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GAS CHROMATOGRAPHIC STUDY OF FREE POLYOLS AND ALDOSES IN CATARACTOUS HUMAN LENS TISSUE

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SUMMARY

An analytical procedure for the qualitative and quantitative analysis of human lens tissue for polyol and aldose content is described. Profile samples are obtained by direct derivatization of lyophilized lenses. The components are analyzed as per-O-acetylpolyols (from the polyols) and per-O-acetylaldononitriles (from the aldoses). This procedure converts each component into a single derivative and terminal dissymmetry for each aldose is retained. The derivatives form in quantitative yield, give good chromatographic peaks, are thermally stable and readily volatilized. They are not subject to adsorption on gas chromatographic columns and are suitable for both qualitative and quantitative analytical studies.

Six non-cataractous lenses and fourteen lenses from patients with senile cataract (in seven instances complicated by diabetic pathology) were analyzed. Thermostable borosilicate glass open-tubular capillary columns, coated with the non-polar phase SE-30, and containing dispersed particles of silanized silicic acid, were used for the gas chromatographic separations.

The results are discussed in relation to what is known from earlier studies of human and animal cataracts.

A gas chromatographic method for determining the polyol and aldose excretion levels of controlled diabetics is also reported along with a typical metabolic profile.

INTRODUCTION

Cataract formation results in a loss of transparency of the lens of the eye. No cure or means of preventing senile cataract, the most common form of the disorder, is known. Most investigations of this circumstance have indicated that cataract formation, both in man[1–4] and in experimental animals[5–13], involves the accumulation of one of several polyols within the tissue of the lens. A diabetic is about five times more likely to have an initial cataract operation than is a nondiabetic of the same age and sex[14]; the polyol in these instances is believed to be glucitol.

It has recently become possible to separate complex mixtures of polyols and aldoses completely and rapidly by gas chromatography (GC)[15]. The recommended GC procedure, based on the classical Wohl degradation of aldoses, includes the use

of high-resolution glass open-tubular capillary columns which possess high theoretical plate efficiencies (125,000–150,000). In this method each sample component forms a single derivative with retention of terminal dissymmetry for each aldose. Polyols are analyzed as per-O-acetylpolyols and aldoses as per-O-acetylaldononitriles. The procedure has been successfully used[16] to determine normal excretion ranges and average excretion values for seventeen polyols and aldoses usually found in the urine of human neonates, juveniles, and adults.

This study includes the results of GC analyses of six human non-cataractous lenses (obtained at autopsy) and fourteen lenses from patients having senile cataract, with seven cases complicated by diabetic pathology. Values are reported in μg of free polyol or aldose per mg of lyophilized lens. Also included are excretion values (mg/g creatinine) for eleven polyols and aldoses found in the urine of five of the subjects whose lenses were analyzed. Urinary analyses of two closely controlled diabetics are included in this group.

EXPERIMENTAL

Reference compounds

Reference aldoses, alditols, *myo*-inositol and glyceryl-1-decyl ether were purchased from Supelco (Bellefonte, Pa., U.S.A.).

Preparation of analytical sample from lens tissue

Adherent tissue (zonular ligament, ciliary muscle) was removed from surgically extracted human lenses. The intact lens was quickly weighed and frozen under an atmosphere of nitrogen. Lyophilization was effected over a 48-h period at 0.5 Torr using a Virtis Model 10-300CF lyophilizer. The dry lens was weighed (Table I), pulverized to a homogeneous powder using a glass rod, and stored under nitrogen, under deep-freeze conditions, until analyzed.

Analytical samples were prepared by adding to 10 mg of powdered lens, 10 mg of hydroxylamine hydrochloride, 0.0315 mg of glyceryl-1-decyl ether (internal reference compound, from a solution of 6.30 mg per 10.0 ml of methanol) dissolved in 50 μl of methanol, 23 mg of sodium acetate, and 1 ml of methanol. The mixture was heated at 60° for 2 h, evaporated to dryness using a nitrogen stream, and treated with 1 ml of acetic anhydride. After this mixture had been heated for 2 h at 110°, it was allowed to stand overnight in a desiccator for complete conversion of the carbohydrates, which react more slowly in tissue preparations than they do as pure reference compounds[17]. Precipitated protein was removed by centrifugation. GC samples were removed from the clear supernatant solution and co-injected with *n*-alkanes when methylene unit (*MU*)[18,19] values were being determined (Tables I and II).

