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GEL FILTRATION OF ABH BLOOD GROUP SUBSTANCES

I. FRACTIONATION OF ABH SUBSTANCES OF HUMAN SALIVA

A. FIORI, G. V. GIUSTI AND G. PANARI
Istituto di Medicina Legale e delle Assicurazioni, Università Cattolica del Sacro Cuore, 00168 Rome (Italy)
AND
G. PORCELLI
Istituto di Chimica, Università Cattolica del Sacro Cuore, 00168 Rome (Italy)

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SUMMARY

Human saliva from secretors of groups A, B, O and AB was gel filtered on Sephadex G-200 and G-100 columns, and on thin layers and the eluates were tested for A, B and H antigens by the haemagglutination inhibition method. A main excluded fraction (fraction 1) was detected in all the samples examined. A part of this fraction was also excluded from Sepharose 4B. The molecular weight of this fractions can be assumed to be between $2 \cdot 10^5$ and $5 \cdot 10^6$. In some samples of saliva, one or two group-specific subfractions were identified (fractions 2 and 3). Fraction 2 is excluded from Sephadex G-50 and probably has a molecular weight between 10 000 and 13 000. Fraction 3 is dialysable and has a lower molecular weight, probably no greater than 1500–2000. Fraction 1 is water-soluble and can be precipitated in alcohol, while fractions 2 and 3 are both water- and alcohol-soluble.

INTRODUCTION

Gel filtration on dextran gels or on crosslinked polyacrylamide has already been used for the purification and/or fractionation of ABH substances from various sources[1–10]. This method therefore seemed particularly useful for the study of ABH salivary substances, in which the presence of alcohol-soluble and alcohol-insoluble fractions has been recently demonstrated[11]. The present study deals with the results obtained by gel chromatography of untreated and alcohol-fractionated human saliva.

MATERIALS AND METHODS

Preparation of the samples

Samples of saliva from seven A, six B, five AB and five O secretors were examined. 10–15 ml of resting saliva, collected without preliminary stimulation, were

centrifuged, titred and then either immediately subjected to gel chromatography or submitted to alcohol or Rivanol fractionation before gel filtration. Secretors were considered those donors whose saliva clearly inhibited the specific serum at a dilution of 1/10 or greater.

The titre of the blood group specific substances on boiled and on unboiled samples was determined by the inhibition technique using freshly collected, diluted and centrifuged saliva, according to a slightly modified version of the method of BOORMAN AND DODD[12]. The samples examined had the following titres:

group A = 1/10; 1/50; 1/100; 1/400; 1/500; 1/1000; 1/2000
group B = 1/100; 1/100; 1/200; 1/400; 1/500; 1/1000
group O = 1/10; 1/50; 1/100; 1/100; 1/300
group AB = A 1/10, B 1/100; A 1/50, B 1/100; A 1/200, B 1/100; A 1/400, B 1/400; A 1/400, B 1/1000.

Untreated saliva, freshly collected and centrifuged, was used for thin-layer gel filtration experiments.

The samples to be examined by gel filtration on columns, in the first experiments were dialysed 24 h against Tris–HCl buffer, pH 7.3, (Tris–HCl 0.005 *M* containing 0.9% NaCl) and then concentrated to 3–4 ml in an air current at 4°. In successive experiments, dialysis was omitted to prevent loss of the lowest molecular weight fraction, and concentration was effected only on saliva from weak secretors.

In some experiments samples of saliva, usually 10 ml, were treated with 4.4 ml of 0.5% aq. Rivanol. After standing for 1 h the precipitated material was collected by centrifugation, washed five times with water, resuspended in water and, together with the supernatant, dialysed overnight against water in order to remove any excess Rivanol. After dialysis the precipitate became partially water soluble.

Samples of saliva (usually 10 ml) from the same donors were treated with absolute methyl alcohol or ethyl alcohol (1:4). After standing for 1 h, the precipitated material was collected by centrifugation, dried and suspended in saline. As a part of the precipitate was soluble in saline, the insoluble component was separated by centrifugation and once again suspended in saline for serological examination. The alcoholic supernatant solution was brought to dryness and dissolved in saline. The supernatants from Rivanol and ethanol or methanol precipitation, as well as the water-soluble part of the precipitate, were submitted to serological tests and then to gel filtration.

Gel filtration on columns

Gel filtration on Sephadex G-100 (fine) was performed using 1.5 × 36 cm columns, and 0.05 *M* Tris–HCl buffer (pH 7.3), which contained 0.9% NaCl. Flow rate was maintained at 15–20 ml/h and 1–1.5-ml fractions were collected.

