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GEL FILTRATION OF ABH BLOOD GROUP SUBSTANCES

III. ABH GEL FILTRATION PATTERN OF SOLUBILISED RED CELL STROMA

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SUMMARY

Human red cell stroma were solubilised with the nonionic detergent Triton-X-100. By gel filtration of the solubilised material on Sephadex G-200 and G-100, three group specific fractions of each of the A, B and H substances were detected. Fraction 1 is water-soluble and can be precipitated with alcohol, while fractions 2 and 3 are water- and alcohol-soluble. The three active fractions had the same gel filtration and thin-layer chromatographic behaviour as fractions 1, 2 and 3 detected in saliva and reported in a previous paper. The probable identity of the stromal fraction 3 with the blood group specific glycolipid isolated by many investigators from erythrocytes is suggested.

INTRODUCTION

It has been shown in a previous work[1] that seven gel filtration patterns (Types I–VII) of the A, B and H substances can be detected in human saliva. Only a small number of subjects have no salivary ABH antigens (Type VIII, *i.e.* true non-secretors). The various patterns are formed by three serologically active fractions of each antigen, termed fractions 1, 2 and 3, which can be easily detected by gel filtration on small Sephadex G-100 columns owing to their quite different molecular weights. Each of these fractions is secreted in saliva alone or associated with one or both of the other fractions[1].

It was of interest to determine whether the three naturally occurring fractions of the ABH substances were also present in the red cell stroma. From the data presented in this report, it seems evident that three active fractions of each of the A, B and H substances are normally present in red cells and have the same gel chromatographic and solubility behaviour as the salivary ABH fractions.

MATERIALS AND METHODS

Preparation of the samples

The blood of 31 donors (nine group A; sixteen O; four B and two AB) was

examined. 10 ml of whole blood were usually employed. The blood was centrifuged for 30 min at 3000 r.p.m. and the red cells were washed three times with saline. The washed cells were haemolysed in 10 vol. of 0.005 *M* phosphate buffer (pH 7.4) and centrifuged for 40 min at 20 000 × *g*. The supernatant was carefully decanted and the ghost button was resuspended in the same volume of fresh buffer. The operation was repeated three or more times until the supernatant was free of haemoglobin. Finally, the clear white precipitate surrounding the small central-buff-coloured portion of the button was aspirated and suspended in 2 ml of 0.05 *M* Tris–HCl buffer (pH 7.3). In some cases the ghosts were resuspended in saline or in 0.005 *M* phosphate buffer.

The solubilisation of red cell stroma was then performed by adding 20 μl of concentrated Triton-X-100 (BDH, Ltd.) to each millilitre of suspension. The suspension clarified immediately on shaking. The solubilisation of the ghosts was controlled by a phase-contrast microscope. The solution was directly employed for gel filtration experiments or submitted to methanol fractionation of the blood group substances, followed by gel filtration.

Gel filtration

Gel filtration was performed on Sephadex G-200, G-100, G-50, G-25 and Sepharose 4B columns as previously described[1,2]. Bed dimensions of 36 × 1.5 cm or 20 × 1.2 cm; 32 × 2.5 cm; 80 × 2 cm; 38 × 2.5 cm; 32 × 2.2 cm were used, respectively. 0.05 *M* Tris–HCl buffer (pH 7.3) containing 0.9% NaCl was employed as eluant for Sephadex G-200, G-100, G-50 and G-25, and 0.025 *M* phosphate buffer (pH 7.2) for Sepharose 4B. In some particular experiments on Sephadex G-50 and G-25, the Tris–HCl buffer was replaced by distilled water.

Column packing and the void volumes were checked using a freshly prepared dilute solution of Blue Dextran 2000 (Pharmacia, Uppsala). Cytochrome *c*, insulin, glucagon, bacitracin, oxytocin and sucrose were used as reference substances of known molecular weight.

The fractions eluted from Sephadex G-200, G-100, G-50 and G-25 were directly examined by the technique of haemagglutination inhibition. Only for the tubes containing haemolytic material was an additional treatment required as described below.

The contents of the tubes from Sepharose 4B gel filtration experiments were made hysotonic by adding 0.1 ml of a 14.6% solution of NaCl to 2 ml of eluate and then were submitted to serological analysis. The fractions obtained from Sephadex G-50 and G-25 with distilled water as eluant were used for some TLC experiments.

