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CHROMATOGRAPHY OF SUGARS IN BODY FLUIDS

III. STEPWISE DETECTION OF SUGARS WITH ANILINE CITRATE ON PAPER AND THIN-LAYER CHROMATOGRAMS

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SUMMARY

The paper chromatographic detection method of White and Hess for urinary aldoses using aniline citrate has been studied and modified. As the reactivity of individual sugars is determined mainly by their molecular weight and structure, our modification is based on a gradual increase in the reaction temperature (room temperature, 37 or 55 and 105°) and variations in heating time. This technique enables reaction conditions to be obtained that lead to a full display of both the transient and final characteristics of the fluorescence and colour of the individual spots in the sugar spectrum of any analyzed material.

Compared with other reagents based on aniline or other aromatic amines, aniline citrate possesses unusually wide operational flexibility. The procedure is suitable for paper and thin-layer chromatography and is especially valuable in the analysis of urinary and tissue sugar extracts for the identification of largely overlapping spots with different reaction times, the differentiation of oligosaccharides, particularly those with different linkages, and obtaining additional data for the identification of unknown metabolites.

INTRODUCTION

The neutral sugar component of normal human urine consists of wide concentrations of at least 24 aldo and keto sugars [1—6]. Under certain pathological conditions, the number of sugars and their concentrations may be even higher [7—9].

The complexity of urinary sugar components and their close structural similarity lead to overlapping of the individual spots in paper or thin-layer chromatography. Neither repeated development with the same solvent system nor two-dimensional chromatographic procedures [2, 10] work satisfactorily,

especially in the more numerous aldose fraction [5]. The situation is also aggravated by the fact that some aldoses share their positions with ketoses in any solvent system. Their interference cannot be completely overcome even by using highly specific detection reagents for aldoses such as phthalate, oxalate or citrate salts of aniline [1, 2, 10].

This paper describes a detection procedure that improves the identification of incompletely resolved sugar spots on paper and thin-layer chromatograms when complex mixtures of sugars in urine and a large variety of tissues are analyzed. The technique is based on the observation that on a chromatogram sprayed with aniline citrate as detection reagent [10] the individual or groups of spots require different reaction temperatures and times to develop optimal colour and fluorescent properties.

EXPERIMENTAL

Urinary specimens were collected from a severely injured surgical patient and a healthy individual who had been given a large dose of *d*-galactose. The patient was a 42-year-old male, semi-conscious with a fractured leg and gas-gangrene, on parenteral nutrition consisting of glucose and occasional administration of blood and its fractions. Routine examination of the urine showed no significant pathological changes. The healthy individual was a 40-year-old male. Galactose was administered after 11 h of fasting in a dose of 0.5 g per kilogram of body weight (45 g total). Urine was collected in 3-h periods prior to and after the ingestion of galactose. During the collection, the urines were refrigerated and then kept frozen until required.

Sugar standards were mostly commercial preparations. All chemicals were of ACS grade or better. Aniline was re-distilled every 2 months.

Preparation of urine for chromatography

Urines were filtered through filter-paper, pre-washed with water and de-salted by passage through ion-exchange columns [6]. For paper chromatographic, colorimetric and enzymatic analysis, the dry residues obtained after lyophilization of concentrated column effluents were re-dissolved in small amounts of water. For chromatography, the urine extracts were streaked on a 30-mm line on Whatman No. 17 paper in a portion corresponding to a 10- or 20-min aliquot of diuresis [5]. Standards of aldoses, ketoses and sugar alcohols were spotted as small circles in the amounts indicated in the legends of Figs. 1 and 3.

Chromatography and detection

Urine (Figs. 1 and 3) was chromatographed by the ascending technique in at least three solvent systems: (1) *n*-butanol—pyridine—benzene—water (5:3:1:3); (2) ethyl acetate—acetic acid—water (3:1:1); and (3) isobutanol—acetic acid—water (4:1:1) [5].

The spots on the chromatograms were detected with aniline citrate [10] in a modification described in detail under Results. Ketoses were revealed by the orcinol—trichloroacetic acid method [11], modified by replacing the spraying technique with dipping and increasing the heating temperature to 120—125°.

Sugar alcohols and other sugar derivatives were located with periodate—benzidine reagent [2].

The total contents of reducing sugars, glucose and galactose in de-salted urinary preparations were determined as described elsewhere [6].

