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POLYMORPHISM OF A, B AND H SUBSTANCES IN HUMAN URINE

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SUMMARY

The gel-chromatographic behaviour of A, B and H substances from urine was examined and compared with that of previously described ABH salivary fractions. Urinary fractions 1 and 2 exhibited a molecular size smaller than those of salivary fractions 1 and 2. In the urine of 52 subjects a polymorphism of A, B and H substances, with four main types, was observed which is independent of the salivary one and of the so-called secretor and non-secretor status.

INTRODUCTION

Polymorphism of A, B and H substances has recently been described in human saliva [1—4] and seminal fluid [5]. Twenty gel-chromatographic types have been identified in the secretions, which are the result of various associations of five serologically active fractions of each substance, all normally contained in red-cell stroma [6]. The ABH secretory types are probably under genetic control [7].

The ABH group-specific glycoproteins in urine have been studied by Yoshida [8], Freudenberg and Eichel [9, 10], Jorpes and Norlin [11, 12], Jorpes and Thaining [13], Kobayasi et al. [14] and Yasuoka [15], and more recently by Masamune et al. [16], King et al. [17, 18], Price Evans et al. [19] and Lundblad and Berggård [20]. Low-molecular-weight ultrafiltrable group-specific compounds were then detected by King et al. [17] and subsequently identified as oligosaccharides by Lundblad [21—23] and Björndal and Lundblad [24].

In the present study the gel-chromatographic behaviour of the ABH urinary fractions was examined and compared with that of salivary fractions. Moreover, a polymorphism of A, B and H substances was observed and found to be independent of the salivary one.

MATERIALS AND METHODS

Preparation of the samples

Urine. Urine specimens were collected from 52 healthy subjects selected from those previously tested for ABH salivary types [3]. The urine of two children of a couple of true non-secretors included in the group was also examined. 250—350 ml of urine were usually obtained from each subject. In some cases, when a larger amount of group-specific substances was needed for particular analytical work, 2000—4000 ml were processed. Each sample was usually concentrated some hours after collection or, sometimes, preserved at —20° until examined. Desalting by dialysis was as a rule omitted to prevent loss of low-molecular-weight dialysable fractions. Samples were concentrated to a syrup in a rotating evaporator at 30—35°, and then kept at 4° for 5—6 h. The precipitated material was discarded by centrifugation and the liquid further concentrated to 8—10 ml. The final fluid was again maintained at 4° overnight to precipitate other salts, and an aliquot was subjected to gel chromatography. The remaining solution was kept at —20° for subsequent controls. Additional steps in the concentration process were required when 2000—4000 ml of urine were examined. Because such samples were dialysed after the first evaporation, a 100×—200× concentration was easily reached.

Saliva. Saliva specimens were obtained from the same subjects and gel chromatographed for serological assays to check the ABH secretory types as previously described [3]. Only in some cases was the presence of sub-fractions 4 and 5 [4] tested for.

Gel and ion-exchange chromatography

Sephadex G-25 (Pharmacia, Uppsala, Sweden) columns (20 × 1.2 cm) were routinely used for the determination of the individual urinary ABH patterns. Different bed sizes of the same gel (28 × 1.2 cm, or 50 × 1.2 cm) were used when grouping was performed by the automated haemagglutination-inhibition method. Distilled water was used as eluent. The flow-rates were maintained at 10—12 ml/h, and fractions of about 1—1.5 ml were collected.

For other analytical or preparative purpose, gel filtration was carried out on larger Sephadex G-25 columns (42 × 2.5 cm) and on Sephadex G-15 (28 × 1.2 cm), Sephadex G-100 (20 cm × 1.2 cm), Sephadex G-200 (40 × 1.5 cm) and on agarose Bio-Gel A-15m (100—200 mesh) and A-50m (50—100 mesh) (Bio-Rad Labs., Richmond, Calif., U.S.A.), 62 cm × 1.5 cm columns, with distilled water, or 0.05 *M* Tris—HCl buffer (pH 7.3) containing 0.9% sodium chloride, as eluents. Blue Dextran 2000, Dextran 200.000 (Pharmacia), and IgG anti-A antibodies were used as reference substances for the latter gels. The reference substances were detected by UV absorption spectrophotometry at 260 nm, by the orcinol—sulphuric acid test and by haemagglutination of A red cells, respectively.

