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The Distribution of Blood-Group Antigens on Butanol Extraction of Human Erythrocyte 'Ghosts'

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The distribution of protein and blood-group-antigen activity obtained after butanol extraction of erythrocyte 'ghosts' under various conditions is described. Butanol extraction under low-ionic strength conditions results in the recovery of membrane protein in high yield in the aqueous phase. Blood-group-A activity is found in both the aqueous and butanol phases, whereas blood-group-P activity is confined to the butanol phase and blood-group-I and blood-group-MN activity are restricted to the aqueous phase. Much lower yields of protein are obtained in the aqueous phase when high-ionic-strength conditions are used. An appreciable amount of material is precipitated at the interface. Under these conditions blood-group-P activity is found only in the butanol phase, blood group-A activity in the butanol phase and interface material and only blood-group-MN activity in the aqueous phase. In contrast with previous reports no correlation could be demonstrated between the secretor status of the donors and the presence of blood-group-A activity in the aqueous phase after butanol extraction under any of the extraction conditions used. By using butanol extraction under high-ionic-strength conditions it is possible to isolate the blood-group-MN-active sialoglycoprotein in high yield from erythrocyte 'ghosts' by a simple procedure.

The butanol-extraction technique (Maddy, 1966) has been widely used to effect a gross separation of erythrocyte 'ghost' proteins from erythrocyte lipids. There have been several reports of the application of this technique to study the distribution of human erythrocyte blood-group antigens between the organic and aqueous phases (Whittemore *et al.*, 1969; Gardas & Koscielak, 1971; Liotta *et al.*, 1972; Rosse & Lauf, 1970). Blood-group-A and blood-group-B activities have been found in both phases and this observation has led to the proposition that two distinct types of A and B antigen exist. These two forms of A and B antigen are assumed to reflect differences in the nature of the molecules that carry the antigenic determinants. Gardas & Koscielak (1971) reported that the presence of the secretor gene correlated with the appearance of blood-group-A and blood-group-B activity in the aqueous phase obtained after butanol extraction of erythrocyte 'ghosts'. We report here our observations on the distribution of membrane proteins and blood-group-antigen activity under various conditions of butanol extraction of human erythrocyte 'ghosts'.

Materials and Methods

General methods

Protein was determined by the method of Lowry *et al.* (1951) and sialic acid by the thiobarbituric acid method (Warren, 1959). Polyacrylamide-gel electrophoresis in the presence of 0.1% sodium dodecyl sulphate was carried out by the method of Fairbanks *et al.* (1971). The preparation of samples and staining of the gels was as described by Tanner & Boxer (1972). Samples were taken immediately after preparation, treated as described above and stored frozen before electrophoresis.

Erythrocyte 'ghosts'

Samples (10 or 20ml) of fresh blood were drawn from volunteers with the use of acid–citrate–dextrose anticoagulant and used within 1 week. The cells were then washed three times with 0.15M-NaCl. All preparations of erythrocyte 'ghosts' were stored at 4°C. To prepare 'Dodge ghosts' (Dodge *et al.*, 1963) the cells were washed twice with 50ml of 0.103M-Na_2HPO_4 adjusted to pH 7.4 with 0.155M-NaH_2PO_4 (iso-osmotic phosphate buffer), and lysed by dilution into 200ml of cold iso-osmotic phosphate buffer previously diluted with 20.5vol. of cold de-ionized water. The 'ghosts' were collected by centrifugation at 20000*g* for 40min and washed once with the same solution to yield a readily dispersed pale yellowish-pink 'ghost' preparation. 'Koscielak ghosts' were prepared as described by Gardas & Koscielak (1971). The washed cells were lysed into 5vol. of cold de-ionized water (which had been titrated to pH 5.5 with 1M-acetic acid) and kept at 4°C for 30min.

The 'ghosts' were collected by centrifugation at 20000*g* for 5min and repeatedly washed in the centrifuge with the lysing solution until the supernatant was colourless. A red, rather sticky pellet was obtained. Phase-contrast microscopy showed the 'Dodge ghosts' to be freely mobile, discoid, intact erythrocyte 'ghosts', whereas the 'Koscielak ghosts' preparations consisted of highly aggregated small vesicles.