Gel filtration on Sephadex G-200 (fine) was carried out using K 25/45 (bed dimensions, 2.5 × 32 cm) columns (Pharmacia, Uppsala) with the same buffer as eluant, at a flow rate of 15–20 ml/h and 2–3-ml fractions.

Bed dimensions of 2 × 80 cm for gel filtration on Sephadex G-50 and beds of 2.5 × 38 cm for Sephadex G-25 were used, at a flow rate of 15–20 ml/h and 2-ml fractions.

Gel filtration on Sepharose 4B (bed dimensions, 2.2 × 32 cm) was performed

with 0.025 *M* phosphate buffer (pH 7.2) at a flow rate of 15–20 ml/h, collecting fractions of 2–3 ml.

Packing of the columns and the void volumes were checked with Blue Dextran 2000, which had been freshly prepared (Pharmacia, Uppsala). Cytochrome *c* (mol.wt. 12 400), glucagon (mol.wt. 3485), oxytocin (mol.wt. 1007), bacitracin (mol.wt. 1400), and sucrose (mol.wt. 342) were also used as reference substances. The protein content of each fraction was determined by the LOWRY–FOLIN method[13] with an autoanalyser.

The fractions from Sephadex G-100, G-200, G-50 and G-25 were directly examined by the inhibition technique as described below. The fractions obtained from Sepharose 4B were made isotonic by adding 0.1 ml of 14.6% NaCl to 2 ml of eluate and then were submitted to serological analysis.

Thin-layer gel filtration

Thin-layer gel filtration was performed on Sephadex G-200 (superfine) and on Sephadex G-100 (superfine), swollen in 0.05 *M* Tris–HCl buffer (pH 7.3) containing 0.9% NaCl. To obtain 1-mm-thick layers of the gel on glass plates (20 × 20 cm), a strip of tape 1 cm wide was attached along the four edges of the surface of each plate. Into the centre of the glass surface (18 × 18 cm) so delimited, were poured 40 ml of a semifluid slurry of Sephadex that was then spread with a glass rod. A horizontal homogeneous surface was obtained by shaking the plate with caution. The excess water was absorbed by applying over the tape, along the four edges of the plate, four sheets of filter paper (20 × 20 cm). After 20–30 min, a convenient consistency was reached. The gel plates were then equilibrated overnight with Tris–HCl buffer by a descending development at an angle of 15° in a closed chamber. The flow rate was 2 cm/h, as measured by a completely excluded substance (Blue Dextran 2000). After the equilibration was completed, samples of saliva (80–100 μl) were applied to the starting line of the horizontal plate and chromatographed at an angle of 15°. As reference substances, Blue Dextran 2000, bovine serum albumin, chymotrypsin, cytochrome *c*, insulin and oxytocin were used. After completion of the run the uncoloured proteins were detected by covering the corresponding lane of the gel for a few minutes with a filter paper which was then dried for 10 min at 100° and stained with a methanolic solution of Amido Black. Insulin and oxytocin were eluted with distilled water and determined colorimetrically using the Folin–Ciocalteu reagent[14].

For the detection of active blood group substances by means of the inhibition technique, the gel was cut into 1 × 2 cm fractions (usually 16) beginning 1 cm before the starting line and continuing until the exclusion line. Each fraction of the gel was then transferred into separate test tubes and centrifuged; 0.2 ml of saline was added, and the elution prolonged overnight. After centrifugation 0.1 ml of the clear supernatant was transferred into another test tube and serologically examined by the inhibition test.

Haemagglutination inhibition test

Commercial anti-A and anti-B sera usually titred 1/4–1/8 were employed. Anti-H reagent of the same titre was prepared from seeds of *Ulex europaeus* according to BOORMAN AND DODD[12]. 0.1 ml of the antiserum was added to 0.1-ml samples of

the fractions eluted from the columns or from the thin-layer chromatograms. After 30 min of incubation at room temperature, 0.1 ml of a 2% suspension in saline of the appropriate red cells was added. The tubes were then centrifuged at 1000 r.p.m. for 1 min and finally vigorously shaken before reading the agglutination macroscopically. The results were scored in the following way: C = one clump, complete agglutination; +++ = very large clumps; ++ = large clumps; + = several clumps; ± = minimal clumps; and − = no agglutination, *i.e.* complete inhibition.

