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GEL FILTRATION OF ABH BLOOD GROUP SUBSTANCES

IV. FRACTIONS 4 AND 5 OF ABH SUBSTANCES IN HUMAN RED CELLS AND THEIR SECRETION PATTERNS IN SALIVA

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SUMMARY

Two new low-molecular-weight fractions of the ABH substances, fractions 4 and 5, have been identified. They inhibit non-specifically anti-A, anti-B and anti-H sera and have no effect on anti-Rh, anti-M, anti-N and anti-P sera. These fractions are constantly present in human red cell membranes and are secreted in the saliva of some of the subjects who secrete the specific fraction 3 of the ABH system described earlier.

As fraction 3 can be secreted in saliva alone or associated with fraction 4 or 5, or both, each of the secretory types III, IV, VI and VII that have recently been identified can have four sub-types, α, β, γ and δ. The variable association of salivary ABH fractions 1, 2, 3, 4 and 5 results in 20 individual gel filtration patterns.

INTRODUCTION

Three group-specific fractions of the A, B and H substances in solubilized red cell stroma have recently been described and called fractions 1, 2 and 3 (ref. 1). These fractions are secreted in saliva in different combinations and give rise to different gel chromatographic patterns which are a property of the individual person[2,3] and are probably under genetic control[4].

In the course of subsequent studies, attention was drawn to the fact that fraction 3 from the saliva of some individuals of A or B groups inhibited non-specifically the three main antisera of the ABH system. As the presence of other serologically active substances was suspected in these cases, the purification of fractions 3 from saliva and red cell stroma was carried out by gel filtration on Sephadex G-25, and two new fractions have been detected.

MATERIALS AND METHODS

Preparations of the samples

The blood of 30 donors (16 group A; 3 group B; 2 group AB; 9 group O)

was examined. A 10-ml volume of whole blood was usually used in order to obtain about 2 ml of solubilized stroma. Solubilization was carried out with Triton X-100 according to a previously described technique[1]. A 1-ml volume of the final solution was used for a gel filtration experiment.

The saliva of 101 subjects was also examined: 12 samples were obtained from some of the above-mentioned blood donors, and 89 from subjects selected among the 289 individuals whose ABH gel filtration pattern had previously been determined[3]. Of the latter, 60 were secretors of fraction 3, and the others were used as a control. Samples of 1 ml of whole centrifuged saliva were gel chromatographed directly on Sephadex G-100 or G-25 or, in a few cases, on G-15 columns.

The fraction 3 samples, isolated by gel filtration of saliva on Sephadex G-100, were concentrated to 1 ml and then recycled on Sephadex G-25.

In thin-layer, paper and glass-fibre chromatography and in electrophoretic studies, the ABH substances were previously separated by gel filtration or directly isolated from a 30–100 ml sample of saliva. In the latter case, 4 volumes of methanol were added and the solution was maintained at 4° overnight. The precipitate, containing fraction 1, was partially dissolved with saline. The supernatant methanol was evaporated to 1–2 ml under reduced pressure and by gentle heating at 30°.

Gel filtration

Gel filtration was carried out on 20 × 1.2 cm columns of Sephadex G-100 with 0.05 tris–HCl buffer (pH 7.3), containing 0.9% NaCl, as eluent. The flow-rate was maintained at 6 ml/h and fractions of about 1 ml were collected.

Gel filtration on Sephadex G-25 and G-15 columns was carried out on 20 × 1.2 cm and 30 × 1.2 cm beds, respectively, using tris–HCl buffer or, more frequently, distilled water as eluent. The flow-rate was about 8 ml/h and fractions of 1.2–1.4 ml were collected over a period of 10 h for G-25 and 8 h for G-15.

Haemagglutination inhibition test

The inhibition test was carried out as previously described, using undiluted commercial sera (Ortho, Raritan, N.J., U.S.A.) exhibiting a titre of 1/8–1/16 by the test-tube method. Fractions 3, 4 and 5 were tested also with anti-M, anti-N, anti-P and anti-Rh sera (Biotest, Frankfurt/M., G.F.R.) with the same method and with a moist chamber method on lucite plates with 80 U cups each 15 mm in diameter. In the latter case, 0.5% red cell suspensions and appropriate dilutions of the antisera were used.

When distilled water was used as eluent, 15 μl of 9% NaCl solution were added to 0.1 ml of eluate before the inhibition test.

Carbohydrate analyses

Carbohydrates were determined by a slightly modified α-naphthol method[5]. To 1 ml of the eluate from the columns, 3 ml of concentrated sulphuric acid were added dropwise in an ice-bath. The mixture was then heated for 3 min in a boiling water-bath, and the tubes were then cooled under tap water. To each tube, 0.2 ml of a 2% alcoholic solution of α-naphthol was added. After mixing, the optical density was read after 2 h at a wavelength of 560 nm.

