Modification of Sialyl Residues of Sialoglycoprotein(s) of the Human Erythrocyte Surface*

(Received for publication, March 21, 1973)

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SUMMARY

Modification of erythrocyte membrane sialoglycoprotein(s) in isolated form, or when in membrane, is described using sequential sodium periodate oxidation and tritiated sodium borohydride reduction, essentially as suggested by Van Lenten and Ashwell ((1971) J. Biol. Chem. 246, 1889). Conditions of modification were investigated; under the optimal but nondestructive conditions selected, the modification appears specific for sialyl residues and leads to incorporation of tritium into the modified product. 5-acetamido-3,5-dideoxy-L-arabino-2-heptulosonic acid, identified by its susceptibility to neuraminidase and mild acid hydrolysis, and by its identity with the authentic compound in chromatographic behavior, several colorimetric assays, and extent of tritium incorporation.

The selectivity of the modification for the sialoglycoprotein(s) is further demonstrated by carrying out the modification on solubilized membrane proteins; only the sialoglycoprotein(s) incorporates the label. In the modification of the intact membranes some lipid components also become radioactive.

The modification of intact erythrocytes, the membranes, or the isolated sialoglycoprotein(s) results in loss of the M blood group activity, but the A and B blood group activities and the phytohemagglutinin binding sites are not affected. The modification offers a handle for isolation of membrane sialoglycoprotein(s) and study of their disposition, and possibly, for evaluation of the physiological role of the terminal polyhydroxy side chains of their sialyl residues.

MILD PERIODATE OXIDATION OF SEVERAL GYCO PROTEINS RESULTS IN SELECTIVE MODIFICATION OF THEIR SIALYL RESIDUES AND LEADS TO THE FORMATION OF A 7- OR 8-CARBON ALDEHYDE DERIVING (1-5). Subsequent reduction with sodium borohydride of these derivatives gives rise to the corresponding hydroxy analogues; if tritiated sodium borohydride is employed, these analogues then contain tritium. Several investigators have applied these reactions to several biologically active glycoproteins to study the possible functional significance of carbon atoms 7, 8, and 9 of the polyhydroxy positions of sialyl residues (2-5). In our laboratory these reactions were used to modify the sialyl residues of the human erythrocyte membrane sialoglycoprotein and, more generally, to provide a means for investigations of membrane sialoglycoproteins (6). In a previous study we have shown that the sialoglycoprotein can be modified in the intact erythrocyte, in the isolated erythrocyte membrane, or in its isolated form. In the current study we demonstrated further the specificity and selectivity of these chemical modifications, and considered the stoichiometry of the reactions. We also determined the effect of these modifications on certain immunological or serological properties of the membrane and of the isolated sialoglycoprotein.

MATERIALS AND METHODS

All chemicals employed were reagent grade. Tritiated sodium borohydride, NaBD₄ (200 mCi per millicurie) was purchased from New England Nuclear Corp., Boston, Mass. It was diluted with a 16-fold molar excess of carrier sodium borohydride. Each preparation was standardized by reduction of 4-p-nitrobenzamidobutyraldehyde (7). With this standard, specific activities in the range of 8.5 to 13.0 × 10⁶ dpm per μmole of reduced group were obtained with different preparations of the NaBD₄. Radioactivity was determined in a Packard Tri-Carb scintillation spectrometer using the Biosolve or Bray scintillation mixture.

N-Acetyleneuraminic acid (NANA)³ was purchased either from Sigma or Calbiochem and [4-¹⁴C]NANA (50 mCi per mmole), from New England Nuclear Corp. This preparation was diluted with a 8000-fold molar excess of carrier NANA, and its specific activity based on the thioarbituric acid assay was 6.4 μCi per mmole (8). Clostridium perfringens neuraminidase (Type VI) was obtained from Sigma Chemical Co. (1.1 unit per mg). Antiserum A, B, and M were obtained from Ortho Diagnostics.

1 Notation sialoglycoprotein(s) is adopted in the title since uncertainty still exists as to whether the protein is a single protein component or consists of a number of sialolglycopeptides; in the text, for simplification, the notation "sialoglycoprotein" is used.

