Selective Radioactive Labeling of Cell Surface Sialoglycoproteins by Periodate-tritiated Borohydride*

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Low concentrations of sodium metaperiodate induce specific oxidative cleavage of sialic acids between carbon 7 and carbon 8 or carbon 8 and carbon 9. The aldehydes formed can easily be reduced with NaB\textsubscript{3}H\textsubscript{4} to tritiated 5-acetamido-3,5-dideoxy-L-arabino-2-heptulosonic acid or 5-acetamido-3,5-dideoxy-L-arabino-2-octulosonic acid. At 0\textdegree, the periodate anion penetrates the cell plasma membrane very slowly and only externally exposed sialic acids are oxidized. This was shown by (a) limited labeling of the sialoglycoproteins in a preparation of inside-out erythrocyte vesicles; (b) trapping \textsuperscript{14}C-labeled fetuin within resealed erythrocyte ghosts; fetuin was then poorly labeled, whereas the erythrocyte sialoglycoproteins were highly labeled; (c) comparison of labeled glycoproteins of mouse lymphoid cells before and after treatment with neuraminidase. This simple method of specifically introducing a radioactive label into cell surface sialic acids is useful in the study of cell surface sialic acid-containing glycoproteins.

Numerous methods are available to specifically label cell surface proteins and lipids, and much of our knowledge about the molecular organization of the cell surface is based on the use of such methods (1–5). However, very few techniques have been developed to label cell surface carbohydrates, although developments in this field would be of great practical importance (see Refs. 6 and 7 for recent reviews). The galactose oxidase-NaB\textsubscript{3}H\textsubscript{4} method (8, 9) for labeling cell surface galactosyl-N-acetylgalactosaminyl residues has proved very useful and combined with fluorography of electrophoretically separated erythrocyte surface proteins, has permitted detection of numerous minor surface glycoproteins (10).

Incubation of living cells with cytidine monophosphate-N-acetylneuraminic acid is expensive and the specific radioactivities of the labeled products are low; (b) the need for sialyltransferases and the availability of suitable acceptors; and (c) it is possible that cytidine monophosphate-N-acetylneuraminic acid is hydrolyzed and the free acid penetrates into the cell where it could be used for the formation of cytidine monophosphate-N-acetylmuramic acid, which in turn could cause intracellular labeling.

Mild periodate treatment of glycoproteins results in selective modification of sialic acids. After reduction with tritiated sodium borohydride, tritium-labeled 5-acetamido-3,5-dideoxy-L-arabino-2-heptulosonic acid is the major product formed (12–14). AcNeu\textsuperscript{1} is sensitive to cleavage by neuraminidase (13) and is easily released by mild acid treatment (9). Glycoproteins labeled by this technique have often been used to study their turnover in plasma (12).

This method has been used for labeling of membrane glycoproteins. Liao et al. (15) obtained specific labeling of human erythrocyte sialoglycoproteins and carefully characterized the labeled products. The periodate-tritiated borohydride technique has also been applied to the labeling of sialoglycoproteins of erythrocyte variants (16), and very recently, high resolution of erythrocyte sialoglycoproteins was seen after labeling with this method (17). Sialoglycoproteins of various other cells have also been studied (18, 19).

With the exception of the erythrocyte, where all the sialic acid is external, the method has not been considered to be specific for the cell surface and no careful attempts have been made to evaluate its potential use in this respect. We now report that by performing the oxidation at 0\textdegree, using short incubation times and low concentrations of periodate, the label is specific for external sialic acid-containing glycoproteins.

MATERIALS AND METHODS

Preparation of Cells

Erythrocytes—Fresh or recently outdated blood was used as source of human erythrocytes. The cells were washed by centrifugation four times in 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.4 (NaCl/PO\textsubscript{4}).

Lymphoid Cells—Mouse thymuses and spleens (CBA/HT876 mice) were teased apart in cold RPMI 1640 culture medium. Clumps were removed by filtration through cotton wool. Erythrocytes were lysed with an 0.85% aqueous solution of NH\textsubscript{4}Cl and phagocytic cells were removed after incubation with carbonyl iron by treatment with a magnet (20). The spleen cell preparation contained more than 80% lymphocytes, as judged morphologically from May-Grunewald-Giemsa-stained smears. The cells will be referred to in the text as thymocytes and lymphocytes.