Dialyses

Dialyses were performed at 4° using Visking 32/32 dialysis tubing (Scientific Instr. Centre Ltd., London, Great Britain). The dialyzate was concentrated by rotatory evaporation on a water-bath at a temperature of 30–35°.

Chromatographic and electrophoretic techniques

The separation of the five ABH fractions was attempted also by several chromatographic and electrophoretic techniques.

Thin-layer chromatography (TLC). TLC was chiefly performed on silica gel G plates 20 × 20 cm, 250 μm thick, activated at 110° for 1 h. The following solvent mixtures were tried, each with 3 or 4 successive developments: chloroform–methanol–water (65:25:4 or 52:40:8 or 80:30:5); chloroform–methanol (6:4); dioxane–water (9:1); *n*-propanol–5% ammonium hydroxide (7:1); *n*-propanol–ethyl acetate–water (5:1:4). Alumina (E. Merck, Darmstadt, G.F.R.), acid and basic alumina (Woelm, Eschwege, G.F.R.) plates were used with the same solvent and Adsorbosil-3 (Applied Science Lab., State College, Pa., U.S.A.) with the solvent ethylacetate–ethanol–water (50:40:13).

Paper and glass-fibre chromatography. Paper chromatography (on Whatman No. 1 paper) and glass-fibre chromatography (on Whatman GF-83) were carried out by the descending method on 28 × 57 cm sheets and by the ascending method on 24 × 30 cm sheets with *n*-butanol–pyridine–water (60:40:20) or *n*-butanol–acetic acid–water (130:30:50) as solvent.

Paper, cellulose acetate and glass-fibre electrophoresis. These techniques were performed with 0.1 and 0.05 *M* borate buffers at pH 7–9 and a current of 0.5 mA/cm for paper and cellulose acetate and 5 mA/cm for glass-fibre electrophoresis.

The spots were revealed on thin-layer and glass-fibre chromatograms and electropherograms by exposure to iodine vapour and/or by spraying with a solution of 1.6% aqueous orcinol–60% sulphuric acid (1:7.5), and then heating at 120° for 10 min in an oven. Paper chromatograms and electropherograms were treated with PAS–potassium permanganate reagent.

The blood group activity was detected on the untreated chromatograms and electropherograms by scraping off the gel layers or cutting the paper, the cellulose acetate and the glass fibre at various levels. Elution was usually carried out with chloroform–methanol–water (65:25:4). After standing overnight at 4°, the supernatant solution (after centrifugation when necessary) was evaporated to dryness. The residue was dissolved in 0.2 ml of saline and serologically tested.

Polyacrylamide gel electrophoresis. This was performed on 7% 80 × 6 mm gels with phosphate buffer (pH 7.4), 0.06 *M* in the gel and 0.03 *M* in the tank. A current of 10 mA per tube was applied for 60 min. The gels were stained with amido black and by the periodic acid–Schiff (PAS) method.

Blood group activity was detected on untreated gels by cutting the gels into 2-mm slices, and eluting this material in 0.5 ml of saline for 24 h. After centrifugation, the supernatant solution was serologically tested for the presence of ABH substances.

Column chromatography on silicic acid. This procedure was carried out on solubilized red cell membranes on 20 × 1 cm columns of silicic acid (E. Merck). A 2–3-ml volume of solubilized stroma was previously treated with four volumes of methanol. The precipitate was removed by centrifugation and discarded. The super-

natant solution was evaporated to dryness and the residue dissolved with a few millilitres of chloroform–methanol (9:1) and chromatographed. Elution was carried out first with 200 ml of chloroform–methanol (9:1) and then with 200 ml of chloroform–methanol (6:4). The material eluted was evaporated to dryness, the residue dissolved in 1 ml of saline and the solution obtained gel chromatographed on Sephadex G-25.

RESULTS

Gel filtration of solubilized red cell stroma on Sephadex G-100 and G-25

Fractions 1, 2 and 3 of A, B and H substances were identified in each sample of solubilized red cell membranes by gel filtration on Sephadex G-100 columns, in agreement with previous results[1].

A careful and systematic control of the specificity, made by testing all of the tubes from each sample with the three sera (anti-A, anti-B and anti-H), revealed a constant non-specific activity at the elution volume of fraction 3 from A, B and O subjects, *i.e.*, the eluate containing fraction 3 inhibited anti-A, anti-B and anti-H sera irrespective of the blood group of the individual. A slightly stronger activity was observed only against the specific serum. The specificity of fractions 1 and 2 was confirmed. In every case, all fractions showed H activity.