Thin-layer chromatography

Silica Gel G (Merck, Darmstadt) plates (20 × 20 cm), 250 μ thick, activated at 110° for 1 h, were developed once or twice with chloroform–methanol–water (65:25:4) (solvent I) or with petroleum ether–ethyl ether–acetic acid (90:10:1) (solvent II). The samples were applied as separate spots or, when small-scale preparative runs were required, by streaking the sample along the whole origin line. The spots or the bands were revealed by exposure of the plates to iodine vapours. Carbohydrates were detected by spraying the plate with 1.6% aq. orcinol–60% sulphuric acid solution (1:7.5) and heating 10 min at 110°. The blood group specific activity was

determined on the untreated chromatograms by scraping the silica gel layer at various levels and by eluting with solvent I. After standing overnight, the suspension was centrifuged and the supernatant evaporated to dryness. The residue was finally dissolved in 0.2 ml of saline and serologically determined. Protein and carbohydrate tests were also performed in some eluates with the Folin–Ciocalteu reagent[3] and the orcinol reagent[4], respectively.

Haemagglutination inhibition test

The technique described in a previous paper was employed for testing ABH activity[2]. Where haemolysis was present, *i.e.* in the zone of the gel chromatograms in which Triton-X-100 was eluted, the detergent was eliminated as described below.

RESULTS

Sephadex G-100 gel filtration

Gel filtration of solubilised red cell stroma on Sephadex G-100 columns gave a pattern for each of the A, B and H substances as shown in Fig. 1. Three active fractions were detected for each antigen. The first fraction was eluted with the

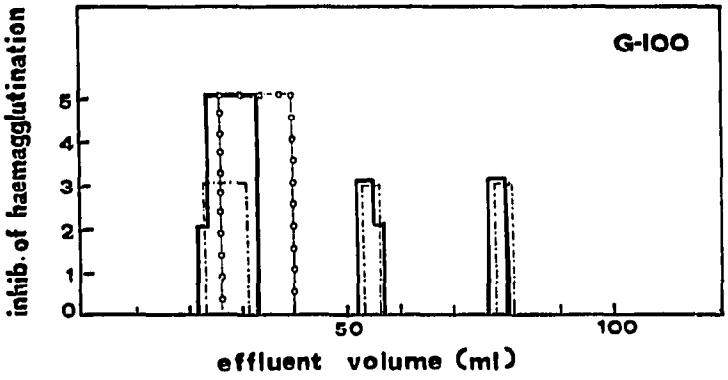

Fig. 1. Gel filtration of solubilised red cell stroma (group A) on Sephadex G-100 (36 × 1.5 cm bed). The solid line indicates the A blood group activity and the broken line the H activity. The results of the inhibition of haemagglutination are scored as follows: 0 = one clump; 1 = +++; 2 = ++; 3 = +; 4 = ±; 5 = − (no agglutination); ○—○ represents the haemolytic effluent.

excluded effluent. Only one or two of the leading tubes of the first peak were free of haemolytic substance and were therefore serologically determined without any preliminary treatment. Because the contents of the other tubes were haemolytic (Fig. 1), Triton-X-100 was eliminated by precipitating the blood group substances. For this purpose, four volumes of absolute ethanol were added to the content of each tube. The supernatant was discarded, and the precipitate was washed with 80% methanol, suspended in saline and serologically tested. Blood group activity was detected both in the insoluble precipitate and in that part of the precipitate which was still soluble in saline. A strong specific inhibition occurred in the leading part of the haemolytic zone which was excluded from the gel (Fig. 1).

A second active fraction was detected with an elution volume of 56–57 ml in 36 × 1.5 cm columns, and of 18 ml in 20 × 1.2 cm columns. A third specific fraction

was identified with elution volumes of 78–80 ml in larger columns and of 27 ml in smaller ones. The two subfractions had a relatively weaker activity, which was confined to two or three tubes.

The three active fractions were termed fraction 1, fraction 2 and fraction 3, respectively, as the gel chromatographically similar fractions of the saliva[1,2].

Each of the A, B and H antigens had the three active fractions in all the blood samples examined. In subjects of group O only fractions 1, 2 and 3 for H substance were identified. In persons of group A, B and AB the three fractions were represented for each of the group antigens as well as for the H antigen. Only slightly quantitative differences in the inhibition test were observed between A_1 and A_2 subjects; in our cases A_1 subjects were detected by absorbed human anti-A serum and also gave a weak agglutination with anti-H from *Ulex europaeus*. The elution volumes for the different antigens in the same sample were sometimes identical or sometimes only similar as also observed in saliva[1,2].