Butanol extraction

This was done at 4°C on freshly prepared 'ghosts'. The 'ghosts', derived from 10ml of whole blood, were suspended in 50ml of the required buffer, centrifuged, and the pellet was made up to 5.5ml with the same buffer. Part of this suspension (4.5ml) was used for butanol extraction by the method of Gardas & Koscielak (1971). Ice-cold *n*-butanol (2.25ml) was added to each sample, the mixture was vigorously shaken for 20s and then kept on ice for 15min. The phases were separated by centrifugation at 40000*g* for 15min. The aqueous phase was carefully removed and 2.5ml of the aqueous buffer with which the 'ghosts' were originally equilibrated was added to the butanol phase and interface material. The mixture was shaken vigorously for 20s, centrifuged at 40000*g* for 10min and the aqueous phase was pooled with the previously obtained aqueous phase. Cold butan-1-ol (0.5vol.) was added to the pooled aqueous phases, the mixture was shaken for 20s and then kept for 5min on ice. The aqueous phase was removed and the butanol phase and any interface was pooled with the previously obtained butanol phase and interface.

The pooled butanol phases and interfaces were dried *in vacuo*. Ethanol (83%, v/v; 3.5ml) was added to the dry material and the mixture was heated in boiling water for 3min. The ethanol-soluble material was decanted into a pointed centrifuge tube and left overnight at −20°C. The precipitate was collected by centrifugation at −10°C for 30min at 20000*g*, resuspended in 5ml of acetone at −20°C and the centrifugation repeated. The final washed precipitate was dried under a stream of N_2.

The aqueous phase was dialysed overnight at 4°C against two changes of 2 litres of phosphate-buffered saline (0.15M-NaCl in 10mM-phosphate buffer, pH7.0). The dialysed solutions were concentrated to a volume of 2.5–3.5ml (for every 10ml of whole blood used in the preparation) at 4°C by coating the dialysis bags with dry Sephadex G-100 and were stored frozen. These solutions were assayed for antigen activity as soon as possible after their preparation (usually within 2–3 days).

Preparation of erythrocyte sialoglycoprotein

'Dodge ghosts' were prepared from 200ml of packed outdated erythrocytes. The final 'ghosts' were washed with 20mM-sodium phosphate buffer, pH7.0, and made up to 180ml with the same buffer. Butanol extraction of these 'ghosts' was as described above. The aqueous phase was dialysed overnight at 4°C against two changes of 8 litres of 0.1mM-sodium phosphate buffer, pH7.0. This solution was concentrated by pressure dialysis under N_2 over an Amicon PM-10 membrane to 20ml and the sample was applied to a column (2.5cm × 80cm) of Sepharose 6B, previously equilibrated with a solution containing 50mM-Tris–HCl, pH8.0, 0.1mM-EDTA and 0.2M-NaCl. The relevant fractions were pooled, dialysed exhaustively against water at 4°C and freeze-dried to yield 53mg dry weight of the sialoglycoprotein preparation.

Serological methods

Human anti-A, anti-B, rabbit anti-M and anti-N antisera were from the Blood Group Reference Laboratory, London, U.K. Human anti-I (fry) antiserum was the gift of Miss M. G. Kenwright, London, U.K. Goat anti-P_1 antiserum was kindly given by Professor O. Prokop, Berlin, G.D.R. and anti-P (ellis) antiserum was from Dr. J. Darnborough, Cambridge, U.K. Four auto-anti-I sera were used. All other antisera were available from the Regional Blood Transfusion Centre, Bristol, U.K. Cell typing was performed by the standard serological techniques appropriate for the particular antiserum used [saline, albumin layering, indirect anti-human globulin methods (Boorman & Dodd, 1970)]. Manual haemagglutination-inhibition tests were performed as described by Anstee *et al.* (1973).

