MODIFICATION AND INTRODUCTION OF A SPECIFIC RADIOACTIVE LABEL INTO THE ERYTHROCYTE MEMBRANE SIALOGLYCOPROTEINS+

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Received June 1, 1972

SUMMARY

A specific radioactive label was introduced into the sialoglycoproteins of the erythrocyte membrane by sequential sodium periodate oxidation and tritiated sodium borohydride reduction. This was achieved whether the sialoglycoproteins were isolated present *in situ* within the intact erythrocyte, or the isolated erythrocyte membranes. The label is found in the oligosaccharide chains of the sialoglycoproteins predominantly in residues which were formerly those of bound sialic acid. The method appears selectiv for the sialoglycoproteins and certain, as yet unidentified lipid components.

INTRODUCTION

The important role of cell surface protein, carbohydrate and lipid components in many functional phenomena of cellular function has received wide attention (1,2). The surface sialoglycoproteins of the erythrocyte are among the few such membrane components which have been isolated in pure form (3-6). To facilitate studies of these proteins and to develop procedures for studies on related proteins in other cells we have modified and introduced a specific label into the oligosaccharide chains of these sialoglycoproteins. This was achieved whether the sialoglycoproteins were isolated, present *in situ*, either as components of the erythrocyte, or the isolated erythrocyte membrane. A Smith degradation procedure employed here was modified from one described by Van Lenten and Ashwell (7); this involves a mild periodate oxidation of sialic acid residues followed by tritiat

+This work was supported by USPHS Grants #AM-05821, #GM-16389, #AM-12791 and #HE-13979 from the National Institutes of Health.

[1]National Institutes of Health Development Award #GM-5998.

[2]Recipient of NIH Career Award, #AM-19,435.

sodium borohydride reduction of the aldehyde product obtained after the periodate treatment.

MATERIALS AND EXPERIMENTAL PROCEDURES

Human Erythrocytes, Erythrocyte Membranes and Erythrocyte Membrane Sialoglycoproteins

Erythrocytes and erythrocyte membranes were prepared by the procedure of Dodge, Mitchell and Hanahan (8) as described (4,5). The pure sialoglycoprotein was prepared from erythrocyte membranes by aqueous pyridine solubilization followed by ethanol fractionation (4,5).

Tritiated Sodium Borohydride

Tritiated sodium borohydride $^{3}[H]$ $NaBH_4$ (200 μc/μmole) was purchased from New England Nuclear Corporation, Boston, Mass. For these studies it was diluted with a 16 fold molar excess of carrier sodium borohydride or sodium borodeuteride*. Each preparation was standardized by reduction of 4-p-nitrobenzaminobutyraldehyde (9). A specific activity of 8.5-13.0 x 10^6 dpm/μmole of reduced group was usually obtained (see TableII).

Modification and Introduction of Tritium Label into Sialoglycoproteins when Present In Situ Within the Intact Erythrocytes or the Isolated Membranes

In a typical experiment, to 50 ml portions of either packed erythrocytes or isolated membranes in isotonic sodium phosphate buffer at pH 7.4 (8) were added 1 ml of 0.1 M sodium metaperiodate in the same buffer; this represents approximately a 10 fold molar excess relative to content of sialic acid. Another portion of erythrocytes or erythrocyte membranes was used as controls, omitting the periodate oxidation step. The solutions were frequently agitated, and after 10 minutes at room temperature the oxidation reaction was terminated by addition of 25 ml of the isotonic sodium phosphate buffer and centrifugation; the cells were centrifuged for 10 minutes at 370 x g, and membranes for 20 minutes at 15,600 x g, at 4° C. (In certain experiments the oxidation was terminated by addition of an equimolar quantity of ethylene glycol without apparent

*In some experiments tritiated sodium borohydride was diluted with sodium borodeuteride as carrier, to facilitate subsequent mass spectral analysis which is not in the scope of this communication. The results for this communication with tritiated sodium borohydride and borodeuteride appear identical.

