Solubilization of Human Erythrocyte Membrane Glycoproteins by Triton X-100

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1. The enzymic removal of sialic acid residues from the glycoproteins of the human erythrocyte decreases the solubilization of membrane glycoprotein by Triton X-100. 2. The solubilization of asialoglycoprotein by Triton X-100 may be restored by the addition of borate. 3. Use of this non-ionic detergent in the presence of borate, as a general procedure for the mild solubilization of membrane glycoproteins deficient in sialic acid residues, is discussed.

Membrane-bound glycoproteins have been the subject of much contemporary research, especially with respect to the elucidation of their biological function. This increasing interest in the biological significance of glycoproteins at the cell surface has created a demand for general methods for the solubilization of membrane glycoproteins under mild chemical conditions. A number of membrane-solubilization procedures have been devised (Maddy & Dunn, 1976), and, in particular, erythrocyte stroma has received considerable attention.

Non-ionic detergents are especially valuable as chemically mild and efficient chaotropic agents. Furthermore, the components solubilized by these agents may be used directly in various separation procedures and characterization techniques, including isoelectric focusing, isotachopheresis, affinity chromatography and immunological investigations.

In the present paper the factors that affect the solubilization of the glycoproteins of human erythrocyte stroma by the non-ionic detergent Triton X-100 are reported. We find that the reversible binding of borate to *cis*-vicinal diols promotes the solubilization by Triton X-100 of asialoglycoproteins. The relevance of these data to providing a general method for membrane-glycoprotein solubilization is discussed.

Materials and Methods

Chemicals

Triton X-100 [Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K., or BDH, Poole, Dorset, U.K.] was deionized by passage through mixed-bed ion-exchange resins, and the actual concentration of detergent determined (Wright & Plummer, 1973). All chemicals were of analytical-reagent grade unless otherwise stated.

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Neuraminidase (EC 3.2.1.18)

Purified neuraminidase from *Vibrio comma* (formerly *Vibrio cholerae*) filtrate was obtained from Behringwerke A.-G., Marburg/Lahn, Germany, as an aqueous solution containing 500 units/ml, where 1 unit of activity is defined as the amount of enzyme that releases 1 μ g of *N*-acetylneuraminic acid in 15 min at 37°C from α_1 acid glycoprotein in an appropriate medium at pH 5.5. This preparation is stated by the manufacturer to be free of phospholipase C (lecithinase C), and neither proteinases nor aldolase activity could be demonstrated.

Preparation and neuraminidase treatment of human erythrocyte stroma

Human erythrocyte stroma were prepared by the procedure of Dodge *et al.* (1963) from blood (group O, rhesus-positive, MN) kindly provided by the Regional Blood Transfusion and Immunohaematology Centre, Cambridge, U.K. After exhaustive dialysis against 0.145 M-NaCl containing 6.8 mM-CaCl₂ and 50 mM-sodium acetate, pH 5.5, at 4°C, quantities of the stroma were incubated at 37°C with sufficient neuraminidase to release all the available *N*-acetylneuraminic acid in the membranes within 90 min. Appropriate controls were performed by incubating suspensions of stroma, under the same conditions, in the absence of neuraminidase. Treated and control samples of stroma were then removed from the above suspending media by centrifugation for 60 min at 4°C and 100 000g (r_{av} 9.86 cm). These samples were resuspended in and exhaustively dialysed against distilled water adjusted to pH 8.6 with 1 M-NaOH. Finally portions of dialysed, treated and control stroma were further dialysed exhaustively against 50 mM-sodium borate buffer, pH 8.6. In this latter stage a control was performed by continuing dialysis of portions of enzyme-treated and untreated stroma against further quantities of distilled water (pH 8.6). All dialysis procedures were performed in sealed vessels and care was taken to

check that the pH of the dialysis fluid remained constant. To determine the effect of pH on the solubilization of stromal constituents, portions of stroma were dialysed against distilled water adjusted by the required pH with 1M-HCl or 1M-NaOH, or appropriate phosphate or borate buffers.