The contents of the tubes of fraction 3 of each sample, pooled, concentrated to 1 ml and recycled on Sephadex G-25, showed one peak of specific inhibitory activity at the elution volume of 40 ml and two additional peaks of non-specific activity at 52 and 64 ml. These peaks were termed fraction 4 and fraction 5. No inhibitory activity was observed in fractions 3, 4 and 5 against anti-M, anti-N, anti-P and anti-Rh sera.

Each sample of solubilized stroma was also submitted to direct gel chromatography on Sephadex G-25. In this case, the excluded effluent was highly haemolytic, owing to the use of Triton X-100 for solubilization. In the remaining eluate, three peaks of ABH activity were detected, with the same elution volumes and the same

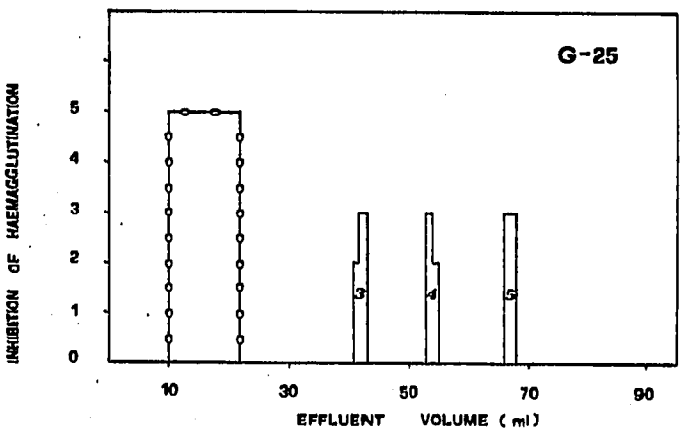

Fig. 1. Gel filtration of solubilized red cell stroma (group A) on a 20 × 1.2 cm Sephadex G-25 column. The results of the inhibition of haemagglutination are scored as follows: 0 = one clump; 1 = +++; 2 = ++; 3 = +; 4 = ±; 5 = — (no agglutination). ○—○ represents the haemolytic effluent.

serological behaviour as those observed in recycling on Sephadex G-25 the samples of fraction 3 obtained by gel filtration of stroma on Sephadex G-100 (Fig. 1).

The group-specific peak at 40 ml from Sephadex G-25 columns, recycled on Sephadex G-100, was eluted at 26 ml, like the specific fraction 3.

Gel filtration of saliva on Sephadex G-100 and G-25

The re-examination on Sephadex G-100 of the saliva from 89 subjects whose ABH salivary pattern had been previously determined[3] gave the following new results, owing to the careful control of the specificity of the serological tests.

A small number (15 cases) of secretors of fraction 3 showed a non-specific serological activity at the elution volume of this fraction, while in 40 subjects fraction 3 exhibited a specific activity. In three cases (one group AB, one group A and one group B) H activity only was represented as fraction 3, and in two AB group subjects, A, B and H activity was detected (see the footnote to Table I). No serological activity was detected at the elution volume of fraction 3 in the saliva of the 29 control cases, in agreement with the determinations previously reported[3].

Specific samples of fraction 3 from Sephadex G-100, when recycled on Sephadex G-25, gave rise to one peak only of specific activity at the elution volume of 40 ml. Recycling on Sephadex G-25 of non-specific samples of fraction 3 allowed the separation of a specific peak at 40 ml from one or two additional peaks at 52 and 64 ml elution volumes. These two new peaks exhibited a non-specific inhibitory activity against anti-A, anti-B and anti-H sera, irrespective of the blood group of the subject. They have been termed fraction 4 and fraction 5, like the similar fractions detected in solubilized red cell stroma.

The same results were obtained by direct gel filtration on Sephadex G-25 of untreated samples of saliva from the same individuals. In this case, a strong group-specific activity was frequently found in the excluded effluent, due to fractions 1 and/or 2 (Fig. 2), as demonstrated by recycling this peak into Sephadex G-100.

Table I summarises the results obtained for the 60 secretors of fraction 3.

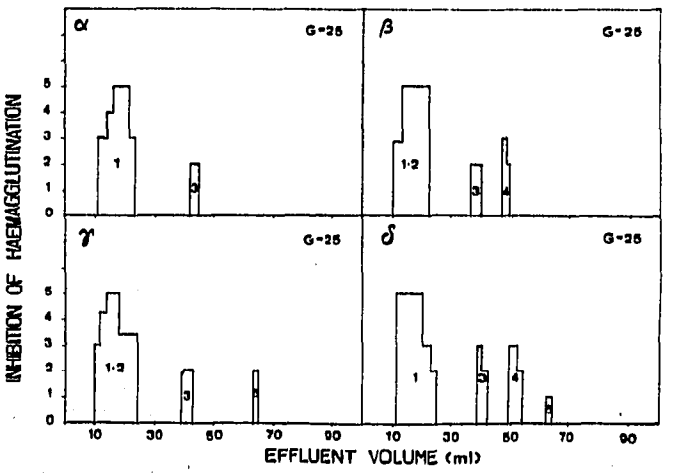

Fig. 2. The four salivary patterns of 3, 4 and 5 ABH sub-fractions as determined on 20 × 1.2 cm Sephadex G-25 columns from four secretors of fraction 3.

