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CHROMATOGRAPHY OF SUGARS IN BODY FLUIDS

I. PREPARATION OF URINE FOR PAPER CHROMATOGRAPHIC ANALYSIS

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SUMMARY

The ion-exchange method of WHITE AND HESS for the urine desalting and its several modifications were employed as a model to study some factors important for the preparation of high quality sugar concentrate for paper chromatography. The main investigated features included the urine:resin ratio; desalting by passage through columns *vs.* by mixing and shaking; and the effect of acetate and OH forms of the anion-exchange resin on saccharides.

At least three times more urine may be desalted with the WHITE AND HESS system composed of Amberlite IR 120 (H form) and IRA 410 (acetate form) than had been previously described. These resins or their monobed mixture Amberlite MB-3 (acetate form) gave the best results as demonstrated by high yield and good reproducibility in both colorimetric and enzymatic determinations of sugars and qualitative paper chromatography, using volumes of urine as much as fifty times greater than those proposed in the original work.

The quality of desalting is most sensitively indicated by the levels of urea. Sensitive but less reliable are fluorescing urinary pigments. Inorganic salts are more readily removed than other interfering substances.

INTRODUCTION

Almost the entire present knowledge on the composition of human urinary sugar content in health and disease can be ascribed to paper chromatographic studies. However, because of the lack of standardization both in analytical procedures and also to a certain degree in dietary conditions, the reported numbers of urinary sugars are erratic and frequently controversial[1].

Among the variables influencing the number of sugars found in normal urine by a paper chromatographic procedure are the volume of applied urine, presence and concentration of non-sugar constituents and the concentration of the strongest com-

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ponent of the sugar spectrum. In normal urine, the last is always glucose, constituting 18–33% of the copper reducing power of desalted urine obtained after overnight fasting[2].

In screening the urine for various strong glycosurias by paper chromatography[3–5] (PC) and thin-layer chromatography[6,7], the urine is frequently used without any pre-treatment. The performances of both techniques can be markedly extended by removing the interfering substances[8,9] represented by mineral salts, pigments, urea, creatinine, uric acid, ascorbic acid and other organic acids and amines. For the past twenty years, pre-treatment has been done by deionization with different ionex resins in various experimental arrangements[9–14].

In the present study, we have investigated the effect of several factors determining the quality of desalting in order to ensure PC detection of the largest number of sugars occurring in the urine regularly or occasionally.

EXPERIMENTAL

Urine of a healthy 25-year-old female weighing 48 kg was collected over a period of four days without dietary restrictions. After each voiding, the urine was immediately frozen and at the end of the collection all portions were thawed and pooled. After filtration through a filter paper, the whole volume was divided into small aliquots that could be used within one working day. The frozen specimens were stored at −15° without preservatives.

[2-^{14}C]Galactose (45μCi) and [1-^{14}C]glucose (90μCi) dissolved in 5–10 ml saline were given intravenously in a single dose to overnight fasting healthy individuals. The urine was collected in 2-h periods and preserved as normal urine.

Preparation of the ionex resins

Ionex resins Amberlite IR 120 (H form), Amberlite IRA 410 (both of medium porosity, wet mesh size 20–50) and Amberlite MB-3 (indicating mixture of Amberlite IR 120 (H form) and Amberlite IR 120 (OH form), mesh size 20–50) were employed.

Before use, Amberlite IR 120 was washed with distilled water with stirring, until the intensive coloration of washings disappeared entirely.

Amberlite IRA 410, supplied in chloride form, was converted into the OH form in portions of 1.50 kg in a 58-mm diameter column, by treatment with 6% NaOH solution, until the acidified effluent showed only traces of chlorides with $AgNO_3$. Then the resin was washed with 40 l of distilled water. Resin obtained in the OH form was converted further into the acetate form by passing 15 l of 6% acetic acid through the column. This was followed by washing with 40 l of distilled water.

Amberlite MB-3 (OH form) was converted into the acetate form by treatment with acetic acid in the same way as described above. In this process, its indicating property was suppressed.

Desalting procedures

The column method of WHITE AND HESS[10] (Table I, procedure 1), its modified version, regularly used in this laboratory (procedures 3a and 3b) and the shaking procedure of MENZIES AND SEAKINS[14] (procedure 8) were employed as standard

TABLE I

OUTLINE OF DESALTING PROCEDURES

Procedure	*Resin*	*Principle*	*Volume of desalted urine*	*Water for elution (ml)*	*Method*
1	Amberlite IR 120 (H) + Amberlite IRA 410 (acetate), 100 + 100 ml	Mixed bed, single column	20 ml	300	WHITE AND HESS[10]
2a	IR 120 (H) + IRA 410 (acetate), 100 + 100 ml	Mixed bed, single column	30-min diuresis (31.5 ml)	300	The same as procedure 1 but urine load increased by 57.5%
2b	IR 120 (H) + IRA 410 (acetate), 100 + 100 ml	Mixed bed, single column	60-min diuresis (63 ml)	300	The same as procedure 1 but urine load increased by 215%
3a	IR 120 (H) + IRA 410 (acetate), 100 + 100 ml	Separate columns	30-min diuresis	350	Modification of procedure 1[a]
3b	IR 120 (H) + IRA 410 (acetate), 100 + 100 ml	Separate columns	60-min diuresis	350	The same as procedure 3a
4	IR 120 (H) + IRA 410 (acetate), 100 + 100 ml	Mixing and shaking	30-min diuresis + 6 vols. of water	—	Mixing and shaking method based on column procedure 2a[b]
5	IR 120 (H) + IRA 410 (acetate), 100 + 100 ml	Mixing and shaking	30-min diuresis + 6 vols. of water	—	A modified version of procedure 4[c]
6a	Amberlite MB-3 (OH), 200 ml	Single column	30-min diuresis	350	Modified procedure with commercially supplied resin in OH form[d]
6b	Amberlite MB-3 (OH), 200 ml	Single column	60-min diuresis	350	The same as procedure 6a
7a	Amberlite MB-3 (acetate), 200 ml	Single column	30-min diuresis	350	Amberlite MB-3 (OH form) converted into acetate form, as described in EXPERIMENTAL
7b	Amberlite MB-3 (acetate), 200 ml	Single column	60-min diuresis	350	The same as procedure 7a
8	Amberlite MB-3 (acetate), 8 ml	Shaking and mixing	15-min diuresis (15.75 ml)	—	Biodeminrolit acetate resin originally used by MENZIES AND SEAKINS[14] was replaced by functionally identical Amberlite MB-3 (acetate)[e]

[a] Procedure routinely used in this laboratory with a 45-min aliquot of normal urine.