difference**.) They were then washed with 2 more portions of 25 ml of the isotonic buffer and centrifuged as above. The cells or membranes were then reduced with tritiated sodium borohydride by adding to each 7 mg dissolved in 2 ml of the isotonic sodium phosphate buffer. The reduction was allowed to proceed at pH 7.4 for 20 minutes, at room temperature, with frequent agitation. To terminate the reduction 25 ml of the isotonic sodium phosphate buffer were added and the cells or membranes centrifuged as above and washed two more times with isotonic sodium phosphate buffer. The erythrocytes were then lysed with hypotonic sodium phosphate buffer (~ 0.01 M pH 7.4) and membranes prepared. These membranes, or the membranes which were directly oxidized and reduced were dialyzed against distilled water prior to isolation of their surface glycoproteins.

Modification and Introduction of Tritium Label into Isolated Sialoglycoproteins

To 10 mg of sialoglycoproteins in 5 ml of water was added 0.6 ml of 0.2 M sodium metaperiodate. (This represents approximately a 15 fold molar excess relative to the content of sialic acid.) The oxidation was allowed to proceed for 10 minutes at 4° and was terminated by addition of 0.6 ml of 0.2 M ethylene glycol** or 0.6 ml of 0.1 M glucose. The solution was dialyzed against water and then against 0.1 M sodium phosphat buffer pH 7.1. 3.5 mg of tritiated sodium borohydride was then added and reduction allowed to proceed for 30 minutes at pH 7.1. The reduction was terminated by acidificatio with 2N HCl to pH 4. After decomposition of excess tritiated sodium borohydride the pH was brought to pH 6.0 with NaOH and the solution dialyzed exhaustively against H_2O and lyophilized. In a control experiment the sialoglycoproteins were similarly treated but the sodium periodate oxidation step was omitted.

Polyacrylamide Gel Electrophoresis

The procedure described by Fairbanks, Steck and Wallach (10) was used. Electrophoresis was performed on parallel gels; one was stained with Coomassie stain for proteins, the other with the Schiff stain for carbohydrates. Radioactivity was determine in the Coomassie stained gel using the procedure of Tishler and Epstein (11). The gels

**In more recent experiments we have avoided the use of ethylene glycol to prevent the possible reaction of formaldehyde, formed by periodate oxidation of ethylene glycol, from reacting with the protein or other membrane components.

were sliced promptly since the modified sialic acid which contains the radioactive label is labile in acetic acid.

RESULTS

Mild periodate oxidation followed by reduction with tritiated sodium borohydride leads to modification and introduction of tritium label into the surface sialoglycoproteins of the erythrocyte. This can be accomplished readily whether the isolated proteins, the erythrocytes, or the isolated membranes are subjected to the sequential oxidation and reduction procedures. This is shown in figure 1 where the distribution of the labelled membrane proteins is shown. Whether membranes were labelled directly, or prepared from erythrocytes which had been labelled, the radioactivity appears predominantly in the modified surface sialoglycoproteins as evidenced by the correlation of radioactivity with the Schiff staining protein bands. Other membrane protein components are apparently not labelled significantly. However, some membrane lipid components in both the periodate treated and control reduced membranes show an uptake of tritium; these tritium containing lipid components appear close to the front of the gels (fig. 1A and B). Moreover, as shown in Table I they are extractable into chloroform-methanol (2:1) and can be chromatographed on thin layers of silica gels in chloroform, methanol, H_2O (75:25:4). Since the proteins of control erythrocytes or membranes, not treated with sodium periodate, but reduced, show essentially no uptake of radioactivity it can be concluded that periodate oxidation gives rise to protein bound residues which become labelled.