RESULTS

Sephadex G-100 and G-200 columns

All the samples of saliva filtered through Sephadex G-100 and G-200 columns contained a main fraction of blood group specific substance which was completely excluded from the gel. When zone broadening took place (*e.g.* in strong secretors) a control was made on the last fractions of the active peak. These fractions were pooled, dialysed overnight against distilled water, then concentrated to a small volume (2–3 ml) by an air current at 4° and recycled on the same column. A sharp

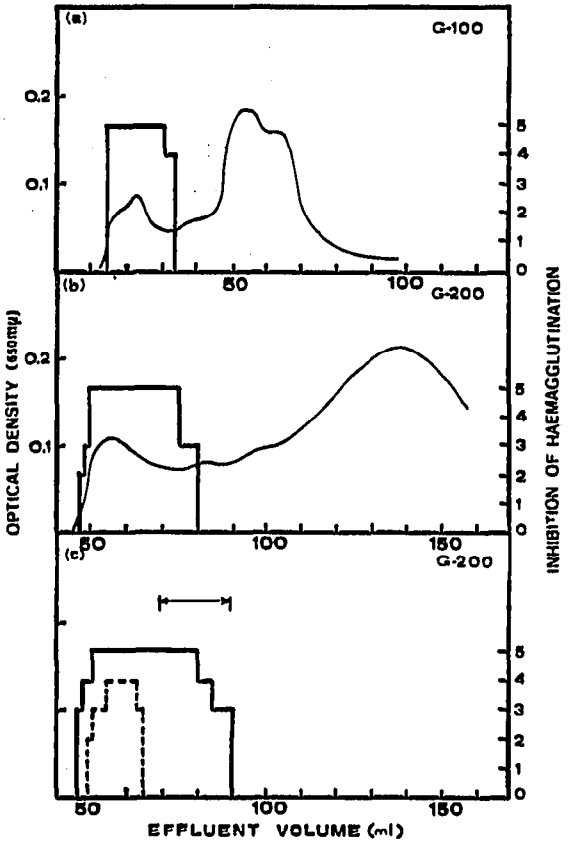

Fig. 1. Gel filtration of human saliva on Sephadex G-100 and G-200 columns. The serological activity is indicated by the thick line and the protein concentration by the thin line. The results of the inhibition of haemagglutination are scored as follows: 0 = one clump; 1 = +++; 2 = ++; 3 = +; 4 = ±; 5 = − (no agglutination). (a) Gel chromatogram of the saliva from a subject of group A (A titre: 1/200); (b) saliva from a group B subject (B titre: 1/800); (c) saliva from a group A_1 subject (A titre: 1/1000). The broken line shows a recycling experiment on the effluent indicated by the arrow.

excluded active peak was obtained. Fig. 1 shows the elution diagram of some typical experiments.

Sepharose 4B columns

The gel filtration on Sepharose 4B columns was performed with the aim of studying the spread of molecular weight of the active fraction of the blood group excluded from Sephadex G-100 and G-200. For this purpose specimens of whole saliva, having only the fraction excluded from Sephadex G-200 and G-100, were submitted to gel filtration on Sepharose 4B. Also excluded fractions isolated by means of a preliminary gel filtration through Sephadex G-200, then dialysed and concentrated to 2–3 ml, were examined in the same way.

All the samples examined were eluted as a broad zone of serological activity. The leading part of this zone was excluded from the gel, as shown in Fig. 2, where

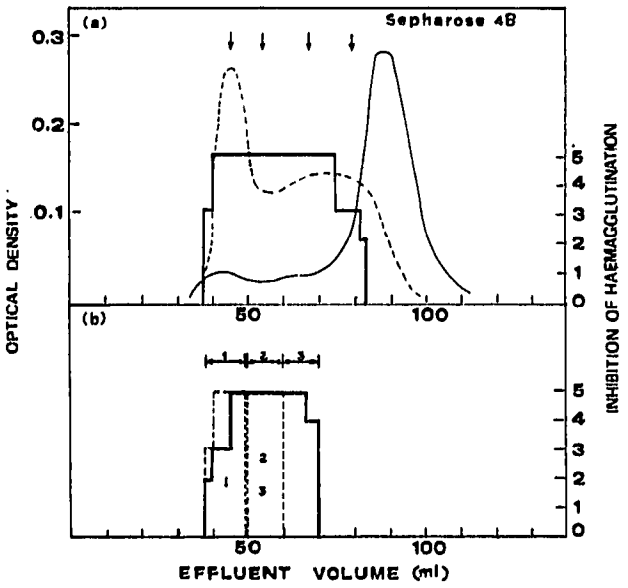

Fig. 2. Gel filtration on Sepharose 4B of a sample of saliva from a subject of group O (H titre: 1/200). The broken line of (a) is the UV absorption curve at 260 mμ of Blue Dextran 2000. In (b) recycling experiments are recorded. The arrows indicate the serological titration reported in Table I.

the elution curve is compared with that of Blue Dextran. The void volume is indicated by the leading peak of the UV absorption curve (at 260 mμ) of the Blue Dextran. The inhibition titre was determined on the eluate by the double dilution method. Maximum activity was found to coincide with the excluded part of the active zone.

