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GEL FILTRATION OF ABH BLOOD GROUP SUBSTANCES

II. INDIVIDUAL GEL CHROMATOGRAPHIC PATTERNS OF ABH SUBSTANCES IN THE SALIVA OF SECRETORS AND NON-SECRETORS

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SUMMARY

The saliva from 605 donors was gel chromatographed on Sephadex G-100 and was examined serologically for ABH blood group specific fractions termed fractions 1, 2 and 3. All secretors had the main active excluded fraction (fraction 1) alone or associated with fraction 2 and/or fraction 3. The saliva from non-secretors lacked fraction 1 and had fraction 2 and/or fraction 3. In some non-secretors, no active fraction was detected (true non-secretors). Eight gel filtration patterns for each of the A, B and H antigens were therefore identified.

A standardised technique on small Sephadex G-100 columns was developed and the frequencies of each gel filtration type determined in the last 199 secretors and 32 non-secretors were examined. The gel filtration pattern of H substance in group A, B and AB subjects was also studied. In some of these subjects, gel filtration patterns of the single antigens had been found which differ from each other. The ABH salivary gel filtration pattern of a single subject is a stable individual characteristic and probably is genetically determined.

INTRODUCTION

In a previous paper[1] it has been shown that the saliva from secretors contains a main active fraction of blood group specific substances (fraction 1) which is excluded from Sephadex G-200 and G-100 and only partially excluded from Sepharose 4B. It has also been reported that saliva from secretors can contain one or two blood group specific subfractions of low molecular weight (fraction 2 and fraction 3). The subfractions appeared to be associated with each other and with fraction 1, giving four gel filtration ABH patterns.

In this paper the results of a more extensive study are presented in which the saliva of non-secretors is also included. Several individual ABH gel filtration patterns were detected among secretors and non-secretors and the frequency of this character determined.

MATERIALS AND METHODS

Preparation of the samples

The samples of saliva were collected without preliminary stimulation and immediately centrifuged at 3000 r.p.m. for 10 min. Gel filtration of the supernatant was performed no later than 12 h after or, otherwise, the supernatant was preserved at −20° until gel chromatographed.

The titre of blood group substances was determined before gel filtration on unboiled saliva, using a slight modification of the technique described by BOORMAN AND DODD[2]. Secretors were considered the subjects whose saliva inhibited the specific serum at a dilution of 1/10 or more.

Gel filtration

Thin-layer gel filtration on 1-mm-thick layers of Sephadex G-100 (superfine) was performed on 20 × 20 cm plates prepared as previously described[1]. After the run was completed, the gel chromatogram was cut into several 1-cm sections, eluted with 0.2 ml of saline and the eluate serologically determined for A, B and H substances. To allow this determination, three separate spots of the same saliva were usually run on the same plate.

Gel filtration on columns was carried out on 36 × 1.5 cm beds of Sephadex G-100 with 0.05 *M* Tris–HCl buffer (pH 7.3) containing 0.9% NaCl as eluant. Flow rate was maintained at 15–20 ml/h and fractions of 1–1.5 ml were collected. 2.5–3-ml samples of saliva were usually used for gel filtration.

For routine purposes, smaller columns were found to be more practical. In fact 20 × 1.2 cm beds, with a flow rate of 6 ml/h and 1-ml fractions, allowed a satisfactory separation of the three active specific fractions. 1-ml specimens of saliva were gel filtered through these small columns, using a timed-flow fraction collector with six concentric rows of 100 holes each; in this way six samples were examined simultaneously.

Haemagglutination inhibition test

The inhibition test was performed using undiluted commercial sera (Ortho Pharm. Co., Raritan, N.J.) titred 1/4–1/8 and having a high binding constant (K_0) between 10^6–10^8 and a heterogeneity index of about 0.5. In this way the macroscopic reading of the agglutination after a very short centrifugation (30 sec at 1000 r.p.m.) was facilitated by the formation of one unbroken clump in the tubes where no inhibition occurred. An anti-H serum titred 1/8 was prepared in our laboratory from *Ulex europaeus* seeds, using the method of BOORMAN AND DODD[2].