Sephadex G-200 gel filtration

An easier detection of fraction 1 was realised by gel filtration on Sephadex G-200. This dextran gel allowed a nearly complete separation between the main excluded fraction (fraction 1) and the haemolytic zone (probably a Triton–lipoprotein complex) which is retained (Fig. 2). Only in the determination of H substance was a

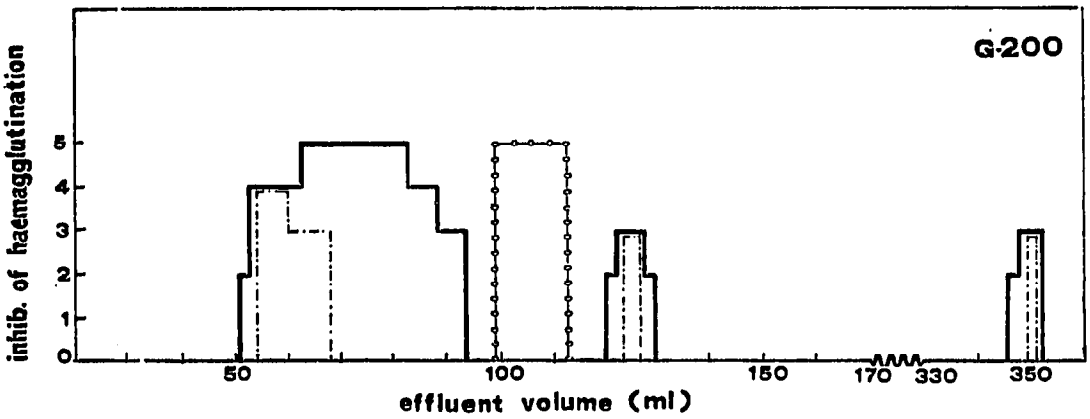

Fig. 2. Gel filtration of solubilised red cell stroma (group A) on Sephadex G-200. The haemolytic effluent (○—○) is completely separated from the blood group specific fractions A and H.

negligible overlapping of the haemolytic zone with the last part of the excluded peak frequently observed.

Fraction 1 had an elution volume of about 65–70 ml, and the haemolytic zone was located immediately after it, having an elution volume of approx. 108 ml and usually covering 20 ml. The elution volumes of fractions 2 and 3 were 125 and 350 ml, respectively.

The haemolytic zone was investigated for the presence of the specific active substances. For this purpose, the content of each tube exhibiting haemolysis was brought to dryness and absolute methanol was added to the residue. The precipitate was washed with methanol, dried and suspended in 0.4 ml of saline and serologically determined. Specific inhibition was observed in a few tubes of the last part of the excluded peak only in some group O samples.

In some cases another control was performed by eliminating Triton-X-100 through TLC on silica gel plates. The contents of the haemolytic tubes were concentrated on the whole origin of the plate by the streaking method and developed with solvent I. Triton-X-100 was detected by iodine vapour as a large yellow band near the front. The remaining area of the chromatogram was divided into three zones which were eluted with solvent I and serologically determined, giving constantly negative results.

Relationship between ABH patterns of red cell stroma and saliva

To study the relationship between the ABH gel filtration patterns of the solubilised red cell stroma with those of the saliva, the stroma of some subjects whose saliva lacked fraction 1 (so-called non-secretors), and had fraction 2 and/or fraction 3, were examined by gel filtration on Sephadex G-200 and G-100. In each subject all three fractions were found in solubilised stroma, irrespective of the secretor status of the individual.

Sepharose 4B gel filtration

The relation between fraction 1 detected in solubilised stroma and fraction 1 from saliva was also investigated by gel filtration on Sepharose 4B. The results of a

