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ABO(H) Blood Group Antigens of the Human Erythrocyte Membrane: Contribution of Glycoprotein and Glycolipid*

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Summary. Formaldehyde-fixed human erythrocytes were extracted with sodium dodecyl sulfate and with three other solvent systems, at least two of which are known to remove glycolipids quantitatively. The extracted cells possessed the ability to absorb the ABO blood group-specific antibody at about one-third the level of unextracted cells. Treatment of fresh cells with pronase also reduced the ability of the cells to absorb the antibody, further supporting the presence of ABO blood group active glycoprotein in the membrane. Trypsinization of red cells, while removing PAS-1 and partly PAS-2, did not lead to any decrease in the activity. Papainization also did not diminish the activity, although PAS-1, PAS-2, and PAS-3 were removed from the cells. Thus, both glycolipid and glycoprotein contribute to ABO antigens of erythrocytes. Also, none of the three major glycoproteins of the membrane bears this activity.

The chemical nature of ABO(H) blood group substances of the human erythrocyte membrane has been debated for the past fifty years. Early attempts at their purification led to isolation of lipoidal substances (*see* Kabat, 1956). Since these were difficult to handle, subsequent work on the chemistry of blood group antigens was done on preparations obtained from tissue secretions. These were glycoprotein in nature, and their immunochemical specificity was found to reside in sugar residues (*see* Watkins, 1966; Marcus, 1969). Later, blood group active glycolipids were isolated from erythrocytes (*see* Hakomori & Jeanloz, 1970); however, efforts were already under way to purify glycoprotein antigens from the membrane. Current opinions on the nature of erythrocyte blood group substances vary widely. According to some workers (Gardas & Kościelak, 1974; Anstee & Tanner, 1975; Dejter-Juszynski, *et al.*, 1978) glycolipids exclusively make up the blood group antigens of the membrane, while some others (Whittemore *et al.*, 1969; Järnefelt *et al.*, 1978) suggest that glycoproteins are the only constituents possessing this activity. The majority of workers, however, seem to be of the opinion that both types of antigen molecules exist in the membrane.

Since blood group glycolipids can tenaciously bind to proteins (Sweeley, 1969), it is crucial that the isolated glycoprotein preparation be free of contaminating glycolipid. All the procedures employed so far for the purification of glycoprotein antigens either yield substances that have been shown to be contaminated with glycolipid or do not give uniform results (Table 1). There have been other approaches (Pinto da Silva, Douglas & Branton, 1971; Takasaki & Kobata, 1976), but the conclusions are open to doubt (*see* Discussion).

Since the approach involving purification of glycoproteins has not provided any conclusive evidence about the occurrence of ABO glycoprotein antigen in the RBC membrane, we have used an indirect approach based on two observations from the literature: (i) Erythrocytes fixed with bifunctional reagents become resistant to lysis by treatments such as hypotonic shock, sonication, and extraction by organic solvents or detergents (Berg, Diamond & Marfey, 1965; Dutton, Adams & Singer, 1966; Marinetti *et al.*, 1973). (ii) Erythrocyte membrane glycolipids are not cross-linked to proteins if the bifunctional reagent is less than 6 Å in size (Ji, 1974). Thus if formaldehyde is used to fix red blood cells, the membrane glycolipids would remain uncross-linked and could be extracted from the cells using methods that are known to remove them quantitatively. If after their removal

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Table 1. Summary of literature in which glycoproteins bearing ABO blood group antigenicity have been identified