Preservation of detected chromatograms

Darkening of chromatograms treated with aniline citrate or orcinol—trichloroacetic acid can be prevented by keeping them from contact with air at a temperature below 0°. Storage in plastic folders in a deep-freezer or box of solid carbon dioxide enables the detection of aldoses to be interrupted at any stage (overnight or for periods of several days or even weeks).

RESULTS

Preparation of developed chromatograms for detection

As the chromatographed material (Figs. 1 and 3) was of biological origin and contained UV-absorbing and fluorescing spots, before staining the chromatograms were viewed under the light from two UV lamps. Of several brands tested, Mineral-Light UVS-11 (main wavelength 260 nm) and Black Ray UVL-21 (360 nm) lamps, both from Ultraviolet Products (San Gabriel, Calif., U.S.A.) [7], were the most suitable. Their light was strong enough to display the fluorescent and UV-absorbing properties satisfactorily and yet did not affect the surface of the paper even after prolonged exposure. The shape and intensity of the fluorescing and absorbing spots were marked on the back of the sheet, which was of value in checking the regularity of the solvent flow after each run and in pre-determining the locations of the expected sugar spots and fluorescent spots of non-sugar substances. After staining, most fluorescent and UV-absorbing spots disappeared entirely and only a few showed colours. UV-absorbing spots usually seen in the urine are recorded in Fig. 3.

Spraying technique

Whatman No. 1 and similar thin papers were sprayed with aniline citrate on the front. On Whatman No. 3 and thicker papers, optimal intensity of the spots was achieved by applying a gentle spray on the back so that the paper was uniformly saturated on both sides without the formation of shiny spots.

Changes in colour properties of spots during stepwise detection

The detection procedure with aniline citrate using Whatman No. 17 filter-paper was divided into five stages and is outlined in Table I. Standard sugars and urinary spots with a distinct colour and/or fluorescence in the detection stages I—III are recorded in Fig. 1, while spots evident in stages IV and V are shown in Fig. 2.

The colour development of the sugar spots in its initial stage has a number of characteristic features common to all sugars, regardless of the final colour. At first there is a weak fluorescence of an indefinite light colour, changing within 15—30 min (at room temperature) into greenish yellow, yellowish green or similar shades characteristic of the sugar or its class. The change in fluorescence is preceded by the appearance of a yellow spot, which is at first most visible in