Preparation of analytical sample from urine

Urine samples from patients were collected over a 24-h period just prior to cataract extraction. No preservative was used, but the sample was kept at 4° during the collection period and stored under deep-freeze conditions as soon as possible after the volume had been determined. The pH of a 20-ml aliquot of urine was adjusted to 4.5 using 10% aqueous acetic acid. This sample was poured onto a packing of DEAE-Sephadex A-25 Medium (Pharmacia, Uppsala, Sweden) contained in a

10 × 1.3 cm I.D. glass column. Basic and neutral compounds were eluted by washing the packing with 100 ml of deionized water which had been glass-distilled from potassium permanganate. The eluate was collected in a 250-ml round-bottomed flask and lyophilized to constant weight. The dry residue was dissolved in 10 ml of methanol. A 1-ml aliquot of the methanolic solution was heated with 5 mg of hydroxylamine hydrochloride and 12.5 mg of sodium acetate at 60° for 30 min in a PTFE-lined screw-cap vial. The product was dried at 40° using a nitrogen stream, and derivatized in 1 h using 300 μl of acetic anhydride at 100°. When an internal reference compound was required, 200 μl of a solution of glyceryl-1-decyl ether (6.30 mg/10.0 ml of methanol) were added prior to heating with hydroxylamine hydrochloride. Also, in these instances 100 μl of pyridine was substituted for the sodium acetate catalyst, and the dry residue from these reactions was treated with 300 μl of acetic anhydride and 50 μl of pyridine at 100° for 1 h.

Gas chromatography

Column preparation. Sixty-meter borosilicate glass capillaries (1.0 mm O.D., 0.3 mm I.D.) were drawn from 1.25-m tubes (7.8 mm O.D., 3.8 mm I.D.) using a Hupe-Busch glass drawing and coiling apparatus (now available from Hewlett-Packard). Novotný and Zlatkis[20] have described the general procedure. Pyrex tubes to be drawn were successively rinsed with acetone, methylene chloride, 1% aqueous potassium hydroxide, and methanol, and dried under vacuum. The diameter of the capillary coil was 12 cm.

Using the method of German and Horning[21], glass capillaries silanized with dimethyldichlorosilane (DMCS) and coated with SE-30 containing Silanox 101 (Cabot, Boston, Mass., U.S.A.) were prepared. These were conditioned under carrier gas (nitrogen) flow by temperature programming at 1°/min from 25–280°, holding at 280° for 2 h, lowering to 200°, programming at 1°/min to 300°, and holding at 300° for 1 h. The theoretical plate efficiencies of the resulting columns (for *n*-tetracosane at 250°) ranged from 125,000–150,000.

Instrumental conditions. Separation of both the lens and the urinary polyols and aldoses as per-O-acetylpolyols and per-O-acetylaldononitriles was carried out with a Hewlett-Packard Model 5710A gas chromatograph which had been modified to include a previously described[22] glass inlet system and to accept 60-m glass capillary columns. A Fisher Recordall Series 5000 recorder was used and the flame ionization detector was modified (see next section) for use with glass capillary columns. Temperature programming was employed at 1°/min from 150°. Other GC conditions included: sample volume, 2 μl; split ratio, 5:1; precolumn-inlet splitter temperature, 250°; detector block temperature, 300°; column inlet pressure, 18 p.s.i., resulting in a carrier gas (helium) linear velocity of 16–18 cm/sec (200°); hydrogen flow-rate, 30 ml/min; air flow-rate, 240 ml/min; nitrogen make-up gas to the detector, 20 ml/min.

Detector modification. The HP Model 18765A flame ionization detector (standard equipment on the Hewlett-Packard Model 2710A gas chromatograph) is a highly satisfactory detector for packed columns. The suggested carrier gas flow-rate through a 2 m × 6.4 mm O.D. conventionally packed column is 30 ml/min, and the recommended burner flow-rates are 30 ml/min hydrogen and 240 ml/min air.