After sonication of interface fractions [in 1ml of phosphate-buffered saline (Dulbecco 'A'; Oxoid Ltd., London E.C.4, U.K.) pH7.3], much insoluble material remained and the supernatants only were examined for blood-group activity after centrifugation at 900*g* for 10min at 4°C. Manual haemagglutination-inhibition assessments are not satisfactory for the calculation of yields of blood-group activity in the separated phases because of the inherent inaccuracies of the system. A quantitative system of haemagglutination inhibition was therefore preferred. Manual assessments gave an essentially similar distribution of blood-group activity to that obtained by quantitative assessment.

Quantitative haemagglutination-inhibition assessments were done by using the method of Gibbs *et al.* (1961), modified for use in a Technicon Autoanalyser system. The agglutination obtained with various dilutions of antibody is calculated as a percentage of the theoretical maximum agglutination. A plot of percentage agglutination against antibody dilution on probit/treble cycle log paper gives a value for the amount of antibody necessary to give 50% agglutination in the system (1 HD_{50} unit);

$5HD_{50}$ units of antibody are then sampled at the same time as various concentrations of inhibitor. A plot of percentage agglutination in the presence of inhibitor against inhibitor concentration gives a value for HID_{50} (which is the amount of inhibitor that is equivalent to $4HD_{50}$ units of antibody).

Results

Distribution of antigens

Each of the butanol-extraction experiments (Table 2) were carried out on six individual donations of blood. In each experiment three of the donations were from blood-group-A_1 secretors and three were from blood-group-A_1 non-secretors (Table 1). Three separate extraction experiments were carried out with butanol (Table 2). In Expts. 1 and 2 intact 'ghosts' were prepared by the procedure of Dodge *et al.* (1963) ('Dodge ghosts') and then equilibrated either with 1mM-sodium phosphate buffer, pH7.0 (Expt. 1) or with 20mM-sodium phosphate buffer, pH7.0 (Expt. 2) before butanol extraction by the method of Gardas & Koscielak (1971). In Expt. 3, 'ghosts' ('Koscielak ghosts') were prepared by the method of Gardas & Koscielak (1971), equilibrated with 20mM-sodium phosphate buffer, pH7.0 and extracted as described by these authors. The recoveries of protein in the aqueous phase were 65 (±4)%, 4 (±1)% and 32 (±5)% in Expts. 1, 2 and 3 respectively.

When the butanol extraction was done on 'Dodge ghosts' at very low ionic strengths then the recovery of protein in the aqueous phase was quite high. In contrast, the presence of 20mM buffer in the aqueous medium dramatically lowered the recovery of protein in the aqueous phase. An appreciable amount of material was found precipitated at the interface in this latter situation. Sodium dodecyl sulphate–polyacrylamide-gel electrophoretograms (Plate 1*b*) show that the aqueous phase of Expt. 1 yielded a protein pattern which confirms that all the proteins that are present in the intact erythrocyte 'ghosts' are retained in the aqueous phase although it is depleted in protein E. In contrast, the aqueous phase derived from Expt. 2 yields essentially only one weak band on staining for protein which corresponds in mobility to the erythrocyte sialoglycoprotein (Plate 1*d*). This band also stains with the periodic acid–Schiff's reagent and is the erythrocyte sialoglycoprotein (Plate 1*e*). The 'ghosts' obtained by the 'Koscielak procedure' gave variable gel-electrophoresis patterns which appeared to show evidence of protein degradation (Plate 1*f*), were grossly contaminated with haemoglobin and had no morphological resemblance to the 'Dodge ghosts'. Although there is an apparently high recovery of protein in the aqueous phase obtained on butanol extraction of these 'ghosts', gel electrophoresis showed that much of this is haemoglobin, and few other discrete bands that could be ascribed to 'ghost' proteins were found. The interface in this case also contained a large amount of protein (Plate 1*h*).

The distribution of blood-group-A activity in the original 'ghosts', and in the fractions obtained after extraction, was estimated by automated quantitative haemagglutination inhibition and the results are shown in Table 2. In Expt. 1 a good overall recovery of blood-group-A activity was obtained. Most of this activity was found in the aqueous phase, only about 10% being in the butanol phase. In contrast, identical 'ghosts' extracted in the presence of 20mM-sodium phosphate buffer (Expt. 2) showed no recovery of blood-group-A activity in the aqueous phase, whereas the butanol phase contained significantly more blood-group-A activity than did similar material derived from Expt. 1. Under these conditions a good overall recovery was again obtained, appreciable amounts of blood-group-A activity being found in the interface material. 'Koscielak ghosts' extracted under the latter conditions (Expt. 3)

Table 1. *Antigenic profile of intact erythrocytes from donors used in experiments*

Cell and saliva typing were done by using standard serological techniques. N.t. denotes 'not tested'.