When the modified surface sialoglycoproteins are isolated from the treated cells or membranes, preparations of specific activities shown in Table II are obtained. Their gel electrophoretic patterns and distribution of radioactivity are shown in figure 2. In this figure and Table II are also included preparations of modified sialoglycoproteins which we first isolated and then periodate oxidized and reduced. In each case the sialoglycoproteins became radioactive. No such uptake of tritium is noted in the control sialoglycoproteins. Using standardized tritiated sodium borohydride it is possible to compute the number of groups which become tritium labelled under each condition. As can be seen in Table II the sialoglycoproteins obtained from treated intact erythrocytes

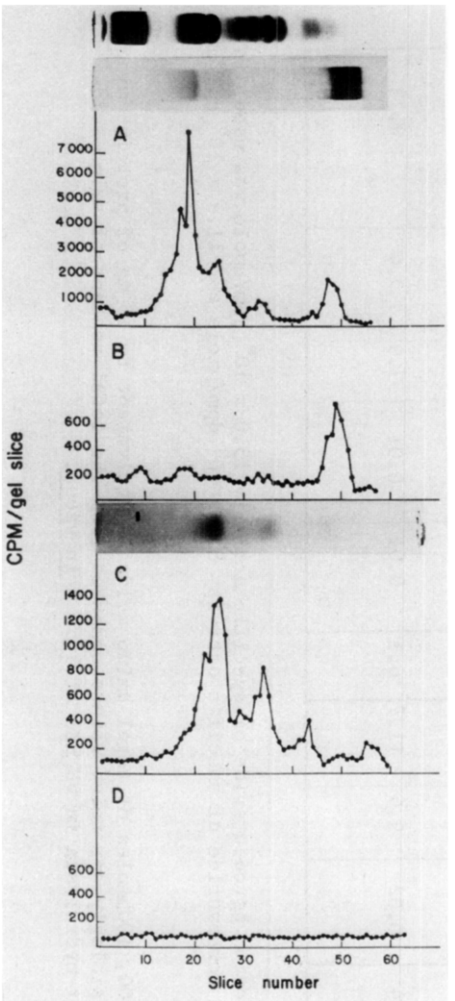
TABLE I

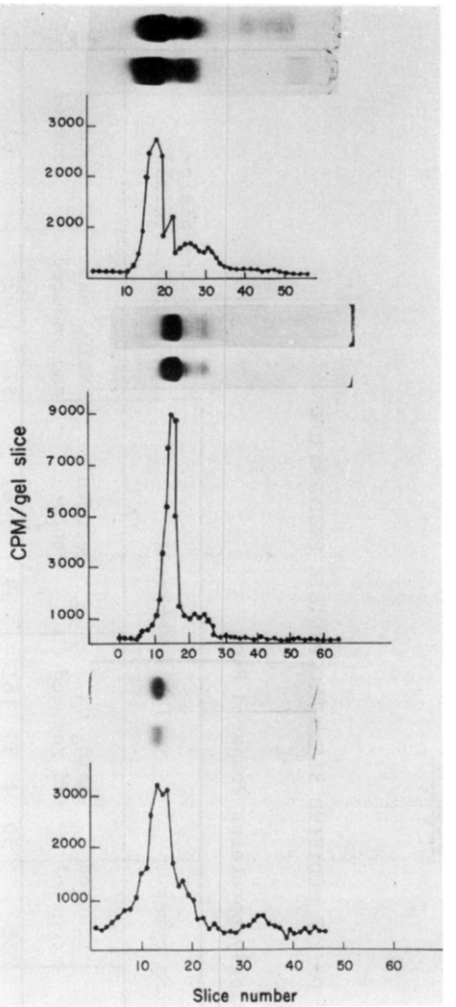
RADIOACTIVITY OF MEMBRANES TREATED WITH SODIUM PERIODATE AND/OR TRITIATED SODIUM BOROHYDRIDE EXTRACTABLE INTO CHLOROFORM-METHANOL (2:1)

	cpm/ml membranes	
	Periodate Oxidized & Reduced	Reduced Controls
Radioactivity before extraction	660,000	18,000
Radioactivity in chloroform-methanol	130,000	11,100