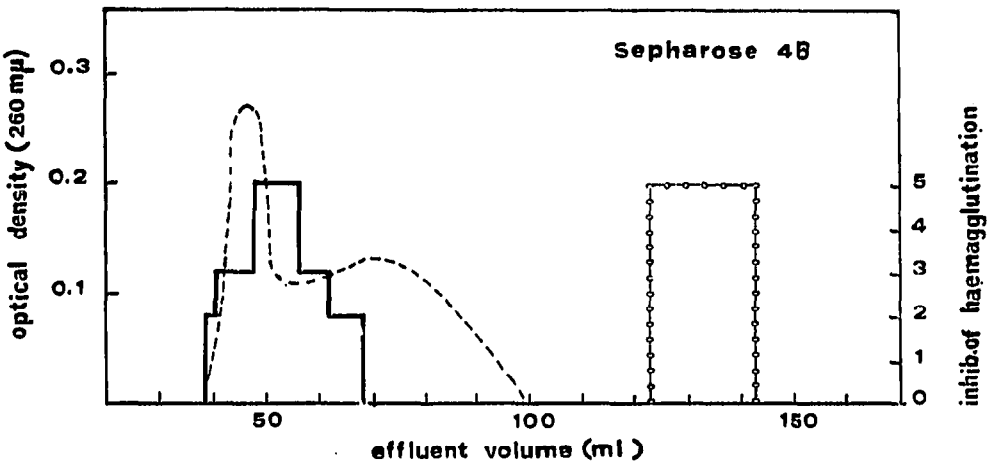

Fig. 3. Gel filtration of solubilised stroma (group O) on Sepharose 4B. The broken line represents the elution profile of Blue Dextran 2000. (○—○) indicates the hemolytic effluent.

typical experiment are shown in Fig. 3. The elution curve of the active substance from red cell stroma was practically the same as that for fraction 1 from saliva. Only a part of the active substance was excluded from Sepharose 4B; the remaining part was retained as shown by recycling experiments. The haemolytic material was eluted later. Broadening of the haemolytic zone was observed in detecting the H substance, so that the active fraction 1 was almost immediately followed by the haemolytic effluent.

Fraction 1

Samples of fraction 1 from Sephadex G-200 columns were treated to obtain the group specific substance free from lipids. The excluded effluent was brought to

dryness and 80% methanol was added to the residue. The precipitate was suspended in chloroform–methanol (1:1). The precipitate again formed was dried and washed with chloroform. The final precipitate was only partially soluble in saline. The undissolved part had a strong group specific activity but the aqueous supernatant also was clearly active.

TLC on Silica Gel G of the aqueous supernatant and of the chloroform–methanol and chloroform extracts was performed using solvent I (for neutral lipids) and solvent II (for phospholipids). After exposure of the plates to iodine vapour, yellow spots were located only in the chromatograms of chloroform–methanol and chloroform extracts. On the other hand, the aqueous supernatant exhibited a strong positive reaction with the orcinol–sulphuric acid reagent at the starting line (Fig. 4a).