2 The abbreviations used are: NANA, N-acetyleneuraminic acid; O-methyl-NANA, O-methyl ketoside of N-acetyleneuraminic acid; NANA₇-tet, 7-carbon analogue of NANA or 5-acetamido-3,5-dideoxy-L-arabino-2-heptulosonic acid; NANA₇-tet, 7-carbon analogue of NANA or 5-acetamido-3,5-dideoxy-L-arabino-2-heptulosonic acid.
and phytohemagglutinin was obtained from Wellcome Research Laboratories. Unialt silicic acid was purchased from Clarkson Chemical Co., Inc., Williamsport, Pa.

**Human Erythrocytes, Erythrocyte Membranes, Erythrocyte Membrane Solubilized Proteins, and Erythrocyte Membrane Sialoglycoprotein**—Human erythrocytes and erythrocyte membranes were prepared by the method of Dodge et al. (9) as described previously (10). The sialoglycoprotein was prepared using the procedure of Blumenfeld et al. (6) and Blumenfeld and Zvilichovsky (10). The water soluble membrane proteins were obtained by solubilization of membranes in 33% aqueous pyridine and dialysis (10); membrane proteins soluble in 0.1% sodium dodecyl sulfate were prepared by adding solid sodium dodecyl sulfate with stirring to a suspension of membranes in 0.005 M sodium phosphate buffer, pH 7.4 (hypotonic).

**Modification of and Introduction of Tritium Label into Erythrocyte Membranes, and Isolated Sialoglycoprotein**—The procedure described by Blumenfeld et al. (6) was followed. Sodium periodate was used in an amount not exceeding a 5-fold molar excess over the amount of NANA, and the oxidation was terminated by addition of glucose in one-half the molar quantity of periodate. In other experiments, as indicated under "Results" (see Figs. 2 and 3), sodium periodate concentration and pH of reduction with sodium borohydride were varied.

**Modification of and Introduction of Tritium Label into Sialoglycoprotein When in Soluble Membrane Protein Fraction**—To 30 mg of either 0.1% sodium dodecyl sulfate-soluble erythrocyte membrane proteins or water-soluble proteins obtained by the pyridine method in 10 ml 0.005 M sodium phosphate buffer at pH 7.4 was added 0.2 ml of 0.1 M sodium metaperiodate in the same buffer. The solutions were agitated frequently. After 10 min at room temperature, the oxidation was terminated by addition of 0.1 ml of 0.1 M glucose. The solution was dialyzed against cold distilled water and then against 0.005 M sodium phosphate buffer. The solution then was treated for 30 min at room temperature with 5 mg of tritiated sodium borohydride at pH 8.0. To terminate the reduction, the solution was acidified to pH 4 with 2 N acetic acid. After decomposition of excess tritiated sodium borohydride the pH was adjusted to 6 with 1 N NaOH. The solution was dialyzed exhaustively against distilled water. A control experiment was carried out as above except the step of oxidation with sodium periodate was omitted.

**Enzymatic or Acid Hydrolysis**—One milliliter of a solution of modified membrane, sialoglycoprotein, or modified sialoglycoprotein was prepared in 0.5 M sodium acetate buffer, pH 5.0. To this was added 0.32 or 0.64 unit of neuraminidase and the reaction mixture incubated at 37°C for periods of time indicated in Fig. 1.

Acid hydrolysis was carried out in 0.05 N H2SO4 at 80°C, for 1 hour, and the solution neutralized rapidly (it was found that the modified product obtained from standard NANA was labile in acid).

The amount of free NANA or modified NANA was determined by the thiobarbituric acid procedure (8) applied to unfraccionated incubation mixtures or to released products isolated as described below.

**Isolation of NANA or Modified NANA**—The products released by enzymatic or acid hydrolysis of the sialoglycoprotein were chromatographed on a Sephadex G-75 column (1 x 25 cm) using distilled water as eluant. One-milliliter fractions were collected. The products released by acid hydrolysis were neutralized before application to the column. The eluate was monitored by thiobarbituric assay or content of radioactivity, or both.

The released NANA or modified NANA was present in the retarded peak. The fractions of that peak were pooled and lyophilized.

NANA or modified NANA released from treated membranes was isolated in the supernatant fluid obtained by centrifugation of the incubation mixture at 15,000 x g for 20 min.