Three 0.1-ml amounts from the contents of each test tube were transferred into separated test tubes and tested for inhibition with anti-A, anti-B and anti-H sera, respectively, as described in a previous paper[1]. The thin-layer gel chromatograms obtained with three separate spots of the same saliva were eluted as previously described[1] and examined serologically using anti-A, anti-B and anti-H sera.

Analyses

The proteins and the peptides of the eluate were determined by the LOWRY–FOLIN method[3], in most cases using an autoanalyser. The fucose content was measured

by the method of DISCHE AND SHETTLES[4] on 0.5–0.7 ml taken from the content of each tube.

RESULTS

This work deals with the saliva from 605 healthy adults (501 secretors and 104 non-secretors). As many subjects were examined a number of times, over a thousand gel chromatographic determinations will be discussed.

Thin-layer gel filtration

In the first phase of the research, most cases were studied by thin-layer gel filtration on Sephadex G-100. However, subsequent controls showed certain drawbacks in the method. In some experiments on very viscous saliva from secretors, the excluded fraction seemed to be absent, while it was detectable on columns where the viscosity of the sample had no great effect on the elution volumes. In many other cases fractions 2 and 3 had been erroneously undetected, probably due to the loss of the active material during the elution step. As a consequence, the frequency of the ABH gel filtration patterns which had been calculated on secretors on the basis of the results obtained by thin-layer gel filtration (given in a preliminary communication in 1969, ref. 5) must now be considered invalid.

Gel filtration on columns

A standardised gel filtration technique on 20 × 1.2 cm columns was used and only 231 cases (199 secretors; 32 non-secretors) were utilised in calculating the frequencies of ABH gel filtration patterns. Samples taken at random were also gel filtered on 36 × 1.5 cm columns as a control.

Secretors. All the secretors examined (501 subjects) had the main active fraction excluded from Sephadex G-100 (fraction 1). The elution volume of this fraction in 36 × 1.5 cm columns was the same as determined in a previous study[1], *i.e.* about 25 ml. In the smaller columns the elution volume was about 8–9 ml.

In weaker secretors the blood group activity of fraction 1 was weaker than in stronger secretors, and the active peak covered only a few tubes in column gel filtration. A large zone of activity was on the contrary observed in strong secretors. In some of these subjects, especially of group B, zones of activity from the 22th to the 60th ml in 36 × 1.5 cm beds were detected. In these cases the last part of the active fraction was recycled to confirm its exclusion from the gel. For this purpose the contents of the last tubes of fraction 1 were pooled, dialysed 24 h against distilled water, concentrated to 2 ml and recycled. The blood group activity was always present in the excluded effluent. Fraction 2 and fraction 3 were also found to be present in the saliva of many secretors, either singly or together.

The elution volume on Sephadex G-100 beds (36 × 1.5 cm) was about 55 ml for fraction 2 and 80 ml for fraction 3. In the smaller columns the elution volumes were 18 ml and 26 ml, respectively. It should be noted that the elution volumes of fraction 2 and fraction 3 of the same saliva on a single gel chromatogram frequently exhibited an incomplete overlapping of A, B and H activity. In some cases these slight differences in the elution volumes of the subfractions were clearer, *i.e.* A, B and H activities were displaced with respect to each other at times by as much as

one test tube. Some examples taken from small column determinations could better illustrate these findings.

Case No. 410: group A, saliva A (A-1,2,3/H-1,2,3)
fraction 2 = A activity at the 18th ml; H: 17th ml
fraction 3 = A activity at the 27th ml; H: 26th ml
Case No. 398: group AB, saliva AB (A-1,2,3/B-1,2,3/H-1,2,3)
fraction 2 = A activity at the 14th and 15th ml; B: 17th ml; H: 17th–18th ml
fraction 3 = A activity at the 23rd–24th ml; B: 23rd–24th ml; H: 24th–25th ml
Case No. 482: group AB– saliva AB (A-1,2,3/B-1,2,3/H-1,2,3)
fraction 2 = A activity at the 16th–17th ml; B: 15th–16th ml; H: 16th–17th ml
fraction 3 = A activity at the 24th–25th ml; B: 25th–26th ml; H: 26th–27th ml