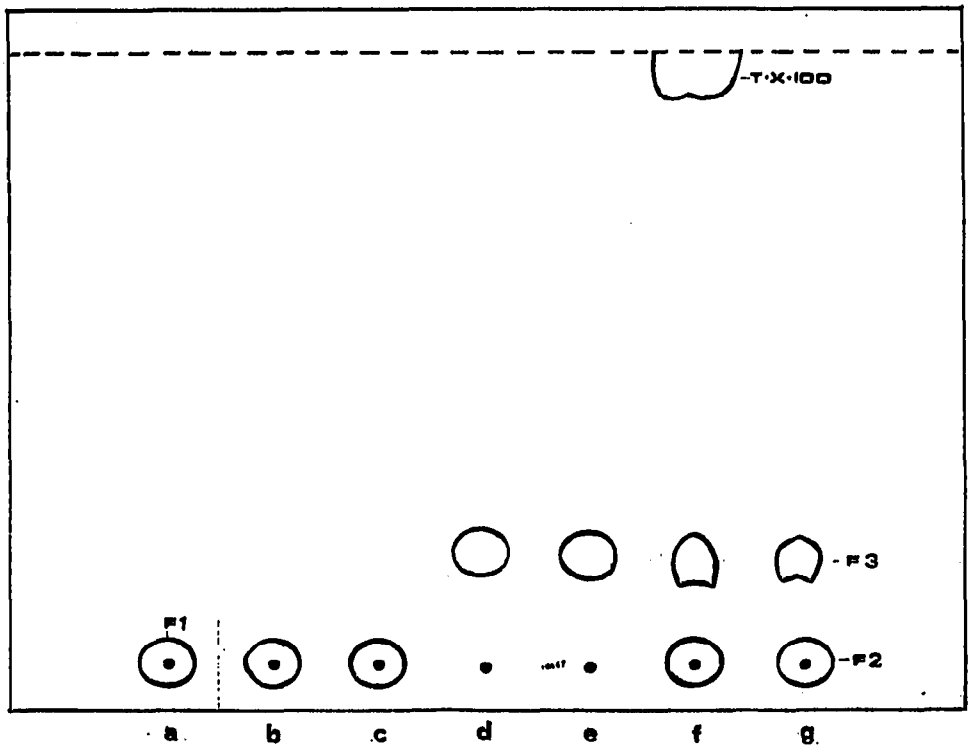

Fig. 4. Thin-layer chromatography on Silica Gel G of ABH blood group specific fractions Solvent: chloroform–methanol–water (65:25:4). (a) lipid-free fraction 1 from solubilised stroma; (b) fraction 2 and (d) fraction 3 from solubilised stroma; (c) fraction 2 and (e) fraction 3 from saliva; (f) fractions 2 and 3 from methanol extraction of solubilised stroma. Triton-X-100 on the solvent front; (g) fractions 2 and 3 from methanol extraction of saliva of Type IV.

In the same place the untreated chromatogram had a clear specific activity. The orcinol and the serological tests were negative on the chromatograms of chloroform–methanol and chloroform extracts.

Fraction 2

Fraction 2 was also characterised by gel filtration on Sephadex G-50. The effluent from Sephadex G-100 gel filtration of solubilised stroma was pooled in the zone containing fraction 2. After dialysis overnight against distilled water and concentration to 2 ml, the samples were gel filtered on a Sephadex G-50 column.

The blood group specific activity was detected only in the excluded effluent. On the other hand cytochrome *c* was retained, as reported in a previous work[2].

Fraction 3

Fraction 3 was characterised in a similar way by gel filtration on Sephadex G-25. Blue Dextran 2000, cytochrome *c*, insulin, glucagon, bacitracin, oxytocin and sucrose were used as references of known molecular weight. The blood group specific activity had an elution volume of 104–105 ml, *i.e.* the same elution volume as fraction 3 from saliva[2].

Samples of fraction 3 isolated by gel filtration on Sephadex G-100 were dialysed against distilled water. The concentrated dialysate exhibited a clear specific activity while the retenate was free of blood group activity. Fraction 3 from solubilised stroma is therefore dialysable as fraction 3 from saliva[2].

Fractions 2 and 3

The TLC behaviour of fractions 2 and 3 from solubilised red cell stroma was determined and compared with that of fractions 2 and 3 from human saliva. Samples of fractions 2 and 3 from saliva of type IV (*i.e.* containing fractions 1, 2 and 3) and from solubilised stroma were isolated by gel filtration on Sephadex G-100. Fraction 2 was then dialysed against distilled water, brought to dryness and finally dissolved with 80% methanol. Fraction 3 was desalted by gel filtration on Sephadex G-25, then concentrated and dissolved with 80% methanol. The samples were chromatographed on silica gel by a double development with solvent I. The spots were revealed with iodine vapour followed by spraying with the orcinol–sulphuric acid reagent. The blood group specific substances were also detected on untreated chromatograms by the serological test on the eluates. For this purpose the silica gel was scraped, eluted with solvent I, the eluate was centrifuged and the solvent evaporated. The dried residue was dissolved with 0.2 ml of saline and serologically tested. Fraction 2 from saliva and from stroma did not move from the starting line, while fraction 3 migrated about 3 cm (Fig. 4b, c, d, e).

A similar investigation was carried out on fractions 2 and 3 extracted directly from saliva of Type IV and from solubilised stroma by treatment with four volumes of absolute methanol. Since it was known from a previous work[2] that the salivary fraction 1 can be precipitated in alcohol and fractions 2 and 3 are alcohol soluble, it seemed obvious that stromal fractions 1, 2 and 3 behave in the same manner. The precipitates from the methanol treatment of saliva and of solubilised stroma were therefore discarded. Some of the methanol extracts were then brought to dryness, the residue was dissolved with saline and gel chromatographed on small 20 × 1.2 cm Sephadex G-100 columns. Fractions 2 and 3 were identified at the usual elution volumes both in salivary and stromal extracts.

Some of the other methanol extracts were concentrated to 0.2–0.3 ml and then chromatographed on silica gel plates by two developments with solvent I. The spots were revealed as described above. The results are recorded in Fig. 4f, g. Triton-X-100 migrated as a large spot with the solvent front and gave a yellow colour with iodine vapour. Fraction 2 did not move from the starting line and fraction 3 migrated about 3 cm.