**Preparation of Standard O-Methyl Ketoside of N-Acetylneuraminic Acid and 5-Acetamido-5-Dideoxy-L-Arabinho-B-Hep-tulosonic Acid**—The procedure of Yu and Ledeen (11) was employed as follows. To 20 mg of NANA or [4-14C]NANA in 4 ml of absolute methanol was added 50 mg of Dowex 50-H resin. The reaction was carried out in a sealed tube at 75°C for 48 hours with occasional shaking. The resin was filtered and washed with methanol. The filtrate and washings were evaporated in vacuo, yielding a yellowish viscous solution. The steps involved in the hydrolysis of the methyl ester were carried out as described by Yu and Ledeen (11). The β-methyl ketoside was oxidized with a 6-fold excess of sodium metaperiodate for 1 hour at room temperature; the oxidation was terminated by addition of an equimolar quantity of barium acetate, and the mixture was filtered. The filtrate was concentrated to a small volume and reduced with a 5-fold excess of tritiated sodium borohydride at room temperature for 1 hour. Reduction was terminated by addition of 1 N acetic acid to pH 4. Borate salts were eliminated by flash evaporation of the reaction mixture with 3 ml of methanol, five times. The β-methyl ketoside of NANA or NANA, was obtained by chromatography of the residue as described below and elution from paper. Standard NANA was obtained by hydrolysis of the β-methyl ketoside in water at 100°C for 60 min.

**Isolation and Characterization of NANA and Products of Modification of NANA**—Descending chromatography on Whatman No. 3MM paper for 16 hours at room temperature was used. The solvent systems employed were: Solvent A, n-butyl acetate-acetic acid-water (3:2:1); Solvent B, n-butyl alcohol-n-propyl alcohol-0.1 M HCl (1:2:1); Solvent C, n-butyl alcohol-acetic acid-water (5:2:2). The paper was dried; a vertical strip was then cut and sprayed with thiobarbituric acid (12) or resorcinol (13), or dipped in AgNO3 (14). The remaining chromatogram was cut into 0.5- to 1-cm horizontal strips; each strip was then placed into a counting vial and its radioactivity was determined.

**Extraction and Fractionation of Lipid Components**—The procedure described by Dittmer and Wells (15) was used with minor modifications. To 8 ml of modified membranes were added 17 volumes of chloroform-methanol (2:1). After agitation of the mixture for 30 min, the organic phase was evaporated to dryness under nitrogen and redissolved in 2 ml of chloroform. It then was chromatographed on a column (1 x 10 cm) of silicic acid. The column was eluted first with 30 ml of chloroform, then with 40 ml of methanol. The fraction eluted with chloroform usually consists of fatty acids and simple steroids, and that eluted with methanol, consists of phospholipids, sphingolipids, and glycolipids. Both fractions were evaporated to dryness and radioactivity was determined. Two-dimensional thin layer chromatography was performed to separate the complex lipids. The developing solvent systems consisted of chloroform-methanol-water-acetic acid (65:43:3:1, v/v), and chloroform-methanol-water (60:35:8, v/v). The lipid components were detected with iodine vapor. The radioactivity of each spot was determined.

**Hemagglutination Inhibition Test**—The test human erythrocytes were suspended in 0.9% NaCl. Hemagglutination titrations were performed by the modified method of Salk (16). That amount of antiserum just causing the agglutination of 50 μl of
2% erythrocytes in a final volume of 150 μl, in 30 min at room temperature, is defined as 1 hemagglutination unit. Hemagglutination inhibition was determined with serial 2-fold dilutions of inhibitor, in 0.9% NaCl solution. Hemagglutination inhibitory activity is defined as the least quantity of protein, in micrograms, that prevents the agglutination of 50 μl of 2% red cells by 1 hemagglutination unit of antiserum.

Polyacrylamide Gel Electrophoresis—The procedure described by Fairbanks et al. (17) and Shapiro et al. (18) was used. Electrophoresis was performed on parallel gels; one was stained with Schiff reagent for carbohydrate, the other with Coomassie stain for proteins. Radioactivity was determined in the Coomassie stained gels using the procedure of Tishler and Epstein (19). Since the modified NANA that contains the radioactive label is labile in acetic acid, the gels were sliced promptly. For such gels the staining procedure of Fairbanks et al. (17) was modified as follows: the gels were stained in 25% aqueous isopropyl alcohol for proteins. Radioactivity was determined in the Coomassie Schiff reagent for carbohydrate, the other with Coomassie stain containing 0.025% Coomassie blue for 15 hours and then desorbed in 10% acetic acid.