[b] Diluted urine was shaken in mechanical shaker for 20 min at 90 oscillations per min in 1-liter flask. After shaking, resin was separated by filtration under suction.

[c] Urine was shaken with each resin separately, starting with Amberlite IR 120.

[d] Amberlite MB-3 (OH form) is composed of Amberlite IR 120 (H form) and Amberlite IRA 410 (OH form) and a color indicator. Before use, resin was washed several times with distilled water.

[e] Conversion of resin into acetate form was carried out as described[14]. 50-ml round-bottom centrifuge tubes were used for shaking.

procedures. Other approaches designed to examine the role of certain methodological factors are given in Table I.

The frozen urine was thawed and warmed up to 30–40° until any eventual precipitate disappeared and deionized in portions corresponding to a 30 or 60-min aliquot of daily diuresis, *i.e.* 31.5 or 63.0 ml. In each procedure, 100 ml of Amberlite IR 120 and 100 ml of Amberlite IR 410 or other resins were placed into two individual columns, or they were mixed and placed into one column, 28 mm diameter, fitted with a Teflon stopcock and fritted disc at the bottom. The cation exchanger was always packed into the upper column and the effluent entered directly into the lower one which was attached with an air-tight joint. The urine was carefully added on the upper bed, just submerged in water. After the urine soaked into the resin, three or four 20–30-ml portions of water were added. These represented the initial portion of a total of 300–350 ml of water used for the elution.

The maximum effluent rate was 2.0 ml/min. The first 40 ml of the effluent were discarded. The remaining part, including the water obtained by draining the columns after desalting, was evaporated on a rotary evaporator at 36–38° in a 1000-ml distillation flask. The concentrate and flask washings were transferred through a small paper filter into a 50-ml centrifuge tube and lyophilized to dryness. The desalted urine containing radioactive metabolites was lyophilized only. Freeze dried residues were stored at −15°.

Testing of individual desalting procedures

The desalting effectiveness was tested by measuring sodium, potassium, chlorides, urea, creatinine, uric acid and osmolarity. The losses of sugars were examined by determining glucose, galactose and total reducing sugars and by PC. Sodium and potassium were quantified by flame photometry (Flame Photometer Model 143, Instrumentation Lab., Inc.), chlorides according to CORTLOVE *et al.*[15] (Chloridometer of Buchler Instruments, Inc., Fort Lee, N.Y., U.S.A.), glucose by the glucose oxidase test, supplied by Worthington Co. (Freehold, N.J., U.S.A.), galactose by the galactose dehydrogenase method, uric acid by uricase assay and urea by the colorimetric method based on urease action and the Berthalot reaction (the latter three obtained as test combinations from Boehringer and Soehne Co., Mannheim, G.F.R.). Total reducing sugars were estimated by the SOMOGYI–NELSON method[16] and creatinine colorimetrically by Jaffe's reaction.

Osmolarity was measured with the osmometer (Advanced Instruments, Inc., Newton Highland, Mass., U.S.A.) and expressed as milliosmoles (mOs/l).

Measurement of ^{14}C-labeled sugar passage through columns

A 20-min aliquot of urine collected 2 h after intravenous administration of ^{14}C-labeled glucose or ^{14}C-labeled galactose from three individuals was desalted as described in desalting procedures (Table I, procedure 3a) and the eluate was collected in 25-ml fractions. The radioactivity was measured in a 1-ml portion of the fraction added to 10 ml of dioxane scintillation liquid in a 15-ml glass vial and expressed in counts per minute (CPM), representing the average of two parallels, counted three times for 10 min. The quenching by water was corrected using [^{14}C]benzoic acid as an internal standard.

The scintillation liquid was composed of 60 g of naphthalene, 4 g of 2,5-

diphenyloxazole (PPO, New England Nuclear Corp.), 200 mg of 1,4-bis-2,2-(5-phenyloxazolyl)benzene (POPOP, Packard Instrument Co.), 25 ml of ethylene glycol, 100 ml of ethanol, and dioxane added up to 1000 ml.

The remaining part of each 25-ml fraction of the eluate was lyophilized to dryness and used for chromatography.

Paper chromatography

Radiochromatography of the column effluent. Fractions of the effluent were subjected to one-dimensional multiple ascending chromatography on Whatman No. 17 paper in butanol–pyridine–benzene–water (5:3:1:3)[2]. After the last irrigation, a 60-mm wide strip carrying the separated sugars was cut out and divided into 10 × 60 mm segments, successively numbered. The radioactivity of each segment was counted separately in a 15-ml vial three times for 10 min, in 10 ml of scintillation liquid composed of 4 g of PPO, 100 mg of POPOP, and toluene added up to 1000 ml. The counts, corrected against the background, were plotted on a cross-section paper and their distributions compared with the positions of standard aldoses and ketoses revealed with aniline citrate[10] and orcinol–trichloroacetic acid reagent[17], respectively.

The sugar spectrum of urines deionized by individual procedures. The performance of individual desalting procedures was also judged by the quality of the sugar separation. Chromatography on Whatman No. 3MM filter paper in ethyl acetate–acetic acid–water (3:1:1) was carried out as reported previously[2]. All samples, except L-1 (Fig. 3) which was applied as a small circle, were streaked on a 20-mm long line in aliquots of diuresis. The spots of saccharides were visualized by spraying with aniline citrate reagent[10].

The presence of fluorescing and absorbing spots, before and after staining, was examined in the light of Mineralight UVS-11 (260 mμ main wavelength band) and Black Ray UVL-21 lamp (360 mμ), both of Ultraviolet Products, Inc., San Gabriel, Calif., U.S.A.[18].

RESULTS

Desalting by column procedures

Procedures 1 to 3b (Table I) based on the same combination of exchangers, demonstrate the effect of gradually increasing urine load and compare the performance of mixed monobed columns and columns with separated resins. The results (Table II, procedures 1 to 3b) show that the values of glucose, galactose and total reducing sugars fluctuate in the same range. The yield of sugars was not affected either by the mixed or separated exchangers or by the three-fold increase in urine load. Removal of inorganic salts was so effective that in most cases the potassium levels could be determined by a flame photometric method only when the deionized material was concentrated twenty-fold. The highest level of sodium found in individual samples was 1.01 mequiv./l (procedure 3a), representing 1% of the original level, while the mean for all specimens desalted in this group (procedures 1–3a) was 0.2%. The weakly ionized nitrogenous metabolites were retained also very satisfactorily. The highest values of creatinine, urea and uric acid found were 0.001 mg/ml, 2.489 mg/ml and

TABLE II

SUGARS AND OTHER METABOLITES IN THE URINE DEIONIZED BY PROCEDURES OUTLINED IN TABLE I

Concentrations given are ranges and their means.