	Reference	Comments
Principal glycoprotein extractant		
Pyridine	Zvilichovsky, Gallop & Blumenfeld, 1971; Tanner & Boxer, 1972	Glycolipid contamination has been shown to occur in the glycoprotein extracted by this method (Blumenfeld & Zvilichovsky, 1972). The glycolipid could be removed by extraction with ethanol-ether (Blumenfeld & Zvilichovsky, 1972)
Phenol	Springer, Nagai & Tagtmeyer, 1966; Howe & Lee, 1969; Howe, Lloyd & Lee, 1972; Liotta *et al.*, 1972; Fukuda & Osawa, 1973	Glycolipid contamination shown in the glycoprotein preparations obtained by this solvent (Blumenfeld & Zvilichovsky, 1972). The contamination could be removed by ethanol-ether extraction
Lithium diiodosalicylate	Marchesi & Andrews; 1971; Yamata, Handa & Yamakawa, 1975	Glycolipid contamination shown (Brennessel & Goldstein, 1974)
Chloroform-methanol	Hamaguchi & Cleve, 1972; Fujita & Cleve, 1975	Glycolipid contamination shown (Brennessel & Goldstein, 1974)
Butanol	Poulik & Lauf, 1965; Whittemore *et al.*, 1969; Gardas & Koscielak, 1971; Yatziv & Flowers, 1971; Anstee & Tanner, 1974	Using this extraction procedure, it has been concluded that only glycolipid (Gardas & Koscielak, 1974; Anstee & Tanner, 1975), only glycoprotein (Whittemore *et al.*, 1969) or both glycolipid and glycoprotein bear blood group activity (Anstee & Tanner, 1974; Poulik & Bron, 1970)
Chloroethanol	Zahler, 1968	Presence of glycolipid not evaluated
Triton X-100	Carey, Wang & Alanpovic, 1976	Presence of glycolipid in the preparation not evaluated
Other methods		
Electron microscopy	Pinto da Silva, Douglas, & Branton, 1971	*See* Discussion
Enzymatic conversion	Takasaki & Kobata, 1976	*See* Discussion

the cells are able to absorb the group-specific antibody, the presence of glycoprotein antigen would be indicated. Also such a glycoprotein, if present, should be cleaved from fresh cells by some proteolytic enzymes leading to a decrease in the antigenic concentration on the cell.

Materials and Methods

Twice-crystallized trypsin was obtained from Sigma; papain was from E. Merck; and pronase was the Sigma "protease". The organic solvents were either of analytical grade or were distilled before use. Other chemicals were of reagent or better grade.

Human B group blood was used throughout. The blood, collected with EDTA as the anticoagulant, was used on the day of collection or within 24 hr of it. The same batch of anti-B serum (titer, 1:256) was used throughout the study.

Blood was centrifuged at approximately $1{,}000 \times g$ for 5 min in the cold. The plasma and buffy coat were removed, and the cells were washed five times in 10 volumes of 0.15 M NaCl. After each centrifugation, some cells from the top of the pellet were discarded to ensure maximal removal of leukocytes.

For fixation with formaldehyde, the washed erythrocytes were suspended at 5% (vol/vol) concentration in 10% neutralized formalin (diluted with 0.15 M NaCl; formaldehyde concentration, 3.7–4.1% (wt/vol)). After 18 hr at room temperature, the cells were washed at least four times in 10 volumes of normal saline. Microscopic examination revealed no aggregation of cells.

1.12×10^{10} cells (equivalent to 1 ml packed fresh cells) were extracted in 50 volumes of the solvent by vigorous mixing for 5 min on a magnetic stirrer. The residue was centrifuged down and further extracted. The extract was filtered through Whatman No. 41 filter paper and stored in the cold. When fixed cells were extracted, a small sample was removed for the assay of antibody absorption. Three solvent systems were used for lipid extraction: (*A*) two extractions with chloroform/methanol (2:1, vol/vol), followed by two extractions in water/ethanol/ether (1:1:4, vol/vol) (Blumenfeld & Zvilichovsky, 1972); (*B*) two extractions in chloroform/methanol (2:1, vol/vol), followed by two extractions in chloroform/methanol (1:2, containing 5% H_2O, vol/vol/vol) (Laine, Stellner & Hakomori, 1974) and (*C*) two extractions in chloroform/methanol (2:1, vol/vol), followed by two extractions in chloroform/methanol (1:2, vol/vol) (Esselman, Laine & Sweeley, 1972). Following the extraction of fixed erythrocytes, the cells were suspended in methanol (or ethanol) for a few hours and centrifuged. They were then washed twice in 50% methanol (or ethanol), twice in 25% methanol (or ethanol), and finally twice in normal saline. This washing procedure was essential to avoid clumping of the cells. In addition to the above three systems, glycolipids were extracted by suspending fixed cells in 1% sodium dodecyl sulfate (SDS) for 1 hr. The cells were then washed over a period of few