Other Analytical Procedures—Fucose was determined by the procedure of Dische and Shettles (20). NANA and NANA2, or NANA3,4,6Id, were determined by the thiobarbituric acid assay (8), resorcinol (21), or direct Ehrlich (22) procedures. Amino acid content was determined with N-methyl benzothiazolone hydrazone using the spectrophotometric and colorimetric procedures of Paz et al. (23). Amino acid analysis was performed on a Jelco model JLC 6AH amino acid analyzer after hydrolysis of the protein, sealed in vacuo in 6 M HCl at 105°C for 22 hours. Protein concentration was computed from the content of amino acids or by the reaction described by Lowry et al. (24).

Results

Sialyl Residues as Sites of Incorporation of Tritium Label in Sialoglycoprotein of Erythrocyte—As shown previously (6), mild oxidation with sodium periodate followed by reduction with tritiated sodium borohydride results in incorporation of tritium label into the sialoglycoprotein of the erythrocyte. This occurs whether the sialoglycoprotein is modified in its isolated form or is present in situ in the intact erythrocyte or the isolated membrane.

As shown in Fig. 1, the modified sialoglycoprotein or modified membrane is a substrate for neuraminidase, the rate of hydrolysis being slower than for the respective controls. This was previously observed by Suttajit and Winzler (3) for modified orosomucoid. One should note that the rate of release of radioactivity parallels the release of products reacting with thiobarbituric acid.

Seventy-six per cent of the tritium label was released from the modified sialoglycoprotein by neuraminidase and 100% by mild acid hydrolysis (see Table I). No release of radioactivity occurred in controls if neuraminidase were omitted. This suggests that in the case of the sialoglycoprotein, sialyl residues were selectively modified; the release from the modified membrane was less extensive due, most probably, to the partial incorporation of label into membrane lipids (see below). When the product released from either the modified sialoglycoprotein, the modified membrane, or its sialoglycoprotein by enzymatic or acid hydrolysis was chromatographed on paper, a single radioactive band was obtained in several solvent systems (see Table II) that reacted with thiobarbituric acid, resorcinol, and with AgNO3 and whose mobility was identical with that of the synthetic NANA. No band with mobility of intact NANA was apparent suggesting that the modification reactions had been complete; the absence of other radioactive bands suggests that no other carbohydrate components had been modified significantly (2, 25). A colorimetric determination of fucose revealed it to be unmodified. In some preparations of sialoglycoprotein a nonradioactive, fast moving band (Band B) staining exclusively with AgNO3 is seen in the modified protein but not in the control; its origin is not clear.

![Fig. 1. Hydrolysis with Clostridium perfringens neuraminidase;](image)

**TABLE I**

Release by neuraminidase of Clostridium perfringens and by acid hydrolysis of bound tritium from modified sialoglycoprotein and modified membranes

<table>
<thead>
<tr>
<th>% Total radioactivity</th>
<th>Neuraminidase</th>
<th>0.05 N H2SO4</th>
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</thead>
<tbody>
<tr>
<td>Modified sialoglycoprotein</td>
<td>76</td>
<td>100</td>
</tr>
<tr>
<td>Modified membranes</td>
<td>55</td>
<td>73</td>
</tr>
</tbody>
</table>

**TABLE II**

Chromatographic behavior of products released from modified sialoglycoprotein and modified membrane or its sialoglycoprotein by neuraminidase or acid hydrolysis

<table>
<thead>
<tr>
<th>Mobility in solvent</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard NANA</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Synthetic NANA</td>
<td>2.00</td>
<td>1.43</td>
<td>1.42</td>
</tr>
<tr>
<td>Modified sialoglycoprotein</td>
<td>1.91</td>
<td>1.43</td>
<td>1.39</td>
</tr>
<tr>
<td>Band A</td>
<td>4.54</td>
<td>1.70</td>
<td>5.00</td>
</tr>
<tr>
<td>Modified membrane</td>
<td>2.00</td>
<td>1.37</td>
<td>2.17</td>
</tr>
<tr>
<td>Sialoglycoprotein isolated from modified membrane</td>
<td>2.00</td>
<td>1.37</td>
<td>2.17</td>
</tr>
</tbody>
</table>

a Mobility relative to NANA. All bands reacted positively with AgNO3, thiobarbituric acid or resorcinol except band B which did not react with thiobarbituric acid or resorcinol.