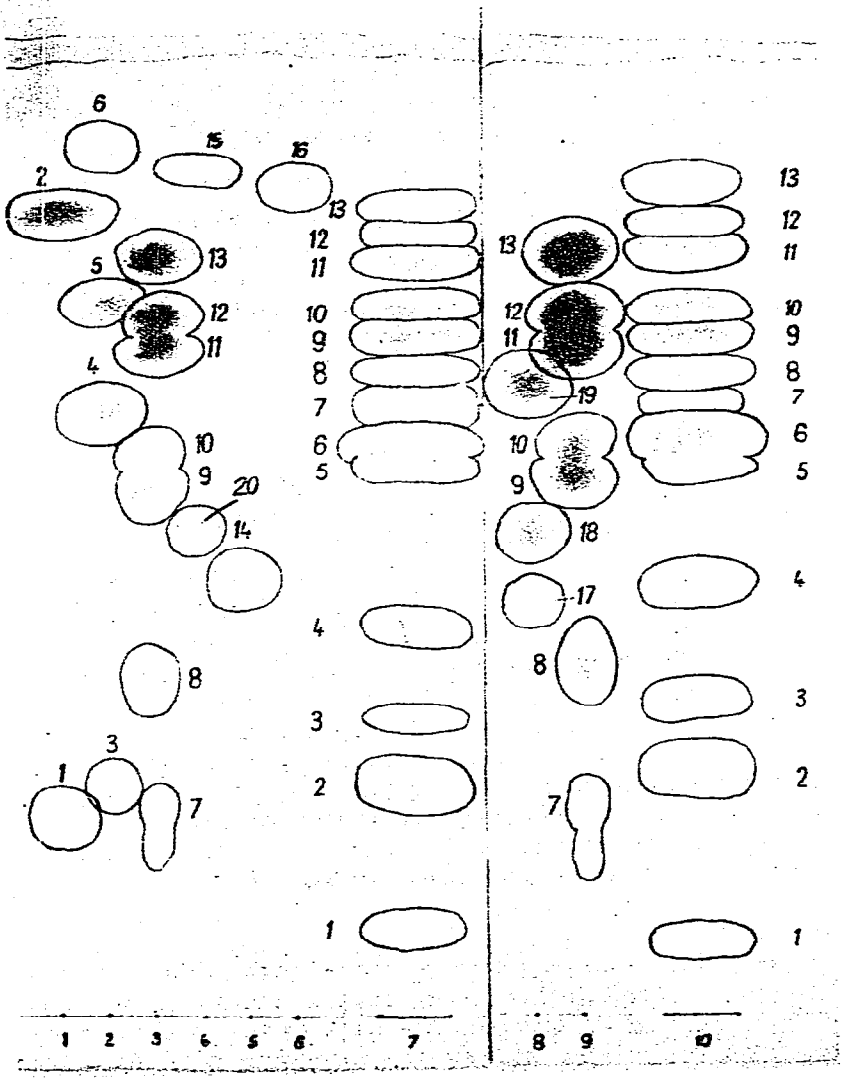

Fig. 1. Paper chromatograms of urinary sugars of a severely injured patient illustrating colour development after stage III (Table I) of the detection procedure using aniline citrate. Ascending chromatography in isobutanol—acetic acid—water (4:1:1) on Whatman No. 17 filter-paper repeated eight times [5]. Ten-minute aliquots of urine specimens collected in two successive 1-h periods were streaked on two chromatograms at positions 7 and 10. Chromatography and detection of both sheets were carried out simultaneously. Standard sugars were spotted in amounts of 100 μg or as indicated in parentheses at positions 1—6 and 8—9: 1 = melibiose (150 μg); 2 = rhamnose (200 μg); 3 = cellobiose (200 μg); 4 = gulose; 5 = lyxose; 6 = glyceraldehyde (200 μg); 7 = lactose; 8 = 3-O-D-galactosyl-D-arabinose (General Biochemicals, Chagrin Falls, Ohio, U.S.A.); 9 = galactose; 10 = glucose; 11 = arabinose; 12 = xylose; 13 = ribose; 14 = mannosamine; 15 = deoxyribose (300 μg); 16 = erythrose; 17 = galactosamine; 18 = galacturonic acid; 19 = mannose; 20 = glucuronic acid. Spots in urine samples: 1 = unknown; 2 = isomaltose + lactose; 3 = unknown; 4 = tentatively identified as glucosyl-xylose; 5 = galactose; 6 = glucose; 7 = allose; 8 = mannose; 9 = arabinose; 10 = xylose; 11 = ribose + fucose; 12 = ribulose + xylulose; 13 = unknown with yellow-orange fluorescence and colour.