TABLE I

SALIVARY GEL FILTRATION PATTERNS ON SEPHADEX G-100 AND G-25 OF 60 SECRETORS OF FRACTION 3

Sub-type	*Type III (fractions 1,3)*			*Type IV (fractions 1,2,3)*			*Type VI (fraction 3)*			*Type VII (fractions 2,3)*			*Total*
	Blood group												
	A	*B*	*O*	*A*	*B*	*O*	*A*	*B*	*O*	*A*	*B*	*O*	
α (fractions 3)	3	3	5	10	1	15		—		2		1	40
β (fractions 3, 4)	1		1			1			1		—		4
γ (fractions 3, 5)		—		2	1	2		—			—		5
δ (fractions 3, 4, 5)		—		3		3		—			—		6
													55*

* Among the remaining five secretors of fraction 3, three were group AB and three secreted H-3, but neither A-3 nor B-3. Their patterns were the following: AB(A-1,2,3/B-1,2,3/H-1,2,3/-); AB(A-1, 2,3/B-1,2,3/H-1,2,3/-); AB(A-1,2/B-1,2/H-1,3/-); B(B-1/H-1,3/-); A(A-1/H-1,2,3/-). They were therefore all sub-type α.

TABLE II

ABH GEL CHROMATOGRAPHIC PATTERNS IN RED CELL STROMA AND SALIVA OF 12 BLOOD DONORS

Donor's initials	*Blood group*	*Red cell stroma ABH pattern*	*Salivary ABH pattern*
M.T.F.	A_1B	A-1,2,3/B-1,2,3/H-1,2,3/-4,5	A-1,2/B-1/H-1,2
G.G.	A_1	A-1,2,3/H-1,2,3/-4,5	A-1,2/H-1,2
F.A.	A_2	A-1,2,3/H-1,2,3/-4,5	A-1/H-1
A.P.	A_1	A-1,2,3/H-1,2,3/-4,5	A-1,2/H
G.A.	A_1	A-1,2,3/H-1,2,3/-4,5	A-1,2,3/H-3
F.M.	A_1	A-1,2,3/H-1,2,3/-4,5	A-1/H-1
E.P.	A_2	A-1,2,3/H-1,2,3/-4,5	A-3/H-3
P.B.	B	B-1,2,3/H-1,2,3/-4,5	B-1,2,3/H-1,2,3
M.P.	B	B-1,2,3/H-1,2,3/-4,5	B-2,3/H-2,3
C.D.F.	B	B-1,2,3/H-1,2,3/-4,5	B-1/H-1,2,3
R.G.	O	H-1,2,3/-4,5	H-1,2
Z.D.	O	H-1,2,3/-4,5	H

The comparison of stromal and salivary ABH G-100 and G-25 gel chromatographic patterns in 12 blood donors showed no apparent correlation (Table II).

Carbohydrate analyses

The α-naphthol reaction was performed on the eluates from Sephadex G-25 for the purpose of studying the correspondence between the serological and chemical results.

In the analysis of Sephadex G-25 gel chromatograms from red cell stroma, four carbohydrate-positive peaks were recorded at the same elution volumes of the