days by suspension in 100 volumes of normal saline several times. 0.1% sodium azide was included in the washing medium.

The organic extracts were concentrated on a rotary evaporator and dried *in vacuo* over P_2O_5. The residue was taken up in a small volume of chloroform/methanol (1:1, vol/vol). The extracts usually had a light yellow-brown color, with those obtained from fixed cells being darker.

Inhibition of hemagglutination was used to assay the blood group substance on the cell membrane. Two-tenths ml of a suspension of erythrocytes (2.25×10^8 cells/ml, equivalent to 2% (vol/vol) suspension) in Tris-buffered saline, pH 7.4 (TBS), was serially two-fold diluted. The anti-serum was diluted in TBS to 4 hemagglutination units, and 0.2 ml of the diluted serum was added to each tube. After standing at room temperature for 1 hr (which was found to be sufficient for the absorption of antibody), the cells were centrifuged, and 0.2 ml of the supernatant was transferred from each tube. To this, 0.2 ml of a 2% suspension of fresh, washed B group cells was added. After about 2 hr at room temperature, agglutination was scored microscopically. The tube showing approximately 50% free cells was taken as the end point.

Enzyme treatment was as follows: Washed cells were suspended in 2 volumes of the enzyme solution in TBS, and incubated for 1 hr at 37 °C. The cells were centrifuged and washed four times in 10 volumes of normal saline in the cold. They were suspended at 2% (vol/vol) concentration for the assay of antibody absorption, or were used for ghost preparation.

The supernatant after pronase digestion (no visible hemolysis) was twice extracted with 8 volumes of ethanol/ether (1:4 vol/vol) or with 10 volumes of chloroform/methanol (2:1, vol/vol). The organic phases were dried *in vacuo*. The pronase solution was pre-incubated at 37 °C for 1 hr to inactivate any glycosidases present.

For the preparation of erythrocyte ghosts, the procedure of Dodge, Mitchell and Hanahan (1963) was used, except that Tris buffer was used instead of phosphate buffer for lysis and washing. Polyacrylamide gel electrophoresis and staining were done essentially according to Fairbanks, Steck and Wallach (1971).

Protein was estimated according to the procedure of Lowry *et al.* (1951), using crystalline bovine serum albumin as the standard. "Total" sugars were estimated by the phenol-sulfuric acid method (Dubois *et al.*, 1956) with glucose as the standard. In order to correct for the contribution due to color of the extract, a no-phenol control was included and substracted from the reading.

Results

Formaldehyde-Fixed Cells

After 18 hr in 10% formalin, the erythrocytes were found to be resistant to a variety of treatments, as found by earlier workers using different bifunctional reagents (Berg *et al.*, 1965; Dutton *et al.*, 1966; Marinetti *et al.*, 1973). The cells could withstand several rounds of extraction in mixtures of chloroform and methanol. They were also resistant to treatment with 1% SDS. After these treatments the shape of the cells was not affected (Fig. 1), but there was a slight reduction in their size. Cell counts before and after extraction showed that few (less than 10%) cells were broken. The organic-solvent extracts contained small quantities of protein (around 350 μg from 1.12×10^{10} fixed cells, and about 205 μg from the same number of unfixed cells). When the extracts were concentrated and analyzed on SDS-polyacrylamide gel electrophoresis, diffuse Coomassie blue staining material was seen over the region normally occupied by ghost proteins (not shown). No individual bands were present; in the normal cell extract, however, hemolyzate proteins could be seen as distinct bands. The periodic acid-Schiff staining also showed diffuse distribution covering the same region as seen with Coomassie blue-reactive substances. In both extracts, positive staining was also seen directly behind the tracking dye, indicating the presence of glycolipid. Thus, during extraction no protein was selectively removed. The protein present presumably arises from the lysed cells and is probably extensively degraded. The surprising point is its solubility, although in small amounts, in these solvents.