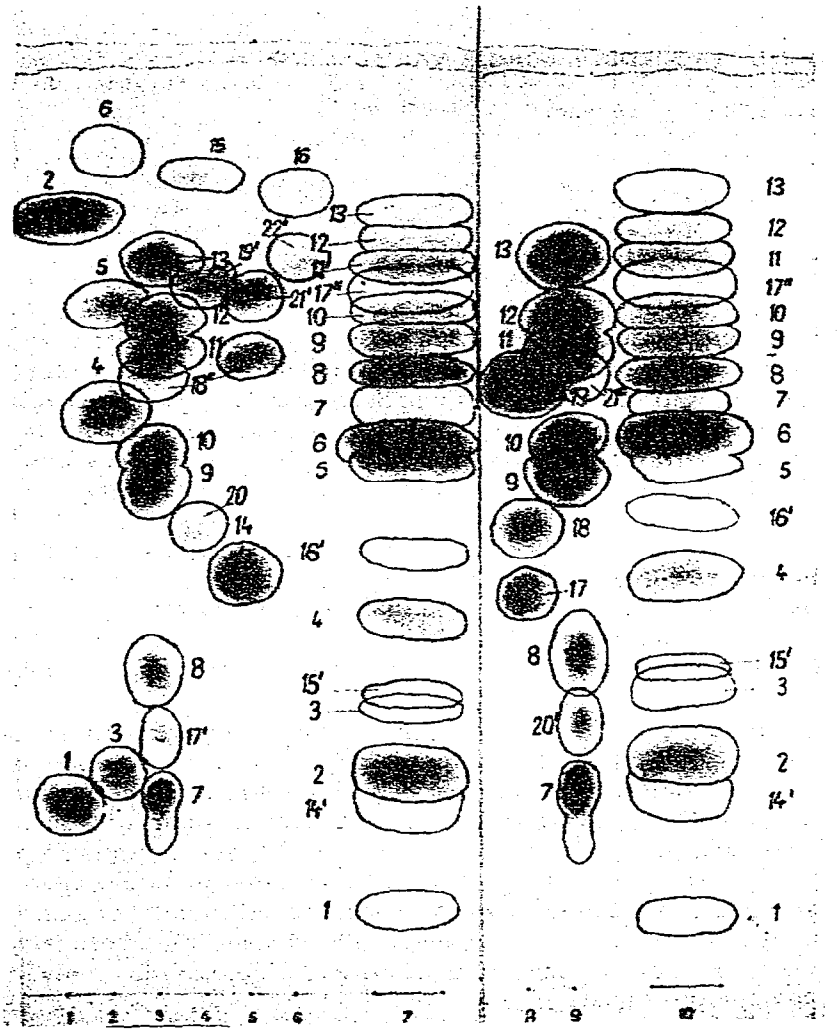

Fig. 2. Chromatograms of sugars in the urine of a severely injured patient after stage V of the detection procedure (Table I). Chromatograms are identical with those illustrated in Fig. 1. Spots recorded after stage IV are designated with numbers with one superior prime and spots recorded after stage V with two superior primes. Standard sugars at positions 1—6 and 8—9: 17' = maltose; 18'' = fructose; 19' = N-acetylglucosamine; 22' = N-acetylmannosamine; 20' = maltose; 21' = N-acetylgalactosamine; 21'' = fructose. Urinary specimens at positions 7 and 10 : 14' and 16' = unknowns, no colour; 15' = unknown with weak light brown colour and light fluorescence; 17'' = N-acetylglucosamine.

a transparent light. Starting from the centre, it changes gradually into the colour characteristic of the particular sugar, which is pink to cherry red for pentoses and greenish brown or yellow for hexoses and their oligosaccharides. Subsequent heating at 55° for an appropriate time causes intensification of the characteristic fluorescence and colour. Continuation of heating or even extend-

TABLE I

SCHEME OF DETECTION PROCEDURE FOR URINARY SUGARS

Stage	Temperature (°C)	Duration
I	24—26	2 h
II	55	10 min
III	55	60 min
IV	55	60 min
V	105—110	10 min

ed exposure at room temperature at this stage leads to a loss of brilliance and darkening. The colour due to pentoses changes into purple with a brown tone and that of aldohexoses into sepia brown. Final heating at 110° turns all sugar spots into various shades of brown with little individual differences. At the same time, the initially hardly discernible light yellow tint of the background becomes gradually darker with a light bluish fluorescence absorbing light fluorescing spots.

Table II summarizes the fluorescence and colours of the sugars included in Figs. 1 and 2. The onset of the stage I occurred by the time the chromatogram was dry. However, while the paper was still damp, in its lower part some standards (Figs. 1 and 2) such as mannosamine (spot 14), glyceraldehyde (6), erythrose (16) and aldopentoses (5, 11, 12, 13), especially ribose (13), were already yellow.

The colours and fluorescence of sugar derivatives often differ markedly from those of the parent sugar. At 55°, deoxyribose (15) yields an intense lemon yellow fluorescence and colour, both very resistant to deterioration by further heating, while the ribose (13) spot is red. The greenish brown colour of glucose and galactose differs from the pink of glucuronic acid (20) and the orange of galacturonic acid (18). Both can be more easily differentiated by fluorescence, which is strong purple for glucuronic acid and its lactone but orange for galacturonic acid. N-Acetylation of amino sugars drastically changes their reactivity and their intense yellow colour changes to light brown, which is visible only at the much higher temperatures of stage V.

TABLE II

FLUORESCENCE AND COLOUR PROPERTIES OF SUGAR SPOTS RECORDED IN FIGS. 1 AND 2

Stage numbers as in Table I.

Spot No.	Sugars*	First fluorescence stage No.	Peak of fluorescence stage No.**	First colour stage No.	Peak of colour stage No.**
(A) Standards					
1	Melibiose	II	III (g-y)	II	IV (g-br or y-br)
2	Rhamnose	I	III (g-y)	II	IV (br)
3	Cellobiose	II	III (g-y)	II	IV (g-br or y-br)
4	Gulose	I	III (g-y)	II	III (g-br)
5	Lyxose	I	II (y-g)	I	II (r)