Dialysis and ultrafiltration

Dialyses and ultrafiltrations were carried out at 4° in Visking 32/32 tubing

(Scient. Instr. Centre, London, Great Britain). XM 100 and UM 10 Diaflo membranes with an Amicon Model 52 ultrafiltration cell (Amicon, Oosterhout, The Netherlands) were also used with nitrogen at differential pressures of 0.68 kg/cm^2 and 3 kg/cm^2, respectively.

Haemagglutination-inhibition tests

The manual test-tube method previously described was used for all specimens [3].

In many cases the results obtained by the manual method were checked by the automated technique suggested by Sturgeon and McQuiston [25] and by Sturgeon et al. [26] slightly modified [27].

With the automated method some drawbacks were observed. First, there was interference of urinary pigments in the photometric determination of haemoglobin. With short Sephadex G-25 beds (20 × 1.2 cm) these pigments were eluted as a yellow band at an elution volume of 25 ml, the same as urinary group-specific fraction 2. There was separation with 28 × 1.2 cm beds and this was improved in larger columns where the pigments separated into two yellow bands, at elution volumes of 205 and 260 ml, respectively, and a red band at 235 ml.

Another drawback of the automated technique applied to eluates of urine was the irregular "agglutination base". This was caused by the slight haemolytic activity of the eluates, which could not be avoided even when they were carefully made isotonic. The haemolysis effect increased in the fractions eluted immediately before the urinary pigments.

Carbohydrate analyses

Qualitative analyses of neutral sugars and amino sugars were carried out on purified urinary ABH substances.

Purification of urinary fraction 1 was difficult owing to the presence in urine samples of a number of high-molecular-weight carbohydrate-containing compounds [28, 29]. Different methods were tried on 4000-ml samples of urine, i.e. modifications of the original methods proposed by King et al. [18] and by Kobayasi et al. [14].

Most satisfactory results were obtained by the following technique. Three 4000-ml samples of urine containing group A fraction 1 and two samples containing B fraction 1 were concentrated to 300 ml, dialysed, concentrated to 4 ml and gel chromatographed on a 42 × 2.5 cm Sephadex G-25 column. The excluded effluent was recycled on Sephadex G-200. The serologically active, excluded peak was gel filtered through Bio-Gel A-50m and the active fraction finally hydrolysed with 2 *M* hydrochloric acid for 3 h at 110°. The hydrolysate was concentrated to dryness over sodium hydroxide and phosphorus pentoxide in a vacuum dessicator. As a control, two 4000-ml samples were taken from a group A and from a group B subject lacking ABH specific substances in urine.

Neutral sugars. Neutral sugars were detected by paper chromatography and by gas—liquid chromatography.

Paper chromatography was carried out on Whatman No. 1 paper (57 × 29.5 cm) with 1-butanol—pyridine—water (6:4:2) as solvent. The reducing sugars were detected by a silver-dip reagent [30].

A modification of the method of Sweeley et al. [31] was adopted for GLC. The gas chromatograph was a Model Fractovap 2300 from Carlo Erba (Milan, Italy), equipped with a dual-flame detector system and a Speedomax Leeds & Northrup recorder.

The separation of trimethylsilyl ethers of sugars was carried out on Chromosorb W (60—80 mesh) coated with 1.5% OV-17, obtained from Carlo Erba. Glass columns were 6 ft. × 1/8 in. I.D. The columns were conditioned at 250° without gas flow for several hours and then at the same temperature with normal gas flow for an additional 2 h. The flow of the carrier gas (nitrogen) was 30 ml/min. The temperature of operation was 200°. Samples of 1 μl were used from a total of 50 μl of the silanized extract.