Extraction of Glycolipids

Of the three extraction systems employed, two (systems *B* and *C*, *Materials and Methods*) are known to extract glycolipids quantitatively from red cell membrane (Esselman *et al.*, 1972; Laine *et al.*, 1974). System *A* (*Materials and Methods*) should also work equally well, since extraction with ethanol/ether removes the glycolipid contamination from glycoprotein preparations obtained by phenol- or pyridine-extraction procedures (Blumenfeld & Zvilichovsky, 1972). All three systems are found to extract around 95% of glycolipids (Table 2), as determined by the phenol-sulfuric acid reaction. An additional extraction in each of the three solvent systems did not yield any more lipid-linked sugars, indicating complete extraction of glycolipids prior to this step.

Antibody Absorption by Glycolipid-Depleted Cells

By the inhibition of hemagglutination assay, fresh and formaldehyde-fixed cells showed identical end-points, in tube 7 or 8 in blood samples obtained from different individuals. After extraction, the end-point shifted to tube 3 or 4 (Table 4). Each of the three extraction systems and extraction by SDS brought the value down to the same tube, showing that the three systems and SDS are equally effective in the extraction of glycolipids. An additional extraction, which did not remove any more glycolipid (above), also did not decrease the titer further. Treating the extracted cells with 1.0 M NaCl also did not reduce the titer any more (Table 3). The slightly different end-points seen in different individuals probably reflect individual variations in the glycolipid/glycoprotein antigens on their cells.

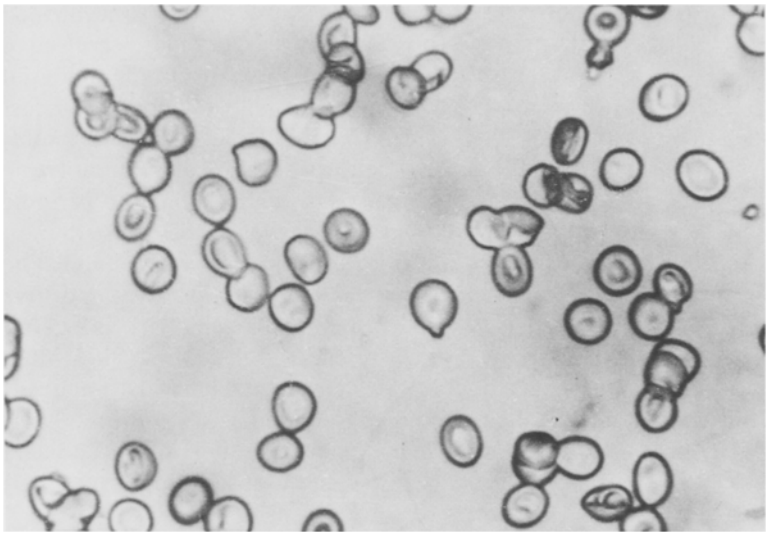

Fig. 1. Formaldehyde-fixed erythrocytes after extraction of their glycolipids (×960)

Table 2. Extraction of lipid-bound sugar by the three extraction systems

	Lipid-bound sugar in extract (%)
Fresh cells	100.0
Fixed cells	
Extraction system *A*	92.3±2.84
Extraction system *B*	97.5±2.50
Extraction system *C*	96.2±1.85

Mean ± SE. Average of three parallel extractions by each solvent system (*Materials and Methods*). Erythrocytes from the same batch were used for all extractions.