In Table I the results of such a determination on four critical tubes of the case given in Fig. 2a are reported. A recycling of pooled fractions of the active zone was found necessary to ascertain if only one part of this zone was excluded. For this purpose three pools were prepared from the active zone. The first pool (40–50 ml) was made with the effluent of the leading part of the curve; the second (51–58 ml) and the third (59–68 ml) with the remaining part of the curve (Fig. 2b). The pooled solutions were dialysed 24 h against distilled water, then concentrated by an air current at 4° to 2–3 ml and separately recycled on Sepharose 4B. As shown by

TABLE I

SALIVA A GEL FILTERED ON SEPHAROSE 4B (SEE FIG. 2a)

To each dilution, 0.1 ml of serum anti-A titred 1/8 was added and, after 30 min, 0.1 ml of 2% red cells suspension. Agglutination is indicated by C (one clump), +++, ++, +, ±. No agglutination is indicated by —.

Sample (Test tube no.)	*Dilution of 0.1 ml of the eluate*							
	1/1	*1/2*	*1/4*	*1/8*	*1/16*	*1/32*	*1/64*	*1/128*
16 (46 ml)	—	—	—	±	±	+	+	+++
20 (55 ml)	—	—	±	+	+	++	C	C
24 (68 ml)	—	—	±	+	++	C	C	C
32 (80 ml)	+	++	C	C	C	C	C	C

Fig. 2b, the first pool is again excluded. The second pool had the same elution volume (50–59 ml) as in the first cycle. On the contrary the elution volume of the third pool was changed, becoming the same as the second pool (50–57 ml).

Eight samples of saliva (4 of group O; 3 of group A and 1 of group B) exhibited one or two additional active peaks on Sephadex G-100 and G-200 columns. The elution volumes of these two blood group subfractions were the following: 105 ml and 340 ml, respectively, on Sephadex G-200 and 55 ml and 78–80 ml on Sephadex G-100, with variations of 2–5 ml in relationship to the packing of the columns. The subfractions, especially the first one, had weak activity so that the 0.1 ml usually employed in serological tests sometimes gave complete inhibition, but more frequently incomplete inhibition (±; +).