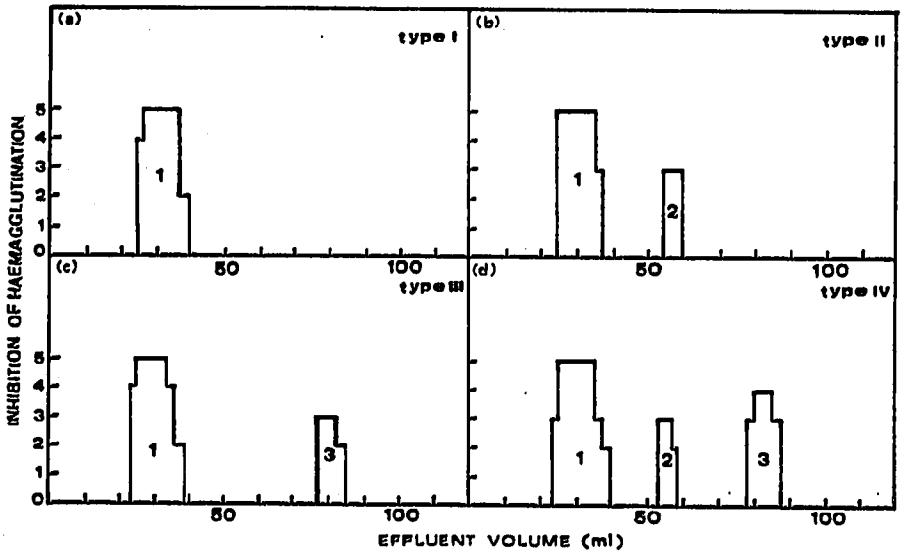

Fig. 1. The four salivary ABH gel filtration types as determined on a Sephadex G-100 36 × 1.5 cm column in the saliva from four secretors of group A. The blood group activity is indicated by the solid line. The results of the inhibition of haemagglutination are scored as follows: 0 = one clump; 1 = +++; 2 = ++; 3 = +; 4 = ±; 5 = — (no agglutination).

The four ABH gel filtration patterns which were observed in group A, B, O and AB secretors are shown schematically in Figs. 1 and 3a. In Table I the frequency of the four ABH gel filtration patterns in the saliva from 199 group A,B and O secretors, as determined by the standardised small column method, are reported. In Table IIa, the salivary gel chromatographic patterns of AB subjects are given, and in Table IIb are the H patterns of each subject. In two persons the B substance was absent, in agreement with the data given by some authors[6,7].

In conclusion, each of the A, B and H substances can be present in the saliva from secretors in four types of gel filtration patterns that have been called Type I, Type II, Type III and Type IV (see Table III).

So-called non-secretors. The saliva of so-called non-secretors does not contain the excluded fraction, *i.e.* fraction 1. This had been determined in the first phase of the present study and was later confirmed by the standardised small column method. In the preliminary experiments non-secretors had also been examined for fractions 2

TABLE I

FREQUENCIES OF THE VARIOUS GEL FILTRATION PATTERNS IN 199 SECRETORS AND 32 NON-SECRETORS
The numbers in parentheses are the percentages.

Gel filtration pattern	*Blood group*			*Totals*
	A	*B*	*O*	
Secretors				
Type I (fraction 1)	16 (17.58)	6 (20.68)	29 (26.12)	51 (22.07)
Type II (fractions 1, 2)	9 (9.89)	3 (10.34)	11 (9.90)	23 (9.95)
Type III (fractions 1, 3)	17 (18.68)	5 (17.24)	17 (15.31)	39 (16.38)
Type IV (fractions 1, 2, 3)	35 (38.46)	10 (34.48)	41 (36.93)	86 (37.22)
Subtotals	77 (84.61)	24 (82.72)	98 (88.36)	199 (84.14)
Non-secretors				
Type V (fraction 2)	4 (4.39)	—	4 (3.60)	8 (3.46)
Type VI (fraction 3)	3 (3.29)	—	1 (0.90)	4 (1.73)
Type VII (fractions 2, 3)	7 (7.69)	2 (6.89)	6 (5.40)	15 (6.49)
Type VIII (no fraction)	—	3 (10.34)	2 (1.80)	5 (2.16)
Subtotals	14 (15.37)	5 (17.23)	13 (11.70)	32 (13.86)

and 3, but frequently negative results had been obtained due to the use of thin-layer gel filtration. These data have therefore been discarded.