Hexosamines were detected by paper chromatography and GLC after the hydrolysis procedure suggested by Lundblad [21].

RESULTS

The group-specific fraction excluded from Sephadex G-200

The non-dialysable, non-ultrafiltrable fraction of the A, B and H substances [18] was found in the concentrated urine of some subjects only (see Tables I and II). This serologically active fraction, which has been called urinary fraction 1 (uF1), was excluded from Sephadex G-25 columns of various sizes and also from Sephadex G-100 and G-200 columns. The data were obtained by gel chromatographing concentrated urine in amounts ranging from 1.5 (in small columns) to 3—4 ml (in larger columns). uF1 was precipitable with four volumes of ethanol or methanol and the precipitate was readily soluble in saline or in diluted aqueous sodium acetate solution.

As this behaviour is the same as that of the high-molecular-weight salivary fraction 1 (sF1), a further chromatographic comparison between the two substances was made on Bio-Gel A-15 m, and a clear-cut difference resulted from these experiments. Specimens of saliva (3 ml) containing group A or B sF1 were gel filtered on the agarose bed, and sF1 was eluted at 52 ml. On the other hand, uF1, isolated by exclusion chromatography on Sephadex G-200, had an elution volume of 170 ml on Bio-Gel A-15m (Fig. 1). This result was confirmed by direct gel filtration on the same gel of some samples of concentrated urine containing uF1.

TABLE I

DISTRIBUTION OF THE FOUR ABH URINARY PATTERNS

Urinary fraction	Blood group			Total	Percentage
	A	B	O		
Type I uF1	5	2	2	9	18.7
Type II uF2	2	1	2	5	10.5
Type III uF1/uF2	5	6	1	12	25
Type IV uF—	3	2	17	22	45.8

TABLE II

COMPARISON OF THE ABH URINARY AND SALIVARY PATTERNS

Subject	Group	Salivary patterns		Urine
		so-called secretors	so-called non-secretors	
S.P.	B	B-1,2		uF1—uF2
M.G.	B	B-1,2		uF1—uF2
P.G.	A		A-2,3	uF—
G.V.G.	A	A-1		uF1
F.A.	O	H-1		uF—
D.M.D.	O	H-1,2		uF—
G.V.	O	H-1		uF—
T.F.	B		B-	uF—
B.L.	A	A-1,2,3		uF1
F.A.	B	B-1		uF1
S.A.	AB	A-1/B-1		uF2
S.V.	O		H-3	uF—
C.G.	O		H-2,3	uF—
R.G.	O	H-1,3		uF1—uF2
N.D.	O	H-1,3		uF—
A.P.	A		A-	uF1—uF2
A.M.	O		H-	uF1
M.G.	O	H-1,2,3		uF1
M.A.	O	H-1,3		uF2
R.R.	A	A-1,3		uF1—uF2
B.D.	O	H-1,3		uF—
B.M.	O	H-1		uF—
C.A.	O	H-1		uF—
B.S.	A		A-2,3	uF2
D.C.F.	O	H-1,2,3		uF—
C.L.	O	H-1		uF—
F.R.	A	A-1,2,3		uF1
G.G.	O	H-1		uF2
L.L.	A	A-1		uF1—uF2
D.A.P.	B	B-1,2,3		uF1—uF2
G.M.	B		B-	uF2
T.M.	B	B-1,2,3		uF1—uF2
A.V.	B	B-1,3		uF1—uF2
S.P.	B	B-1,3		uF1
G.M.	AB	A-1/B-1		uF1—uF2
P.R.	O	H-1,2,3		uF—
N.A.	A		A-	uF—
B.F.	A	A-1		uF1
F.S.	O	H-1		uF—
C.G.	A	A-1,3		uF2
V.R.N.	AB	A-1/B-1		uF1—uF2
D.M.L.	O	H-1		uF—
M.C.	O	H-1		uF—
C.S.	O	H-1		uF—
S.A.	A	A-1,2,3		uF—
F.A.	B	B-1,2,3		uF—
C.G.	B	B-1,2,3/4,5		uF1—uF2/3,4,5
F.M.T.	AB	A-1,2/B-1		uF1—uF2/3,4,5
B.A.	A	A-1,2,3/4,5		uF1—uF2/3,4,5
P.V.	O	H-1,3		uF—/3,4,5
C.A.	A		A-2	uF1—uF2/3
N.F.	A	A-1,3		uF1/3