b The commercially purchased standard NANA was first purified from other minor derivatives (for example, O-glycolyl) by chromatography on Solvent C. The mobility of pure NANA = 1.

c The bands contained tritium in all three solvents.

d Upon treatment with neuraminidase, all preparations of modified sialoglycoprotein showed exclusively the presence of Band A; some preparations showed in addition Band B. Treatment with 0.05 N H2SO4 for 1 hour at 80° resulted in additional bands (not indicated here) which reacted only with AgNO3 and were also present in acid-treated controls.

e Released by neuraminidase.

f Released by treatment with 0.05 N H2SO4 for 1 hour at 80°.
Effect of sodium periodate treatment on the sialoglycoprotein. The standard O-methyl-[4-14C]-NANA was oxidized with sodium periodate and then reduced with sodium borohydride, pH 8.0, for 2 hours; reduction was performed with a 30-fold molar excess of NaBH₄ over NANA. The modified sialoglycoprotein, when compared to the modified control treated with neuraminidase, was shown to have a higher mobility. The Coomassie-stained gel patterns of modified sialoglycoprotein and the modified control treated with neuraminidase showed that the reaction was specific and could be used for quantitative conversion of NANA to NANA. The reactivity in the thiobarbituric acid reaction when the assay is performed as described by Warren (8), with the oxidation step at room temperature, was used to further ascertain that a quantitative conversion of NANA to O-methyl-NANA occurred. In Table IV is shown the specific activity of O-methyl-[4-14C]-NANA, which was used to further ascertain that a quantitative conversion of NANA to O-methyl-NANA occurred. The modified sialoglycoprotein was treated with neuraminidase, when compared to the modified control, revealed a broader, less intense Schiff-staining band of higher mobility. The Coomassie-stained gel patterns of modified membranes treated with neuraminidase and the modified control were altered, suggesting membrane protein degradation probably due to prolonged incubation conditions; but the major Schiff-staining band was present showing a lesser intensity and a considerable decrease in radioactivity.

Stoichiometry of Modification—As noted by Spiro (25) and Van Leuten and Ashwell (26), the product resulting from modification of sialyl residues, similar to that described here, reacts in several colorimetric assays used for intact NANA but with different yields of color. Color equivalents of similar magnitude were obtained for the modified sialoglycoprotein (see Table III). In addition, a comparable reactivity in the colorimetric assays was observed for products of modification of standard O-methyl-NANA. On the basis of the color value in the thiobarbituric acid assay of NANA, obtained by sequential oxidation and reduction of O-methyl-NANA, one may conclude that sialyl residues of the sialoglycoprotein are quantitatively converted to NANA. The determinations with resorcinol and Ehrlich's reagent are less specific and cannot be used for strict quantitation. In view of the apparently reduced color value of the periodate oxidized and periodate oxidized and then reduced NANA in the thiobarbituric acid assay, O-methyl-[4-14C]NANA was used to further ascertain that a quantitative conversion of NANA to O-methyl-NANA occurs. In Table IV is shown the specific activity of O-methyl-[4-14C]NANA, and O-methyl-[4-14C]-NANA using the thiobarbituric acid assay. It can be concluded that NANA, and O-methyl-NANA show close to a 50% decrease in reactivity in the thiobarbituric acid reaction when the assay is performed as described by Warren (8), with the oxidation step at room temperature. However, when the oxidation is carried out at 37° the reactivity of O-methyl-NANA with thiobarbituric acid is increased.

An attempt was also made to quantitate the conversion of NANA to O-methyl-NANA using tritiated sodium borohydride calibrated by reduction of p-nitrobenzamidobutyraldehyde. If, as usually assumed, in the reduction of this standard 1 eq of tritium is incorporated (7), it was found that in the reduction of NANA, to NANA, only approximately 0.5 eq of tritium is incorporated; the value of this magnitude was found in the reduction of both the standard O-methyl-NANA, and the periodate treated sialoglycoproteins.