Carrier gas (helium) flow-rate through an open-tubular glass capillary column rarely exceeds 2 ml/min. It was therefore necessary to modify the standard flame noz-

zle of the detector as indicated in Fig. 1. (Extensive modification of another type of flame ionization detector has been reported previously[23].)

The modification consisted of drilling the bottom end of the original flame nozzle stem (1) to a diameter which could accept a concentric 1/8 in. length of stainless-steel hypodermic tubing (1/32 in. O.D., 0.020 in. I.D.). When this tube (A) was to be joined to the end of the modified flame nozzle, it was first inserted all the way into the newly drilled portion of the nozzle, then withdrawn 1/32 in., and positioned there until the nut which anchors the two-piece ferrule union (2) to the base of the detector was tightened. This arrangement made it possible to introduce sufficient make-up gas (nitrogen) into the flame so that the original ion collector of the detector could be utilized.

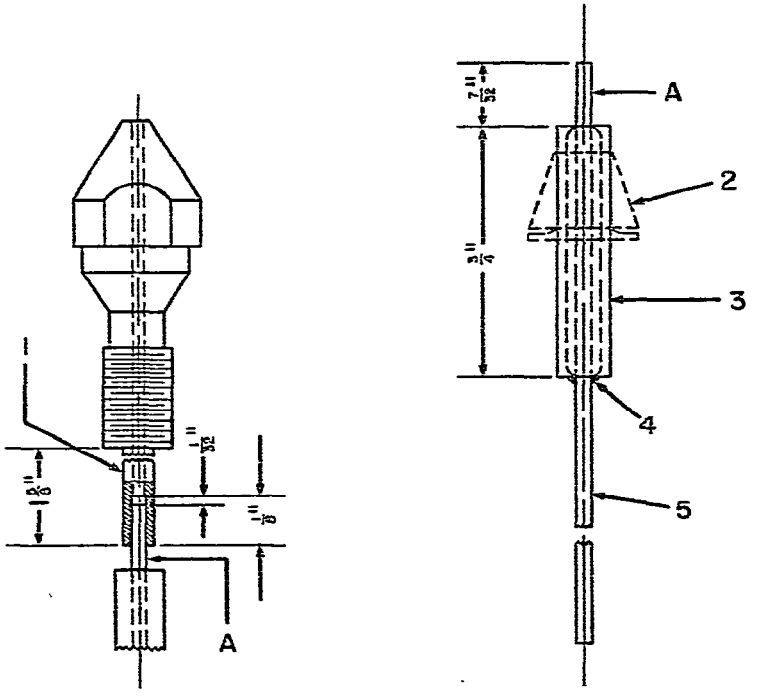

Fig. 1. Flame nozzle assembly for modified flame ionization detector: (1) original flame nozzle stem; the bottom end was drilled to accept a concentric 1/8 in. length of hypodermic tubing, (2) stainless-steel two-piece Swagelok union ferrule by which assembly A is attached to the base of the detector, (3) stainless-steel tubing (1/8 in. O.D.; 0.020 in. wall thickness) with both ends rolled closed, and then drilled to accept 1/32 in. tubing, (4) silver-soldered joint, (5) stainless-steel hypodermic tubing (1/32 in. O.D.; 0.020 in. I.D.; 2 1/2 in. long).

Quantitative analysis. Calculations were made by hand; area measurements were made by multiplying the height of the peak by the width at half height. The chart speed and pen line width are variables that affect the results; in our work the height was measured to the outside of the pen line, but the width at half height was measured to the inside of the pen line. A magnifying scale was used for the width measurement. Usually a chart speed of 1.25 cm/min was used for quantitative calculation

charts and 0.5 cm/min for illustrations. The precision of this method is about 2% (standard deviation).

The quantitative calculations were based on previously reported molar response factors (MRF), calculated in relation to the detector response observed for the diacetyl derivative of glyceryl-1-decyl ether[16].