The results of only 33 cases will be reported here which have all been obtained by the small column method. No variation in the gel filtration pattern was observed

TABLE II

GEL FILTRATION PATTERNS FOR A, B AND H SUBSTANCES IN 10 AB SECRETORS AND 1 NON-SECRETOR (NS)

AB case No.	*a*				*b*	
	A		*B*		*H*	
	Type	*Fractions*	*Type*	*Fractions*	*Type*	*Fractions*
1 (NS)	VII	2, 3	VIII	—	VII	2, 3
2	IV	1, 2, 3	IV	1, 2, 3	IV	1, 2, 3
3	III	1, 3	VIII	—	III	1, 3
4	II	1, 2	I	1	III	1, 3
5	I	1	IV	1, 2, 3	II	1, 2
6	IV	1, 2, 3	IV	1, 2, 3	IV	1, 2, 3
7	IV	1, 2, 3	I	1	III	1, 3
8	I	1	I	1	I	1
9	III	1, 3	III	1, 3	III	1, 3
10	II	1, 2	I	1	II	1, 2
11	III	1, 3	I	1	III	1, 3

TABLE III

TYPES OF GEL FILTRATION PATTERNS FOR EACH OF THE A, B AND H SUBSTANCES IN SECRETORS AND SO-CALLED NON-SECRETORS

Type	*Gel filtration pattern (fractions)*	*Symbol for each antigen*		
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-

in random samples of saliva examined on 36 × 1.5 cm beds. The data showed that the saliva from some non-secretors contained only fraction 2. Fraction 3 was present in other non-secretors and both fractions 2 and 3 in others. Finally, a small number of subjects had no active fraction and so behaved as true non-secretors.

The gel filtration patterns, as detected in columns and thin-layer experiments on non-secretors, are shown schematically in Fig. 2 and Fig. 3b. In Table I the frequencies are reported of the different gel filtration patterns in 32 non-secretors; in Table II (No. 1) the pattern of an AB non-secretor is given.

In conclusion, each of the A, B and H substances was found to be secreted in saliva of non-secretors according to three types of gel filtration patterns, which have been called Type V, Type VI and Type VII. The term Type VIII was used for the absence in the saliva of the considered blood group substance (Table III). The number of secretors examined being relatively small, it was not surprising that in our samples Type V and Type VI among group B subjects and Type VIII in group A

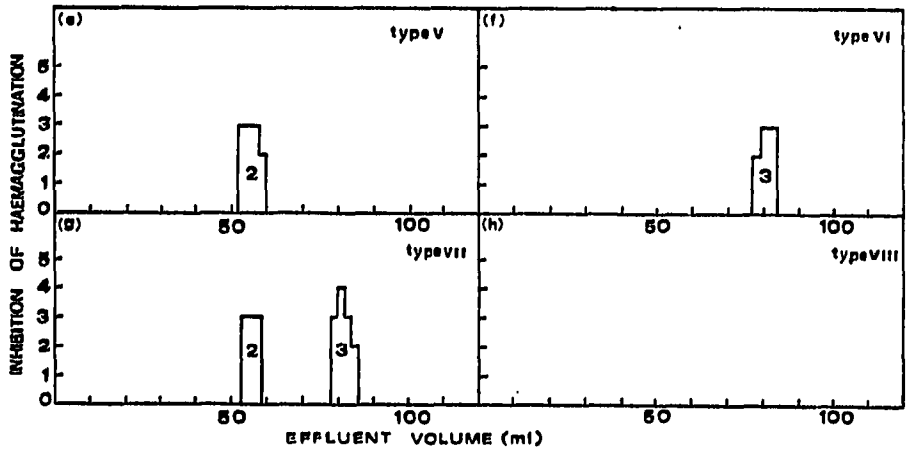

Fig. 2. The salivary ABH gel filtration types of non-secretors as determined on Sephadex G-100 36 × 1.5 cm beds. e, g, h = non-secretors of group A; f = non-secretor of group B.