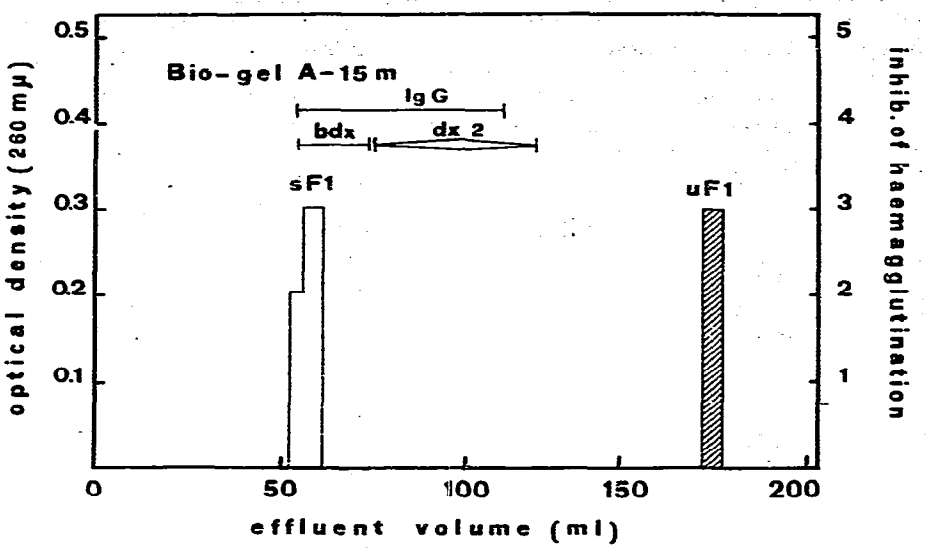


Fig.1. Gel filtration on Bio-Gel A-15m (60 x 1.5 cm) of Blue Dextran (bdx), group A salivary fraction 1 (sF1), group A urinary fraction 1 (uF1) anti-A IgG antibodies and Dextrans 200,000 (dx 2). The results of the inhibition of haemagglutination, in this figure and in Fig. 2, are scored as follows: 0 = one clump; 1 = +++; 2 = ++; 3 = +; 4 = ±; 5 = — (no agglutination).

To obtain further information about the molecular size of uF1, 500 ml of urine containing group A uF1 were dialysed and ultrafiltered through an XM-100 Diaflo membrane. The ultrafiltrate was concentrated to a few milliliters and, as well as the retentate, filtered on Sephadex G-200. Serological activity was found in the excluded effluent of the ultrafiltrate only. The experiment was repeated with a PM-10 Diaflo membrane and the group-specific activity was detected in the retentate.

The specific serological activity of uF1 eluted from short columns of Sephadex G-25 was variable when studied by the manual method. In most cases an inhibition score of 3 (i.e. agglutination +) was observed in 4—5 elution tubes. In other cases, the inhibition score was 4 (±) or 2 (++), the antiserum being selected to give inhibition 0 (clump) in negative tubes. Comparable results were obtained with the automated method.

Qualitative analysis of sugars. Qualitative analysis of sugars of uF1 were carried out with the chief aim of ascertaining the presence of glucose. The most reliable results were obtained by the purification procedure which had, as final step, gel chromatography on Bio-Gel A 50m.