Mass spectrometry

Mass spectra were obtained using an LKB 9000 gas chromatograph–mass spectrometer (LKB Produkter, Stockholm, Sweden) fitted with a 3 m × 4 mm I.D. glass column packed with 2% SE-30 on Gas-Chrom P (100–120 mesh, acid-washed, DMCS-treated); column temperature, 180°; flash heater temperature, 230°; molecular separator temperature, 260°; ion source temperature, 250°; electron energy, 70 eV.

RESULTS AND DISCUSSION

Glass open-tubular capillary column separations

Analyses of six non-cataractous lenses and fourteen lenses from patients having senile cataract (seven of the cases complicated by diabetic pathology) are reported in Table I. The age range in both the non-patient and the patient group was from 52–81 years. A typical gas chromatogram is given in Fig. 2 for lens 5. Polyol and aldose contents of the lyophilized lenses were determined according to a slightly modified version of the method of Pfaffenberger *et al.*[16]. GC separations were made using thermostable borosilicate glass open-tubular capillary columns, coated with the non-polar phase SE-30, and containing dispersed particles of silanized silicic acid. Identities were confirmed by retention behavior and by mass spectral data obtained using an LKB 9000 gas chromatograph–mass spectrometer. Fig. 3 shows the mass spectra of *myo*-inositol from lens 12, and of an authentic reference sample (both analyzed as the corresponding per-O-acetylpolyol).

Free polyols and aldoses in human lenses

By far the largest free polyol component of human lens tissue is *myo*-inositol. This is evident in Fig. 2. Amounts of this component ranged (in μg/mg lyophilized lens) from 2–19 for non-cataractous lenses, 3–23 for uncomplicated senile cataractous lenses, and 7–29 for senile cataractous lenses from patients with diabetes.

All lenses contained a trace (>0.1 but <1.0 μg/mg lyophilized lens) of glucose. One non-cataractous lens, obtained at autopsy from an individual 81 years of age, contained a trace of glucitol, two of the uncomplicated senile cataractous lenses contained a trace of glucitol, and all but two of the senile cataractous lenses from patients with diabetes contained at least a trace of glucitol. Lenses 2 and 4 contained 2 and 1 μg glucitol per mg lyophilized lens, respectively.

All except two lenses (non-cataractous) contained a trace of an unidentified compound with $MU = 19.79$, and all except four lenses contained an unidentified compound with $MU = 20.95$. The first unknown was not one of the simple aldohexoses which elute within an MU range[16] of 19.02–19.56 under the analytical conditions employed here. The second unknown was not mannitol[15], galactitol[16], *scyllo*-inositol[24], or 1,5-anhydroglucitol[25]. (Study of the two unidentified compounds is continuing.)

TABLE I

POLYOLS AND ALDOSES* DETECTED IN HUMAN LENS TISSUE

	Cataractous lenses														*Non-cataractous lenses*					
	Diabetic source							*Non-diabetic source*												
Lens number	1	2	3	4	9	11	13	5	6	7	8	10	12	14	15	16	17	18	19	20
Age (years)	52	69	71	58	74	77	58	67	73	54	64	81	72	65	58	74	81	63	52	70
Dry weight (mg)**	67	58	88	65	80	59	68	69	67	55	72	51	81	61	70	86	86	73	54	64
Glucose ($MU = 19.12 \pm 0.01$)***	T‡	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
Unknown 1 ($MU = 19.79$)	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	X‡‡	T	T	T	T
myo-Inositol ($MU = 20.50 \pm 0.03$)	15	16	12	7	29	14	21	11	3	3	19	4	23	8	2	16	19	12	2	15
Glucitol ($MU = 20.68 \pm 0.01$)	X	2	T	1	T	X	T	T	X	X	X	X	T	X	X	X	T	X	X	X
Unknown 2 ($MU = 20.95 \pm 0.01$)	T	T	T	T	T	T	T	T	T	X	T	X	T	T	X	T	T	T	X	T

* Values are in μg/mg lyophilized lens.