Solubilization of erythrocyte stroma by Triton X-100

Portions of dialysed erythrocyte stroma were removed from suspension by centrifugation for 1 h at 4°C and 100000g (r_{av} , 9.86cm) and resuspended at a known protein concentration (3.0mg/ml) in the appropriate buffer containing Triton X-100 (concentrations of up to 1%, v/v, were examined). After extraction at 4°C for 30min the extracts were centrifuged for 1 h at 4°C and 100000g (r_{av} , 9.86cm). Material remaining in the supernatant fluid after centrifugation was considered solubilized.

Chemical analyses

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin prepared as

Fig. 1. *pH-dependence of erythrocyte-membrane-sialo-glycoprotein solubilization by 1% (v/v) Triton X-100* Portions of erythrocyte stroma were dialysed against distilled water adjusted to the required pH as detailed in the Materials and Methods section. Dialysed stroma were then suspended at a protein concentration of 3 mg/ml in 1% (v/v) Triton X-100 in distilled water adjusted to the same pH. The stroma were incubated at 4°C for 30min, followed by centrifugation for 1 h at 100000g (r_{av} , 9.86cm). Analyses for sialic acid (□) and protein (○) were performed in duplicate as described in the text. Material remaining in the supernatant fluid was considered solubilized.

Fig. 2. *Effect of pH on the extent of solubilization of erythrocyte sialo- and asialo-glycoproteins by 1% (v/v) Triton X-100 in the presence of borate and phosphate buffers*

(a) The preparation of the erythrocyte stroma and the various chemical analyses are described in detail in the Materials and Methods section. Stroma were treated with 1 (v/v)% detergent, as described in the text, in the presence of 50mm-borate buffer (□, sialic acid; ○, protein) or 50mm-phosphate buffer (■, sialic acid; ●, protein), the total available protein for each point being 3.0mg/ml. (b) Neuraminidase-treated stroma were prepared from erythrocytes as detailed in the text. The stroma were treated with 1% (v/v) detergent in the presence of 50mm-borate buffer (△, hexosamine; ○, protein) or 50mm-phosphate buffer (▲, hexosamine; ●, protein), the total available protein for each point being 3.0mg/ml.

a protein standard by Armour Pharmaceutical Co., Eastbourne, East Sussex, U.K. The determination was found to be independent of the concentration of Triton X-100, as noted by Wright & Plummer (1973), the yellow precipitate formed on addition of the Folin-Ciocalteu phenol reagent being removed by centrifugation for 5 min at room temperature at 1000g (r_{av} 11.0 cm). Phospholipids were extracted by the procedure of Bligh & Dyer (1959). After digestion for 15 min at 180°C in a mixture of 70% (w/v) perchloric acid, 30% (v/v) hydrogen peroxide and water (5:2:1, by vol.), phosphorus was determined by the method of Zilversmit & Davis (1950).

Sialic acid was determined by the 2-thiobarbituric acid method of Warren (1959) after hydrolysis in 0.05M-H₂SO₄ at 80°C for 1 h, with *N*-acetylneuraminic acid (Sigma type IV) as a standard. Hexosamine was determined by the procedure of Boas (1953), with D-glucosamine hydrochloride (Sigma) as a standard. Samples used for the determination of hexosamine were hydrolysed by the Boas (1953) technique in 4M-HCl for 4 h at 100°C, and the hydrolysates freed of amino acids and neutral sugars by chromatography on Dowex 50 (X8; H⁺ form). Under the same conditions, standard solutions of D-glucosamine hydrochloride were quantitatively recovered (98%).

Neutral sugars were assayed by the anthrone reaction as described by Roe (1955) with D-galactose (glucose-free preparation from Sigma) as a reference material.

Fucose was assayed by means of the Dische-Shettles cysteine/sulphuric acid reaction (Dische & Shettles, 1948) with L-fucose (Sigma) as a standard.

Care was taken to correct for non-specific chromophores.

Results and Discussion

Chemical analysis of the stroma

The human erythrocyte stroma used were found to contain *N*-acetylneuraminic acid (126 nmol/mg of protein), hexosamine (190 nmol/mg of protein), hexose (425 nmol/mg of protein) and fucose (35 nmol/mg of protein). These values compare favourably with those published by others (Rosenberg & Guidotti, 1968; Tanner & Boxer, 1972).