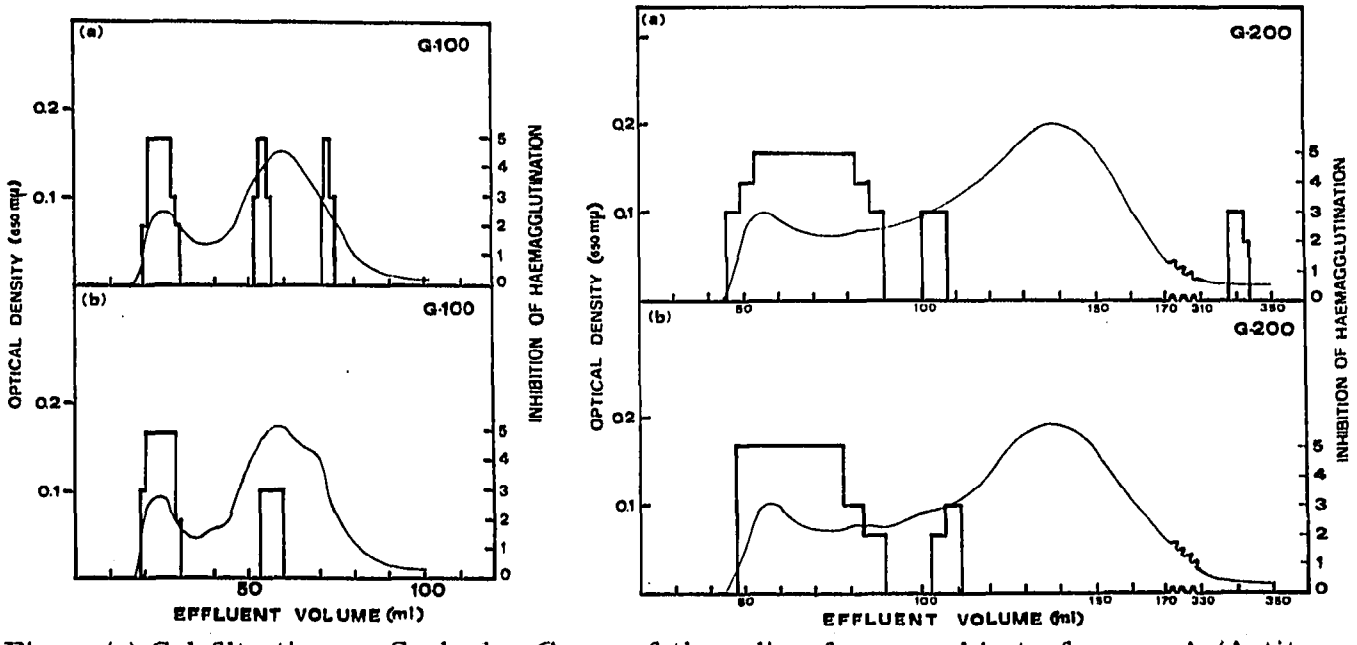

Fig. 3. (a) Gel filtration on Sephadex G-100 of the saliva from a subject of group A (A titre: 1/2000) with three group specific fractions; (b) The saliva from a secretor of group A (A titre: 1/800) with two group specific fractions.

Fig. 4. Gel filtration on Sephadex G-200 of the same samples of Fig. 3a and b.

Figs. 3 and 4 show the elution curves of salivas having one or two retained active fractions. In Table II a typical titration experiment is given in which the inhibition activity of the main excluded peak and that of the two subfractions are compared. Reproducible results were obtained in the same secretors reexamined at various times. No differences were observed in saliva preserved at —20° for a number of weeks.

TABLE II

GEL FILTRATION OF SALIVA A, TITRE 1/100

3 ml gel filtered on Sephadex G-100 column (36 × 1.5 cm); flow rate 8 ml/h.

Sample (Test tube no.)	*Dilution of 0.1 ml of the eluate*							
	1/1	*1/2*	*1/4*	*1/8*	*1/16*	*1/32*	*1/64*	*1/128*
Excluded effluent								
9	C	C	C	C	C	C	C	C
10	±	+	+	+ +	+ + +	C	C	C
13 (18 ml)	—	—	—	—	±	+	+ + +	C
16	—	—	—	—	—	±	+	+ + +
19 (24 ml)	—	—	—	—	—	—	+	+ + +
22	—	—	—	±	+	+ + +	C	C
26	—	±	+	+ + +	C	C	C	C
Non-excluded effluent								
54	C	C	C	C	C	C	C	C
55	+	+	+ + +	C	C	C	C	C
56 (62 ml)	±	+	+	+ +	C	C	C	C
57	+	+	+ +	C	C	C	C	C
58	C	C	C	C	C	C	C	C
70	C	C	C	C	C	C	C	C
71	+	+	+ + +	C	C	C	C	C
72 (80 ml)	—	—	—	—	±	+	+ + +	C
73	+	+	+ +	+ + +	C	C	C	C
74	C	C	C	C	C	C	C	C

Thin-layer chromatography on Sephadex G-200 and G-100

Thin-layer chromatography on Sephadex G-200 and G-100 allowed an easy separation of the two subfractions from the excluded fraction. As thin-layer gel filtration on Sephadex G-200 had no real advantages in comparison with Sephadex G-100, the latter was preferred throughout this work.

The main active fraction (fraction 1) was detectable in all the subjects examined. It migrated as the Blue Dextran spot, *i.e.* with the exclusion front. The faster subfraction (fraction 2) migrated 8–9 cm from the starting line (7–8 cm on Sephadex G-200) and had the same mobility as cytochrome *c*. The slow subfraction (fraction 3) was detected in a zone located in the first 2 cm after the starting line.

The R_{cy} values (ratio of the migration distance of the active fraction to that of cytochrome *c*) were the following:

fraction 1 (excluded) R_{Cy} 1.65
fraction 2 R_{Cy} 1
fraction 3 R_{Cy} 0.10

In Fig. 5a and b, thin-layer gel chromatograms are given which were obtained from the same saliva as in Figs. 3 and 4; in Fig. 5d, a chromatogram from a saliva containing the 3 fractions, while in Fig. 5c the chromatogram is recorded of the same saliva as in Fig. 1a which was lacking in the subfractions.

The advantage of the thin-layer gel filtration is the possibility of running four samples of saliva in small amounts together with the reference substances. On the other hand the elution step is tedious and unpractical for routine work. In addition, false negative results when searching for subfractions could be obtained, due to the loss of material during elution.