Procedure	*Number of desalted specimens*	*Glucose (μg/ml)*	*Galactose (μg/ml)*	*Reducing sugars (mg/ml)*	*Na (mequiv./l)*	*K (mequiv./l)*	*Cl (mequiv./l)*	*Creatinine*[a] *(mg/ml)*	*Urea (mg/100 ml)*	*Uric acid (μg/ml)*	*Osmolarity*[a] *(mOs/l)*
1	6	26.6–33.3	—	0.180–0.215	0.09–0.10	0.0	0.21–0.23	0.001	0.26–0.35	1.90–2.15	5.8–6.1
		30.3		0.197	0.095		0.22		0.29	1.98	6.0
2a	6	28.4–35.6	4.25–4.50	0.183–0.205	0.04–0.05	0.0	0.14–0.19	0.001–0.001	0.25–0.27	1.75–2.15	5.6–5.8
		31.6	4.35	0.197	0.045		0.17	0.001	0.26	1.94	5.7
2b	6	30.2–33.3	3.95–4.42	0.199–0.215	0.03–0.04	0.0	0.28–0.32	0.001–0.001	0.22–1.32	1.70–2.83	5.5–6.0
		31.8	4.20	0.207	0.035		0.30	0.001	1.00	2.13	5.8
3a	7	25.9–33.2	3.91–4.53	0.176–0.215	0.20–1.01	0.0	0.17–0.23	0.001–0.001	0.89–0.97	1.60–2.35	6.2–8.1
		28.9	4.12	0.204	0.605		0.20	0.001	0.94	1.93	7.2
3b	8	29.1–33.5	3.75–4.00	0.188–0.210	0.22–0.45	0.0–0.03	0.28–0.30	0.001–0.001	1.53–2.49	1.55–2.05	7.3–11.4
		31.1	3.90	0.198	0.34	0.02	0.29	0.001	1.72	1.90	9.4
4	6	20.6–22.4	2.52–2.74	0.175–0.200	0.06–0.10	0.0	0.15–0.24	0.001–0.001	58.53–60.61	1.95–3.40	25.6–3.02
		21.3	2.55	0.188	0.08		0.185	0.001	59.60	2.90	27.9
5	6	18.1–22.6	2.43–2.71	0.156–0.178	1.70–2.02	0.14–0.17	0.19–0.20	0.003–0.004	59.08–60.30	2.15–3.40	32.0–35.2
		21.1	2.65	0.163	1.86	0.16	0.20	0.004	59.54	2.79	33.6
6a	6	0.15–0.28	—	0.003–0.018	0.02–0.03	0.0	0.16–0.21	0.0–0.0	0.62–3.19	0.10–0.55	0.2–2.1
		0.21		0.009	0.025		0.19		1.90	0.30	1.15
6b	6	0.15–0.21	—	0.004–0.005	0.02–0.02	0.0	0.22–0.28	0.0–0.0	0.88–17.88	0.00–0.15	3.0–4.1
		0.16		0.004	0.02		0.25		6.99	0.04	3.55
7a	6	28.2–31.4	3.44–4.04	0.189–0.203	0.05–0.07	0.0	0.20–0.22	0.001–0.001	0.60–0.72	1.80–2.20	5.7–5.8
		29.2	3.82	0.196	0.06		0.21	0.001	0.66	1.99	5.8
7b	6	30.3–31.3	3.93–4.24	0.159–0.184	0.01–0.02	0.0	0.18–0.20	0.001–0.001	0.57–19.66	1.70–2.25	5.8–7.1
		30.7	4.05	0.171	0.015		0.19	0.001	5.29	2.03	6.5
8	6	17.9–21.1	2.45[b]	0.195–0.210	0.51–0.51	0.1–0.15	0.60–0.75	0.0–0.0	297.48–326.30	38.5–46.0	92.0–92.1
		20.2		0.202	0.51	0.13	0.68		310.17	42.61	92.0
Original urine		—	—	—	106.50	25.72	105.90	0.444	1071.20	210.11	466.0

[a,b] Values obtained from 3 and 6 pooled samples, respectively.

3.40 μg/ml, respectively, compared with 0.444 mg/ml, 1071.2 mg/ml and 210 μg/ml, respectively, of the original urine.

The three-fold urine load led to a slight elevation of urea in some samples (Table II, procedure 2b) but its concentration was still in the limits not detectable by the chromatography (Fig. 3, L-2B), unlike in procedure 3b where the exchangers in separate columns released a detectable, yet not interfering amount of urea.

Commercial monobed resin of Amberlite MB-3 converted into acetate form was tested with two different volumes of urine (Table II, procedures 7a and 7b). Compared with the mixed bed prepared in our laboratory, its effectiveness was of the same degree only in the experiment with a 30-min diuresis.

The deionization power of the resins in procedures 1–3b, 7a and 7b is reflected in very low values of osmolarity which sensitively respond to the increase of unremoved metabolites. The value of the highest specimen in this group is 11.4 mOs/l compared with the original level of 466 mOs/l.

The use of Amberlite MB-3 in the OH form (Table II, procedures 6a and 6b) resulted in drastically reduced yields of glucose and other reducing sugars with both aliquots of urine. In comparison with the exchanger in the acetate form, the losses on sugars amounted to 99%. Also, interfering anionic substances such as chlorides and uric acid, especially in the experiment with the smaller volume of urine, were removed more thoroughly.

Desalting by mixing and shaking

The results obtained with this desalting approach are given in Table II (procedures 4, 5 and 8). Procedures 4 and 5, employing the same amount of urine and resin as in column procedure 2, provided markedly lower yields of glucose and galactose. Compared with corresponding column procedures, the level of urea remaining in the urine was high (0.26 in procedure 2a *vs.* 59.60 mg/100 ml in procedure 4). Shaking of the urine with resins separately, first with the cation-exchange resin, did not improve the performance (Table II, procedure 5).

A recently recommended[14] mixing method using undiluted urine and 12.5 times less resin than in procedures 4 and 5 was tested in procedure 8. The yields of glucose and total reducing sugars were very similar to those in the above procedures, but the levels of urea and uric acid were reduced only by 71.1 and 80%, respectively.