** Each lens was lyophilized 48 h.

*** All methylene unit (*MU*) values are for the corresponding per-O-acetylaldononitriles (from aldoses) and the per-O-acetylpolyols (from polyols) as determined on an SE-30 open-tubular glass capillary column temperature programmed at 1°/min from 150°.

‡ "T" signifies <1.0 μg/mg but >0.1 μg/mg lyophilized lens.

‡‡ "X" signifies that this compound was not present above 0.1 μg/mg lyophilized lens.

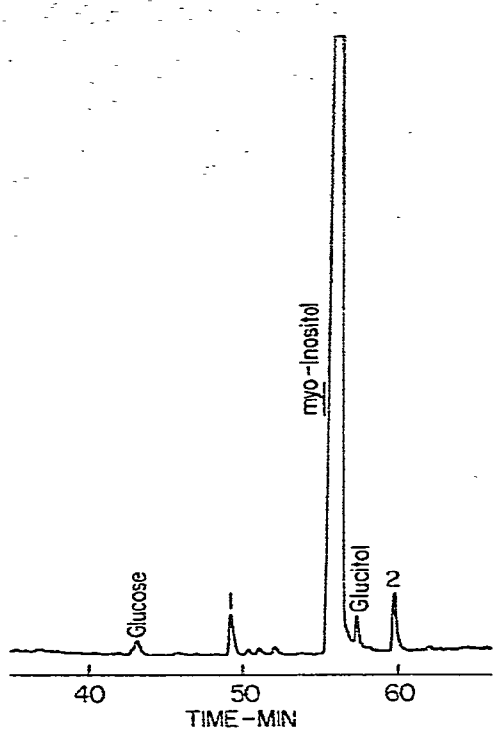

Fig. 2. Profile of the free aldoses and polyols found in human cataractous lens tissue (lens 5 of Table I). Separation of the components as per-O-acetylaldononitriles ("Wohl" derivatives from aldoses) and per-O-acetylpolyols (from polyols) was carried out on a 60-m SE-30 open-tubular glass capillary column temperature programmed 1°/min from 150°. For further conditions see text.

Structure of the human lens

The lens is an isolated organ; fibers of the zonule attach it radially to the ciliary body. The lens contains no internal blood vessels or nervous tissue. Its cells depend on the circulating aqueous humor both for needed compounds and for the removal of the products of catabolism. Trauma, various forms of radiation[26], and metabolic changes within the lens can damage it directly. Changes in the composition of the aqueous humor can upset lens metabolism and damage it indirectly[3].

Osmotic balance of the lens

A single layer of cells encases the lens and forms a capsule which permits diffusion of small molecules such as glucose, amino acids, salts, vitamins, and other substances from the aqueous humor. Amino acids are mainly responsible for maintaining osmotic balance of the lens[10]. An excess of potassium ions and a deficit of sodium ions is maintained within the tissue; potassium and chloride ions make osmotic contributions[3]. Free polyols and aldoses also affect the osmotic balance. Polyols are believed to modify the usual osmotic balance[10] of the lens and to initiate loss of its transparency.