Methanol or ethanol treatment

Methanol or ethanol treatment of saliva gave rise to a precipitate which was partially soluble in saline. The water-insoluble material was suspended in saline

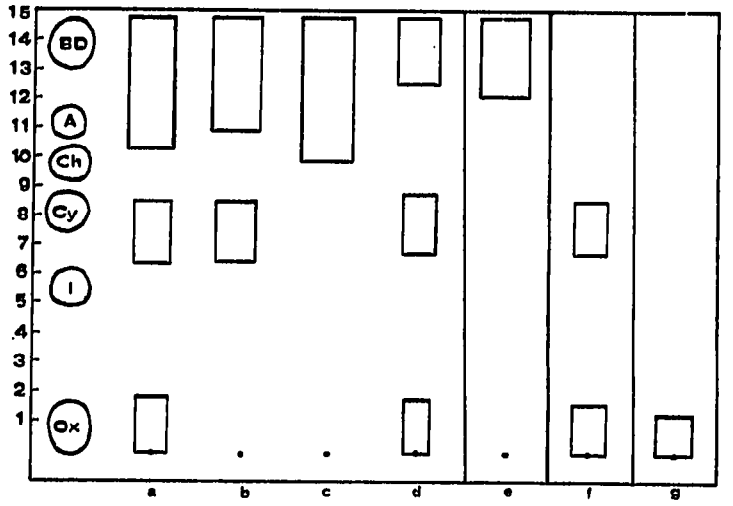

Fig. 5. Thin-layer gel filtration of saliva on Sephadex G-100. Reference substances: BD = Blue Dextran 2000; A = albumin; Ch = chymotrypsin; Cy = cytochrome *c*; I = insulin; Ox = oxytocin. The zones of blood group activity are represented by rectangles. (a) and (b): the same samples of Figs. 3 and 4; (c) the saliva of Fig. 1a; (d) another sample of saliva (group B) with three active fractions. For (e), (f) and (g) see explanation in RESULTS under *Methanol or ethanol treatment* and *Fraction 3*.

and serologically tested. It exhibited a strong activity. The water-soluble part of the precipitate was gel filtered on Sephadex G-200, G-100, Sepharose 4B columns and on thin layers of Sephadex G-100. Only the excluded blood group specific substance (fraction 1) was detectable on Sephadex G-200 and G-100, also in those samples of saliva containing subfractions (Fig. 5e). The elution profile on Sepharose 4B was very similar to that of untreated saliva, but the serological activity was very much lower.

The alcoholic supernatant was brought to dryness and dissolved in saline. It exhibited a clear blood group specific activity in those salivas having active subfractions (*i.e.* fractions 2 and 3). These specimens were gel filtered on columns

and on thin layers. The two subfractions were detected and they had the same elution volumes and R_{cy} values (Fig. 5f) as subfractions detected in untreated saliva.

The Rivanol treatment gave a precipitate which was partially soluble in saline after removal of Rivanol by dialysis. The solutions had blood group activity. Their main component in gel filtration was fraction 1. Gel filtration of the aqueous supernatant from Rivanol precipitation contained fractions 2 and 3.

Fraction 2

The behaviour of fraction 2 in dialysis experiments and in gel filtration on Sephadex G-50 columns was also investigated. For this purpose, salivas of secretors containing fraction 2 were gel filtered on Sephadex G-100 columns. The eluate from 48–58 ml was pooled and dialysed 24 h against distilled water. No blood group activity was detected in the dialysate whilst the retenate was clearly active. The latter was concentrated to 2 ml and gel filtered on a Sephadex G-50 column. Blood group activity was detected only as an excluded peak (elution volume 80 ml), while the reference substance cytochrome *c* was retained and had an elution volume of 104 ml.

Fraction 3

Specimens of saliva (30–100 ml) containing fraction 3 were dialysed 24 h in Visking tubes against distilled water, with three changes. The dialysate was evaporated to 2–5 ml. Only the dialysate exhibited a clear blood group activity. It was gel filtered on Sephadex G-100 and gave the same elution volume and R_{cy} value as fraction 3 of the untreated saliva (Fig. 5g).

The dialysate was also gel filtered on a Sephadex G-25 column. The elution volumes of the group specific substance (104 ml) and of some substances of known molecular weight are recorded in Fig. 6.