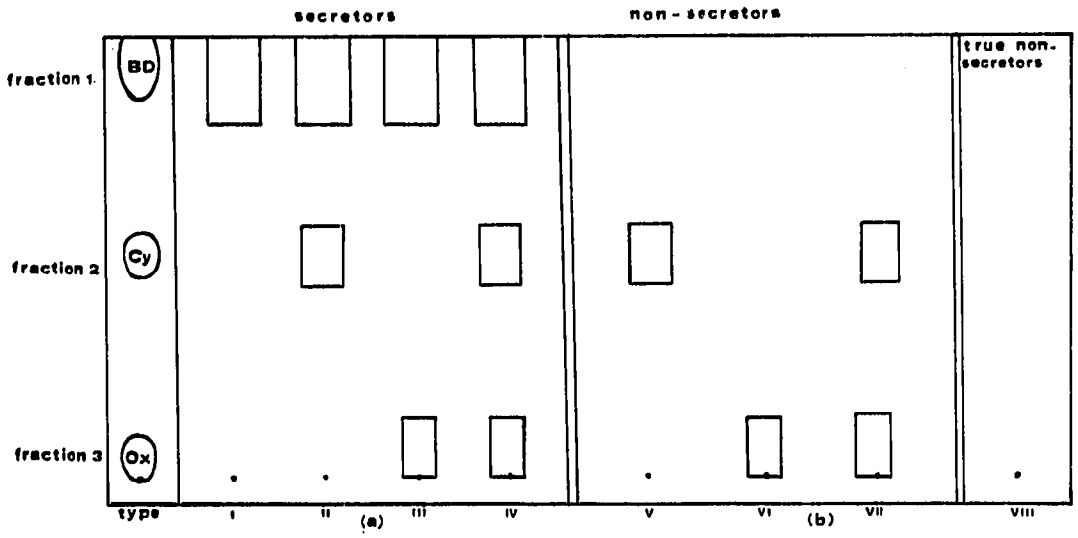

Fig. 3. Schematic representation of the eight ABH gel filtration types determined by thin-layer chromatography on Sephadex G-100 (superfine). Reference substances: BD = Blue Dextran 2000; Cy = cytochrome *c*; Ox = oxytocin.

subjects were absent. The eight types of gel filtration patterns which are possible for each of the A, B and H substances are given in Table III. Types V and VI for group B and Type VIII for group A are, of course, hypothesised.

H substance in the saliva of group A, B, O and AB subjects

The secretion titre of the H substance was determined in all the samples of saliva before gel filtration. The results of this preliminary study will be reported elsewhere[8]. The frequencies of the salivary gel filtration pattern of H substance in group O secretors and non-secretors are given in Table I.

The gel filtration patterns of the H substance were also studied in the saliva of group A, B and AB subjects. In these salivas the H substance was also found to be secreted according to the seven gel filtration types. Type VIII, which is the term used for the absence of any active fraction, was also detected. The various combinations of gel filtration patterns of A and B substances with the H substance patterns are reported in Table IV and Table V, respectively. The gel filtration patterns of H

TABLE IV

CORRELATION BETWEEN A AND H GEL FILTRATION PATTERNS
The number of cases is recorded.

	H-1	*H-1, 2*	*H-1, 3*	*H-1, 2, 3*	*H-2*	*H-3*	*H-2,3*	*H-*
A-1	13	1	2					
A-1, 2	1	7		1				
A-1, 3	3		14					
A-1, 2, 3	5			27		3		
A-2					4			
A-3						3		
A-2, 3							7	
A-								

TABLE V

CORRELATION BETWEEN A AND H GEL FILTRATION PATTERNS
The number of cases is recorded.

	H-1	*H-1, 2*	*H-1, 3*	*H-1, 2, 3*	*H-2*	*H-3*	*H-2, 3*	*H-*
B-1	3		3					
B-1, 2		1			2			
B-1, 3			4			1		
B-1, 2, 3	2			7			1	
B-2								
B-3								
B-2, 3							2	
B-								3

substance in the saliva of group AB subjects are given in Table IIb. The results indicate, as a general rule, a close relationship between the salivary patterns of the A and B substances and those of the H substance in the so-called non-secretors. In the saliva of secretors, a similar relationship was also found in most cases, but in 26 cases among A, B and O subjects the H substance gel filtration pattern was different from that of the group specific A or B substances. In seven of these cases, the H substance had only fractions 2 and 3 and lacked fraction 1.