Fractionation of T and B Lymphocytes

Spleen lymphocytes were fractionated into T and B cells by free flow cell electrophoresis as described previously (21). After fractionation...
tion more than 90% of the cells were viable as judged by trypan blue exclusion tests. The T cell fraction contained less than 5% of surface immunoglobulin-carrying cells (B cells) and the B cell fraction less than 2% of 6-antigen-carrying (T) cells.

Membrane Preparations

Isolation of erythrocyte membranes has been described previously (8). Resealing of erythrocyte ghosts containing fetuin was done as follows. Isolated, packed ghosts were incubated with an equal volume of 0.3 M NaCl, 0.005 M MgCl₂, 0.02 M sodium phosphate, pH 7.4, containing 2 mg/ml of [¹⁴C]fetuin (specific activity 45,450 cpm/mg of protein). After incubation at 25°C for 10 min, the membranes were washed three times in NaCl/P0₄, 1 mM MgCl₂ buffer (22). Further washings did not reduce the specific radioactivity of the [¹⁴C]fetuin-containing resealed ghosts.

Inside-out erythrocyte vesicles were prepared according to Stock and Kant (22). The vesicles were fractionated on a Dextran T 110 continuous gradient, density 1.01 to 1.05 g/ml, made in 0.5 mM sodium phosphate, pH 5.0, with a Spinco SW 27 rotor at 25,000 rpm for 16 h in a Beckman L4 ultracentrifuge. The resealed inside-out vesicles were collected from the top of the gradient. The degree of contamination by right-side-out vesicles and unsealed ghosts was assayed by incubation with Vibrio cholerae neuraminidase in Dulbecco's NaCl/P0₄ containing Ca²⁺ ions either in the presence or absence of 1% Triton X-100 (22).

Chemicals

Sodium metaperiodate was from Merck AG, Darmstadt, Germany. Acrylamide and N,N'-methylene-bisacrylamide were obtained from Eastman Kodak Co., Rochester, N. Y. 2,2-Di-phenylglyoxal (PPO) and p-bis-(2-phenylglyoxalyl)benzene (POP0) were obtained from New England Nuclear, Boston, Mass. Phenylmethylsulfonyl fluoride and crystalline N-acetylneuraminic acid were from Sigma. Triton X-100 was purchased from British Drug Houses Chemicals Ltd., Poole, England. Bovine fetuin (99% pure) was obtained from Grand Island Biologicals. Tritiated sodium borohydride (8.2 Ci/mmol) and [¹⁴C]formaldehyde (4.54 mCi/mmol) were obtained from the Radiochemical Centre, Amersham, England. The NaBH₄ preparation was handled as described previously (18).

Enzymes

Galactose oxidase with a specific activity of 130 units/mg of protein was purchased from Kabi AB, Stockholm, Sweden. It displayed no protease or neuraminidase activities when measured as described previously (8). Vibrio cholerae neuraminidase (500 units/ml) was obtained from Behringwerke AG, Marburg-Lahn, Germany. It was free of protease activity.

Labeling of Proteins with [¹⁴C]formaldehyde

This was done essentially according to Rice and Means (23). The 5 mg of fetuin or the standard proteins used for electrophoresis were dissolved in 0.05 ml of 0.5% NaHCO₃. Then 3 µl (6 µCi) of [¹⁴C]formaldehyde was added. After 3 min at room temperature, the proteins were reduced three times with 5 µl of NaBH₄ (5 mg/ml) at 1 min intervals to stabilize the Schiff bases formed. 1 ml NaCl/P0₄ was added and the samples were dialyzed at 4°C for 48 h against distilled water.

Polycrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed according to Laemmli (24) as described previously (10, 16, 18) using either cylindrical or slab gels. The acrylamide concentration in the separating gels was 8%. The treatment of the slab gels for fluorography (23) and slicing and counting of the cylindrical gels has been described (10, 16).

Chemical Determinations

Protein was measured according to Lowry et al. (26) with bovine serum albumin as standard. Sialic acid was quantitated by the thiobarbituric acid method (27) with crystalline N-acetylneuraminic acid as standard. For determination of total sialic acid, hydrolysis was performed in 0.1 H₂SO₄, at 80°C for 1 h.