Both paper and gas—liquid chromatography disclosed in all samples the presence of glucose, galactose, fucose and hexosamines. No sugar was detected on the material from the subjects lacking group-specific substances in urine.

The low-molecular-weight ABH urinary fractions

In 6 cases only (see Table II, Nos. 47, 48, 49, 50, 51 and 52) low-molec-

ular-weight, dialysable, water- and alcohol-soluble fractions were found in urine which had the same gel-chromatographic behaviour with Sephadex G-25 as fractions 3, 4 and 5 of saliva and red cells [4].

Another fraction, which was alcohol- and water-soluble, and ultrafiltrable but not dialysable, was detected in a number of subjects, alone or associated with uF1. As the elution volume (20 ml) of this fraction on Sephadex G-100 columns was only slightly different from that of salivary fraction 2 (10 ml), it was called urinary fraction 2 (uF2). However, a further study disclosed some differences between uF2 and salivary fraction 2 (sF2). sF2 was not ultrafiltrable, as demonstrated by experiments on specimens of whole saliva containing sF2. On Sephadex G-25, sF2 was excluded while uF2 was retained. The elution volumes of uF2 with this latter gel were the following: 25 ml on columns 20 cm × 1.2 cm (void volume 17 ml); 28 ml on columns 28 cm × 1.2 cm (void volume 25 ml), 178 ml on columns 42 cm × 2.5 cm (void volume 90 ml). uF2 was excluded from Sephadex G-15.

Serological activity. The specific serological activity of uF2 according to the manual test was sometimes stronger than that of uF1 contained in the same sample. An almost complete inhibition (agglutination ±) in two or three tubes, and more frequently a 3 (+) score, was observed.

Qualitative analysis of sugars. The qualitative analysis of sugars of uF2 was carried out on a group A uF2 isolated from a 5000-ml sample of urine which had been dialysed, concentrated to a few millilitres and filtered on a large Sephadex G-25 column. The active fraction was purified on Sephadex G-15 and hydrolysed with 2 *M* hydrochloric acid. Paper chromatography and gas—liquid chromatography disclosed the presence of glucose, galactose, fucose and hexosamines.

Individual gel-chromatographic patterns

Four main gel-chromatographic patterns were found in the urine of the subjects examined, as shown in Fig. 2. In some individuals only uF1 was found in the urine, in others only uF2, and in some others both uF1 and uF2. In a fourth group of subjects no active ABH fraction was detected. Only 6 persons had some additional small fractions (see Table II).

The data were all obtained by the manual serological method. In many cases, samples of the same subject taken at different times were examined, with identical results. A number of specimens was also checked by the automated technique. Fig. 3 (a,b,c) shows some of the findings.

The different distributions of the four main types are reported in Table I, where the symbol uF— indicates the absence of both uF1 and uF2.

In Table II the ABH urinary and salivary patterns of each subject are compared. Sub-fractions 4 and 5 were determined only in the saliva of those subjects who had fractions 3, 4 and 5 in the urine.

Some individuals, whose saliva contained sF1 and were therefore called secretors, had only uF2 (e.g. Nos. 19, 28, 40) or no fraction (e.g. Nos. 5, 6, 7, 15, 45, etc.) in the urine. Additional evidence of these findings was given by the study of a family (Fig. 4; the parents are Nos. 16 and 17 in Table II). The couple and the children had no group-specific fraction in their saliva, all being true non-secretors, and had uF1 or uF1/uF2 in the urine.