Passage of urinary sugars through ioncx-exchange columns

The passage of [2-^{14}C]galactose and its metabolites is illustrated in Fig. 1. The measurement of the radioactivity in successive 25-ml fractions of the column effluent revealed that the initial two fractions did not contain any radioactivity. The third 25-ml fraction constituted 0.72% of the total activity passed through the column during the entire collection. Its level sharply increased in the fourth fraction to 11.49%, reached a peak in the fifth (27.89%) and sixth (28.18%) portions and declined markedly in the successive five portions (16.04, 7.22, 4.11, 2.34 and 1.20%, respectively). Starting with the 14th fraction, the activity stabilized in the range 0.06–0.08% and remained unchanged in the following 13 fractions. The total volume of the eluate was 685 ml, resulting from 85 ml of urine, 500 ml of water for eluting and 100 ml of water released by draining the columns.

The data obtained after administration of 90 μCi of [1-^{14}C]glucose and [2-^{14}C]-

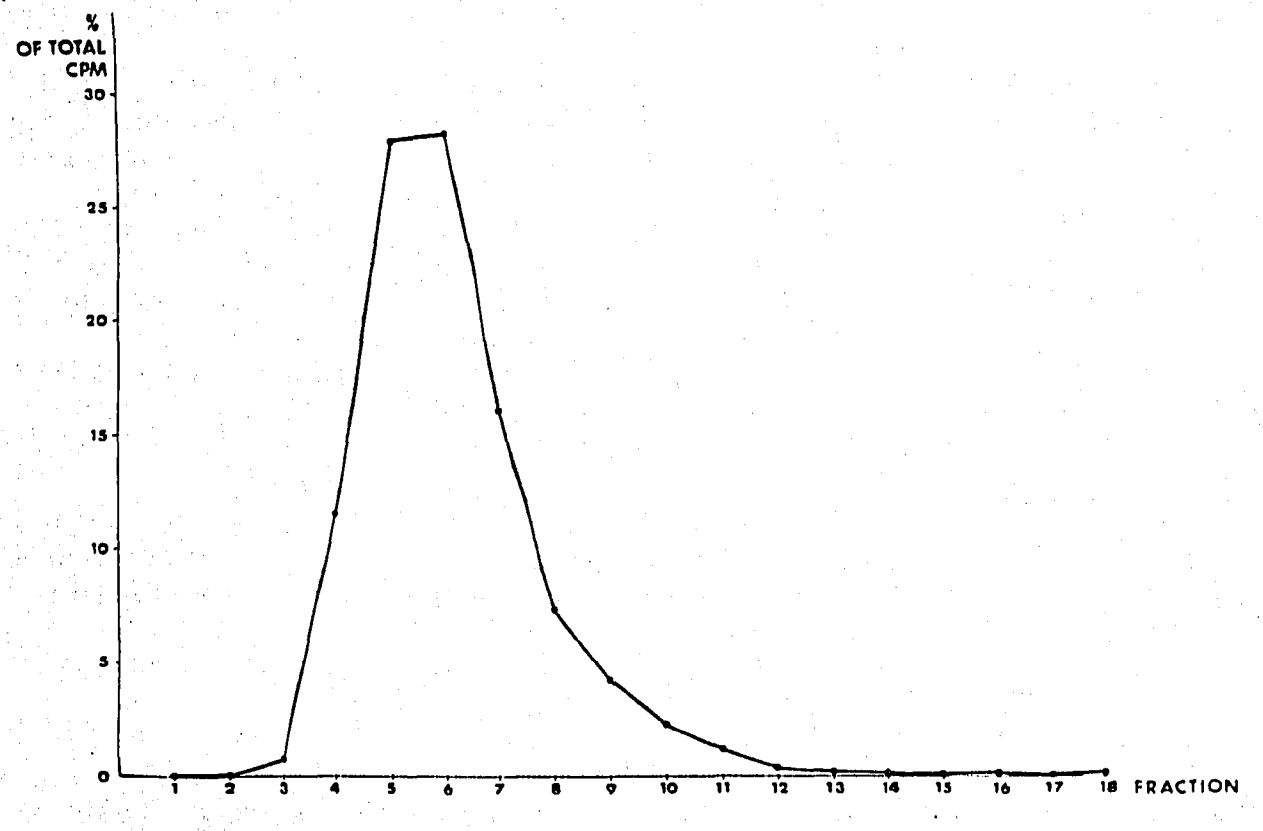

Fig. 1. Passage of [2-^{14}C]galactose and its metabolites through columns of Amberlite IR 12((H form) and Amberlite IRA 410 (acetate form). The effluent was collected in 25-ml fractions Counts per minute (CPM) are expressed as percent of total radioactivity obtained in 27 fractions

glucose were in a very good agreement with those described above. Under the sam conditions, the recoveries of standard unlabeled glucose and galactose in six experi ments were 98.7 ± 2.6% and 97.7 ± 3.3%, respectively.

Chromatography of labeled sugars in the eluate fractions

The chromatographic separation of three fractions illustrated in Fig. 2 indicate that the initial as well as the final fractions have essentially the same compositio and that even the quantitative relations between individual components remai practically the same through the whole desalting process. The sugar pattern in thi urine resembled closely that of the normal urine shown in Fig. 3 (L-2B). Among th [2-^{14}C]galactose metabolites, we have reported[19] glucose, tagatose, talose, galacticol galactonolactone, several oligosaccharides and unchanged galactose as the mai components.

Paper chromatography of urine desalted by different procedures

Fig. 3 represents individual samples after the chromatography in ethyl acetate acetic acid solvent and detection with aniline citrate. The urines were applied in 10-min aliquot of diuresis, except the samples L-1, L-6 and L-8. 0.2 ml of urine, corre sponding to an 11.4-sec aliquot, was used for the sample L-1 in order to demonstrat the desalting technique of WHITE AND HESS[10] in combination with multipl