To ascertain that the reduction of NANA, proceeded to completion the content of aldehydes was determined in O-methyl-[4-14C]NANA, before and after reduction with sodium borohydride. As shown in Table V the reduction proceeds to completion. It thus appears that less tritium is incorporated in the reduction of NANA, than in the reduction of p-nitrobenzamidobutyraldehyde. The reason for this difference is not clear and may reside in structural differences between the two aldehydes, but it is probable that the use of O-methyl-NANA, for calibration of NaBH₄ is more justified here. Using the specific activity value of NaBH₄, calibrated in this manner leads to the conclusion that a quantitative conversion of NANA to NANA occurs in the reduction of the sialoglycoprotein.
Table VI
Comparison of specific activity of NANA isolated from modified O-methyl NANA and the modified sialoglycoprotein

<table>
<thead>
<tr>
<th></th>
<th>Specific activity (dpm/mole)</th>
</tr>
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<tbody>
<tr>
<td>Standard p-nitrobenzamido butanol&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13.9 x 10^4</td>
</tr>
<tr>
<td>NANA isolated from modified O-methyl NANA</td>
<td>7.3 x 10^4</td>
</tr>
<tr>
<td>NANA released by neuraminidase from the modified sialoglycoprotein&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.3 x 10^6</td>
</tr>
</tbody>
</table>

<sup>a</sup> Obtained by reduction of standard p-nitrobenzamido butaldehyde; the tritiated sodium borohydride used for reduction of this aldehyde was the same as that used for reduction of periodate-treated standard O-methyl-NANA or sialoglycoprotein.

<sup>b</sup> Number of micromoles was estimated from absorbance at 200 nm using molar extinction coefficient $E = 11.3$ (7).

<sup>c</sup> Number of micromoles estimated from thiobarbituric acid assay; the extinction of O-methyl-NANA was used.

<sup>d</sup> The same specific activity is found in the intact isolated modified sialoglycoprotein.

Fig. 3. Reduction with tritiated sodium borohydride of sodium periodate-oxidized sialoglycoprotein (a→c) and intact membranes (d). Molar ratio of sodium borohydride to NANA, 30:1; b, c, and d, in 0.008 M sodium phosphate buffer, pH 8.0; in b at the indicated time, pH value was adjusted to pH 12.0 and additional NaBH₄ was added (final molar ratio, 60:1); a, in 0.008 M sodium phosphate buffer, pH was adjusted to pH 9.2.

Fig. 2. Effect of oxidation of sialoglycoprotein (left) and erythrocyte membranes (right) with varying concentrations of sodium periodate, upon subsequent reduction with tritiated sodium borohydride. Conditions of oxidation: 0.008 M sodium phosphate buffer, pH 7.4, 10 min, 25°C; conditions of reduction: 30-fold molar excess NaBH₄/NANA in 0.008 M sodium phosphate buffer, final pH 8.0, 1 hour, 25°C.

(see Table VI). This conclusion is consistent with results obtained upon chromatography and in the thiobarbituric acid assays (see Table III).

Conditions for Modification of Sialoglycoprotein and Intact Membrane—For modification of the sialyl residues, optimum and nondestructive conditions were sought. As seen in Fig. 2, in the case of the sialoglycoprotein, oxidation with sodium periodate, using mild conditions, leveled off rapidly when the molar ratio of periodate to NANA was nearly 2; the oxidation thus appeared selective for sialyl residues.

The oxidation of the intact membrane was less specific since lipid components also were oxidized (see below). Oxidation of membranes by use of concentrations of sodium periodate greater than 0.01 M was avoided since, even though the modified sialoglycoprotein could be readily isolated, changes in gel patterns of the other membrane proteins were noted.

As shown in Fig. 3, reduction of sodium periodate-treated sialoglycoprotein occurred readily and extension of time or use of higher pH values did not cause significantly greater incorporation of radioactivity. A 10 to 20% variation in the uptake of tritium among different preparations was noted and perhaps reflected differences in contents of sialyl residues (10). Reduction was usually conducted at pH 5 for 30 min at room temperature, and more alkaline conditions were avoided to prevent β-elimination of seryl- and threonyl-bound oligosaccharides and possible cleavage of peptide bonds (7). As shown previously (6) and found here, reduction of control samples, i.e. of sialoglycoprotein, intact membranes or erythrocytes, or solubilized membrane proteins not previously treated with sodium periodate, did not result in significant incorporation of radioactivity.