The decrease in titer at different extraction steps is shown in Table 4. It is clear that extraction merely with chloroform/methanol (2:1, vol/vol) is not enough to remove all glycolipid (Marchesi & Andrews, 1971; Yamata *et al.*, 1975)

Effect of Proteolytic Digestion on the Blood Group Activity of Erythrocytes

Trypsin and papain up to concentrations of 5 mg/ml and 10 mg/ml, respectively, had no effect on the titer; whereas treatment with 0.5 mg/ml pronase consistently reduced the titer by 2 or 3 tubes (Table 5). Using higher concentration, up to 5 mg/ml, of pronase, which makes the cells rather fragile, no further reduction in the titer was observed.

The pronase-digest contained only traces of lipid-bound sugar (Table 6). Thus the possibility that the decrease in activity might have been due to removal of glycopeptide-bound glycolipid is ruled out.

Removal of Pronase-Sensitive Glycoprotein and of Glycolipid

The evidence indicated that, in addition to glycolipid, pronase-susceptible glycoprotein carries blood group activity. If both are removed, no activity should be left on the cell. When cells pretreated with pronase were fixed with formaldehyde and extracted to remove glycolipid, the cells consistently showed some residual activity (Table 7). The cells devoid of any blood group activity should show the titer of 1; in these cells the titer was 2. This shows that either pronase, under the conditions, does not remove all the glycopeptides possessing blood group activity or that a population of glycoproteins exists that is not digested by pronase.

PAS-1, PAS-2, and PAS-3 in Relation to the Blood Group Activity

As described above (Table 5), treatment of erythrocytes with trypsin or papain had no effect on the blood group activity of the cells. When ghosts prepared from these enzyme-treated cells were analyzed on SDS-polyacrylamide gels, PAS-1 and partly PAS-2 were missing on the trypsinized cells and all the three major glycoproteins were absent on the papainized cells (Table 8). Since there is no decrease in activity following treatment with either of the enzymes, evidently none of the three glycoproteins is associated with ABO blood group activity. Pronase also cleaved the three glycoproteins, but there was reduction in the activity.

Table 3. Antigenic titration of cells before and after glycolipid extraction

Cells		Titer (tube showing 50% free cells)
Fresh		7
Fixed		7
Fixed, extracted by system (Expt. No.)		
A	1	4
	2	4
	3	4
B	1	4
	2	4
	3	4
C	1	4
	2	4
	3	4
SDS		4

The results described were obtained with erythrocytes from the same batch. In addition, other experiments gave the following results (titer after extraction/titer before extraction): (i) by extraction procedure *A*: 3/7, 4/8 and 5/8; (ii) by extraction procedure *B*: 3/7.

Table 4. Removal of blood group activity at different stages of extraction

		Titer
Fixed cells		8
Extraction stages:		
Chloroform/methanol (2:1, vol/vol)	1	6
	2	5–6
Water/ethanol/ether, (1:1:4 vol/vol/vol)	1	5
	2	4
1.0 M NaCl		4

Extraction system *A* (*Materials and Methods*) was used.

Table 5. Effect of proteolytic digestion on the blood group activity of cells

Enzyme	Concentration (mg/ml)	Titer
Untreated		7
Trypsin	1.0–5.0	7
Papain	1.0–10.0	7
Pronase	0.5–5.0	5

The cells were incubated at indicated concentrations for 1 hr at 37 °C.