TABLE II *(continued)*

Spot No.	Sugars*	First fluorescence stage No.	Peak of fluorescence stage No.**	First colour stage No.	Peak of colour stage No.**
6	Glyceraldehyde	I	I (l.y)	I	II (b-br)
7	Lactose	II	III (g-y)	II	IV (g-br)
8	Galactosyl-arabinose	I	II (y-g)	I	III (r)
9	Galactose	I	III (g-y)	II	III (y-br)
10	Glucose	I	III (g-y)	II	III (y-br)
11	Arabinose	I	II (y-g)	I	II (r)
12	Xylose	I	II (y-g)	I	II (r)
13	Ribose	I	II (y-g)	I	II (r)
14	Mannosamine	I	II (y)	I	III (y-br)
15	Deoxyribose	I	II (y)	I	II (y)
16	Erythrose	I	II (y)	I	II (y)
17	Galactosamine	I	II (y)	I	III (y-br)
18	Galacturonic acid	I	II (w.o)	I	III (o-r)
19	Mannose	I	III (g-y)	II	III (y-br)
20	Glucuronic acid	I	III (p)	I	III (o-r)
17'	Maltose	III	IV (260nm, bl; 360nm, g-y)	IV	V (l.br)
18''	Fructose	IV	V (g-y)	IV	V (l.br)
19'	N-Acetylglucos-amine	IV	IV (y)	IV	V (l.br)
21'	N-Acetylgalac-tosamine	IV	IV (y)	IV	V (l.br)
21''	Fructose	IV	V (g-y)	IV	V (l.br)
22'	N-Acetylman-nosamine	IV	IV (y)	IV	V (l.br)
(B) Urinary sugars					
1	Unknown	I	I (g-y)	—	I-V (n.c.)
2	Isomaltose and lactose	II	III (y)	II	IV (r-br)
3	Unknown	II	III (g-y)	—	II-V (n.c.)
4	Glucosylxylose	I	II (y-g)	I	III (r)
11	Ribose and fucose	I	II (y-g) and III (g-y)	I	II (r) and III (br)
12	Ribulose and xylulose	I	II (l.y)	II	III (o-pk)
13	Unknown	II	IV (y-o)	III	IV (y-o)
14'	Unknown	IV	IV (g-y)	—	IV—V (n.c.)
15'	Unknown	IV	IV (g-y)	V	V (l.br)
16'	Unknown	IV	IV (g-y)	—	IV—V (n.c.)
17'	N-Acetylglucos-amine	IV	V (y)	V	V (l.br)

*The numbers against the names of the sugars are identical with the numbers of the spots in Figs. 1 and 2.

**Abbreviations for colours: b = beige; bl = blue; br = brown; g = green; l. = light; n.c. = no colour; o = orange; p = purple; pk = pink; r = red; y = yellow; w. = weak.

The colour and fluorescence of oligosaccharides seem to be dependent not only on the sugar component, e.g., red for galactosylarabinose (8) compared with greenish yellow or brown for lactose (7), but also by the kind of linkage. Melibiose (1), with a β-1,6- bond, gave a reddish brown colour with a yellow fluorescence, both distinctly different from the green-yellow colour and similar fluorescence of isomeric lactose (7), with a β-1,4- bond. Unlike other reducing oligosaccharides, maltose and its derivatives, with an α-1,4- bond (i.e., maltotriose, panose, etc.), reacted at considerably higher temperatures, developing a weak greenish brown colour. This drawback was compensated for by their highly sensitive and specific blue fluorescence at 260 nm and greenish yellow fluorescence at 360 nm.

Application of stepwise detection in the identification of urinary sugars

The spot of urinary glucose (6) in the final detection stage (Fig. 2, samples 7 and 10) overlapped considerably the spot of galactose (5), yet they could be distinguished in stage I or II by the yellow fluorescence and/or colour of uneven intensity, which are stronger with glucose. In Figs. 1 and 2, fucose, which is always present in the urine, shared its position with ribose (11). The latter is the fastest reacting pentose and was easily identified by the red colour and green-yellow fluorescence in stage I, while fucose first became apparent after heating for 10 min at 55°. Its fluorescence altered that of ribose and, after heating for 60 min (stage III), due to the admixture of fucose, there was a pronounced difference between the spots of standard and urinary ribose in the fluorescence and especially in the colour. After final heating at 110°, only an experienced eye could discern the presence of two sugars.