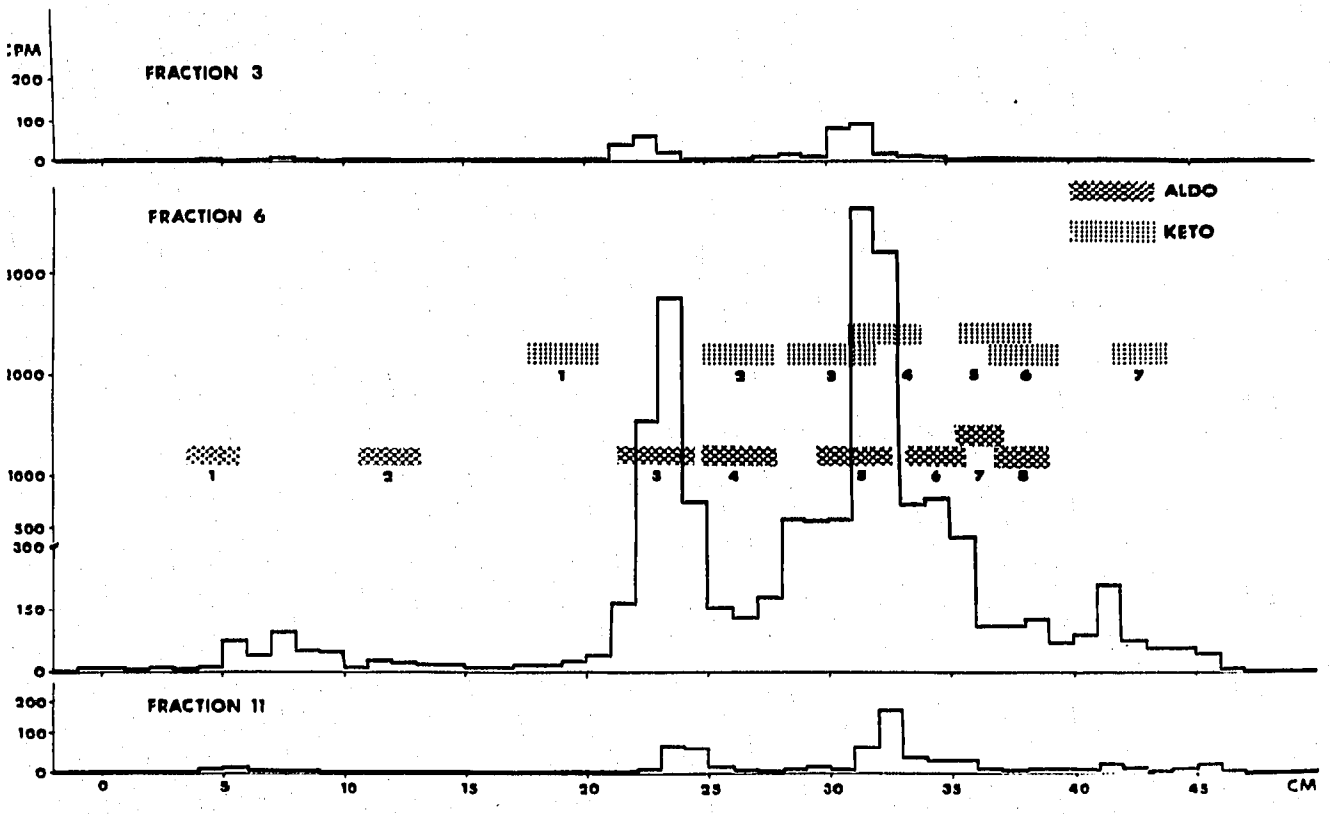

Fig. 2. Radiochromatogram of [2-^{14}C]galactose and its metabolites in eluate fractions. Aldo-standards: 1 = lactose; 2 = 3-O-β-D-galactosyl-D-arabinose (General Biochemicals, Inc.); 3 = galactose; 4 = glucose; 5 = arabinose; 6 = xylose; 7 = fucose; 8 = ribose. Keto-standards: 1 = sucrose; 2 = mannoheptulose; 3 = sedoheptulose + fructose; 4 = tagatose; 5 = xylulose; 6 = ribulose; 7 = dihydroxyacetone. Ascending chromatography repeated four times on Whatman No. 17 filter paper in *n*-butanol–pyridine–benzene–water (5:3:1:3)[2]. The first largest peak was tentatively identified as galactose and galacticol and the second as tagatose.

chromatography. Out of twelve saccharide spots, four were revealed by the color, *i.e.* glucose (10), arabinose (13), xylose (14) and an unknown metabolite (19), and the rest by the more sensitive fluorescence.

Specimen L-6B was necessary to apply in a 60-min aliquot because of the detrimental effect of Amberlite IRA 410 in the OH form on sugars. Only traces of glucose (10), mannose (11), arabinose (13) and two unknown metabolites (19 and 24), but a surprisingly intensive spot of fucose (16) could be revealed.

In sample L-8, large amounts of interfering urea allowed only a 4-min aliquot to be applied in order to ensure a fair resolution of the major part of the sugar spectrum.

Except in samples L-1 and L-2B, the presence of urea could be established in all samples. The concentration of 180 μg of urea in sample L-3B did not influence the chromatography at all. However, 550 μg in sample L-7B masked the spot 22, and 4.40 mg in L-6B caused distortion of the shape and migration of several neighboring spots. This unfavorable effect became even more pronounced in samples L-4 and L-8 containing 6.25 and 13.06 mg urea, respectively. The disturbing effect of uric acid