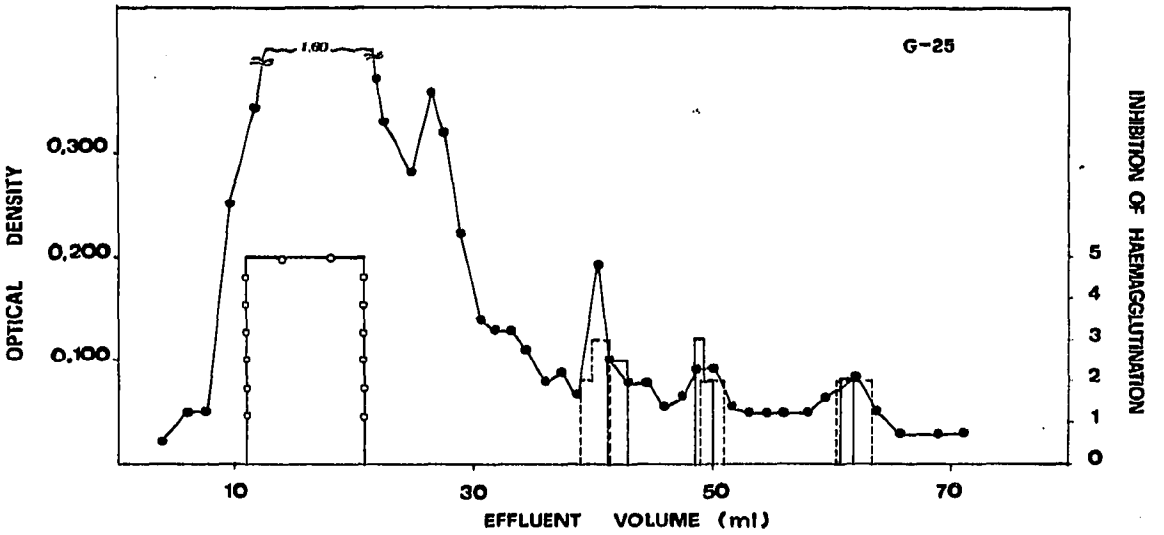

Fig. 3. Carbohydrate analysis (α-naphthol reaction) on the eluate from gel filtration of solubilized red cell stroma of group A on Sephadex G-25. ———, Inhibition of the anti-A serum; — — —, inhibition of the anti-H serum; ○—○, haemolytic material; ●—●, optical density at 560 nm.

excluded effluent (fractions 1 and 2) and of fractions 3, 4 and 5. In Fig. 3, a typical result is shown. In the carbohydrate analysis of Sephadex G-25 gel chromatograms from different ABH types of saliva, the reaction was positive only where a peak of

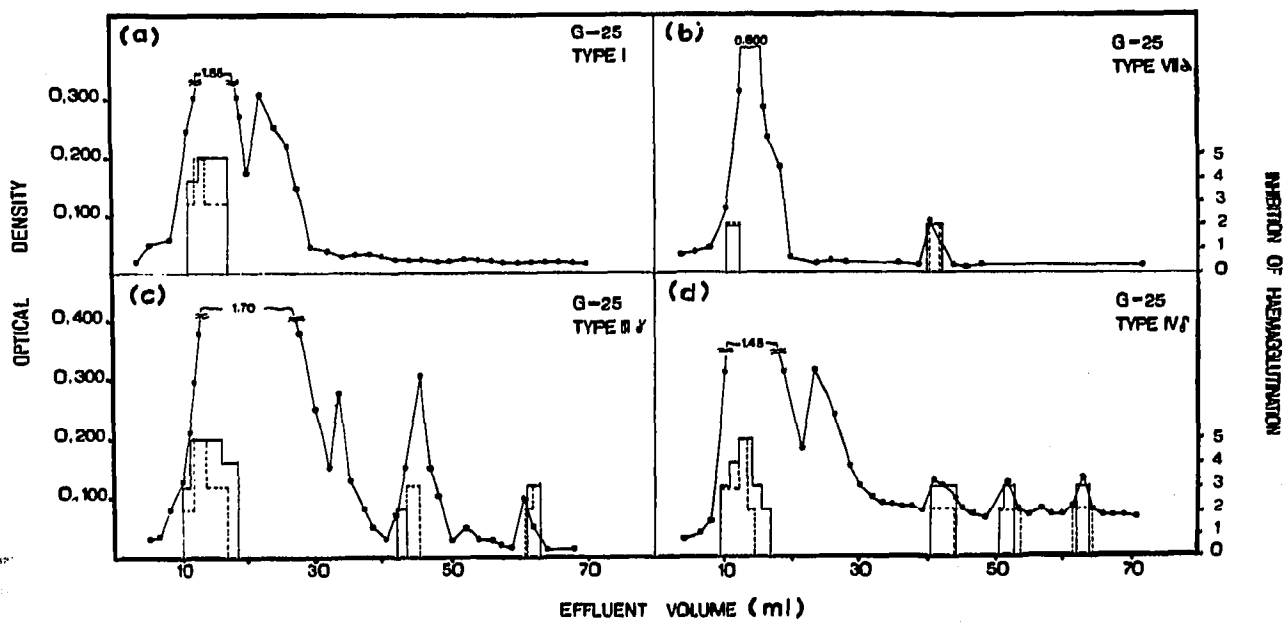

Fig 4. Carbohydrate analysis (α-naphthol reaction) on the eluate from gel filtration of some ABH types of human saliva (type I; type VII α; type III γ; type IV δ). Symbols as in Fig. 3.

serological activity was detectable. The results of some experiments are reported in Fig. 4a–d.