DISCUSSION

Three blood group specific fractions of each of the A, B and H substances were detected by gel filtration of solubilised red cell stroma. The fractions have been called fraction 1, fraction 2 and fraction 3, respectively. The same nomenclature used to indicate the active fractions of ABH substances identified in saliva of secretors and non-secretors[1] was adopted because the blood group specific fractions from red cell stroma had a gel chromatographic behaviour identical to the active fractions from saliva.

Gel filtration on Sephadex G-200 is preferable for studying fraction 1 of solubilised stroma because the haemolytic material is completely separated from this and the other fractions, so that no particular treatment is required for the serological determination in the relevant tubes. Since a large volume of effluent is required to elute fraction 3 from Sephadex G-200 columns, we have found it very practical, throughout the work, to detect fractions 2 and 3 by gel filtration on small 20 × 1.2 cm beds of Sephadex G-100.

A polydispersity of molecular weight for stromal fraction 1 was demonstrated in the same way as for salivary fraction 1 (ref. 2). Gel filtration of solubilised stroma on Sepharose 4B showed that only the leading part of the first active peak, *i.e.* of fraction 1, is excluded. The exclusion limit for polysaccharides on Sepharose 4B is $5 \cdot 10^6$ ($20 \cdot 10^6$ for proteins); therefore it can be assumed that the molecular weight of that part of fraction 1 which is excluded from Sepharose 4B is up to or over $5 \cdot 10^6$. The final part of the active peak is retained, as shown by recycling experiments, and apparently has a lower molecular weight. Stromal fraction 1 was also completely excluded from Sephadex G-200 (exclusion limit for dextran: 200 000) as well was fraction 1 from saliva.

Fraction 2 was not dialysable and had the same elution volumes on Sephadex G-200 and G-100 as salivary fraction 2. It was also excluded from Sephadex G-50. It is therefore reasonable to assume that its molecular weight is of the same order as that of salivary fraction 2.

Fraction 3 was dialysable and had elution volumes on Sephadex G-200, G-100 and G-25 as salivary fraction 3. For this fraction it also seems reasonable to assume a molecular weight near to that of the salivary fraction 3.

The evidence of a relationship between the three active fractions of stromal origin and the three salivary fractions leads us to the conclusion that the solubilisation of the red cell stroma by the nonionic detergent Triton-X-100 does not produce artifacts with respect to ABH blood group substances. In fact these are brought in solution in three active fractions which have a gel chromatographic behaviour identical to active blood group fractions occurring naturally in untreated saliva.

The red cell stroma of the subjects examined always contained three fractions of the corresponding antigen, irrespective of their secretor status. In particular, fractions 1, 2 and 3 were detected in solubilised stroma of subjects who only had fraction 2 in their saliva, or fraction 3 or both fractions 2 and 3 but lacked salivary fraction 1.

The donors of group O had in their stroma only three fractions of H substance. Donors of groups A and B had three fractions for A or B antigens and three fractions for H and finally donors of the AB group had three fractions for A, B and H sub-

stances. In contrast with the findings of GREEN[5] and WHITTEMORE *et al.*[6] and in agreement with the data reported by LIOTTA *et al.*[7], H activity was also detected in solubilised stroma from A_1 and A_1B subjects. This is not surprising since it is well known that a weak reactivity to anti-H lectin is usually also exhibited by A_1 erythrocytes.

The present study gives only general information on the nature of the blood group substances from red cells. However some preliminary considerations can be made, also on the basis of more recent literature in this field.

The A, B and H substances had been extracted from erythrocytes chiefly by ethanol until solubilisation procedures of the stroma were worked out in more recent years. This leads to the conclusion that the substances from red cells were alcohol-soluble and chemically different from the water-soluble substances of secretions (see ref. 8).

The work carried out by YAMAKAWA AND IIDA[9], YAMAKAWA *et al.*[10–12], HAKOMORI[13], RADIN[14], HAKOMORI AND JEANLOZ[15], HANDA[16] and KOSCIELAK[17,18] established that A and B substances from red cells are glycolipids. More recently it was demonstrated by HAKOMORI AND STRYCHARZ[19] that the material extracted with ethanol from red cell stroma is a sphingoglycolipid with a single carbohydrate chain. On the other hand, some attempts have been made to obtain the ABH substances from red cells by means of extractions with aqueous solutions of diluted ethanol[20–23]. The data of STEPANOV *et al.*[23] were assumed by KABAT[8] as an indication of the similarity between ABH group specific substances from saliva and those from red cells.