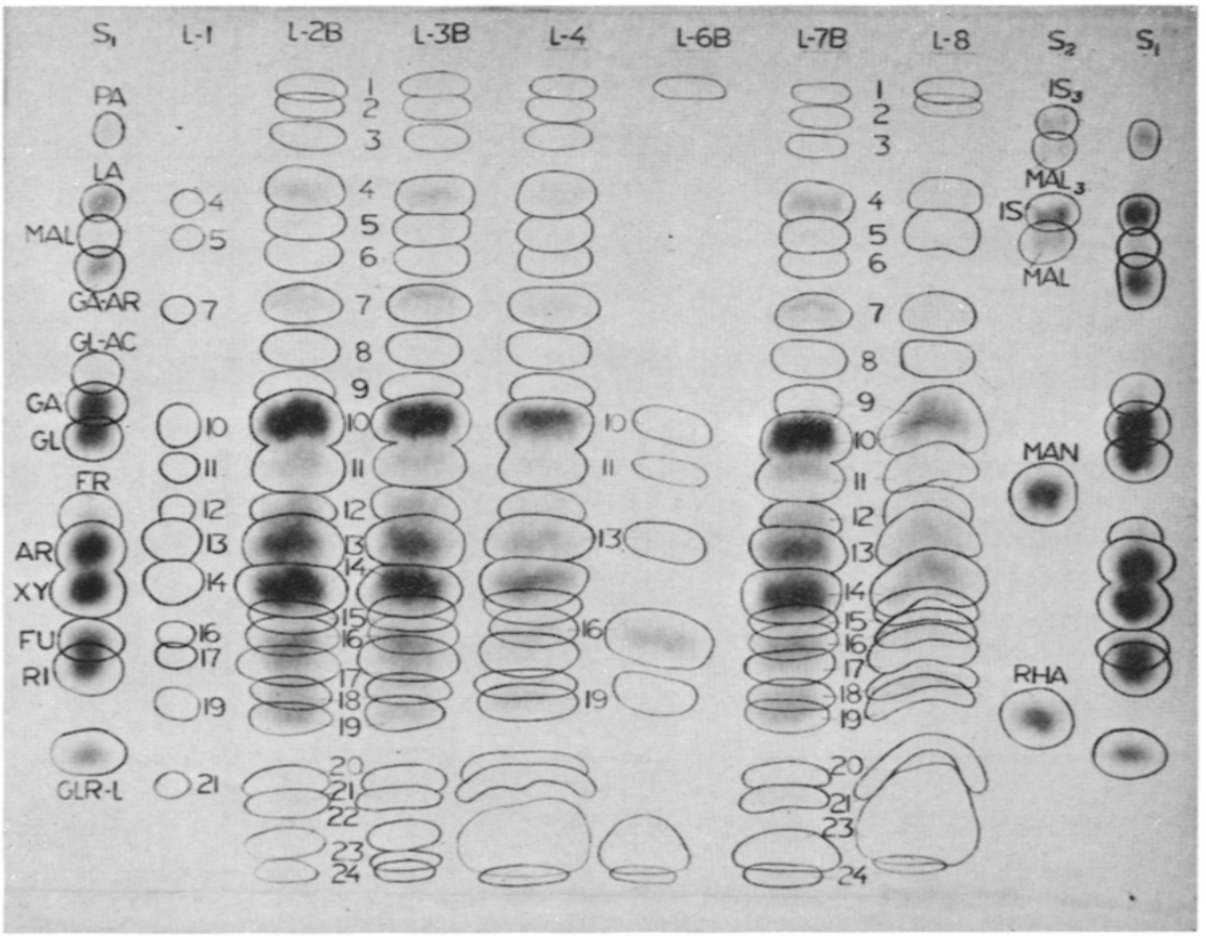

Fig. 3. Spectrum of aldoses in urines desalted by different procedures. Numbers on urine samples are identical with desalting procedures given in Tables I and II. Standards were applied in 100–150 μg quantities, urinary specimen L-1 in a 12-second aliquot of urine (0.2 ml), L-6B and L-8 in 60- and 4-min aliquots of diuresis, respectively, and the remaining samples in 10-min aliquots (10.5 ml) Spots were revealed with aniline citrate reagent and their sizes were recorded by fluorescence. Standards: S_1—PA = panose; LA = lactose; MAL = maltose; GA-AR = galactosylarabinose; GL-AC = glucuronic acid; GA = galactose; GL = glucose; FR = fructose; AR = arabinose; XY = xylose; FU = fucose; RI = ribose; GLR-L = glucuronolactone. S_2—IS_3 = isomaltotriose; MAL_3 = maltotriose; MAL = maltose; MAN = mannose; RHA = rhamnose.
Urinary spots: 1 = unknown (blue fluorescence); 2 = unknown (greenish-yellow fluorescence); 3 = unknown (blue fluorescence); 4 = lactose + isomaltose; 5 = maltose; 6 = unknown (greenish-yellow fluorescence); 7 = unknown (red, tentatively identified as a glucosylxylose); 8 = unknown (light yellow, greenish-yellow fluorescence); 9 = galactose; 10 = glucose; 11 = mannose; 12 = fructose; 13 = arabinose; 14 = xylose; 15 = N-acetylglucosamine; 16 = fucose; 17 = ribose; 18 = unknown (brownish-red, greenish-yellow fluorescence, probably unspecific reaction of the common spot of xylulose + ribulose); 19 = unknown (intensive yellow-orange); 20 = unknown (pale orange); 21 = unknown (pink, whitish fluorescence); 22 = unknown (yellow); 23 = urea; 24 = unknown (brownish, slightly visible before spraying).
Blue fluorescence of maltose and its derivatives was recorded at wavelength 260 mμ. At 360 mμ it changes to greenish-yellow or yellow. Except for glucuronolactone the quality of fluorescence of other saccharides was not influenced substantially by changing the wavelength of the exciting light.

The number of spots reacting with aniline citrate and detected by their color and/or fluorescence varied in individual samples from 12–24. The intensity of the entire sugar spectrum was always in good agreement with the corresponding values of total reducing sugars and glucose (Table II). Accordingly, there was no noticeable difference in the intensity of spots in samples L-2B, L-3B and L-7B.