Discussion

In contrast to short fixation times with formaldehyde where only a few erythrocyte membrane proteins are cross-linked (Steck, 1972), prolonged fixation cross-links all membrane proteins. Formaldehyde cross-links via amino groups, and there is a great preponderance of these groups on the inner side of the membrane (Whitely & Berg, 1974). The cross-linked network formed between proteins and some molecules of aminophospholipids (Marinetti *et al.*, 1973) is evidently strong enough to withstand harsh treatments such as extraction with organic solvents or ionic detergents. Because of the small size of the formaldehyde molecule, the glycolipids situated in the outer layer of the bilayer (Gahmberg & Hakomori, 1973; Steck & Dawson, 1974) cannot be cross-linked to glycoproteins (Ji, 1974). An advantage of this approach is that the topographical arrangement and the orientation of proteins in the membrane are not disturbed.

The identical residual blood group activity left on the extracted cells indicates that all the four extraction procedures work equally well. Two of these procedures (systems *B* and *C*, Laine *et al.*, 1974; Esselman *et al.*, 1972, respectively) and SDS extraction are known to remove glycolipids quantitatively. An additional extraction by any of the three solvent systems does not further decrease the ability of the cells to absorb the antibody, nor is there any further extraction of lipid-bound carbohydrate. Treatment of the extracted cells with 1.0 M NaCl also did not decrease the titer. Glycolipids can be bound to membrane proteins by ionic bonds (Sweeley, 1969; Sharom & Grant, 1978), which should be broken by solutions of high ionic strength. Therefore we think that the less than 100% recovery of glycolipids found in our experiments is probably due to high background color of the extracts. It seems probable that the no-phenol blank that was subtracted from the readings to correct for the background color might in fact be over-correcting. To test for the possible presence of glycolipid on the extracted cells, we have tried to look for the presence of sphingosine (Lauter & Trams, 1962); however, this has not been possible due to deep color of the acid hydrolyzate of the extracted cells. The partial removal of ABO blood group activity after pronase digestion of cells further supports the presence of glycoprotein antigens in the membrane. We have checked that glycolipid is not removed together with pronase-digested glycopeptides in more than trace amounts. Poly(glycosyl) ceramides bearing ABO antigens have been isolated from human erythrocytes (Gardas & Kościelak, 1973; Kościelak *et al.*, 1976). Could these be responsible for the activity remaining on the cell after glycolipid extraction? We expect that these substances would be extracted by the three solvent systems used; and SDS would most certainly remove them. Also, treatment of cells, after extraction

Table 6. Removal of lipid-bound sugar during pronase digestion

Extraction by	Sugar present (μg)	Percentage of total lipid-bound sugar
Chloroform/methanol	3.0	0.96
Ethanol/ether	4.5	1.45

One ml packed cells were digested with pronase (1 mg/ml). The digest was centrifuged at 15,000 × *g* for 20 min and was extracted as described in *Materials and Methods*.

Table 7. Blood group activity of pronase-treated fixed cells before and after extraction of glycolipids

Cell		Titer
Fresh		7
Fixed		7
Pronase-treated		5
Pronase-treated, fixed, and extracted by system	*A*	2
	B	2

Table 8. Removal of PAS-1, PAS-2, and PAS-3 and the effect on blood group activity of cells

Enzyme	PAS-positive component on gel			Activity
	PAS-1	PAS-2	PAS-3	
Trypsin	–	–/+[a]	+	Unaffected
Papain	–	–	–	Unaffected
Pronase	–	–	–	Reduced

[a] Indicates reduction in staining intensity.

in any of the three systems, with 1.0 M NaCl does not affect the titer. The reduction in activity by pronase also rules out this possibility.

In the hemagglutination inhibition assay a change in titer beyond two tubes can be regarded as significant. The fall in titer after the removal of glycolipid is by 3 tubes (from 7 to 4, Table 3). The decrease in titer following pronase digestion is from tube 7 to 5 (Table 5). The latter may not be regarded as significant. However, the consistent decrease obtained in a number of experiments together with the fact that extraction of glycolipid, from pronase-treated cells, reduces the titer to 2 (Table 7) indicates that the removal of activity by pronase is real. If pronase did not remove any blood-group glycoprotein, further extraction of glycolipid should have reduced the titer to 4 rather than to 2. Since shift in titer following glycolipid removal is from 7 to 4, and that following glycoprotein removal is from 7 to 5 in the twofold dilution assay, it follows that there are roughly twice as many glycolipid as glycoprotein antigenic sites. This clearly has to be a crude estimate as the method used cannot be considered more than semi-quantitative. It is also possible that there may be individual variations in the relative proportions of the two types of ABO antigens, as is the case with the concentration of B group antigen on the cells of different individuals (Economidou, Hughes-Jones & Gardner, 1967).