Group AB subjects presented a still clearer example of the possible independence of A, B and H gel filtration patterns in the same subject. In this group two cases were observed in which the three ABH substances exhibited a completely different pattern (Table IIa and b, case No. 4,5,7).

The problem of the individuality of the H substance with respect to the A and B substances of a given saliva was also investigated. As the anti-H serum is known to react also with A and B substances, the hypothesis could be advanced that in a subject typed for example as A-1,2,3/H-1,2,3 the H pattern was due to a non-specific inhibition of anti-H serum by the three A active fractions.

The following type of experiment was developed in which saliva lacking fraction 1 was used to ensure a complete saturation of blood group antigens by the antibodies. 0.5 ml of anti-A serum titred 1/4 was added to 0.7 ml of saliva A-2,3/H-2,3. After incubation for 1 h, the solution was gel filtered through a small Sephadex G-100 column with the usual buffer. The haemagglutination inhibition test was then performed with both anti-A and anti-H sera as usual. Only fractions 2 and 3 of the H substance were detected with the usual elution volume. The same saliva was incubated with anti-H serum (0.7 ml: 0.5 ml) and then gel filtered on Sephadex G-100. The inhibition test revealed the disappearance of the H fractions while A fractions 2 and 3 were still present.

Constancy of the ABH salivary gel filtration patterns

The use of the standardised method on 20 × 1.2 cm Sephadex G-100 beds permitted a careful control of the technical reproducibility of the data. In fact the elution volumes of each fraction showed negligible variations (*e.g.* 1 tube) in all the experiments performed on a given column. In addition, the constancy of the secretory pattern was verified in the saliva of a number of subjects (32 medical students) who were examined many times in various physiological conditions.

The influence of the preservation of the samples of saliva before gel filtration on the reproducibility of the data was also investigated. Saliva maintained at 4° for 48 h, or at −20° for several months, preserved its original gel filtration group specific pattern. On the other hand preservation at 4° for 3–4 days or more resulted in a decrease of blood group activity and in erroneous data with respect to fractions 2 and 3.

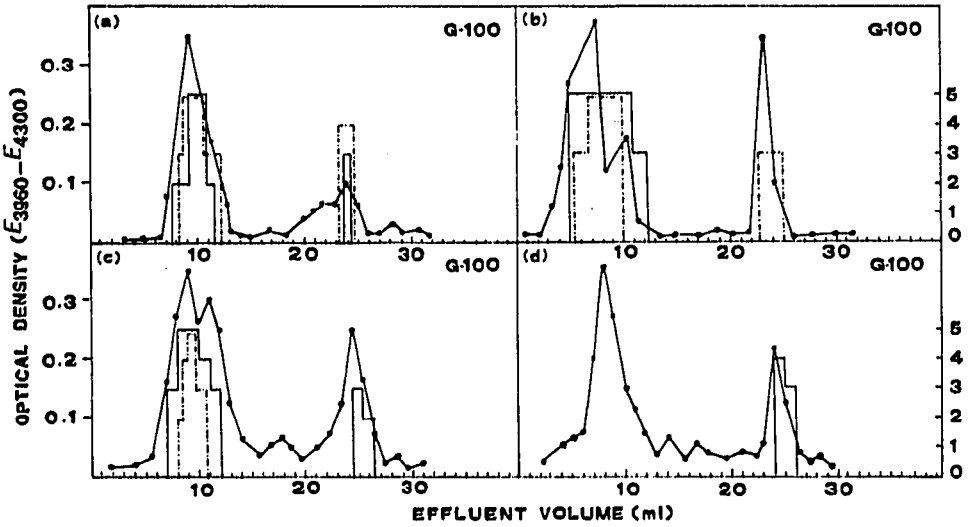

Fig. 4. Some results of fucose analyses on the eluates from gel filtration of saliva on Sephadex G-100 columns (20 × 1.2 cm). The solid line represents the group specific activity of the main antigen of the subject and the broken line the specific activity of the associated H substance. The curve indicates the optical density for fucose. (a) saliva A-1,3/H-1,3; (b) saliva B-1/H-1,3; (c) saliva A-1,3/H-1; (d) saliva H-3.