Solubilization of human-erythrocyte-membrane constituents

The solubilization of erythrocyte-membrane constituents by Triton X-100 is shown in Figs. 1 and 2 and Table 1. Table 1 summarizes the results obtained with three different batches of stroma, and additional preparations were used for experiments described in the Figures. A number of workers (Mazia & Ruby, 1968; Miller, 1970; Yu *et al.*, 1973; Kirkpatrick *et al.*, 1974) have examined the solubilization of human erythrocyte membranes by this non-ionic detergent, and our present results on the solution properties of the erythrocyte sialoglycoprotein correlate well with those studies. The sialoglycoproteins of the erythrocyte stroma were readily solubilized in 1% (v/v) Triton X-100 at pH 7.0, all the available sialic acid being solubilized as long as the membrane-protein concentration was not in excess of 4 mg/ml;

Table 1. *Effect of borate on the detergent solubilization of neuraminidase-treated erythrocyte membranes*

This Table summarizes the percentage solubilization of various membrane constituents by 1% (v/v) Triton X-100 obtained under different conditions with three separate batches of erythrocyte stroma, prepared as described in the text. Solubilization of sialoglycoprotein is measured as sialic acid. The detergent solubilization of asialoglycoprotein (measured as hexosamine) and phospholipid, for the same batches of stroma following treatment with neuraminidase, is also summarized. The chemical analyses and solubilization procedures are detailed in the Materials and Methods section. Abbreviation used: nd, not determined.

Solubilization conditions	Solubilization (%)					
	Preparation ... I		II		III	
	Sialic acid	Protein	Sialic acid	Protein	Phospholipid phosphorus	Protein
Untreated membranes						
Distilled water, pH 8.6	81	46	nd	nd	66	46
50mM-Borate buffer, pH 8.6	89	74	94	59	74	86
50mM-Borate buffer, pH 7.4	99	nd	97	71	nd	nd
50mM-Phosphate buffer, pH 8.6	81	58	88	37	nd	nd
Neuraminidase-treated membranes	Hexosamine	Protein	Hexosamine	Protein	Phospholipid phosphorus	Protein
Distilled water, pH 8.6	0	19	0	nd	63	33
50mM-Borate buffer, pH 8.6	91	49	85	40	61	56
50mM-Borate buffer, pH 7.4	57	30	72	27	nd	nd
50mM-Phosphate buffer, pH 8.6	57	30	58	24	nd	nd

Fig. 1 shows the pH-dependent solubilization of sialoglycoprotein (as *N*-acetylneuraminic acid) and total protein by the detergent. An increase or decrease in pH of material solubilized under optimal conditions of pH caused isoelectric precipitation of sialoglycoprotein. The proportion of these components remaining in the solution was equivalent to the amount found by solubilizing intact membranes by detergent under the appropriate suboptimal conditions of pH. That the isoelectric precipitation of the membrane components was unaffected by Triton X-100 accords with the findings by Hallam & Wrigglesworth (1976).

In the present study we extended the use of Triton X-100 to sialic acid-free erythrocyte membranes and find that asialoglycoproteins are not readily solubilized by this detergent. As may be seen from Table 1, in which the effect on the solubilization of erythrocyte-membrane proteins at pH 8.6 of 1% (v/v) Triton X-100 is displayed, in excess of 80% of the sialoglycoproteins of the cell are readily solubilized. This value is obtained with all concentrations of detergent examined in excess of 0.2% (v/v). The result is achieved independently of whether the pH is adjusted with NaOH or maintained with 50mm-borate buffer. As may be seen from Table 1, an identical extent of solubilization was obtained with 1% (v/v) Triton X-100 kept at pH 8.6 with 50mm-phosphate buffer. However, as expected from the results in Fig. 1, the use of 1% (v/v) Triton X-100 with borate buffer at pH 7.4 results in an increase in solubilization of sialoglycoprotein. The enzymic removal of sialic acid from the erythrocyte stroma completely suppresses the solubilization of asialoglycoprotein (measured as hexosamine), as shown in Table 1. This effect could be reversed by treatment with 50mm-borate under conditions (pH 8.6) shown by Krejci *et al.* (1949) to significantly enhance the anodic mobility of a blood-group-related polysaccharide. With concentrations of Triton X-100 in excess of 0.5% (v/v), nearly 90% of the available hexosamine is solubilized, suggesting that the charge state of the glycoprotein is an important condition for solubilization by this detergent. It is noteworthy that at pH 7.4, where the binding of borate to *cis*-vicinal diols would be expected to be lowered (Krejci *et al.*, 1949), the solubilization of hexosamine is decreased.