A water-soluble blood group specific substance was also detected in the red cell haemolysate and electrophoretically characterised in agar gel by FIORI *et al.*[24]. POULIK AND LAUF[25] reported the isolation of group A specific material from stroma exposed to pH 2–2.2 and extracted with a two phase system (butanol–water). The material was excluded from Sephadex G-200. A similar technique was employed by WHITTEMORE *et al.*[6] and A, B and H activities were detected in aqueous extracts. The material was free of lipids and the extracted blood group substances were considered a glycoprotein with a molecular weight greater than $3 \cdot 10^5$ or probably greater than $1 \cdot 10^6$. LIOTTA *et al.*[7] extracted from red cell stroma a phenol-soluble and water-soluble A specific substance, using the phenol method of MORGAN AND KING[26]. The group specific material was excluded from Sephadex G-200.

More recent studies have given strong support to the hypothesis that blood group specific substances from red cells are glycoproteins as well as group substances from secretions. ZAHLER *et al.*[27] demonstrated A activity in glycolipids as well as in erythrocyte membrane proteins solubilised with 2-chloroethanol and gel filtered on Sephadex LH-20 to separate lipids.

The solubilisation of the red cell stroma was realised by GREEN[5] by means of 2% sodium dodecyl sulphate. Removal of the detergent was obtained by passing the solubilised membranes over a Dowex column. After removal of the detergent, the group specific material was found to be excluded from Bio-Gel P 300.

In the present study a simple method is proposed for the isolation of blood group substances from red cell stroma. A complete solubilisation of stroma is obtained by the nonionic surfactant Triton-X-100 and the detergent is separated from the active material by gel filtration on Sephadex G-200. Gel filtration on Sephadex G-100 separates the haemolytic material from only the active fractions 2 and 3. By this

method, loss of the material is prevented and artifacts are minimised. 4 to 5 ml of packed red cells are sufficient to obtain active material for microanalytical determinations and serological tests. By this method it has been determined that fraction 1 excluded from Sephadex G-200 is water-soluble and is precipitable, and in great part denaturable, by 80% methanol or ethanol. It should be considered a glycoprotein–lipid complex from which lipids can be removed without loss of the specific activity of the molecule. This fraction is therefore the same as that which has been recently isolated from red cell stroma[5–7,25,27]. Its easy denaturation by ethanol explains the unsuccessful attempts to extract this fraction from red cell stroma by many workers on the subject.

The active fractions 2 and 3 which have been isolated from solubilised stroma have gel and thin-layer chromatographic characters quite similar to fractions 2 and 3 from saliva. Both are water- and alcohol-soluble and are mainly carbohydrates in nature. Fraction 2 is not dialysable while fraction 3 is dialysable. Of course, a complete characterisation of these fractions requires further chemical study.

It seems evident, from our data and from the recent studies[5–7,25,27], that the substance chiefly responsible for the ABH blood group specific activity of the red cells is not the low-molecular-weight glycolipid isolated by the Japanese workers[9–12] and recently analysed by HAKOMORI AND STRYCHARZ[19], but on the contrary is a glycoprotein which has a molecular weight within the range of $2 \cdot 10^5$ to $5 \cdot 10^6$ as the group specific glycoproteins from secretions[2]. In the red cells, the glycoprotein probably is linked to the lipids of the membrane. The problem arises whether the glycolipid isolated by the Japanese workers is an artifact. The experiments reported here would support the hypothesis that the group specific glycolipid is the stromal fraction we called fraction 3. In fact this fraction is easily extracted from red cells by ethanol or methanol and can be eluted from silicic acid by chloroform–methanol. Fraction 2 can be extracted in the same way but it is strongly adsorbed to silicic acid (Fig. 4). Finally, fraction 1 is precipitated and in great part denatured by ethanol. As the methods used for the purification of the ethanol extracts of red cell stroma involved column chromatography on silicic acid and elution with chloroform–methanol[9–19], it is probable that only fraction 3 was present in the purified solutions.

On the basis of these considerations and taking into account the structure of the glycolipid as determined by HAKOMORI AND STRYCHARZ[19], it seems reasonable to think that this compound is the same as stromal fraction 3 and also the same as the salivary fraction 3, with respect to the group specific part of the molecule. The difference between fraction 3 from red cells and saliva might consist only, in our opinion, in the fact that in the red cells the group specific substance is linked to the lipids of the membrane.

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