Labeling of Cells or Membranes with Periodate-oxidized Sodium Borohydride

Washed, packed erythrocytes (1 ml) or unsealed ghosts or membrane vesicles derived from 1 ml of cells were incubated in NaCl/P0₄, in a total volume of 2 ml either at 22°C or on ice with indicated concentrations of sodium metaperiodate for different times. After incubation, 0.2 ml of 0.1 M glycine in NaCl/P0₄, was added, the samples washed twice with NaCl/P0₄, by centrifugation, and reduced with 0.5 M of tritiated sodium borohydride per tube for 90 min at room temperature in a total volume of 2 ml of NaCl/P0₄. The cells or membranes were washed three times by centrifugation and membranes were isolated from the cells. Resealed, labeled ghosts containing fetuin were washed in NaCl/P0₄, 1 mM MgCl₂, three times, lysed in 5 mM sodium phosphate buffer, pH 8.0, and centrifuged. The supernatants were concentrated, and these and the membranes were counted for radioactivity in Bray's solution (28) in a Wallace-LKB 8100 liquid scintillation counter.

For labeling of mouse lymphoid cells 180 × 10⁸ thymocytes and 90 × 10⁶ B cells were divided into three equal aliquots. One tube of each cell type was used for labeling with the galactose oxidase method. To another tube was added 12.5 units of neuraminidase and the tubes incubated in 1 ml of Dulbecco's NaCl/P0₄, containing Ca²⁺ ions for 30 min at 37°C. Another set of tubes was incubated in the same way but without neuraminidase. The cells were washed twice by centrifugation in NaCl/P0₄, and suspended in 1 ml of NaCl/P0₄. The cells were then treated with periodate at a final concentration of 1 mM on ice for 5 min, 0.2 µl of 0.1 M glycine in NaCl/P0₄, was added to quench the reaction, and the cells were washed three times in NaCl/P0₄. After they were suspended in 0.5 ml of NaCl/P0₄, the cells were reduced with 0.5 M of tritiated sodium borohydride per tube for 30 min at room temperature and washed three times in NaCl/P0₄. At this stage, more than 90% of the cells were viable as shown by exclusion of trypan blue. Then 0.2 ml of NaCl/P0₄, containing 5% of phenylmethylsulfonyl fluoride (as protease inhibitor) was added on ice. After 15 min, the samples were centrifuged for 10 min at 4000 rpm at 4°C to remove nuclei and the supernatants taken for counting and electrophoresis (8, 16).

Cell Surface Labeling by Galactose Oxidase Method

This was done as described previously (8, 16). Cells or membranes were incubated in 2 ml of Dulbecco's NaCl/P0₄, with 12.5 units of neuraminidase and 5 units of galactose oxidase for 30 min at 37°C, washed, and reduced with tritiated sodium borohydride as for the periodate-oxidized samples.

Identification of Tritiated 5-Acetamido-3,5-dideoxy-L-arabino-2-heptulosonic Acid

Erythrocyte membranes, labeled by periodate-NaBH₄, were treated with 0.1 M H₂SO₄ at 80°C for 1 h. The samples were neutralized with NaOH, centrifuged at 4000 rpm for 10 min, and the supernatants passed through Sephadex G-50 columns (1 × 20 cm) made with distilled water. Aliquots were counted for radioactivity, and the radioactive fractions pooled and lyophilized. N-Acetylneuraminic acid standard, tritiated AcNeu² prepared according to Liao et al. (15), and the radioactive compounds from the erythrocyte samples were chromatographed on Whatman No. 5MM paper with 1-butanol-1-propanol-water (6:2:2 v/v) as solvent. The N-acetylneuraminic acid standard was visualized after staining with alkaline silver nitrate (29). The paper strips containing the radioactive samples were cut into 1-cm pieces, placed into scintillation vials, incubated with 1 ml of water for 60 min, and the radioactivities determined using Bray's solution.

RESULTS

Labeling of Erythrocyte Membranes with NaBH₄, after Periodate Treatment - Labeling with NaBH₄ is dependent on the concentration of periodate (Fig. 1A). With 1 ml of packed cells, the maximal incorporation of radioactivity is obtained with 1 to 2 µmol periodate which is 6 to 12 times more periodate to sialic acids. The oxidation is rapid. There is already an appreciable oxidation after 30 s which is essentially complete after 10 min (Fig. 1B).