Mechanisms initiating cataract formation

Kinoshita and coworkers have demonstrated that lens swelling can be caused

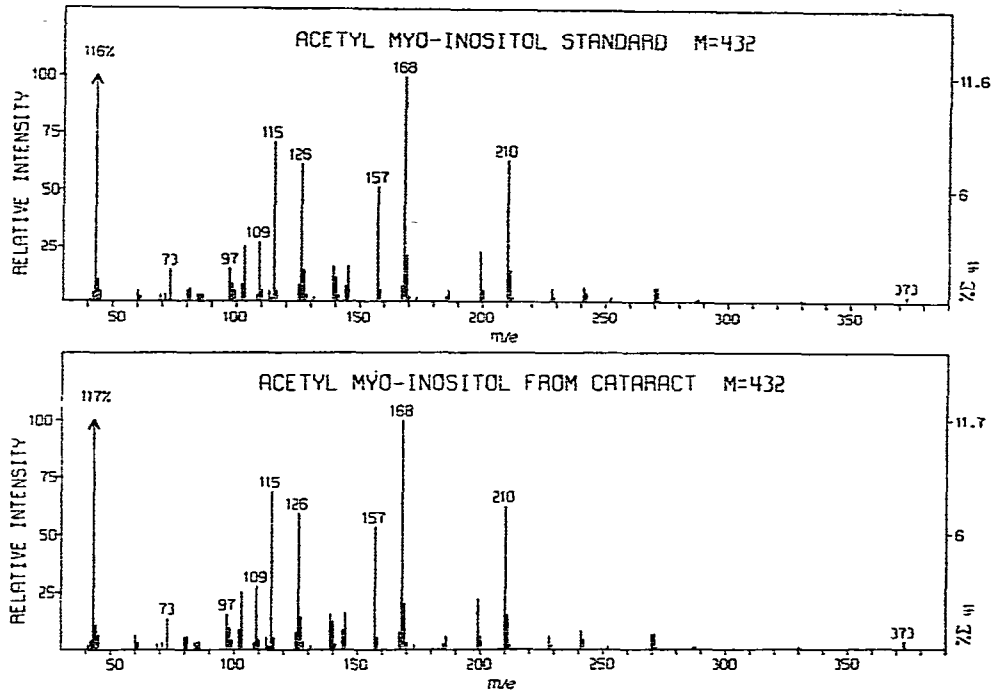

Fig. 3. Mass spectra of authentic *myo*-inositol and *myo*-inositol found in cataractous lens 12 (both samples analyzed as the corresponding per-O-acetylpolyol). The most abundant ion fragment at *m/e* 43 (CH_3CO^+) is not taken as the base peak; the ion fragment at 168 is used. Instrumental conditions are given in Experimental.

by any of the three cataractogenic sugars: glucose[9], galactose[8], and xylose[12]. If the lens is exposed to one of these aldoses, the sugar is converted into the corresponding sugar alcohol by the enzyme aldose reductase (EC 1.1.1.21), which requires for its activity the reduced coenzyme nicotinamide-adenine dinucleotide phosphate (NADPH). With this system, glucose is converted into glucitol, galactose into galactitol, and xylose into xylitol.

The sugar alcohol, because it is not further metabolized effectively and is not able to penetrate readily the lens capsule, accumulates in higher than normal amounts[5]. The hypertonicity this creates is corrected by an influx of water[3]. The osmotic changes which occur initially from polyol accumulation do not seriously alter the state of viability of the lens, and in the early stages of sugar cataract formation the process is reversible. If normal osmotic balance is not restored, lens opacity develops. At this stage there is a gradual breakdown of the permeability barrier and the electrolytes and polyols, including *myo*-inositol[27,28], become freely permeable and only the larger proteins are retained. After this stage becomes fully established, quantities of free sugar alcohol found in the cataract are insignificant and free *myo*-inositol levels are significantly altered from normal. This agrees with the wide range of values obtained experimentally in this study for *myo*-inositol.

Senile cataract accompanied by diabetic pathology

It is accepted that blood sugar fluctuates in diabetics and that the control

achieved by therapy may vary over both short and long periods. Caird *et al.*[29] showed that control of diabetes was below average in effectiveness in diabetics who required cataract extraction. He and his coworkers[30] later demonstrated a direct relationship between degree of control of diabetes and frequency of lens opacity in men of middle age.

The low concentration of glucitol observed in the cataractous lens has been explained[1] by the facts that diabetes is usually well controlled before an operation and the cataractous lens (advanced) is abnormally permeable and has lost glucitol to the aqueous humor by diffusion[3].

Excretion values for urinary polyols and aldoses

Table II contains excretion values (in mg/g creatinine) for eleven polyols and aldoses found in the urine of five of the subjects whose lenses were analyzed. (The peak-numbering system used in Table II and Figs. 4 and 5 is the same one employed in previous[15,16] publications.) The excretion values of samples LU5, LU14 and LU15 are typical of the adult human over 50 years of age[16]. No obvious difference can be seen between the non-cataractous subject (LU15) and the two patients having senile cataracts (LU5 and LU14).