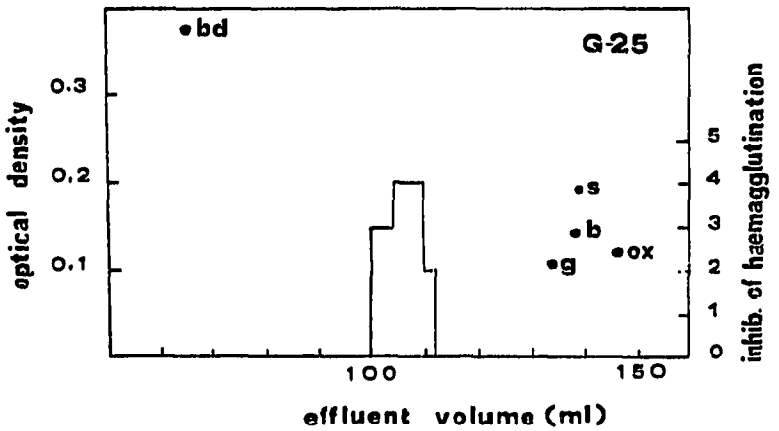

Fig. 6. Gel filtration on Sephadex G-25 of the fraction 3 isolated by dialysis of the saliva from a subject of group B. Reference substances: bd = Blue Dextran; g = glucagon; b = bacitracin; s = sucrose; ox = oxytocin.

DISCUSSION

In a previous paper it has been reported that some specimens of human saliva

can be fractionated into two main fractions by treating dried saliva with absolute methanol or ethanol. One fraction is denatured in the process and becomes water-insoluble; the other is water- and alcohol-soluble[11].

The present work is a development of these findings. In addition, some information is given on the polydispersity of the molecular weight of blood group substances from secretions.

Fraction 1

All the samples from secretors' saliva, which were examined without alcoholic treatment, had a main active peak which was excluded from Sephadex G-200 and G-100 columns and migrated with the exclusion front in thin-layer gel filtration experiments. These data are in agreement with gel filtration experiments of some other workers on ABH substances[2,4,7,9,10].

From the gel filtration behaviour of the main active fraction (fraction 1) it can therefore be assumed that this is the same substance as that isolated from secretions by the usual methods[15]. In fact, purified blood group substances from secretions are glycoproteins containing a high percentage of carbohydrate (about 85%) and have molecular weights greater than $2 \cdot 10^5$ (ref. 16), being therefore excluded from Sephadex G-200 (exclusion limit for dextrans: 200 000) as well as fraction 1 of untreated saliva. It is well known that the carbohydrate content has a strong effect on the gel filtration behaviour of proteins[17–19]. Gel filtration shows that some glycoproteins have a more expanded structure than the typical globular proteins. This may well be due to a greater hydration on solution of the carbohydrate chains[18,19]. Owing to their very high carbohydrate content blood group substances behave similarly to dextrans with regard to gel filtration behaviour.

Fraction 1 from gel filtration on Sephadex G-200 of untreated saliva also contains the larger macromolecules which form the sparingly soluble part of the group specific glycoproteins extracted from secretions by the phenol method[16,20]. These larger macromolecules probably are those excluded from Sepharose 4B.

The excluded fraction (fraction 1) is almost completely denatured, without loss of activity, when air dried and then treated by absolute methanol or ethanol. The treatment of fluid saliva with methanol or ethanol at the ratio of 1:4 gives a precipitate of fraction 1, part of which is irreversibly denatured and can not be gel chromatographed; part remains water-soluble and therefore can be studied by gel chromatography. No particular advantage is obtained by Rivanol precipitation which is incomplete and also more laborious.

The gel filtration studies on fraction 1 provided additional information on the polydispersity of the molecular weight of this fraction of the ABH blood group substances from secretions.

Values of molecular weight varying from $2 \cdot 10^5$ to $2 \cdot 10^6$ have been reported by some authors[15,16,21] from sedimentation velocity experiments carried out on ABH substances from ovarian cyst fluid.

DUNSTONE AND MORGAN[16] also studied the sparingly soluble part of the precipitate from phenol treatment of ovarian cyst fluid. This material exhibited no sharp boundaries, probably due to the high degree of polydispersity. The $S^0_{20,w}$ values of the fastest sedimenting component were greater than 15 S. This is considerably

higher than those generally found ($S^0_{20,w}$ 10–12 S) for the upper molecular range of values determined for the water-soluble blood group specific substances.

More recently, CREETH AND KNIGHT[22] gave qualitative evidence for a considerable spread of molecular weight of a B glycoprotein from ovarian cyst fluid, as demonstrated by the absence of a true plateau region in sedimentation velocity experiments.