Secretor status	Donors	Antigen A_1	B	Le^a	Le^b	Saliva*	P_1	M	N
Secretors	U.C.	+	−	−	+	+	+	+	−
	R. B.	+	−	−	+	+	+	−	+
	P. H.†	+	−	−	+	+	+	−	+
Non-secretors	A. W.	+	−	+	−	−	−	−	+
	R. C.	+	−	+	−	−	+	−	+
	A. F.	+	−	+	−	N.t.	N.t.	N.t.	N.t.
	J. E.	+	−	+	−	N.t.	N.t.	N.t.	N.t.
	H. D.	+	−	+	−	N.t.	N.t.	N.t.	N.t.

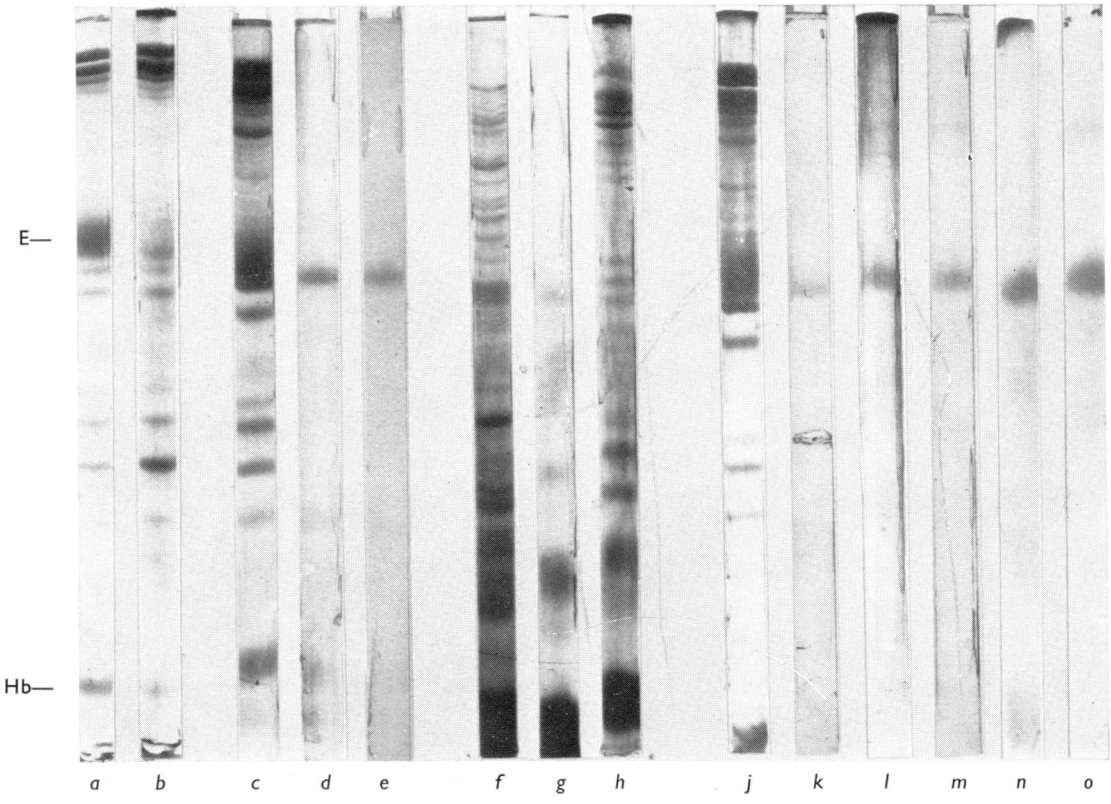
* + or − denotes presence or absence of blood-group-A activity in saliva samples.
† Weak A_1 reactor.