Table I shows that with low concentrations of periodate most of the radioactivity was released by 0.1 M H₂SO₄, at 80°C for 60 min. Somewhat more radioactivity was released from membranes treated with periodate at 0°C. All the released label co-migrated with standard AcNeu² on paper chromatography and with an R₅ value of 1.34 to 1.00 for N-acetylneuraminic acid.
In Fig. 2 are shown the fluorography patterns of slab gels of membrane proteins from periodate-oxidized and NaB³H₄-reduced erythrocytes. The major bands correspond to the sialoglycoproteins PAS 1 to 3. Without periodate treatment, no radioactive band is obtained (Fig. 2b). High concentrations of periodate gave weaker bands (Fig. 2, h and i). Short incubation times gave weaker bands, but the differences are not very remarkable (Fig. 2, j to o).

Comparison of Erythrocyte Glycoproteins Labeled by Galactose Oxidase and Periodate Techniques—Fig. 3 shows a sodium dodecyl sulfate-polyacrylamide gel electrophoresis slab gel pattern of glycoproteins labeled after galactose oxidase and periodate treatments. By the galactose oxidase technique combined with neuraminidase treatment, in addition to the sialoglycoproteins, Band 3 and the minor glycoproteins are labeled, and these are not as well labeled after periodate oxidation. A further difference is the slower mobility of PAS 2 after neuraminidase plus galactose oxidase treatment than after periodate treatment (Fig. 3, a and b).

Labeling of Fetuin by Periodate-NaB³H₄ within Resealed Erythrocyte Membranes—Penetration of periodate through erythrocyte membranes was studied by incorporating the sialic acid-rich glycoprotein fetuin within resealed ghosts. Quantitation of trapped fetuin was possible by using ¹⁴C-labeled fetuin. Table II shows the incorporation into memb-

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**Table I**

<table>
<thead>
<tr>
<th>Periodate concentration</th>
<th>Labeled at 22°</th>
<th>% released</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 mM</td>
<td>54.5</td>
<td>61.8</td>
</tr>
<tr>
<td>0.5 mM</td>
<td>68.2</td>
<td>61.0</td>
</tr>
<tr>
<td>1 mM</td>
<td>67.5</td>
<td>68.5</td>
</tr>
<tr>
<td>2 mM</td>
<td>65.2</td>
<td>68.5</td>
</tr>
<tr>
<td>5 mM</td>
<td>51.1</td>
<td>70.5</td>
</tr>
<tr>
<td>10 mM</td>
<td>43.0</td>
<td>68.5</td>
</tr>
<tr>
<td>20 mM</td>
<td>41.1</td>
<td>59.4</td>
</tr>
</tbody>
</table>
Radioactive Labeling of Cell Surface Sialoglycoproteins

Incorporation of tritium from NalYH, into periodate-treated resealed membranes and trapped fetuin

<table>
<thead>
<tr>
<th>Periodate concentration</th>
<th>Membranes</th>
<th>Fetuin</th>
<th>Fetuin/membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>mm</em></td>
<td><em>cpm/ug N-acetylneuraminic acid</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>At 22°</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>6,080</td>
<td>1,520</td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>53,400</td>
<td>17,100</td>
<td>0.33</td>
</tr>
<tr>
<td>0.5</td>
<td>90,000</td>
<td>19,800</td>
<td>0.22</td>
</tr>
<tr>
<td>1</td>
<td>104,000</td>
<td>14,600</td>
<td>0.14</td>
</tr>
<tr>
<td>2</td>
<td>105,000</td>
<td>12,100</td>
<td>0.11</td>
</tr>
<tr>
<td>5</td>
<td>81,000</td>
<td>9,300</td>
<td>0.11</td>
</tr>
<tr>
<td>At 0°</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>6,000</td>
<td>970</td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>28,100</td>
<td>3,410</td>
<td>0.12</td>
</tr>
<tr>
<td>0.5</td>
<td>52,800</td>
<td>4,610</td>
<td>0.08</td>
</tr>
<tr>
<td>1</td>
<td>60,000</td>
<td>5,090</td>
<td>0.08</td>
</tr>
<tr>
<td>2</td>
<td>64,500</td>
<td>6,010</td>
<td>0.09</td>
</tr>
<tr>
<td>5</td>
<td>66,400</td>
<td>6,420</td>
<td>0.09</td>
</tr>
</tbody>
</table>

Sialic acid released by neuraminidase from erythrocyte membranes

The 200 μg of membrane protein was incubated with 25 units of *Vibrio cholerae* neuraminidase for 30 min at 37° in 1 ml of Dulbecco's NaCl/PO, containing Ca²⁺ ions and the liberated sialic acid determined.