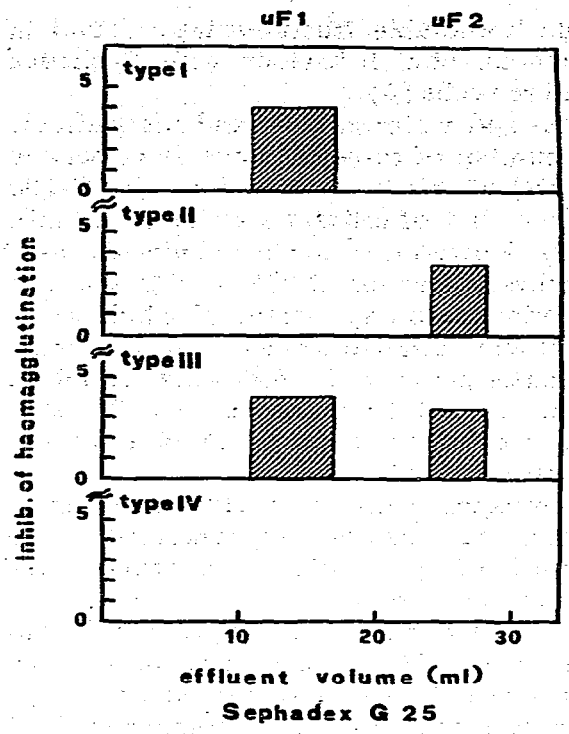
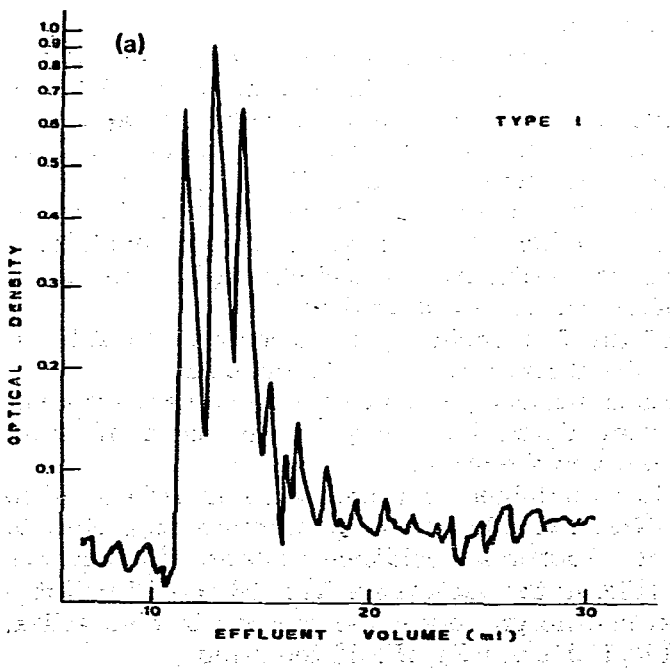
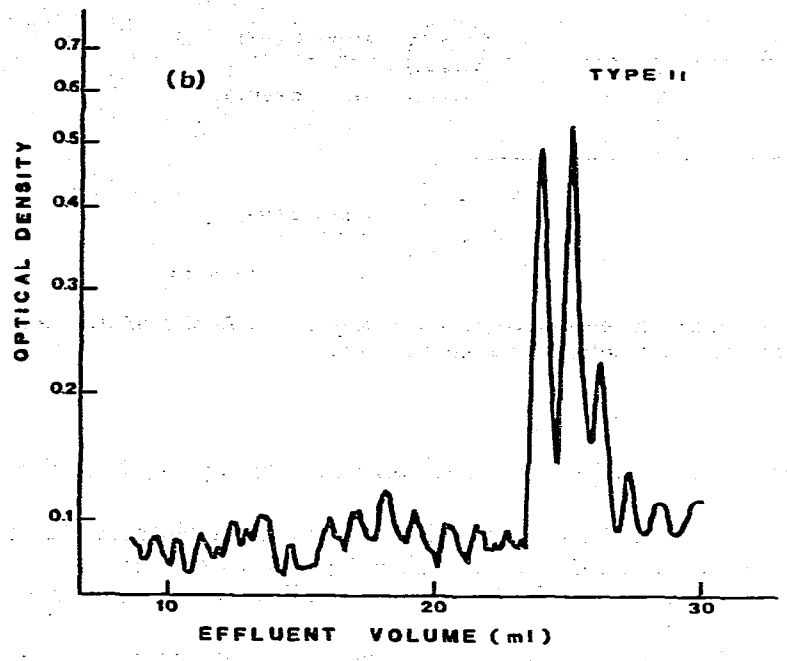


Fig.2. Schematic representation of the four urinary ABH gel-filtration types determined on 20 × 1.2 cm Sephadex G-25 columns.