able 2. *Distribution of blood-group-A activity in fractions obtained after butanol extraction under various conditions*

Blood-group-A-activity units are the reciprocal of the dilution of the fraction which is equivalent to $4HD_{50}$ units of human anti-A serum (HID_{50}) as determined by automated quantitative haemagglutination inhibition (see the Materials and Methods section). A_1 cells were used as indicator cells. All fractions were also tested in an identical system containing $5HD_{50}$ units of human anti-B antiserum and group-B erythrocytes as a confirmation of serological specificity. The three experiments described were performed on separate occasions with different indicator A_1 cells. The values of HD_{50} for the anti-A serum were 1/100, 1/38 and 1/85 for Expts. 1, 2 and 3 respectively. Blood-group-A activity is presented as units of activity obtained from 1 ml of 'ghosts' starting material. The recoveries of greater than 100% blood group activity which were found in some cases may reflect the different organization of the antigen in erythrocyte 'ghosts' and in the various extracted phases Expt. 1: 'Dodge ghosts' extracted in the presence of 1 mM-sodium phosphate buffer, pH 7.0. Expt. 2: 'Dodge ghosts' extracted in the presence of 20 mM-sodium phosphate buffer, pH 7.0. Expt. 3: 'Koscielak ghosts' extracted in the presence of 20 mM-sodium phosphate buffer, pH 7.0.

No. of expt.	Donor	Secretor status	Blood-group-A activity (units/ml of 'ghosts')				Blood-group-A activity recovered (%)			
			Initial 'ghosts'	Aqueous phase	Interface*	Butanol phase	Aqueous phase	Interface	Butanol phase	Total recovery
Expt. 1	U. C.	+	17.5	23.0	—	1.2	131	Nil	7	138
	P. H.	+	8.0	2.4	—	1.4	30	Nil	17	47
	R. B.	+	18.0	12.0	—	1.7	67	Nil	9	76
	A. W.	−	22.0	11.0	—	2.6	50	Nil	12	62
	R. C.	−	24.0	17.0	—	2.2	71	Nil	9	80
	A. F.	−	13.0	13.0	—	1.6	100	Nil	12	112
Expt. 2	U. C.	+	3.4	Nil	1.2	1.6	Nil	36	46	82
	P. H.	+	2.7	Nil	0.4	0.9	Nil	17	34	51
	R. B.	+	4.0	Nil	0.5	1.5	Nil	12.5	38	50
	A. W.	−	3.8	Nil	0.9	1.6	Nil	23	41	64
	R. C.	−	3.0	Nil	1.0	2.4	Nil	33	80	112
	J. E.	−	3.4	Nil	1.3	1.4	Nil	38	41	79
Expt. 3	U. C.	+	9.0	Nil	0.2	4.2	Nil	3	47	49
	P. H.	+	4.4	Nil	< 0.2	1.2	Nil	<5	27	32
	R. B.	+	10.0	Nil	0.3	1.8	Nil	3	18	21
	A. W.	−	6.2	Nil	0.5	2.2	Nil	9	35	44
	J. E.	−	11.0	Nil	0.4	2.2	Nil	3	20	23
	H. D.	−	8.6	Nil	0.8	2.1	Nil	9	24	33

* No interface material observed in Expt. 1.



EXPLANATION OF PLATE 1

Gel electrophoresis of fractions obtained on butanol extraction under various conditions, and purification of sialoglycoprotein