Although the above results would point to the importance of the charge state of the glycoprotein in membrane-solubilization studies, it is difficult to deduce from these experiments to what extent salt effects are a contributing factor. In the case of 50mm-phosphate buffer over the pH range 6–9, slightly smaller amounts of sialoglycoprotein and considerably smaller quantities (<40%) of the total protein are solubilized than when 50mm-borate buffer is used (Fig. 2a). With neuraminidase-treated

stroma the solubilization of asialoglycoprotein in the presence of 50mm-phosphate buffer over the pH range 7–9 is considerably less than that obtained with borate buffer (Fig. 2b). Although the use of borate buffer at pH 8.6 (Krejci *et al.*, 1949) results in the solubilization of over 80% of the membrane hexosamine, larger amounts can be achieved at pH 8.0 (Fig. 2b). Certainly the use of phosphate buffer, the salt of a tribasic acid, but one that does not complex with *cis*-vicinal diols, might be expected to provide a control for estimating salt effects. However, in view of the reports that phosphate has been shown to bind strongly to, and alter, the anionic electrophoretic properties of glycoprotein and protein (Weiss & McGale, 1968; Tuech & Morrison, 1974), caution should be exercised in relating the amounts of glycoprotein solubilized by phosphate to salt effects. In order to control directly for 'salting-in', the effect of solubilizing sialo- and asialo-glycoproteins of the human erythrocyte with detergent in the presence of concentrations of 50–250mm-NaCl was measured on a separate batch of erythrocyte stroma. The presence of NaCl has no effect on the almost complete solubilization of sialoglycoprotein by Triton X-100. With the sialic acid-free membranes, the amount of hexosamine solubilized by 1% (v/v) Triton X-100, adjusted to pH 8.0, in the presence of up to 150mm-NaCl remained constant at 40%, rising to 54 and 57% in the presence of 200mm- and 250mm-NaCl respectively. When the detergent was used with 50mm-phosphate buffer, at pH 8.0, 55% of the available hexosamine was solubilized, a result that would suggest that no more than 15% of the observed solubilization is due to phosphate binding.

Although particular attention has been directed in the present studies towards the solubilization of membrane glycoprotein, the concomitant solubilization of phospholipid was also measured (Table 1). At 1% (v/v) Triton X-100, either in the presence of 50mm-borate buffer, pH 8.6, or detergent adjusted to this pH with NaOH, 66 and 74% respectively of the available phospholipid phosphorus was solubilized. After neuraminidase treatment, 61 and 63% of the stromal phospholipid was found to be soluble. When the effects of concentrations up to 1% (v/v) Triton X-100 on phospholipid solubilization were examined, the shape of the solubilization curves were similar whether untreated or neuraminidase-treated cells had been used, though the curve was less steep than that obtained with glycoprotein.

Conclusions

Non-ionic detergents are particularly attractive membrane-solubilizing agents because of the ability to fractionate material by a variety of conventional biochemical techniques as well as being able to

perform immunological reactions in their presence. In agreement with the work of Yu *et al.* (1973) on erythrocytes and with Butters & Hughes (1974) on nucleated cells, Triton X-100 readily solubilizes membrane glycoproteins, especially those that are sialylated. Butters & Hughes (1974) attribute the relative ease with which membrane glycoproteins may be solubilized to their exposed position in the membrane. From the work presented here, with the human erythrocyte as a model system, it is suggested that the charge on the glycoproteins also appears to be an important consideration.

The greatly enhanced solubilization of membrane asialoglycoprotein by complex-formation with borate represents a novel method for examining these compounds. In particular this procedure has the potential for solubilizing glycoprotein from membranes of other cell types, particularly those low in sialic acid.

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