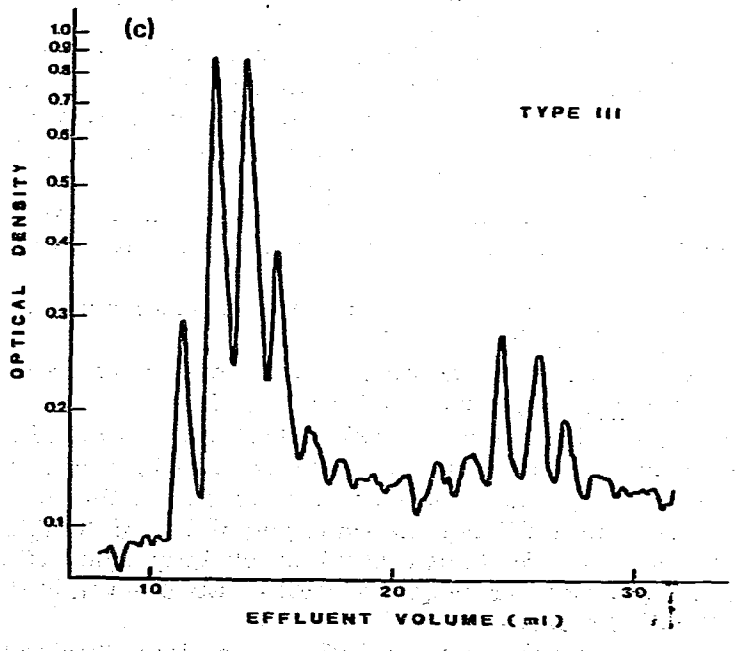


Fig.3. Some examples of urinary ABH gel-chromatographic patterns (Sephadex G-25, 28 x 1.2 cm), determined by the method of automated inhibition of haemagglutination. The peaks that rise from the irregular base line indicate the haemagglutination inhibition. In (a) a group A type I (uF1 only) urine is recorded; in (b) a group B type II (uF2 only) and in (c) a group B type III (uF1/uF2).

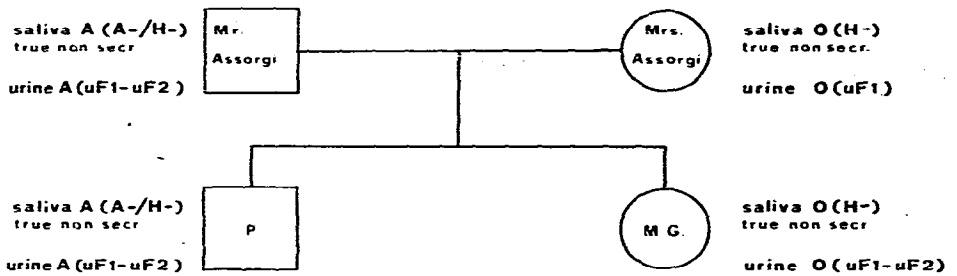


Fig.4. The ABH salivary and urinary gel chromatographic patterns of a family of true non-secretors. All the members had group-specific substances in urine.

DISCUSSION

Comparison of present findings with previous data on urinary A, B and H substances

Urinary fraction 1. uF1 of ABH substances is the same alcohol-precipitable [11], non-dialysable and non-ultrafiltrable [18—20] group-specific glycoprotein as identified by Yoshida in 1928 [8] and studied by a number of authors [9—11, 13, 14, 16, 18—20].

Urinary group-specific fraction 2. uF2 is the same non-dialysable, ultrafiltrable fraction first described by King et al. [17] and exhaustively purified and studied by Lundblad [21—23] and by Björndal and Lundblad [24], whose gel chromatographic data are comparable with ours.