Electrophoresis was on 5.5% (w/v) polyacrylamide gels containing 0.1% sodium dodecyl sulphate. E denotes protein E and Hb denotes haemoglobin. (*a*) 15 μl of intact 'Dodge ghosts' used in Expt. 1; donation A.F.; Coomassie Blue stain. (*b*) 20 μl of aqueous phase derived from Expt. 1; donation A.F.; Coomassie Blue stain. (*c*) 20 μl of intact 'Dodge ghosts' used in Expt. 2; donation A.W.; Coomassie Blue stain. (*d*) 40 μl of aqueous phase derived from Expt. 2; donation A.W.; Coomassie Blue stain. (*e*) Sample as (*d*), but with carbohydrate stain. (*f*) 20 μl of 'Koscielak ghosts' used in Expt. 3; donation A.W.; Coomassie Blue stain. (*g*) 50 μl of aqueous phase derived from Expt 3; donation A.W.; Coomassie Blue stain. (*h*) 30 μl of interface material derived from Expt. 3; donation A.W.; Coomassie Blue stain. (*j*) 25 μl of intact 'ghosts' used for the preparation of the sialoglycoprotein; Coomassie Blue stain. (*k*) 40 μl of sample as (*j*), but with carbohydrate stain. (*l*) 100 μl of aqueous phase obtained during preparation of sialoglycoprotein; Coomassie Blue stain. (*m*) Sample as (*l*), but with carbohydrate stain. (*n*) 50 μl of sialoglycoprotein preparation; Coomassie Blue stain. (*o*) Sample as (*n*), but with carbohydrate stain. Although the mobilities of the bands are comparable within each group of gels, they vary between each group, as the groups were run on separate occasions.

(*Facing p.* 384)

gave a decreased overall recovery of blood-group-A activity. However, the distribution of this activity between the two phases was similar to that obtained in Expt. 2. In none of the experiments was there any difference in the distribution of blood-group-A activity when 'ghosts' derived from cells of secretors or non-secretors were used. These results were also confirmed by using a manual haemagglutination technique with both A_1 and A_2 cells as the indicator (results not shown).

Comparison of these results with gel electrophoretograms of the fractions obtained in each experiment (Plate 1) shows that blood-group-A activity is recovered in the aqueous phase under conditions that also yield the bulk of the membrane proteins in the aqueous phase (Expt. 1). In the cases where protein is found in the interface (Expts. 2 and 3) blood-group-A activity is also associated with the interface.

The butanol phase from all the experiments contained strong blood-group-P activity, and this was absent from the aqueous phases. Blood-group-I activity was not demonstrated in the butanol phase in any experiment. Some samples of aqueous-phase solutions from Expt. 1 gave weak inhibition of anti-I sera, but in no case was inhibition observed with aqueous-phase materials derived from Expts. 2 and 3. The presence of blood-group-I activity in the aqueous phases of all the samples obtained in Expt. 1 was confirmed after concentrating the aqueous phases five times by freeze-drying. A similar assay after five-fold concentration, under identical conditions, of the aqueous-phase materials obtained in Expts. 2 and 3 did not demonstrate blood-group-I activity in any of these samples.

Blood-group-MN activity was always found in the aqueous phase but not in the butanol phase irrespective of the conditions of extraction. This is consistent with the gel electrophoretograms which demonstrate that the aqueous phase obtained in Expts. 2 and 3 contains only the erythrocyte MN sialoglycoprotein and residual haemoglobin.

Purification of sialoglycoprotein

These observations suggested that under certain conditions the butanol-extraction procedure could usefully serve as a simple one-step method for the purification of the erythrocyte sialoglycoprotein. 'Dodge ghosts' from group B-, MN- and P_1-positive cells were subjected to butanol extraction under the conditions of Expt. 2. Gel electrophoresis of the 'ghosts' and aqueous-phase fractions (Plate 1*j–m*) demonstrate that the sialoglycoprotein is found essentially free from other proteins in the aqueous phase; 8% of the initial 'ghost' protein, 55% of the 'ghost' sialic acid and 82% of the 'ghost' blood-group-M activity (as determined by quantitative haemagglutination inhibition) was recovered in the aqueous phase. Gel filtration through Sepharose 6B was used to purify this material further (Fig. 1). The major sialic acid-containing peak obtained corresponds to the erythrocyte sialoglycoprotein (Plates 1*n* and 1*o*) and the pooled material contained 24.5% dry wt. of sialic acid; 36% of the initial 'ghost' sialic acid and 51% of the 'ghost' blood-group-M activity were recovered in this peak. It was shown by a manual haemagglutination-inhibition technique with three haemagglutinating doses of antiserum that this material neutralized anti-M antiserum at