Another partially overlapping group of urinary spots included xylose (10), N-acetylglucosamine (17″), ribose and fucose (both 11, Fig. 2). The differentiation of N-acetylglucosamine (17″) was relatively easy as it started to produce fluorescence and a light brown colour when the fluorescence of all of the above-mentioned sugars was extinguished. Mannose (8) and arabinose (9) (Fig. 2) were partially overlapped by the always present fructose, which reacted non-specifically with aniline citrate. [Urinary fructose is not recorded in Figs. 1 and 2. Its position is indicated by its standard (18″) in Fig. 2.] In the urine, differentiation is facilitated by the pink colour of arabinose reacting in stage I, unlike mannose, which appears as a yellow to brown spot in stage II. During stage IV, arabinose and mannose lose their characteristics while fructose shows peak fluorescence and a light brown colour of the part of its spot that does not overlap in stage V. Apart from fructose, other ketoses such as ribulose and xylulose also react non-specifically with aniline citrate. Both share the same position in the solvent used (12, Figs. 1 and 2) and react in stages I and II. Unlike neighbouring ribose and fucose, they initially display a characteristic pale yellow colour, which in stage III turns orange-pink.

Most of the spots marked on the chromatograms as "unknown" are permanent features of the sugar spectrum, located predominantly in the disaccharide region. Spot 2 in Fig. 2 and spot 4 in Fig. 3 showed typical qualities of disaccharides, yielding peak colour and fluorescence at 55° in stage IV. Its red-brown colour differs from those of standard lactose (7) and cellobiose (3) but is similar to that of melibiose (1). In severely ill patients, the spot is usually

formed by small amounts of lactose and large amounts of isomaltose; in healthy subjects, lactose prevails. Another unknown but characteristic feature of human urine is the intense spot 4 (Figs. 1 and 2). It reacts earlier than hexosyldisaccharides but later than hexoses. In stage II it yields a pink to red colour and fluorescence. Both properties are identical with those of standard galactosylarabinose. It was tentatively identified as glucosylxylose.

Unidentified urinary spots 14', 15' and 16' (Fig. 2, samples 7 and 10) appeared during stage IV in the region of disaccharides and showed a weak green-yellow fluorescence. Additional heating at 110° changed only spot 15' to a weak light-brown colour.

Unknown spot 13 was the only one that did not share its yellow-orange colour with any standard tested. The metabolite yielded a feeble yellow fluorescence after heating for 10 min at 55° (stage II), reaching the highest intensity of fluorescence and its yellow-orange colour simultaneously at the end of stage IV. Unlike other urinary spots, both properties were unusually stable on further heating.

In addition to simple aldoses and ketoses, de-salted urine frequently contains low-molecular-weight neutral or weakly ionized fragments of glycolipids and glycoproteins and always at least 15 sugar alcohols, which on the chromatograms share positions with reducing sugars. The relative positions of some sugar derivatives and sugar alcohols are demonstrated in Fig. 3, samples 6—10.

Unidentified spots 17f, 23f and 26f (Fig. 3) could be located only after spraying with aniline citrate by their fluorescence, which is identical with or similar to that produced by standard carbohydrates.

Urinary sugar extracts prepared by ion-exchange techniques are not entirely free of non-carbohydrate compounds such as spots 10a, 16a, 20a, 24a and 26a. They are a characteristic feature of urinary sugar chromatograms and can easily be detected by their absorption at 260 nm. They have been described in detail elsewhere [6].

The chromatogram in Fig. 3 is an example of an experimental situation in which unknown metabolites may be expected. A 45-g oral dose of galactose given to a healthy person produced several changes in the urinary sugar pattern. Unidentified spot 3 (Fig. 3) is a metabolite with chromatographic properties characteristic of a disaccharide. Unknown spot 2, of a trisaccharide nature, intensified after administration of galactose. The remaining aldoses in the spectrum were excreted in decreased amounts, in contrast to spot 7, shared by glucose and galactose.

DISCUSSION

White and Hess [10] introduced the aniline citrate detection reagent into sugar chromatography. As with other similar reagents [1,2], the original procedure involved heating the sprayed, still damp chromatogram for several minutes at 100°. This treatment proved to be satisfactory to give an overall picture of the sugar spectrum when a complete separation of a simple mixture is expected or only one or few sugars are in question. On the other hand, it does not have the advantage of revealing the changing colour and fluorescence properties of individual sugars that provide a useful data for identification purposes. In