Molecular size. The molecular sizes of uF1 and uF2 are different from those of salivary fractions 1 (sF1) and 2 (sF2). As far as the larger uF1 group-specific urinary glycoprotein [20] is concerned, this is excluded from Sephadex G-200 with sF1 but is eluted much later than sF1 on Bio-gel A-15m. In addition, uF1 is ultrafiltered through XM-100 Diaflo membrane as is dextran, with a molecular weight of 250,000 daltons, but sF1 is not. Finally, uF1 is retained by PM-10 Diaflo membranes that do not hold dextran with a molecular weight of 100,000 daltons.

Taking the above data together one can surmise that the molecular weight of uF1 could fall within the range 150,000—200,000.

The behaviour of uF1 on Bio-Gel A-15m, compared with that of reference substances of known molecular weight, suggests an adsorption effect.

Urinary fraction 2 (uF2) is an oligosaccharide [21—24], smaller than sF2 and larger than salivary fraction 3 (sF3). In fact, uF2 is ultrafiltrable but not dialysable and is retained on Sephadex G-25, whereas sF2 is neither dialysable nor ultrafiltrable and is excluded from the same gel; sF3 is dialysable and ultrafiltrable, and is eluted from Sephadex G-25 much later than uF2 [1, 3]. From the above data the molecular weight of uF2 could be evaluated as being in the range of 4000—5000 daltons.

The comparison of gel-chromatographic and ultrafiltration data obtained from uF1 and uF2 with those of ABH fractions from red cells [6] leads to the same conclusions as regards molecular size.

Glucose. Glucose has been detected in both urinary fractions 1 and 2, in agreement with Lundblad's results [20—24] and in contrast with previous

analytical data on urinary group-specific fractions [9, 14—16, 18]. These contrasting results should be chiefly ascribable to the different analytical procedure adopted.

The presence of glucose in both group-specific fractions is relevant for their inclusion in the class of glucose-containing ABH substances such as salivary fractions 2, 3, 4 and 5 [32] and of glycolipids of red cells. Therefore urinary fractions have no evident relationship to the glucose-free high-molecular weight salivary fraction 1 (see ref. 33).

Urinary and salivary polymorphism of A, B and H substances

It has been shown in previous papers [1—3] that the so-called secretor and non-secretor status is not dimorphic but polymorphic. Eight main types have been identified in saliva of which one type is represented by a few true non-secretors and the remainder by various associations of the group-specific fractions 1, 2 and 3. These findings have been recently confirmed by Ueda [34]. As salivary fraction 3 is sometimes associated with sub-fractions 4 and 5, or with both these small fractions, sub-types were also identified. Thus the possible patterns are twenty [4].

There is now evidence for a molecular polymorphism of A, B and H substances in urine too. In fact the same ABH pattern was found in several specimens of urine taken at different times from the same individuals, and four types of these constantly reproducible individual patterns were identified, i.e. no ABH group-specific activity, uF1 or uF2 only, both uF1 and uF2 (Fig. 2).

The ABH urinary polymorphism shows no demonstrable relationship to the salivary one. Some individuals whose saliva contains sF1 and can therefore be called secretors according to the old classification, have only uF2 (e.g. Nos. 19, 28 and 40 of Table II) or no fraction (e.g. Nos. 3, 5, 7, 15, 45 of Table II) in their urine. Good additional evidence of these findings is given by the family reported in Fig. 4 where all members are true non-secretors in saliva, but have ABH fractions in their urines.

We have no data at present to explain the presence in urine of a molecule as large as urinary group-specific glycoprotein uF1. The hypothesis could be advanced that biosynthesis and secretion of ABH urinary substances occur in some cells of the urinary system.

The possibility of a genetic determinism will now be considered to explain the distribution of the types within the ABO groups. However, the genetic analysis requires still other information not yet available.

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