Gel filtration on Sephadex G-15 and dialyses

No attempts have been made to determine the molecular weights of fractions 4 and 5, but some experiments on Sephadex G-15 were carried out in order to obtain orientative data. Samples of saliva, containing fractions 1, 2, 3, 4 and 5, when filtered through this gel, showed a large excluded peak of activity and another peak of non-specific activity at the elution volume of 53 ml (Fig. 5). The excluded effluent, recycled on Sephadex G-25, gave an excluded peak (fractions 1 and 2) and two other peaks at the elution volumes of fractions 3 and 4.

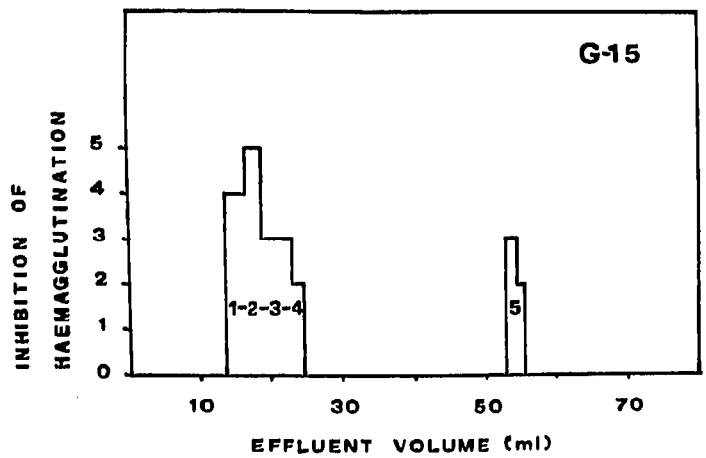

Fig. 5. Gel filtration on Sephadex G-15 (30 × 1.2 cm bed) of a sample of saliva of group A containing the five fractions.

The same specimens of saliva were then directly gel filtered on Sephadex G-25. Fraction 5 separated in this way was recycled on Sephadex G-15, and one peak only of non-specific activity was obtained at the elution volume of 53 ml.

Fraction 5 only was therefore separated on Sephadex G-15, all of the remaining fractions being excluded. In this gel, sucrose and glucose had an elution volume of 24–25 ml.

Dialysis experiments were carried out chiefly on the saliva of some subjects secreting the five ABH fractions. Fractions 2, 3, 4 and 5 were separated from fraction 1 by precipitation with four volumes of methanol. The supernatant solution was evaporated to dryness and the residue was dissolved in distilled water and the solution dialyzed against water. The residue and the dialyzate, reduced to 1–2 ml, were separately gel filtered through Sephadex G-25. Fractions 3, 4 and 5 were detected in the dialyzate and fraction 2 in the residue.

Attempts to separate ABH fractions 1, 2, 3, 4 and 5 by various chromatographic and electrophoretic techniques

Various attempts were made to separate the five ABH fractions by different chromatographic and electrophoretic methods, mostly with unsatisfactory results.

ABH fractions previously separated by methanol precipitation from saliva and solubilized stroma and successive gel filtration on Sephadex G-100 and G-25 were examined, as well as whole saliva and solubilized stroma.

In all cases, fractions 1, 2 and 3 did not move from the starting line in thin-layer, paper and glass-fibre chromatography. Migration with low and medium R_F values, respectively, was obtained only for fractions 4 and 5 with some solvents in thin-layer, paper and glass-fibre chromatography.

The technique of multiple developments on silica gel plates with the solvent mixture chloroform–methanol–water (65:25:4) was preferred, because it also offered the possibility of checking the results reported in a previous paper[1]. These experiments demonstrated that fraction 3 does not move, while fraction 4 migrates about 2–3 cm and fraction 5 about 7–10 cm, both preserving their non-specific serological activity.

No improvements were obtained by paper, cellulose acetate and glass-fibre electrophoresis. In polyacrylamide gel electrophoresis, only fraction 1 was detected at the origin by the periodic acid–Schiff method. The serological tests on the eluates did not give results that could be clearly interpreted.

Column chromatography of solubilized stroma on silicic acid

As the TLC results obtained before the detection of fractions 4 and 5 were used to interpret the results of some workers on the analysis of alcoholic extracts of stroma[1], a revision of the previous work was believed to be necessary and experiments were carried out by column chromatography of solubilized stroma on silicic acid, and gel filtration of the eluates on Sephadex G-25.

No serological activity was observed in the chloroform–methanol (9:1) eluates, while the chloroform–methanol (6:4) eluates contained two active fractions with the same elution volumes and ABH activity as those of fractions 4 and 5.