Selectivity of Modification for Sialoglycoprotein—When intact erythrocytes or the isolated erythrocyte membranes are subjected to sequential mild periodate oxidation and reduction with tritiated sodium borohydride, as shown previously (6), the tritium label predominantly appears in the sialoglycoprotein. In the present study, this kind of chemical modification was extended to solubilized erythrocyte membrane proteins. Specifically, membrane proteins, rendered water-soluble by treatment of intact membranes with aqueous pyridine and dialysis, were sequentially treated with sodium periodate and tritiated sodium borohydride. Fig. 4 shows gel electrophoretic patterns and distribution of radioactivity obtained when the proteins had first been modified in this manner. As observed with the intact erythrocyte or the erythrocyte membrane, only one radioactive band was present with mobility corresponding to the Schiff-staining band of the sialoglycoprotein. The modification thus appeared selective for the sialoglycoprotein. This finding in fact was expected, because this component of the erythrocyte membrane is the major protein with a high content of NANA. Membrane proteins solubilized and modified in 0.1% sodium dodecyl sulfate showed a decreased incorporation of tritium and exhibited no clear gel patterns. Yet, modification of the isolated sialoglycoprotein in 0.1% sodium dodecyl sulfate resulted in a preparation of the same specific activity and electrophoretic mobility as that of the control.

Incorporation of Tritium into Membrane Lipids—As shown in Table I, neuraminidase or mild acid treatment released only a portion of the tritium from intact membranes that had been modified as described. As noted previously, some membrane lipids also incorporate tritium as the result of the periodate and reduction treatments or simply the reduction treatment (6). Extracts of the modified membranes obtained with methanol-
chloroform contained about 30% of total incorporated radioactivity; thus approximately 1% of total lipids had incorporated tritium. Further fractionation of the radioactive lipid components revealed that tritium was not present in the neutral lipid fraction but was associated with compounds which, on thin layer chromatography, had the mobility of phospholipids such as phosphatidyl ethanolamine or phosphatidyl serine. The labeled lipid compounds were not identified further.

**Effect of Modification on Antigenic and Phytohemagglutinin Binding Sites of Erythrocyte Membrane and of Isolated Sialoglycoprotein**—Using the hemagglutination inhibition assay, the sequential oxidation with periodate and reduction with sodium borohydride were found to have had no effect on the activity of the A and B blood group receptors or phytohemagglutinin binding sites of the erythrocyte membrane (whether isolated from modified erythrocyte or modified intact form) or of the isolated sialoglycoprotein. However, the M blood group activity was affected (see Table VII). Control samples, subjected exclusively to tritiated sodium borohydride reduction, were not affected by this treatment.

**DISCUSSION**

A number of procedures have recently been described for chemical modification and introduction of various labels into cell surface components (20-28). The purpose of such modification is to mark a specific component, so that its identification, location, or disposition may be facilitated. A more general purpose is the provision of a label for the cell membrane per se. Most of the reagents used in such procedures show reactivity toward one or more functional side chains of protein components and suffer from lack of selectivity for a particular group of proteins, unless the proteins have a location immediately accessible to the reagents. Use of these reagents, in most instances, does not allow one easily to demonstrate that the modification is specific for the functional groups of a particular protein nor the extent to which a specific modification has occurred.