<table>
<thead>
<tr>
<th>Membranes</th>
<th>Membranes + 1% Triton X-100</th>
<th>Intact/disrupted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact membraones</td>
<td>4.03</td>
<td>41.9</td>
</tr>
<tr>
<td>Ghosts</td>
<td>33.9</td>
<td>30.6</td>
</tr>
</tbody>
</table>

branes of fetuin at 22° and 0°. The specific radioactivities of the membranes are higher after treatment at 22° than at 0°. At 22°, fetuin is clearly labeled by H after treatment with low concentrations of periodate, but at 0°, fetuin is not extensively labeled. Fig. 4 shows the sodium dodecyl sulfate-gel electrophoresis patterns of the fetuin peak fractions obtained after labeling at 22° and 0°. A clearly higher extent of labeling is seen on treatment at 22° than at 0°.

Labeling of Inside-out Erythrocyte Vesicles versus Unsealed Membranes — The inside-out erythrocyte vesicle preparation contained about 10% unsealed or right-side-out vesicles. This was indicated by the accessibility of membrane sialic acids to *Vibrio cholerae* neuraminidase in untreated as compared to Triton X-100-disrupted vesicles (Table III). When inside-out vesicles and unsealed membranes were labeled by the galactose oxidase method combined with neuraminidase treatment, the specific radioactivity in inside-out vesicles was clearly lower than that of unsealed membranes (Table IV). The difference for the periodate-treated membranes was not that clear. With equimolar concentrations of periodate to sialic acid (0.2 μmol with 0.1 mM periodate, Table IV), most radioactivity could be released by 0.1 M H₂SO₄ at 80° for 1 h. With a 10-fold molar excess of periodate to sialic acid, much of the radioactivity was acid resistant. The released radioactivity was all recovered as AcNeu. Fig. 5, A to H, shows the corresponding sodium dodecyl sulfate-polyacrylamide gel electrophoresis patterns. Both after neuraminidase plus galactose oxidase treatment or after periodate treatment, the major labeled peaks correspond to the sialoglycoproteins PAS 1 to 3. Here it can clearly be seen that the labeling is much more pronounced in these proteins from unsealed membranes than from the inside-out vesicle preparations.

Labeling of Lymphoid Cell Glycoproteins — Mouse thymocytes and T and B lymphocytes were labeled after treatments with neuraminidase plus galactose oxidase or periodate. Cells that had been pretreated with neuraminidase were used as controls. The specific radioactivities are given in Table V. Fig. 6 shows that the most radioactive bands obtained by the two labeling techniques exactly correspond to each other. The only obvious exceptions are bands GP9₁ and GP9₂ of Fig. 6, b and c, and e and f, respectively. These bands have previously been shown to derive from the same protein (18). Treatment of intact cells with neuraminidase before labeling removes most