Our gel filtration experiments on Sephadex G-200 confirmed that fraction 1 of untreated saliva has a molecular weight greater than 200 000, as discussed above.

The polydispersity of the molecular weight of this main fraction is suggested by its behaviour on Sepharose 4B. In fact, the elution profile of fraction 1 is similar to that of Blue Dextran 2000 which is known to contain some high-molecular-weight material excluded from Sepharose 4B, and other molecules which are not excluded from this gel. The leading peak of the UV elution profile of the coloured dextran contains the very large molecules and indicates the void volume. The exclusion limit for Sepharose 4B given now (1969) by the manufacturer is $20 \cdot 10^6$ for proteins and $5 \cdot 10^6$ for polysaccharides. Since the gel filtration behaviour of blood group substances is similar to that of polysaccharides, owing to their high carbohydrate content, it seems evident that the leading excluded peak of the salivary active substance has a molecular weight up to or over $5 \cdot 10^6$. The non-excluded part of the active zone is retained in the gel, as demonstrated by recycling experiments. In other words, gel filtration on Sephadex G-200 and on Sepharose 4B supported the results of DUNSTONE AND MORGAN[16] on glycoproteins from ovarian cyst fluid.

It must be emphasised that our data have been obtained mainly on untreated saliva. When the saliva is fractionated by alcohol, the water-soluble part of the precipitate is still excluded from Sepharose 4B but a great loss of substance occurs. It is therefore reasonable to think that many among the procedures used for the isolation of blood group substance produce a considerable denaturation especially of the larger molecules. This could explain the fact that many workers in the field have suggested molecular weights not greater than $1 \cdot 10^6$. It is probable, on the contrary, that the molecular weight of the main salivary blood group substance is within the range of $2 \cdot 10^5$ to $5 \cdot 10^6$.

Fractions 2 and 3

Some salivas contain one or two additional fractions, termed fraction 2 and fraction 3, which are both water- and alcohol-soluble. The blood group activity of these fractions, especially of fraction 2, is very low in comparison with that of fraction 1. In addition, the active peak eluted from the columns is usually very sharp. On the other hand, in thin-layer experiments, the active zones are usually elongated especially in specimens very rich in mucus. Fractions 2 and 3, as well as fraction 1, have a specific inhibitory activity, as controlled in each case by performing the serological test with anti-A, anti-B and anti-H sera. More complex is the problem of the results obtained with anti H serum on the eluates of saliva from A, B and AB subjects. This problem will be considered in a future paper.

The molecular weight of fraction 2 and fraction 3 can be approximately indicated by gel filtration and dialysis experiments. Fraction 2 is eluted with cytochrome *c* in gel filtration on Sephadex G-200 and G-100 columns or thin layers, while gel filtration on Sephadex G-50 columns separates fraction 2, which is excluded,

from cytochrome *c*, which is retained. This could be explained assuming that fraction 2 is a glycoprotein with a high carbohydrate content and therefore behaves on Sephadex G-50 as dextrans for which the exclusion limit of this gel is 10 000. Cytochrome *c* is, on the contrary, a carbohydrate-free protein (mol.wt. 12 400) which is not excluded, as the exclusion limit of Sephadex G-50 for proteins is 30 000. Thus fraction 2 probably has a molecular weight greater than 10 000, but no greater than that of cytochrome *c*. It should also be noted that fraction 2 was not dialysable through dialysis tubes which retained 30–40% insulin (mol.wt. 5734).

Fraction 3 is completely dialysable through a Visking membrane, as are also bacitracin (mol.wt. 1400) and oxytocin (mol.wt. 1007). In gel filtration on Sephadex G-200 and G-100 columns it has elution volumes much greater than fraction 2 (Figs. 3 and 4), migrates with oxytocin in thin-layer gel filtration on Sephadex G-100, and is not excluded from Sephadex G-25. In this gel some peptides have been used as reference substances of known molecular weight (Fig. 6) but the gel chromatographic behaviour of fraction 3, which should be considered mainly a carbohydrate in nature, can be better compared to that of a sugar as sucrose. On the basis of all these considerations, the molecular weight of fraction 3 could be assumed no greater than 1500–2000.

It is interesting to note that naturally occurring ABH substances with low molecular weights have been isolated in recent years in some secretions and also in urine. LUNDBLAD AND BERGGARD[6] reported the isolation from urine of A- and B-specific oligosaccharides. KANAZAWA[3] described several active fractions in saliva chromatographed on CM- and DEAE-cellulose, then filtered on Sephadex G-100. HIRANO *et al.*[23] isolated some ABH glycopeptides from human colostrum.

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