Fucose analyses

Fucose determinations on the eluates from column gel filtration of the various types of saliva were performed with the aim of giving an indirect support to the data obtained by the serological method. Positive reactions were obtained at the elution volumes of the three active peaks, *i.e.* fraction 1, fraction 2 and fraction 3. The fucose reaction is common to A, B and H substances and this could explain some of the results, *e.g.* the presence of a fucose peak with the elution volume of fraction 2 in an A subject who secreted fractions 1 and 3 only, but had fraction H-2.

In Fig. 4 some gel chromatograms are given showing the different results which can be obtained. It should be noted that the fucose peak at the elution volume of fraction 2 frequently had a very low optical density value. In addition, it was sometimes displaced by one tube from the tube containing the active substance. The excluded fucose peak was detectable also in the saliva of the so-called non-secretors lacking an active fraction 1, due probably to some other salivary excluded glycoproteins.

DISCUSSION

Salivary ABH gel filtration patterns

The data presented here show that, besides the well-known ABH blood group

specific fraction excluded from Sephadex G-100 or G-200 (refs. 1, 9, 10), that we termed fraction 1, two additional ABH subfractions occur in the saliva of secretors (fraction 2 and fraction 3). The saliva from non-secretors lacks fraction 1 but can have one or both of the subfractions.

The approximate molecular weights of fractions 2 and 3 have been previously estimated by gel filtration[1] as being between 10 000 and 13 000, and no greater than 1500–2000, respectively. The chemical nature of these subfractions is under investigation.

The three fractions of each of the A, B and H antigens can be variously represented and associated in saliva, thus giving rise to eight gel filtration patterns for each antigen (Figs. 1–3 and Table III). This pattern is a stable character and can be determined by a satisfactorily reproducible method.

As the subjects of group A, B and AB usually also secrete the antigen H in their saliva, the association of the patterns of each antigen in a single subject forms the individual salivary ABH gel filtration pattern. It should be noted that in some subjects gel filtration patterns of the single antigens have been found which differed from each other (see Tables II, IV, V).

Symbols are proposed which indicate the whole ABH gel filtration pattern of a given person, as in the following examples taken from our cases:

O (H-1,2,3)	A (A-1,2/H-1,2)
O (H-1,3)	A (A-1,2,3/H-1,2,3)
O (H-2)	A (A-1,2,3/H-1)
O (H-1)	A (A-1,2/H-1,2,3)
O (H-1,2)	A (A-2/H-2)
B (B-1,3/H-1,3)	AB (A-1/B-1/H-1)
B (B-1/H-1)	AB (A-1,2/B-1/H-1,3)
B (B-1/H-1,3)	AB (A-1,2,3/B-1/H-1,3)
B (B-2,3/H-2,3)	AB (A-1,3/B-/H-1,3)
B (B-/H-)	AB (A-2,3/B-/H-2,3)

The group of the considered subject is indicated with capital letters. The gel filtration pattern of each of the A, B and H antigens is reported in brackets; the gel chromatographic fractions of each antigen are indicated by 1, 2, 3. Since it was interesting to see whether the three active fractions of each of the A, B and H substances were or were not present in the erythrocyte membrane, experiments were carried out on solubilised red-cell stroma. The three fractions were always present, in a water-soluble form, irrespectively of the secretor status of the subject. A subsequent paper in this journal will deal with these findings[11].

As a consequence of the recent studies on the ABH substances of the red cell membrane[11–16] and of the discovery of the water- and alcohol-soluble fractions 2 and 3 from saliva[1] and red cells[11], it appears evident that the distinction of FRIEDENREICH AND HARTMANN[17] between water-soluble and alcohol-soluble ABH substances is no longer valid. We believe that blood group substances from secretions and red cells have the same basic chemical nature; different isolation methods are required owing to the bindings of the group specific substances with other components of the red cell membrane.

New approach to the problem of secretors and non-secretors

Our results suggest that the old problem of secretion of ABH substances in saliva should be reconsidered. Since the discovery of YAMAKAMI[18], it has been known that A and B antigens are present in saliva. LEHRS[19] and PUTKONEN[20] realised that this character is dimorphic and two classes of individuals were identified, *i.e.* secretors and non-secretors. The inheritance according to a Mendelian system was demonstrated by SCHIFF AND SASAKI[21]. Later FRIEDENREICH AND HARTMANN[17] suggested a distinction of ABH antigens into two forms: the water-soluble substances of the secretions and the alcohol-soluble substances from the tissues and the red cells. GRUBB[22] gave evidence that ABH secretion is associated with the Lewis blood groups. The importance of Hh genes was also elucidated[23].