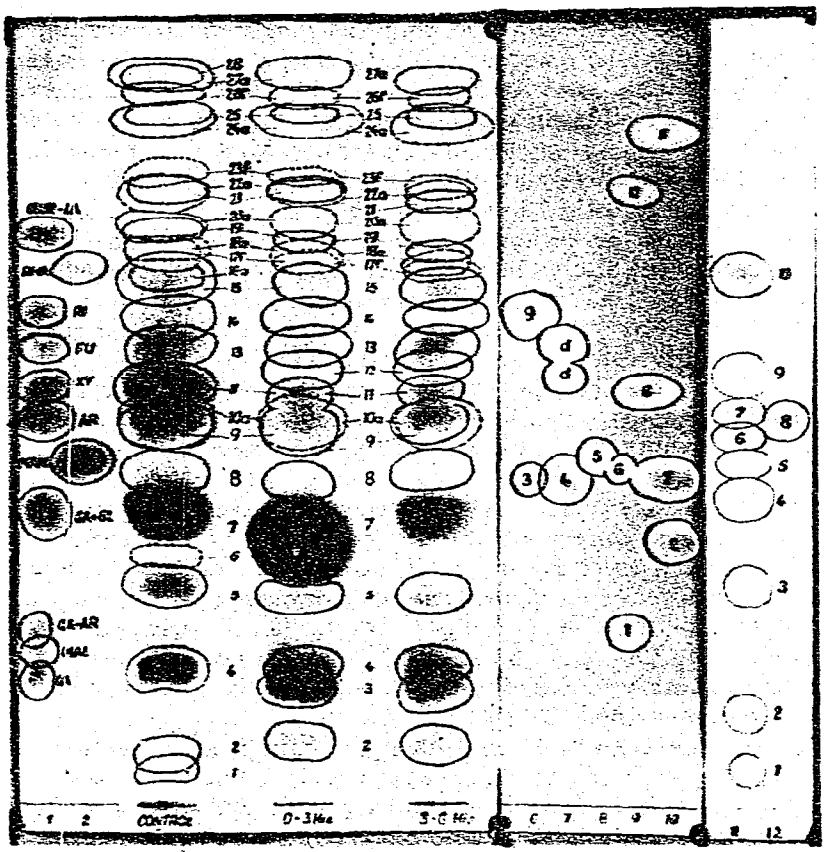

Fig. 3. Chromatogram of sugars in the urine of healthy person after a large dose of galactose. Ascending chromatography on Whatman No. 17 filter-paper in ethyl acetate—acetic acid—water (3:1:1) repeated four times [5]. Detection with aniline citrate as described in the text. Spots marked with a dashed line and "f" were apparent before or after the detection process only by their fluorescence; a dot-and-dashed line and "a" indicate UV light-absorbing spots (wavelength 260 nm) before the detection; a full line shows coloured spots, the size of which is marked by their fluorescence. Standards of aldoses were applied at positions 1 and 2 in amounts of 100—200 μg. Standards of sugar alcohols, sugar acids and their lactones were applied at positions 6—10 in amounts of 200—350 μg. Positions 11 and 12 carried standard ketoses in amounts of 100—200 μg. Control urine was collected in a 3-h period preceding administration of galactose in a dose of 45 g (0.5 g per kilogram of body weight). Samples 0—3 h and 3—6 h represent changes that occurred during the corresponding periods. All urinary samples represent a 10-min aliquot of diuresis with the following amounts of reducing substances determined by the Somogyi—Nelson method and expressed as milligrams of glucose: control, 2.0; 0—3 h, 10.26; 3—6 h, 1.13. At the same time the three urinary specimens contained 1.20, 1.53 and 0.75 mg of pure glucose, respectively, as determined by a glucose-oxidase method [6]. The 0—3-h urine sample contained 7.1 mg of galactose. Standard spots of aldoses at positions 1 and 2: LA = lactose; MAL = maltose; GA-AR = *d*-galactosylarabinose; GA = galactose; GL = glucose; MAN = mannose; AR = arabinose; XY = xylose; FU = fucose; RI = ribose; RHA = rhamnose; GLUR-LA = glucuronolactone. Standards of sugar alcohols (positions 6—10): 1 = myoinositol (200 μg); 2 = perseitol (300 μg); 3 = galactonic acid released from galactonolactone; 4 = gluconic acid released from *d*-gluconolactone (350μg); 5 = mannitol (200 μg); 6 = sorbitol (200 μg); 7 = galacticol

general, the rate of colour development of sugar spots decreases with increasing molecular weight in the following order: tetroses, pentoses, hexoses, heptoses, disaccharides and higher oligosaccharides. Within a homologous series of sugars applied in equimolar amounts, the time of colour development is roughly proportional to the relative speed of migration of the individual member (in non-phenolic solvents) and the fastest-moving sugar reacts first, e.g., in the pairs ribose—arabinose, talose—galactose, mannosamine—glucosamine the second sugar always reacts markedly later than the first. It is also apparent that other structural properties play a substantial role, e.g., the glucose spot appears later than the glucuronolactone spot, which shows up at the same time as that of ribose.