DISCUSSION

The ABH group-specific substances of erythrocytes have been considered, until recent years, to be different from the ABH substances of secretions. Recent gel filtration studies of solubilized stroma[1,6,7] and of stroma extracts prepared with different solvents [8–11] have provided evidence that high-molecular-weight water-soluble and alcohol-precipitable glycoproteins with A, B and H specificities occur and show the same gel chromatographic behaviour as that of ABH glycoproteins from secretions.

This substance (which we have named fraction 1, as the similar salivary fraction) has been detected in all of the stroma examined in this and previous work[1], irrespective of the secretor status of the individual. Thus we were unable to confirm the recent results of Gardas and Koscielak[12], who found that A, B and H specificities in glycoprotein fractions of stroma were present only in the red cells of secretors.

Red cell stroma contains four additional active fractions. Two of these (fractions 2 and 3) were described in a previous paper[1]. The remaining fractions, which we have called fraction 4 and fraction 5, have been detected as impurities in fraction 3 in Sephadex G-100 gel chromatograms, and then separated on Sephadex G-25.

It is possible that fractions 4 and 5 are the same substances as the low-molecular-weight glycolipids that were purified on silicic acid columns by Yamakawa *et al.*[13]

and recently separated into two main fractions (fractions I and II) by Hakomori and Strycharz[14]. In fact, the present study showed that only fractions 4 and 5 have been eluted with chloroform–methanol (6:4) from a short silicic acid column after adsorption of the methanolic extracts of solubilized stroma, under experimental conditions similar to those described by Hakomori and Strycharz. In addition, fractions 4 and 5 are the only two of the five active fractions that show closely similar TLC behaviour to that of fractions I and II of Hakomori and Strycharz.

The molecular weights of fractions 4 and 5 from red cell and saliva are probably very low. Both fractions are completely dialyzable through Visking membranes and have an elution volume much greater than that of fraction 3, whose molecular weight has been previously discussed[2]. Fraction 4, which is retained in Sephadex G-25 (fractionation range 100–5000 and approximate limit for a complete exclusion mol. wt. 5000), is excluded from Sephadex G-15 (approximate fractionation range up to mol. wt. 1500), while fraction 5 is also retained in this gel with an elution volume greater than that of glucose and sucrose. In our opinion, however, in estimating the molecular size of this substance, one should not attach too much importance to the latter results, because the shape and chemical structure of a given compound have a strong effect on its gel filtration behaviour, especially on Sephadex G-15 and G-10.

Because of the non-specific ABH activity of fractions 4 and 5, the problem arises of the true value of their serological properties and of their relevance or otherwise to the ABO system.

Although further purification of these fractions was attempted by gel filtration on Sephadex G-15 and by TLC, we have found no evidence of obtaining purification as complete as that described by Hakomori and Strycharz[14] for their fraction I, which led to the separation of the components I and II with sufficient ABH specificity. Therefore, no definite conclusions can be reached about the lack of specificity of frac-

TABLE III

ABH SALIVARY GEL CHROMATOGRAPHIC TYPES AND SUB-TYPES

Type	*Fractions*	*Gel filtration patterns for each substance*		
Secretors				
I	1	A-1	B-1	H-1
II	1,2	A-1,2	B-1,2	H-1,2
III	1,3	A-1,3	B-1,3	H-1,3
IV	1,2,3	A-1,2,3	B-1,2,3	H-1,2,3
So-called non-secretors				
V	2	A-2	B-2	H-2
VI	3	A-3	B-3	H-3
VII	2,3	A-2,3	B-2,3	H-2,3
VIII	—	A	B	H
Sub-types of Types III, IV, VI and VII				
α	3			
β	3,4			
γ	3,5			
δ	3,4,5			

tions 4 and 5, a property which provisionally is to be considered only as an identification character.

As to the relevance to the ABO system, a certain amount of importance could be attached to the negative results obtained by testing fractions 4 and 5 with anti-M, anti-N, anti-Rh and anti-P sera. Greater importance, in our opinion, should be ascribed to the relationship between the specific fraction 3 and the non-specific fractions 4 and 5, which are secreted only in the saliva of secretors of the specific fraction 3.

On the basis of the present results, fractions 4 and 5 can therefore be considered as belonging to the ABO system as well as fractions 1, 2 and 3 of red cells and saliva.

For this reason, four sub-types, due to fractions 4 and 5, could now be admitted of those ABH gel chromatographic salivary types[3] in which fraction 3 is represented (Types III, IV, VI and VII). These sub-types have been named α, β, γ and δ.