The main problem, especially in disputed paternity cases, was a reliable distinction between secretors and non-secretors. This distinction is very easy in persons having a good titre of ABH antigens in saliva or those lacking specific activity. On the other hand the distinction is difficult between weak secretors and the non-secretors whose saliva is not completely devoid of blood group specific activity. This could explain the fact that the frequency reported for secretors and non-secretors vary with different workers and with different ABO group for the same workers[6,7,24]. To overcome those drawbacks, it has been therefore recommended to use a rigorously standardised quantitative method and a histogram of each individual antiserum[17,25].

The method reported here allows a good qualitative distinction between secretors and non-secretors. In addition, the previously unexplained reasons of the weak activity of many non-secretors seem to be satisfactorily elucidated. Secretors can now be considered those persons who have in their saliva the main active fraction 1, which is excluded from Sephadex G-200 and G-100. This fraction is secreted alone or in association with fraction 2, fraction 3 or with both fractions 2 and 3.

The so-called non-secretors always lack fraction 1. They have fraction 2, fraction 3, or both, these being the cases in non-secretors whose saliva has a very weak group specific inhibitory activity. This activity, apparently, is due to the specific fractions 2 and/or 3.

A few non-secretors lack any active fraction in their saliva, and therefore this saliva is free of group specific activity. These subjects could be termed true non-secretors. Since eight salivary patterns of each of the A, B and H antigens have been identified in place of the previously known dimorphic character, family studies were undertaken in order to determine whether these patterns are inherited molecular phenotypes. The results have shown that this is the case and the details will be reported elsewhere[26].

Recommended method

A brief comment on the drawbacks of thin-layer gel chromatography and on the recommended procedure on small Sephadex G-100 columns seems useful here. Thin-layer gel chromatography was abandoned after some hundred determinations because of the following drawbacks. The method is much too laborious as the plates must be prepared each time; three spots of each specimen of saliva must be run to allow the examination of the three ABH antigens and the elution step is tedious and unpractical. In addition, the method can give rise to serious errors. In viscous salivas

the migration of fraction 1 is sometimes retarded so that the subject can be erroneously considered a non-secretor; fractions 2 and 3 can be erroneously left undetected, due to the loss of the active material during the elution step.

Gel filtration on small 20 × 1.2 cm Sephadex G-100 beds is on the contrary a very practical and valid technique for routine purposes. 1-ml samples of centrifuged, unboiled saliva are sufficient for the detection of the three ABH fractions. The results are not affected by the viscosity of the saliva. If a timed-flow fraction collector with six or more concentric circular rows of at least 50–60 holes is used, six or more Sephadex columns can be employed simultaneously. This is particularly useful for family studies.

The total effluent volume usually collected at room temperature in about 6 h is only 35 ml divided into 32–35 test tubes.

A critical step of the method is the serological identification of fraction 2 and fraction 3 for which an adequate experience and suitable antisera are required due to their weak inhibitory activity. In fact the specific inhibition for fraction 1 is strongly positive in 4–5 tubes, while for fractions 2 and 3 it is confined to 1–3 tubes and is also usually incomplete.

To avoid errors, a correct macroscopic reading of the inhibition of the haemagglutination should be performed by means of a standardised technique. Fresh red cells should be used. Antisera should have low titre, *e.g.* 1/4, to allow antibodies to be absorbed as much as possible by the weaker antigens. At the same time the antisera should have a high binding constant (K_0) which should be between 10^6–10^8 with a heterogeneity index of about 0.5. This is required because the inhibition test is performed on the contents of each tube by an all-or-none method. It is therefore indispensable that the tubes which do not contain active material give a strong agglutination. In other words, after a short centrifugation, a single clump should be formed which is not easily broken when the tube is vigorously shaken. Under the above-mentioned conditions even an incomplete inhibition (±; +) can be assumed as evidence for the presence of an active group specific fraction in the tube under examination.

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