitol, and lactitol. An unstained strip of the paper was cut into 1-in. strips, which were placed in counting vials together with 100 μl H_2O and 15 ml scintillation fluid, and counted. Radioactivity above a background of 50 cpm (17% efficiency) was found only in those areas corresponding to 2% lactitol and 98% galactitol.

Reduction of Milk Oligosaccharides. A solution containing 38 μmoles/ml of anthrone-positive material was prepared. Incubation mixtures containing 10 μl of the milk oligosaccharide solution, 40 μl of water, and 50 μl of [^{3}H]KBH_4 reagent (0.2 M in 0.1 N KOH) were prepared. The reaction mixtures were maintained at 4°C, and at predetermined intervals of time the reaction was stopped by acidification with 150 μl of 1 N HCl, and the samples treated as in the *Standard Assay Procedure.*

TABLE 2
The Reductive Equivalence and Standard Deviation in the Determination of Various Reducing and Nonreducing Sugars

Sugar	Reducing equivalent (%)	S[b]
Glucose	100	0.5
Galactose	91	5.7
Mannose	98	6.1
Fructose	104	9.3
Fucose	106	5.5
Glucose-1-Phosphate	0	—
Glucosamine–HCl	83	0.7
Galactosamine–HCl	88	3.7
N-Acetylgalactosamine	92	3.6
N-Acetylmannosamine	92	9.5
N-Acetylneuraminic Acid	86	2.5
Glucosamine–HCl[a]	98	5.9
Galactosamine–HCl[a]	103	8.3
N-Acetylglucosamine[a]	92	0.8
N-Acetylgalactosamine[a]	91	2.5
N-Acetylmannosamine[a]	96	2.6
N-Acetylneuraminic Acid[a]	102	4.0
Arabinose	107	7.4
Ribose	106	1.0
Xylose	109	2.7
2-Deoxyribose	114	5.2
2-Deoxyglucose	115	6.3
Lactose·H_2O	104	6.2
Melibiose	104	2.2
Turanose	76	17.8
Trehalose	0	—
Sucrose	0	—

[a] These sugars were neutralized and made alkaline by the addition of 0.1 N KOH prior to reduction with the tritiated borohydride. See text for the experimental details.

[b] The standard deviation was calculated for each sugar from the variance of these determinations from their mean and then expressed as a percentage of their mean.

Figure 5 shows a comparison of the rate of reduction of glucose and the milk oligosaccharides.

Rate and Extent of Hydrolysis of A+ Hog Submaxillary Glycoprotein with 0.5 N HCl at 100°C. The hydrolysis mixture was prepared as follows: 100 μl of 1% solution of the A+ (active) hog submaxillary glycoprotein, 400 μl of water, and 500 μl of 1 N HCl. The well-mixed solution was then transferred to 2-ml ampules and sealed. The ampules were placed in a refluxing water bath for predetermined intervals of time. After hydrolysis, aliquots from each ampule (run in duplicate)

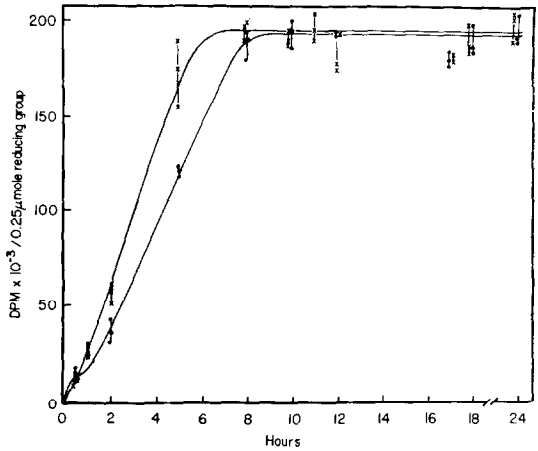

FIG. 5. The rate of reduction of 0.01 M glucose- (×——×) and lactose-free (○——○) milk oligosaccharides by [^{3}H]KBH_4 (0.2 M) at 4°C. Tubes contained 0.25 μmole glucose (0.01 M) or 0.25 μmole reducing groups from milk oligosaccharides (calculated from a previous experiment) and 10 μmole [^{3}H]KBH_4 (0.2 M) in a total volume (with water) of 100 μl. Reactions were stopped, and assays conducted as in the standard procedure.