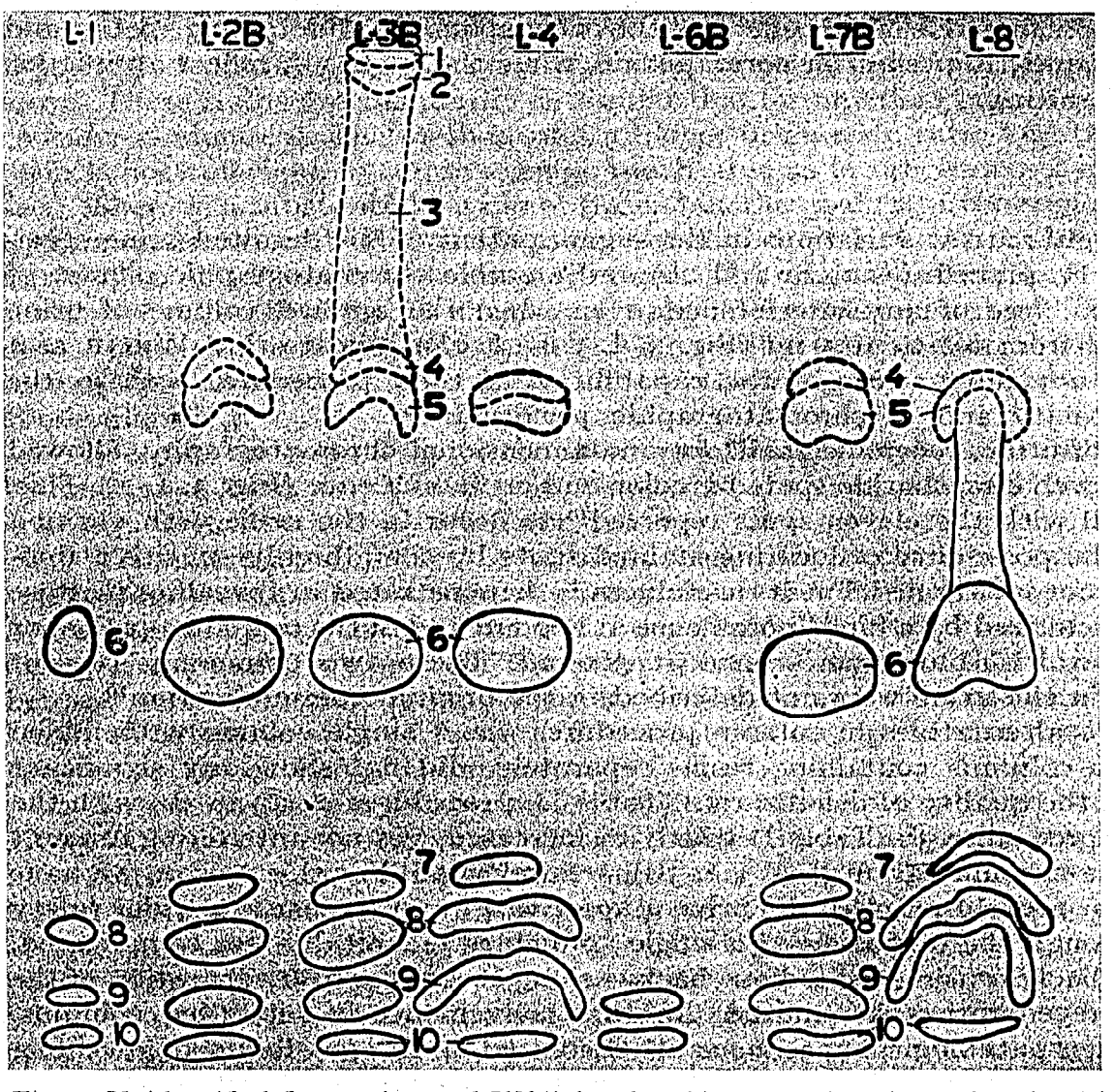

Fig. 4. Unidentified fluorescing and UV light absorbing spots in urines after desalting by different procedures. An outline of desalting procedures is given in Table I. Descending chromatography repeated four times on Whatman No. 3MM filter paper in ethyl acetate–acetic acid–water $(3:1:1)^2$. Full line = absorbing spots, dashed line = fluorescing spots. The figure shows the same chromatogram as that illustrated in Fig. 3 before color detection. Other details are given in EXPERIMENTAL and in the legend to Fig. 3.

UV absorbing and fluorescing spots on chromatograms of urine desalted by different procedures

Upon viewing the chromatogram in UV light (Fig. 3) before detection, several fluorescing and absorbing spots were found which are recorded in Fig. 4. Their number, intensity and shape are dependent on the amount of the applied urine (*e.g.* L-1) and the method used for desalting (*e.g.* L-6B). Spots 5–10 are regular phenomena in the urine. The always most intensively absorbing spot 6 separates the fluorescing spots from the absorbing ones and is preceded by the faintest spot 7. The oval shape of the absorbing spots 7–10 was distorted in shaking procedures L-4 and L-8 proportionally to the concentration of the urea present. Urea itself was not recorded in Fig. 4

in order to show more clearly its effect on the other spots. The fluorescing spots 4 and 5 are very frequently present in normal urine, while the spots 1–3 reflect an inferior quality of desalting.

DISCUSSION

Relatively narrow variations in the sugar content of the desalted urine specimens (Table II, procedures 1, 2a and 2b) and a stable chromatographic picture of urinary sugars, free of any interference, even when the applied volume of urine was increased from 0.2 to 10.5 ml (Fig. 3, L-1 *vs.* L-2B) indicate that WHITE AND HESS[10] were not fully aware of the capabilities of their procedure, both in the desalting capacity and in chromatographic performance.

0.2 ml of urine, recommended[10] for one-dimensional chromatography, allowed us to reveal twelve saccharide spots by color and/or fluorescence (Fig. 3, L-1). This compares well with the eleven spots reported[10] to occur in the urine with varying frequency. The possibility of locating stained spots by their highly sensitive fluorescence was not mentioned, but its usefulness is demonstrated in revealing maltose (spot 5) characterized by a blue fluorescence at 260 mμ and of the unknown pink spot (21) with a whitish-blue fluorescence at 360 mμ. These two commonly present metabolites[2] in the urine were not described in the original communication[10].

The two alternatives in column procedures, *i.e.* a single column with mixed resins or two columns containing resins separately, did not show any significant differences in the quality of deionization (Table II, procedures 2a up to 3b) with the exception of procedure 3b (Table I) where an increased, yet not interfering amount of urea on the chromatogram (Fig. 3, L-3B), was revealed. For this reason, we have reduced in our routine practice the urine aliquot to 45 min to ensure a wider safety margin. Although the operation of a single column is technically less complicated, it has a drawback in using the tedious backwashing technique[10] for separation of the resins when regeneration is required. Another convenience of separated columns, especially when pathological specimens are deionized, is the possibility of revealing the site and thereby also the nature of interfering substances.

The results obtained with monobed mixture Amberlite MB-3 (acetate form) (Fig. 3, L-7B) and the WHITE AND HESS method[10] were very similar. We wonder whether its reduced desalting capacity, apparent only with a 60-min urine aliquot and manifested by a spot of urea (spot 23), was due to the presence of the exhaustion indicator bound to the beads of the cation-exchange component. Otherwise the commercial availability of the resin in the OH form considerably increases its appeal, avoiding the laborious conversion of the chloride form.

Shaking procedures yielded lower amounts of glucose and galactose, and urea was removed less satisfactorily (Table II, procedures 4 and 5). Both features are reflected on the chromatogram (Fig. 3, L-4). The controversial disproportionately high yield of total reducing sugars can be most probably ascribed to unsatisfactorily removed non-carbohydrate compounds non-specifically reducing the copper reagent. Although the urine:resin ratio and the volume of water remained the same as in the corresponding column procedures 2b and 3b, it seems that the inferior results are due to the mode of the resin application.