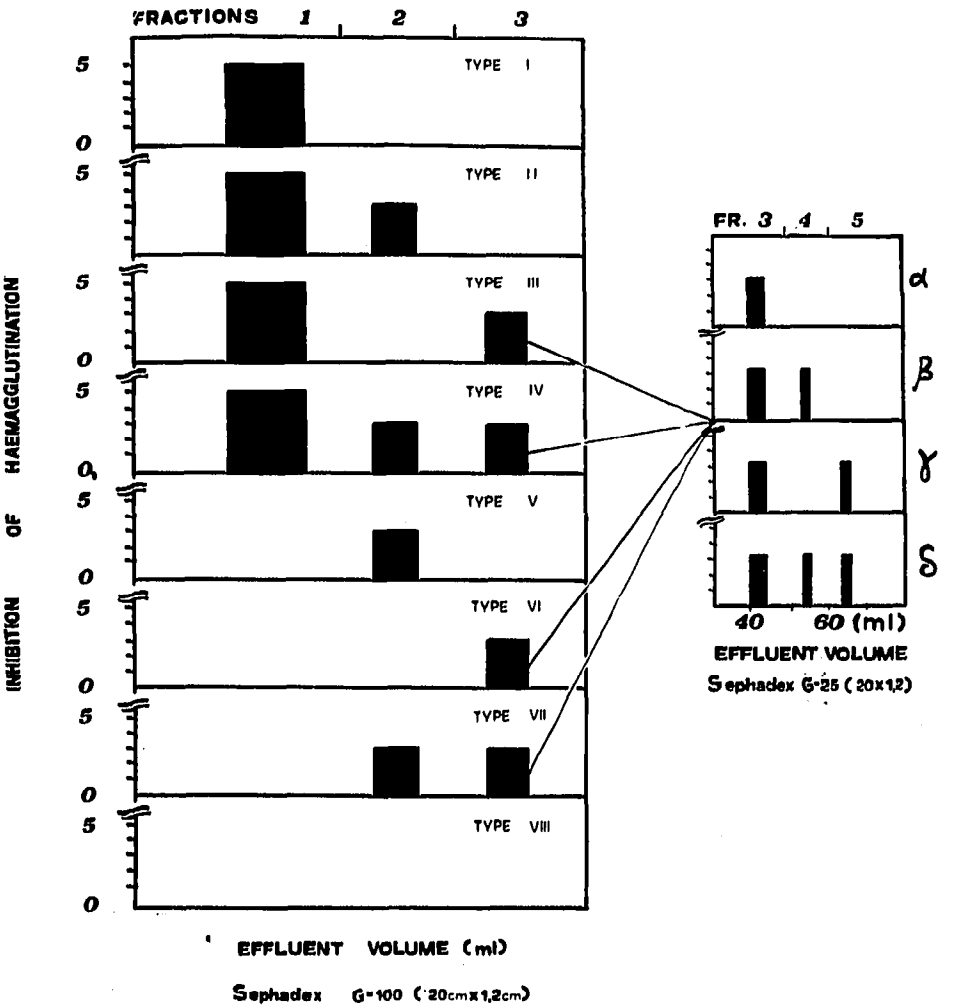

Fig. 6. Schematic representation of the ABH salivary gel filtration patterns that were identified by means of the combined use of Sephadex G-100 and G-25 columns.

As a consequence, the ABH salivary gel chromatographic types and sub-types shown in Table III could be now considered.

Fig. 6 represents the 20 ABH salivary gel filtration patterns that were identified in human saliva by means of the combined use of Sephadex G-100 and G-25 columns.

As the two new fractions, under the experimental conditions used, inhibit the three main sera of the ABO system irrespective of the blood group of the donor, they can be represented by the already proposed symbols[3] without any indication of the group of the fraction, as in the following examples:

Type III, group A: A(A-1,3/H-1,3/-4)
Type IV, group A: A(A-1,2,3/H-1,2,3/-4,5)
Type IV, group B: B(B-1,2,3/H-1,2,3/-5)
Type III, group A: A(A-1,3/H-1,3/-).

Studies are in progress on the gel chromatographic ABH types and sub-type of human meconium, seminal fluid and urine.

The following procedure is recommended for the detection of ABH salivary types. A 3–4-ml volume of saliva is freshly collected and centrifuged, and 1–1.2 ml are gel filtered on a 20 × 1.2 cm Sephadex G-100 column and another 1–1.2-ml portion on a 20 × 1.2 cm Sephadex G-25 column. The haemagglutination inhibition test should be performed as soon as possible (not later than 24 h) on the eluates from Sephadex G-25. Saliva can be stored in the frozen state without serious damage for several months but early examination is to be preferred.

When the amount of the sample is very small, for instance in the case of new-born children, 1 ml of saliva can be sufficient for a first gel filtration through Sephadex G-100 followed by concentration to 1 ml of the content of the tubes of fraction 3 and subsequent gel filtration on Sephadex G-25.

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