were centrifuged, and 50 μl of the supernatants treated with 50 μl of 1 N KOH to neutralize the acid and assure alkalinity of the reaction mixture. The next step involved reduction of the free sugars released with 100 μl of [^{3}H]KBH_4 (0.2 M in 0.1 N KOH) at 4°C for 24 hr. The acid-stable radioactivity was measured in the usual manner. The rate of hydrolysis and maximum reductive equivalent is shown in Fig. 6. The

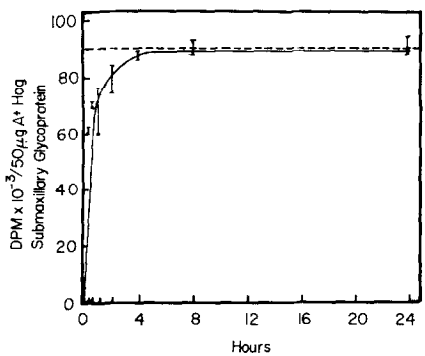

FIG. 6. Rate and extent of hydrolysis of A + hog submaxillary glycoprotein with 0.5 N HCl. See text for experimental details. (-----) The expected maximal amount of incorporation.

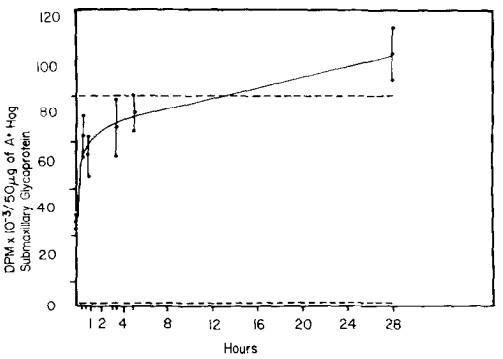

FIG. 7. Rate and extent of hydrolysis of A+ hog submaxillary glycoproteins with glycosidases. See text for experimental details. (- - - - -) The expected amount of incorporation.

expected incorporation of radioactivity is calculated from the amount of *N*-glycolylneuraminic acid, galactose, fucose, and *N*-acetylgalactosamine known to be present in the intact glycoprotein (8).

Rate and Extent of Hydrolysis of A+ Hog Submaxillary Glycoprotein with Glycosidases. An incubation mixture was prepared as follows: 0.25 ml of 1% solution of A+ hog submaxillary glycoprotein, 1.75 ml of 0.5 M phosphate buffer (pH 6.3), and 0.2 ml of the dialyzed enzyme preparation of *Clostridia* glycosidases. The solution was incubated at 37°C, and at predetermined time intervals 100-μl aliquots were removed, and the enzymic hydrolysis stopped by immersion for 2 min in a boiling water bath. Proteins were precipitated by the addition of 400 μl absolute ethanol to yield a final concentration of 80%. The solutions were centrifuged for 10 min at 88,000*g*, and aliquots of 200 μl of the supernate evaporated to dryness *in vacuo*, redissolved in KOH (50 μl of 0.1 N), and reduced with [^{3}H]KBH_4 (50 μl 0.2 M in 0.1 N KOH) at 4°C for 24 hr. Figure 7 shows the results obtained. The data are expressed as dpm $\times 10^3$ incorporated per 50 μg of A+ hog submaxillary glycoprotein to compare with the results obtained by acid hydrolysis of an equivalent amount of this glycoprotein. Both the enzyme blank and substrate blank were negligible.

RESULTS AND DISCUSSION

Most of the parameters of the reaction of the sugars with borohydride had already been determined using the NAD^+ assay (4,7) (see Footnote 2). These were reconfirmed using the radioactive assay procedure. It was further observed[2] that the rate of reduction of different sugars varies, but on completion of the reaction a simple stoichiometry was evident.

with most sugars having the same reductive equivalent. The notable exceptions were the acidic sugars like sialic acid, the hexosamine hydrochlorides, and *N*-acetylhexosamines.

The anomalous behavior of these sugars was eliminated by two measures, firstly by insuring that the solution of the sugar was strongly alkaline before the addition of the $[^3H]BH_4^-$ and secondly by carrying out the incubations at 4°C rather than at 45°C (Fig. 3). At the higher temperatures, the hexosamines, *N*-acetylhexosamines, and sialic acids are unstable and give the chromogen precursors typical of the colorimetric tests for these sugars (16–18). At 4°C the amount of borohydride necessary to achieve maximal reduction of the sugar was found to be 20–50-fold molar excess (Fig. 4).