The worst chromatographic picture was obtained when the performance of

the mixing method of MENZIES AND SEAKINS[14] was examined (Fig. 3, L-8). Since the British Biodeminrolit resin was not available, we employed Amberlite MB-3 (acetate form). These resins are chemically so similar that for practical purposes they may be considered identical. The method uses a higher urine:resin ratio and the amount of resin is not sufficient to retain even as much urea as in procedures 4 and 5 (Fig. 3, L-4). There is a substantial saving of resins but the quality of the sugar extract was not commensurate with the 10-min aliquot of diuresis and it had to be lowered to 4 min in order to obtain a readable chromatogram. The original instructions[14] recommend the use of 0.1 ml of urine for chromatography. Even this amount will disclose a urea spot but with much less interference. Our results raise certain doubts about the assumption of MENZIES AND SEAKINS[14] of the insignificant sugar absorption by Biodeminrolit.

Despite the popularity of the mixing methods[12,14,20–28], our results, supported by findings of MORI AND NAKAMURA[20], show that the yield of sugars by this technique is not quantitative and is of inferior quality.

The detrimental effect of strongly basic ion exchangers, which has been recognized in sugar chromatography for many years[8,9,30–32], became apparent when Amberlite MB-3 was used in the OH form (Table II, procedures 6a and 6b). The disappearance of fructose from the sugar spectrum suggests that urinary reducing ketoses were affected in a manner similar to glucose and other aldoses. On the other hand, an intensive spot of fucose (16) indicates a strong resistance of this methylpentose to the action of the resins.

In our preliminary experiments, we have also used weakly basic Amberlite IR-45 (OH form) and Amberlite IR-4B (OH form), occasionally employed for the deionization of sugar solutions[33] and urine analysis[34–36]. Both ion exchangers were tested in combination with Amberlite IR 120 (H form) in the same experimental arrangement as described in Table I, procedure 3. When the yield of urinary sugars obtained by the WHITE AND HESS method[10] was ascribed a value of 100%, then with IR-45 (OH form) the amount of total reducing sugars was 77.6%, galactose 78.6% and glucose 76.5%. With IR 4B it was 91.4, 90.7 and 90.0%, respectively. TOWER *et al.*[35], using a column, reported a 15–20% loss of ribose with IR-4B and 70% with IR-45. In our case, the losses of sugars were not large enough to be evident on paper chromatograms using a 10-min aliquot of diuresis.

MURPHY *et al.*[37] indicated recently that the loss of sugars by weakly basic anion exchangers is not a simple retention but a reversible reaction between the sugars and resins. The product of this reaction is most likely a Schiff's base or a glycosylamine obtained by the elimination of a molecule of water. Therefore they advise caution in the employment of weakly basic resins. This is also justified by our results.

The extent to which the amount of the retained sugars can be released from the exchangers was the object of a study by MORI AND NAKAMURA[20]. They reported that glucose could be eluted from the columns of Amberlite IR 120 (H) and Amberlite IR 4B with a 98% or higher efficiency by a subsequent passage of an equal volume of water. The column of Amberlite IRA 410 (OH) required a ten times larger volume of water to achieve a complete recovery. Upon testing Amberlite IR 120 (H) they found that at room temperature it hydrolyzed about 2% of sucrose. They did not, however, mention any degradation of sugars by strongly basic resins as described by others[8,9,30–32].

In our experiments with labeled galactose, the elution of sugars from Amberlite IRA 410 (acetate form) and Amberlite IR 120 (H form) was achieved with a volume of water 1.5 times that of the combined volumes of both exchangers (Fig. 1). An incomplete elution or a loss of a part of the eluate during collection would affect all components of the sugar spectrum equally since all possess very similar rates of passage through the column.

Among the interfering substances in the urine, inorganic salts were most easily removed by all tested procedures. Also, in our case, medium pigmented urine did not evoke special problems. In case of a very strong pigmentation, all pigments may not be caught up efficiently. They are then visible on chromatograms by fluorescence and color, located in the area adjacent to the start as spots or frequently as streaks, covering mainly the slowest third of the run. Defined spots may also occur in the front zone of the solvent. Representing a chemically heterogenous group of compounds, they occasionally react non-specifically with the detection reagent and interfere mainly with the faint spots of oligosaccharides.

We have also found that the absorbing spots of an undefined nature (Fig. 4) may interfere after aniline citrate spray in the fluorescence detection of some saccharides present in small concentration; otherwise, they do not seem to influence the separation. Their presence can be utilized for indication of the regularity of the solvent flow, particularly in repeated chromatography, as well as the location of some sugar spots prior to staining.

Uric acid disturbs the chromatography only in very high concentrations and may be revealed in short-wave UV light (260 mμ) as an elongated spot located in acidic solvent system in the second third (Fig. 4, spot 5) and in basic solvents in the slowest third of the chromatogram. Since large amounts of uric acid are encountered only in very poorly deionized sugar extracts, they are generally accompanied by high levels of urea and pigments and there are many disturbances in the flow and shape of the saccharides (Fig. 3, L-8).

The weakly ionized urea is easily removed by a strongly acidic exchanger of the sulphonic type, but because it is present in the urine in abundant quantities, its complete removal is the most serious problem common to all desalting procedures. Therefore its appearance on chromatograms is a sensitive indicator of an impaired deionization procedure (Fig. 3, spot 23, in samples L-3B to L-8). The illustrated position of urea is characteristic for acidic solvent systems and is manifested by white-blue fluorescence before detection. After spraying with aniline citrate it becomes pale yellow, very similar to the color of the unknown spot 22 (Fig. 3). In basic systems such as butanol–pyridine–water (6:4:3), the interference of urea, especially in a larger concentration, is more pronounced since its location between arabinose and xylose can influence a large part of the sugar spectrum. Being aware of its disturbing effect, some investigators[26,34,38,39] have included in the deionization procedures the removal of urea by treating the urine with urease. According to our results, this step can be avoided by selecting an adequate desalting technique.

The use of procedure 3b (Table I) for the desalting of urine allows us to apply on Whatman 3MM filter paper 5–10-min aliquots[2] and in thin-layer chromatography 30–60-sec aliquots of diuresis. We have found that this expression of the urinary volume is of particular advantage in comparative studies as the amount of excreted sugars in specimens collected successively over a few hours is more stable than their

volumes, which may fluctuate as much as 500% or more. The amount of applied urine thus exceeds by more than fifty times the most frequently recommended[1,10,14] volume of 0.1 ml of urine for PC (Fig. 3, L-2B, L-3B). This, in conjunction with multiple chromatography[2], followed by a stepwise detection of sugars, enables us to demonstrate the complexity and particularly the remarkable similarity and regularity of the composition of the urinary sugar spectrum in different individuals.

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