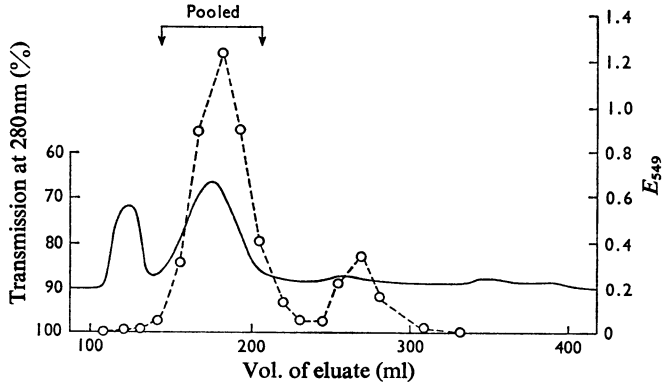


Fig. 1. *Gel filtration of aqueous phase obtained on large scale purification of sialoglycoprotein*

Gel filtration on Sepharose 6B was done as described in the Materials and Methods section. The fractions were monitored for protein at 280nm (——) and for sialic acids at 549nm (----). The fractions indicated were pooled to yield the sialoglycoprotein.

a concentration of 70 μg/ml and anti-N antiserum at 280 μg/ml but had no detectable blood-group-B, -P_1, -P or -I activity at 560 μg/ml. At high loading, gel electrophoresis showed that the preparation still contained residual haemoglobin and traces of other periodate-positive material and, in particular, a band staining for both protein and carbohydrate with an apparent molecular weight of 200000. Although traces of this component are found in gels of the aqueous phase obtained after butanol extraction it cannot be detected in intact 'ghosts'. It is not clear whether this component results from aggregation of the sialoglycoprotein, which is induced by the butanol treatment, or if it is a trace component which is not detectable in gels of intact 'ghosts', but is concentrated into the aqueous phase on butanol extraction.

Discussion

These results clearly demonstrate that the recovery of water-soluble protein after butanol extraction of erythrocyte 'ghosts' is markedly dependent on the ionic milieu during the extraction, as previously noted (Maddy, 1966), and that the recovery of water-soluble blood-group-A activity is also dependent on the exact extraction conditions.

Although we have followed as closely as possible the experimental procedures described by Gardas & Koscielak (1971), we cannot confirm their observation that substantial blood-group-A activity could be recovered in the aqueous phase after butanol extraction in the presence of 20 mM-sodium phosphate buffer. Further, we could not find differences in the amount of blood-group-A activity found in the aqueous phase on butanol extraction under any conditions, of 'ghosts' derived from cells of secretors or non-secretors. Only when extraction was carried out in the presence of 1 mM-sodium phosphate buffer was there a substantial recovery of blood-group-A activity in the aqueous phase.

These results confirm those of Whittemore *et al.* (1969), who found that A-antigen activity is found in both aqueous and butanol phases on butanol extraction. It is noteworthy that Hagamuchi & Cleve (1972), who used a chloroform–methanol system, obtained a similar distribution of blood-group-antigen activity to that reported here and were also unable to demonstrate secretor-status-dependent differences in the distribution of blood-group-A activity in the aqueous phase.

The extraction of blood-group-P activity into the butanol phase irrespective of the extraction conditions is what would be expected from the glycolipid nature of the P_1 antigen reported by Marcus (1971). We have found blood-group-I activity in the aqueous phase obtained from Expt. 1, but not Expts. 2 and 3, indicating that the distribution characteristics of the I antigen are similar to those of the A-antigen activity found in the water phase in Expt. 1. In contrast with blood-group A, blood-group-I activity is not extracted into butanol and these results confirm the findings of Rosse & Lauf (1970).

We have shown that under appropriate conditions the butanol-extraction method can be used as a simple procedure to obtain the erythrocyte sialoglycoprotein without using detergents or other denaturants. The product is obtained in high yield and is free of blood-group-B activity, confirming that this protein does not carry the AB antigens. The contrasting results with the butanol-extraction procedure, obtained by different groups of workers studying blood-group antigens, probably reflect the sensitivity of the butanol-extraction procedure to small differences in the experimental conditions of extraction. It is clear that closely defined conditions are necessary to ensure repeatable results when using this technique.

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