**TABLE VII**

<table>
<thead>
<tr>
<th>Preparation used</th>
<th>Hemagglutination inhibitory activity</th>
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<tbody>
<tr>
<td></td>
<td>Anti-</td>
</tr>
<tr>
<td></td>
<td>sera A</td>
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<tr>
<td>Membranes isolated from erythrocytes</td>
<td></td>
</tr>
<tr>
<td>Not treated</td>
<td>0.22</td>
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<tr>
<td>Treatment with sodium borehydride</td>
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<td>Treatment with sodium periodate</td>
<td>0.22</td>
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<tr>
<td>Modified by treatment with</td>
<td></td>
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<td>sodium periodate and sodium</td>
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<tr>
<td>borohydride</td>
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<td>Intact membranes</td>
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<td>Not treated</td>
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<td>Treatment with sodium borehydride</td>
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<td>Treatment with sodium periodate</td>
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<td>Modified by treatment with</td>
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<td>sodium periodate and sodium</td>
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<tr>
<td>borohydride</td>
<td>0.22</td>
</tr>
<tr>
<td>Sialoglycoprotein preparation</td>
<td></td>
</tr>
<tr>
<td>Not treated</td>
<td>0.02</td>
</tr>
<tr>
<td>Treatment with sodium borehydride</td>
<td>n.d.</td>
</tr>
<tr>
<td>Treatment with sodium periodate</td>
<td>0.02</td>
</tr>
<tr>
<td>Modified by treatment with</td>
<td></td>
</tr>
<tr>
<td>sodium periodate and sodium</td>
<td></td>
</tr>
<tr>
<td>borohydride</td>
<td>0.02</td>
</tr>
</tbody>
</table>

* Hemagglutination unit.

* Amount of protein just sufficient to cause inhibition; content of protein is calculated from the total content of determined amino acids.

* n.d., not determined.

The chemical modification described in this study offers a selectivity for membrane proteins containing sialyl residues. Whether the intact erythrocyte, the isolated membrane or solubilized membrane proteins are treated by the method described, only one class of proteins incorporates the tritium label, namely, the major erythrocyte sialoglycoprotein. Experiments on the isolated protein have now shown that its sialyl residues are the groups modified predominantly.

By use of neuraminidase or mild acid hydrolysis, NANA, which differs from NANA in lacking the two terminal hydroxymethyl groups, was shown to be the product of the modification and, indeed, incorporated the tritium label. This was demonstrated by identity of the product with a sample of the authentic compound, in chromatographic behavior, reactivity in several colorimetric assays, and the extent of tritium incorporation. The modification of the sialoglycoprotein resulted in incorporation of tritium equivalent in quantity to the original content of...
The use of tritiated sodium borohydride calibrated in this manner allows a direct quantitative estimation of NANA. This value was based on calibration of tritiated sodium borohydride using O-methyl-NANA as standard. The use of tritiated sodium borohydride calibrated in this manner showed that the inhibition of phytohemagglutinin-induced agglutination of red cells is not affected by removal of sialic acid from an erythrocyte glycophosphoty protein bearing the phytohemagglutinin receptor sites (32). Suttajit and Winzler observed that mild periodate oxidation followed by sodium borohydride reduction of a number of glycoproteins, including the erythrocyte sialoglycoprotein, leads to reduction of inhibitory activity of influenza virus hemagglutination (3).

The above observations suggest that sialyl residues, and more precisely the two terminal hydroxymethyl groups of sialyl residues, may in part be responsible for a given biological property of a cell. The function of these terminal hydroxymethyl groups in the biological activities of glycoproteins, such as those of ceruloplasmin and several protein hormones, has been studied similarly (2-5). We have used conditions similar to those described here for studying effects on the surface of lymphocytes. Upon oxidation with sodium periodate normal, but not leukemic, lymphocytes undergo blastogenesis and become refractory to further stimulation by phytohemagglutinin; in vivo these cells regenerate. These effects are reversed upon subsequent sodium borohydride reduction (33). Similar studies were reported by Novogrodsky and Katchalski (34) who recently have shown that the blastogenic response to oxidation with periodate is markedly reduced by previous treatment of lymphocytes with neuraminidase or papain (35).

We have shown that sequential oxidation with sodium periodate and reduction with sodium borohydride specifically modify sialyl residues of a membrane sialoglycoprotein and may be used to label selectively such sialoglycoprotein in isolated form or in situ. These reactions thus could serve as a label by which to follow the oxidation of membrane sialoglycoproteins and for studies of their disposition. The evaluation of the physiological consequences of such modifications in membranes implicates at the very least, the terminal polyhydroxy side chains of bound sialyl residues.

Acknowledgment—We wish to thank Miss Linda Bovard for her excellent technical assistance.

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Modification of Sialyl Residues of Sialoglycoprotein(s) of the Human Erythrocyte Surface
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