The lack of complete removal of the receptor activity after pronase digestion and lipid extraction can mean incomplete digestion of the blood group active glycoprotein or the presence of a population of molecules resistant to pronase (Gahmberg, 1976). We favor the first alternative, and visualize that the oligosaccharide chain closest to the membrane sterically prevents hydrolysis of the peptide bonds situated below it, thereby protecting its own removal. It is known that for the complete removal of oligopeptides, very high concentrations of pronase are necessary (Bjerrum & Bøg-Hansen, 1976). We have not gone beyond 5 mg/ml, as the cells become very fragile and lyse during fixation.

Since treatment with trypsin or papain does not affect the antigenic concentration on the cell, it is clear that PAS-1, PAS-2, and PAS-3 do not bear the ABO antigen. Many workers have assigned the activity to PAS-1 (Springer *et al.*, 1966; Marchesi & Andrews, 1971; Zvilichovsky *et al.*, 1971; Howe *et al.*, 1972; Fukuda & Osawa, 1973; Yamata *et al.*, 1975), PAS-3 (Hamaguchi & Cleve, 1972), PAS-2 and PAS-3 (Fujita & Cleve, 1975) or to all three glycoproteins (Carey *et al.*, 1976; Takasaki & Kobata, 1976). Evidently the preparations isolated in these studies must have been contaminated by glycolipid (Anstee & Tanner, 1974, Brennessel & Goldstein, 1974). The lack of participation of PAS-1 in the activity is further confirmed by the fact that the erythrocytes of En(a-) individuals, which completely lack PAS-1 in their membrane (Gahmberg *et al.*,1976; Tanner & Anstee, 1976), do not show diminished ABO blood group activity (Darnborough, Dunsford & Wallace, 1969; Furuhjelm *et al.*, 1969). PAS-1 and PAS-2 appear to be two different physical forms of the same molecule (Schulte & Marchesi, 1978). The transfer of N-acetylgalactosamine to group-O PAS-1, PAS-2, and PAS-3, by the milk transferase (Takasaki & Kobata, 1976) would indicate relative nonspecificity of the enzyme. From the present work it is clear that none of the three glycoproteins is involved in the blood group activity. The blood group activity has been shown to be associated with intramembranous particles of the erythrocyte (Pinto da Silva *et al.*, 1971). The particles may be constituted, besides the sialoglycoprotein and Band 3 protein (Pinto da Silva & Nicolson, 1974), by other proteins or even glycolipid; glycolipid is shown to be associated with membrane protein (Craig

& Cuatrecasas, 1975; Sharom & Grant, 1978). Band 3 protein is easily cleaved by papain (Steck, Fairbanks & Wallach, 1971), and hence it too can be ruled out as carrying the specificity. It thus seems that the ABO blood group activity is associated with a quantitatively minor glycoprotein of the membrane. At least 20 distinct glycoproteins are known to be present in the erythrocyte membrane (Gahmberg, 1976).

The approach described here using formaldehyde-fixed cells can be used to determine if two populations of molecules – glycoprotein and glycolipid – exist for the other blood group antigens or lectin receptors in the cell membrane.

While this manuscript was being prepared, papers by Finne *et al.* (1978) and Krusius, Finne and Rauvala (1978) appeared. These authors have purified an ABO-active poly(glycosyl)-peptide from pronase digest of erythrocytes, indicating the existence of ABO glycoprotein antigens in the membrane.

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