The stepwise detection procedure described above is especially suitable for Whatman No. 17 paper and a complex mixture of sugars containing some unknown substances such as those found in urine. Its timing schedule should be changed when thinner paper is used, as in general the reactions then proceed faster. Simplification of the heating and timing schedule may be advantageous when less complex or well separated mixtures are used. In this instance the colour development at room temperature may be avoided or abbreviated. In one of our alternative modified procedures employing Whatman 3MM paper, the sprayed and dried sheet is maintained at room temperature for 1 h, then heated at 37° for 30—60 min, at 55° for 60 min and finally at 110° for 10 min. On other occasions, particularly when working with di- and higher oligosaccharides, the colour development at room temperature is omitted and the dried or still damp sheet is heated at 55° for 10 or 20 min, then twice at 55° for 60 min and finally at 110° for 10 min.

The procedure is also applicable to thin-layer chromatographic plates coated with silica gel and gives even better results with microcrystalline cellulose. Sugars chromatographed on silica gel do not react at low temperatures or react

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(300 μg); 8 = arabitol (200 μg); 9 = 1,4-*d*-galactonolactone (300 μg); 10 = glycerol (200 μg); 11 = dihydroxyacetone (300 μg). Decomposition products of *d*-galactonolactone are designated by the letter "d". Standard spots of ketoses (position 11 and 12): 1 = stachyose; 2 = raffinose; 3 = sucrose; 4 = mannoheptulose; 5 = sedoheptulose; 6 = fructose; 7 and 8 = tagatose; 9 = impurities of ribulose; 10 = ribulose. Urinary spots: 1 = mixture of unknown saccharides with weak yellow-green fluorescence and light brown colour; 2 = unknown trisaccharide with green-yellow fluorescence and light brown colour; 3 = unknown disaccharide with green-yellow fluorescence and brown colour; 4 = isomaltose and lactose; 5 = tentatively identified as glucosylxylose; 6 = unknown spot apparently of non-carbohydrate nature, with a deep purple fluorescence before and after detection; 7 = glucose and galactose; 8 = mannose with trace amounts of allose; 9 = arabinose; 10 = unknown, always the strongest UV-absorbing spot on the chromatogram visible before but not after detection; 11 = xylose; 12 = N-acetylglucosamine; 13 = fucose; 14 = ribose; 15 = unknown carbohydrate with strong yellow-orange colour and fluorescence; 16 = weak UV-absorbing spot of non-carbohydrate nature; 17 = unknown sugar derivative with white-blue fluorescence; 18a = unknown UV-absorbing spot seen before detection; 19 = glucuronolactone; 20 = unknown, strong UV-absorbing spot; 21 = unknown with a pink colour developed at room temperature with yellow-green fluorescence, after stage five, light brown colour with white fluorescence; 22a = unknown UV-absorbing spot; 23f = unknown with a faint pink colour with room temperature detection, intense white-blue fluorescence after heating at 110°; 24a = unknown strong UV-absorbing spot; 25 = unknown weak orange-brown spot; 26f = unknown with a light fluorescence; 27a = unknown UV-absorbing spot of moderate intensity; 28 = unknown with yellow-orange colour.

to a negligible extent and much higher starting temperatures are required, especially in the presence of borates.

A number of reagents are available for the detection of aldoses [1, 2] but only very few methods are suitable for the detection of the complex range of urinary sugars [1, 5] or other similar mixtures, mainly because of their low specificity or sensitivity. The most widely used reagent for aldoses is aniline phthalate, introduced by Partridge [12], which is considered to be more sensitive than the previously introduced aniline oxalate. Aso et al. [13] reported that different sugars possess different optimal reaction temperatures with aniline phthalate. In our experience, however, this reagent was completely unsuitable for the stepwise detection method, requiring much higher initial heating, which markedly narrowed the range of optimal temperatures of individual sugars. In addition, the fluorescence and colour sensitivity were much more inferior than with aniline citrate reagent. Better results, but still unsatisfactory in all respects, were obtained with aniline oxalate. The popular diphenylamine—aniline—orthophosphoric acid reagent [14, 15] also proved to be unsuitable for good colour differentiation because aldoses and ketoses react simultaneously and lack fluorescence.

The procedure described, in conjunction with a good chromatographic separation technique [5] and an effective de-salting technique [6], enabled us to identify isomaltose as a regular component of human urine [8] and also other previously unknown carbohydrates [5]. In addition, it was instrumental in demonstrating the great complexity and also the remarkable similarity and regularity of the range of urinary sugars in different individuals [5].

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