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1 For nomenclature of mouse lymphoid cell glycoproteins see Ref. 18.
FIG. 5. Sodium dodecyl sulfate gel electrophoresis patterns of erythrocyte membranes and resealed inside-out erythrocyte vesicles labeled by the neuraminidase-galactose oxidase and periodate techniques. Membranes or vesicles containing 72 µg of sialic acid were labeled in all cases with 0.5 mCi of NaB₃H₄ after treatment with neuraminidase plus galactose oxidase or periodate in a total volume of 2 ml. A, unsealed membranes labeled with NaB₃H₄ after treatment with neuraminidase plus galactose oxidase; B, inside-out vesicles labeled after treatment with neuraminidase plus galactose oxidase; C, unsealed membranes labeled after treatment with 0.1 mM periodate for 5 min at 0°C; D, inside-out vesicles labeled after treatment with 0.1 mM periodate for 5 min at 0°C; E, unsealed membranes labeled after treatment with 1 mM periodate for 5 min at 0°C; F, inside-out vesicles labeled after treatment with 1 mM periodate for 5 min at 0°C; G, unsealed membranes labeled without enzyme or periodate treatments at 0°C; H, inside-out vesicles labeled without enzyme or periodate treatments at 0°C. BPB, position of bromphenol blue marker dye; L, lipid peak.
with 1 mM periodate; j, glycoproteins of B lymphocytes labeled after treatment with neuraminidase and 1 mM periodate. The thymocyte glycoproteins labeled after treatment with neuraminidase and 1 mM periodate; i, glycoproteins of T lymphocytes labeled after treatment with neuraminidase plus galactose oxidase; h, glycoproteins of B lymphocytes labeled after treatment with neuraminidase and 1 mM periodate. The thymocyte glycoproteins labeled after treatment with neuraminidase plus galactose oxidase; g, glycoproteins of T lymphocytes labeled after treatment with neuraminidase and 1 mM periodate; f, glycoproteins of T lymphocytes labeled after treatment with neuraminidase and 1 mM periodate; e, glycoproteins of T lymphocytes labeled after treatment with neuraminidase and 1 mM periodate; d, glycoproteins of thymocytes labeled after treatment with neuraminidase; c, glycoproteins of thymocytes labeled after treatment with neuraminidase plus galactose oxidase; b, glycoproteins of thymocytes labeled by NaB\textsubscript{3}H\textsubscript{4} after treatment with neuraminidase plus galactose oxidase; a, pattern of \textsuperscript{14}C-labeled standard proteins; b, glycoproteins of thymocytes labeled by NaB\textsubscript{3}H\textsubscript{4} after treatment with neuraminidase plus galactose oxidase; c, glycoproteins of thymocytes labeled after treatment with 1 mM periodate; d, glycoproteins of thymocytes labeled after treatment with neuraminidase and 1 mM periodate; e, glycoproteins of T lymphocytes labeled after treatment with neuraminidase plus galactose oxidase; f, glycoproteins of T lymphocytes labeled after treatment with 1 mM periodate; g, glycoproteins of T lymphocytes labeled after treatment with neuraminidase and 1 mM periodate; h, glycoproteins of B lymphocytes labeled after treatment with neuraminidase plus galactose oxidase; i, glycoproteins of B lymphocytes labeled after treatment with neuraminidase and 1 mM periodate. The thymocyte preparations used for electrophoresis contained material derived from 7.7 x 10^6 cells, the T cell preparations from 3.75 x 10^6 cells, and the B cell preparations from 2.91 x 10^6 cells. The nomenclature of the labeled glycoproteins is from Ref. 18.

**TABLE IV**

<table>
<thead>
<tr>
<th>Membrane samples containing 72 (\mu)g of N-acetylneuraminic acid were treated with the enzymes or periodate in a total volume of 2 ml and labeled with 0.5 mCi of NaB\textsubscript{3}H\textsubscript{4} per sample.</th>
<th>Neuraminidase plus galactose oxidase</th>
<th>Neuraminidase plus 1 mM periodate</th>
<th>% radioactivity released by mild acid*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inside-out vesicles</td>
<td>86,000</td>
<td>12,500</td>
<td>25,700</td>
</tr>
<tr>
<td>Ghosts</td>
<td>335,000</td>
<td>8,300</td>
<td>38,600</td>
</tr>
</tbody>
</table>

* Membrane samples were treated with 0.1 M H\textsubscript{2}SO\textsubscript{4} for 60 min at 80°C.

**TABLE V**

Incorporation of tritium from NaB\textsubscript{3}H\textsubscript{4} into mouse lymphoid cells

<table>
<thead>
<tr>
<th>Membrane samples containing 72 (\mu)g of N-acetylneuraminic acid were treated with the enzymes or periodate in a total volume of 2 ml and labeled with 0.5 mCi of NaB\textsubscript{3}H\textsubscript{4} per sample.</th>
<th>Neuraminidase plus galactose oxidase</th>
<th>Neuraminidase plus 1 mM periodate</th>
<th>% radioactivity released by mild acid*</th>
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<td>25,700</td>
</tr>
<tr>
<td>Ghosts</td>
<td>335,000</td>
<td>8,300</td>
<td>38,600</td>
</tr>
</tbody>
</table>

* Membrane samples were treated with 0.1 M H\textsubscript{2}SO\textsubscript{4} for 60 min at 80°C.