In our early studies, the $[^3H]BH_4^-$ used was obtained from Amersham/Searle. These preparations had an acid-stable blank of about 0.7%. While normally that might be considered a low content of impurity, with a 20–50-fold molar excess of $[^3H]BH_4^-$ necessary for complete reduction of the sugars, the acid-stable blank becomes a serious problem. A number of different batches of borohydride from Amersham were used and all had a 'high' blank (Table 1). The best procedure to remove that "acid-stable" blank was to pass the reaction products, after reduction with borohydride and removal of excess borohydride by acidification, through a short column of charcoal–Celite as indicated in the Methods section. This was effective in removing all the "acid-stable" blank to give a calibration curve that passed through the origin and which was parallel with the calibration curve without using charcoal (Fig. 2). Where a good sample of $[^3H]BH_4^-$ was not available, a simple mathematical subtraction of the "acid-stable" blank of the borohydride could correct the error. A batch of borohydride obtained from the New England Nuclear Corp. (Table 1) had a negligible acid-stable blank, and the reagent gave a suitable calibration curve without the necessity of using charcoal (Fig. 1).

Using the *Standard Assay Procedure* with the borohydride from New England Nuclear Corp., it was possible to determine the reductive equivalent of a number of monosaccharides, reducing and nonreducing disaccharides. Table 2 shows the results obtained and indicates that nonreducing disaccharides, sucrose, and trehalose and aldoses substituted at position 1 (e.g., glucose-1-phosphate) do not react with borohydride. Within experimental error, the majority of hexoses, pentoses, ketose, and methylpentose react to the same extent as glucose, used as the standard for comparison. In view of the low reducing equivalent of galactose and its contamination with lactose, as was demonstrated by paper chromatography, it was of interest to utilize the radioactive assay to determine the

purity of the galactose. Chromatographic separation of the corresponding radioactive alditols and counting the radioactivity corresponding to galactitol and lactitol indicated a 98% purity of the galactose with 2% contamination with lactose.

The rate of reduction of complex oligosaccharides, such as those found in human milk (Fig. 5), appear to be analogous to that of the simple sugars and reducing disaccharides. Therefore, the extent of incorporation of radioactivity is directly proportional to the number of reducing groups available. Hence the method is ideally suited to the determination of rate of hydrolysis of glycoproteins with acid or enzymes (Figs. 6 and 7). Moreover, as can be seen from the data obtained on mild acid hydrolysis of A+ hog submaxillary glycoprotein, the total reductive equivalent at the completion of hydrolysis corresponds to that calculated from the quantities of individual sugars known to be present in this preparation. This illustrates the analytical versatility of the procedure. Chromatographic separation of the components in the acid hydrolysate presents a quick method of overall analysis of the amounts of each sugar present in the glycoprotein. The advantage of this simplicity is readily apparent. For instance, the determination of the composition of the carbohydrate components within the glycoprotein from A+ hog submaxillary glands required the determination of the sialic acid by the resorcinol method (13,14), galactose and fucose by the anthrone procedure (11), the fucose alone by the cysteine method (12), and the total hexosamine by the Elson-Morgan procedure (15,16). The overall estimate of each component further requires a correction factor for the different molar extinction coefficients of the sugars in the various determinations. For example, *N*-acetylneuraminic acid was used as the standard in the resorcinol test, whereas the sialic acid in hog submaxillary glycoproteins is known to be predominantly *N*-glycolyl (19,20), which gives 134% of the intensity given by *N*-acetylneuraminic acid (8).

The higher maximal reductive equivalence obtained with A+ hog submaxillary glycoprotein after enzymic hydrolysis is due to the small amount of *N*-acetylneuraminic acid aldolase present in the crude *Cl. perfringens* extract used. This enzyme would further cleave the *N*-glycolylneuraminic acid released with the sialidase in the enzyme preparation into *N*-glycolylmannosamine and pyruvate (9,21), thereby doubling the number of carbonyl groups available for reaction with the tritiated borohydride.

The analytical procedure lends itself to any degree of sensitivity required depending on the amount of sugar available for determination and the specific activity of the tritiated borohydride used. The method can, moreover, be used to determine the chain length of a given oligo-

saccharide of known composition, or alternatively, the molecular weight of a given reducing oligosaccharide if it is known to be pure.

The applicability of the method to the analysis of other classes of compounds known to react with borohydride is obvious, e.g., lactones and esters and the uronic acids in polysaccharides after esterification (22). The analysis of alkali-labile glycoproteins and the use of the method to give a fingerprint pattern of oligosaccharides in glycoproteins will be discussed in a subsequent publication.

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