have been least efficiently labelled. This is to be expected since the reactive groups may be least accessible when present in the intact cell. The efficiency of the labelling procedure is presently under investigation in order to achieve the optimal conditions of oxidation and reduction; furthermore, the relationships in the uptake of radioactivity to the colorimetric reactions of the modified sialic acid (7) are being examined. In this study, conditions of periodate oxidation suggested by Van Lenten and Ashwell (7) were used so that sialyl residues could be preferentially oxidized without significant and concomitant oxidation of other moieties. Moreover, reduction conditions were chosen so as to minimize the possible cleavage of peptide bonds as well as the sodium borohydride promoted β eliminations of seryl or threonyl linked oligosaccharides (9). These mild conditions also yield a minimal hemolysis when the intact erythrocytes are used. However, trace amounts of hemoglobin remain bound to membranes prepared from these erythrocytes.

Uhlenbruck and Pardoe (12) and Van Lenten and Ashwell (7) suggest that the periodate oxidation procedure as used here leads predominantly to the oxidation of 3 terminal residues of bound N-acetyl neuraminic acid, and which now upon reduction results in formation of bound heptulosaminic acid (5 acetamido-3,5 dideoxy-L-arabino-2-heptulosonic acid). Moreover, Spiro (13) has shown that sialic acid residues of fetuin are particularly susceptible to periodate oxidation. In this study the label is also present in





left) Fig. 1: Distribution of tritium label in the proteins of the erythrocyte membrane. Gel electrophoresis in 5.6% polyacrylamide gels containing 1% SDS. A) Membranes sequentially treated with sodium periodate and tritiated sodium borohydride; the gels at the top are duplicate Coomassie and Schiff stained gels. B) Control membranes treated with tritiated sodium borohydride. C) Membranes prepared from intact erythrocytes sequentially treated with sodium periodate and tritiated sodium borohydride; the gel at the top is a duplicate Schiff stained gel. D) Membranes prepared from control intact erythrocytes treated with tritiated sodium borohydride. Time of electrophoresis of gels C & D was longer.

ight) Fig. 2: Distribution of tritium label in the isolated sialoglycoproteins of the erythr cyte membrane. Gel electrophoresis in 5.6% polyacrylamide gels containing 1% SDS. From top to bottom: a) Sialoglycoproteins isolated from intact erythrocytes sequentially treated with sodium periodate and tritiated sodium borohydride. b) Sialoglycoproteins isolated from membranes sequentially treated with sodium periodate and sodium borohydride. c) Isolated sialoglycoproteins sequentially treated with sodium periodate and sodium borohydride. The gels at the top are duplicate Coomassie and Schiff stained gels.

TABLE II

RADIOACTIVITY OF THE MODIFIED SIALOGLYCOPROTEIN PREPARATIONS

	Sialoglycoproteins Prepared From										
	Intact Erythrocytes			Isolated Membranes					Isolated Sialoglycoproteins		
	$NaIO_4$ ox. and reduced		reduced control	$NaIO_4$ ox. and reduced			reduced control		$NaIO_4$ ox. and reduced		reduced control
preparation[1]	58	62	58	50	59	61	59	61	57	62	62
specific activity dpm x 10^7/100 μmoles total amino acids[2]	4.7	4.5	0.024	8.2	14.1	8.0	0.018	0.017	10.3	12.3	0.37
μmoles reduced, tritium labelled groups/100 μmoles total amino acids[2]	3.6	3.4	0.02	9.6	11.7	6.1	0.01	0.01	7.9	9.4	0.28

(1) In preparations 57,58,61,62 tritiated sodium borodeuteride of specific activity 13.0 x 10^6 dpm/μmole was used; in preparations 50 and 59 tritiated sodium borohydride of specific activity 8.5 x 10^6 dpm/μmole and 11.9 x 10^6 dpm/μmole respectively, were used.