**DISCUSSION**

Most, if not all cell surface proteins contain carbohydrate, and surface-associated glycoproteins and glycolipids are considered important in a variety of surface-mediated phenomena, as intercellular adhesion (30-32), growth control (33,34), and malignancy (35-37). Therefore, development of techniques for studying these structures are of great importance.

Rather recently it became possible to specifically introduce a radioactive label into cell surface galactosyl and N-acetylgalactosamyl residues of glycoproteins and glycolipids by use of galactose oxidase (8, 9). This method has proved very useful and has been applied to a large number of different cells (8, 9, 16, 18, 19). By combination with neuraminidase, sialic acid-containing surface molecules may tentatively be identified. However, it is possible that removal of sialic acids from the cell surface will change the exposure of glycoproteins and this could lead to misinterpretations. The structure of the sialoglycoproteins is also rather drastically changed by removal of sialic acids, and functional changes may occur (12).

Because of this, a more direct approach would be preferable. Low concentrations of periodate are quite specific for oxidation of sialic acids, and after reduction with tritiated borohydride radioactive AcNeu\textsuperscript{2} is formed (12-14). This method has not been considered to be specific for the cell surface. Only in erythrocytes, where it is known that all sialic acid is external (38), there have not been problems in interpreting the labeling results. Some phospholipids are also labeled after periodate treatment, but these have not been studied (15).

The rationale of obtaining a rather specific labeling of cell surface sialic acids by the periodate-NaB\textsubscript{3}H\textsubscript{4} method is based on oxidation at 0°, low concentrations of periodate, and short reaction times. At this temperature, the membrane is “frozen” and transport of anions may be relatively low. On the other hand, the oxidation of available sialic acids proceeds well at 0°, lower concentrations of periodate, and short reaction times. At this temperature, the membrane is “frozen” and transport of anions may be relatively low. The rationale of obtaining a rather specific labeling of cell surface sialic acids by the periodate-NaB\textsubscript{3}H\textsubscript{4} method is based on oxidation at 0°, low concentrations of periodate, and short reaction times. At this temperature, the membrane is “frozen” and transport of anions may be relatively low.
in the sialoglycoproteins of resealed inside-out erythrocyte vesicles is clearly smaller. In the neuraminidase plus galactose oxidase-treated membranes (Fig. 5, A and B), the difference in labeling is very clear. This is similar to the results of Steck and Dawson (9). In the periodate-treated membranes, the difference between labeling of unsealed versus inside-out membranes is also obvious. It is reasonable to assume that much of the label in region 1 or 3 in the inside-out vesicle preparation is due to the presence of contaminating vesicles with sialic acids readily available. This may be more important for periodate-treated membranes than for neuraminidase plus galactose oxidase-treated membranes because a higher ratio of periodate to available sialic acids will result in more efficient oxidation.

To show a more general application of the periodate-\(\text{NaB}^3\text{H}_4\) method, we have labeled mouse lymphoid cells both by the galactose oxidase and periodate techniques. As previously shown, thymocytes and T and B lymphocytes show characteristic surface glycoprotein patterns when labeled by the neuraminidase-galactose oxidase technique (18). When compared to the patterns obtained after periodate labeling, they are remarkably similar. This strongly supports the surface specificity of the periodate labeling technique. The only major difference is in bands GP9 and GP9\(^\alpha\) and this is due to the neuraminidase treatment, which results in a higher apparent molecular weight. It is interesting to note that removal of sialic acids by neuraminidase increases the apparent molecular weight and this further points out the problems associated with determination of molecular weights of glycoproteins by sodium dodecyl sulfate-gel electrophoresis (40). The controls where intact cells have been pretreated with neuraminidase also show that all major glycoproteins labeled after periodate treatment most probably are surface glycoproteins because during short incubation times, the enzyme should not penetrate the cell plasma membrane extensively.

The periodate-\(\text{NaB}^3\text{H}_4\) method could be especially useful to study cell surface glycopeptides of malignant cells which are known to be highly sialylated (41-43). It may also be possible to specifically label cell surface gangliosides by this technique. The cheap reagents, the short reaction times, and the use of chemical probes instead of more or less labile or impure enzymes are obvious advantages.

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