(2) Assuming an average residue weight of 100, 100μmoles of total amino acids is equivalent to 10 mg of protein amino acids or approximately to 30 mg of the sialoglycoprotein; values computed from the total content of amino acids obtained by an amino acid analysis after hydrolysis in 6N HCl at 105° C in vacuo.

the modified form of sialic acid. After treatment of the labelled glycoprotein with 0.025 N or 0.1 N H_2SO_4 for 60 minutes at 80° C the tritium labelled components are retarded upon gel filtration on Sephadex G75 and emerge in a peak at position of elution of low molecular weight components. Upon cochromatography with authentic heptulosaminic acid (prepared by sequential sodium periodate oxidation and nonradioactive sodium borohydride reduction (7) of the methyl glycoside of N-acetylneuraminic acid (14) and hydrolysis) on Whatmann 3 MM paper, in butanol-acetic acid-water (5:2:2) for 18 hours, the majority of radioactivity appeared in a resorcinol positive spot of faster mobility than N-acetylneuraminic acid. The modified protein can also serve as a substrate for neuraminidase from Cl. Perfingens although the rate of reaction is slower than for the unmodified protein. Approximately 70% of radioactivity becomes retarded on Sephadex G75 after incubation of the modified sialoglycoprotein (2 mg) with 0.32 units of the enzyme at pH 4.7 for 20 hours, at 37° C. These reactions are presently under study and the presence of differently substituted neuraminic acids (15) is being considered.

As seen in figure 1, three radioactive and Schiff staining glycoproteins are present among the proteins of the erythrocyte membrane proteins; the glycoprotein in the band with the slowest mobility appears to be present in the largest amount. This agrees with results of Fairbanks, Steck and Wallach (10). In the isolated membranes (figure 1) and the sialoglycoproteins (figure 2) this band seems to predominate although accompanied by minor bands. It is as yet unknown what relationships prevail among these modified glycoproteins.

DISCUSSION

A procedure of mild periodate oxidation followed by reduction with tritiated sodium borohydride is adapted here in order to introduce a specific radioactive label into the sialoglycoproteins of the erythrocyte surface. A specific reaction occurs whether intact erythrocytes, isolated membranes, or isolated sialoglycoproteins are so treated. The label appears in residues which were formerly those of bound sialic acid. No other membrane proteins become labelled significantly and the method, as described, appears specific for surface sialoglycoproteins. The method may be selective for these proteins since the susceptible residues are those of carbohydrates and predominantly those of si-

alic acid. In this sense the method differs from other labeling procedures recently described (16-18). Some lipid components become radioactive after periodate oxidation and/or tritiated sodium borohydride reduction; they most likely consist of susceptible glycolipids, lipid peroxides, or plasmalogens.

The high specific activity of the surface glycoproteins obtainable by the method described offers a generally useful procedure for modification and introduction of specific label into sialic acid containing membrane components. The sensitivity of the procedure can be much increased by using tritiated sodium borohydride of higher specific activity than described here.

Numerous investigators studied the effect of periodate oxidation on the antigenic properties of the erythrocyte and noted that it resulted in considerable antigenic alteration (see ((19)) for review). In particular Morgan and Watkins (20) showed that the A,B,H (O), Le^a, Rh_oD, M,N and P agglutinogens become inactive, and Hirst (21) reported the loss of virus receptor activity upon treatment with periodate. Since the sialoglycoproteins modified in this study bear several of these antigenic and virus receptor sites, the same protein components were clearly altered in these earlier studies. More recentl Uhlenbruck and Pardoe (12) similarly treated isolated bovine erythrocyte glycoproteins and suggested that such chemical modifications could facilitate studies of carbohydrate antigens.

The more general effect of periodate oxidation on the cell surface components is suggested by the recently described effect of Smith degradation on the conversion of normal gangliosides to those capable of inhibiting malignant cytoagglutination (22). The effect of mild periodate treatment on the transformation of normal (23) and leukemic lymphocytes (24) is also noteworthy. These observations point to a more general use of the chemical modifications described here in facilitating further study of various biological phenomena which involve or are mediated by cell surface glycoproteins and other carbohydrate containing components.

ACKNOWLEDGEMENTS

We wish to express our appreciation to Theresa Koseki for her expert technical assistance and Mr. Charles Gorenstein for his assistance with part of this work.

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