TABLE II

EXCRETION VALUES* FOR URINARY POLYOLS AND ALDOSES

*Peak No.***	*Assignment****	*MU value (±0.01)*	*LU1*§	*LU5*	*LU11*	*LU14*	*LU15*
1	Erythritol	15.73	42	65	44	50	46
2	Threitol	15.91	10	11	10	14	10
7	Ribitol	18.22	T§§	12	8	18	6
9	Arabinitol	18.30	12	48	27	54	33
10	Xylitol	18.47	12	11	10	58	7
12	Glucose	19.13	56	5	32	14	6
13	Galactose	19.41	T	T	T	X§§§	T
14	*myo*-Inositol	20.50	6	25	8	7	16
15	Mannitol	20.60	X	12	92	X	T
16	Glucitol	20.68	4	9	10	X	4
17	Galactitol	20.76	X	4	6	6	9

* Values are in mg/g creatinine.

** The peak numbering system is identical to the one used in ref. 16.

*** Based on methylene unit (*MU*) values; *myo*-inositol was confirmed by gas chromatography–mass spectrometry using an LKB 9000.

§ LU1 is the urinary sample which corresponds to lens 1 etc.

§§ "T" signifies a trace component (<4 mg/g creatinine).

§§§ "X" signifies that this compound is not present in the chromatogram.

Fig. 4 is a metabolic profile of the urinary polyols and aldoses excreted by the patient from whom non-cataractous lens 15 was obtained at autopsy. Excretion values for glucose, *myo*-inositol and glucitol in this sample were 6, 16, and 4 mg/g creatinine, respectively.

Fig. 5 is a urinary metabolic profile for a diabetic patient with senile cataract.

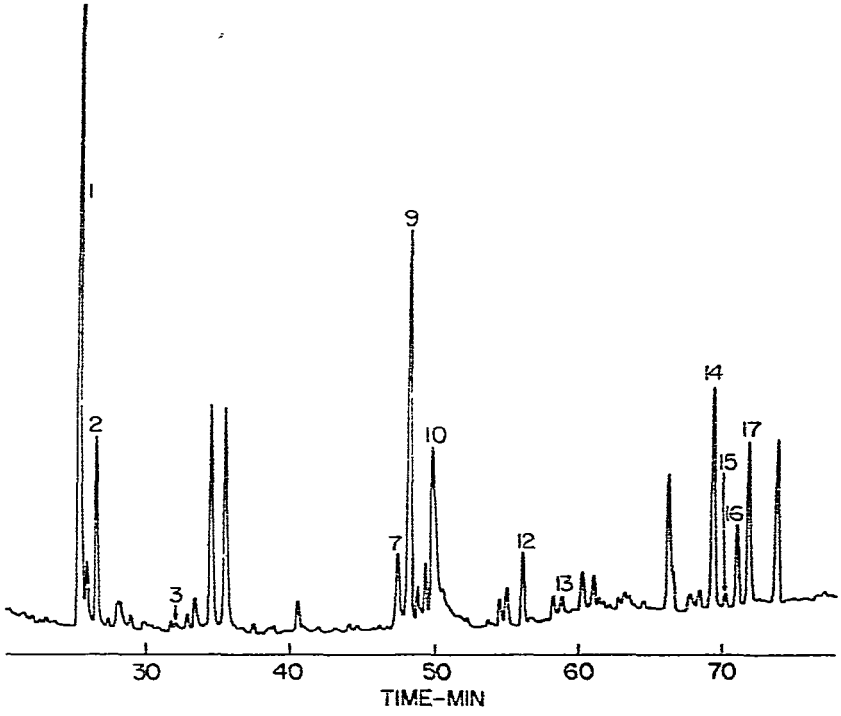

Fig. 4. Metabolic profile of urinary polyols and aldoses excreted by a patient with non-cataractous lens (LU15). Separation was carried out on a 60-m SE-30 open-tubular glass capillary column temperature programmed 1°/min from 150°. For further conditions see text. 1 = Erythritol; 2 = threitol; 3 = ribose; 7 = ribitol; 9 = arabinitol; 10 = xylitol; 12 = glucose; 13 = galactose; 14 = *myo*-inositol; 15 = mannitol; 16 = glucitol; 17 = galactitol.

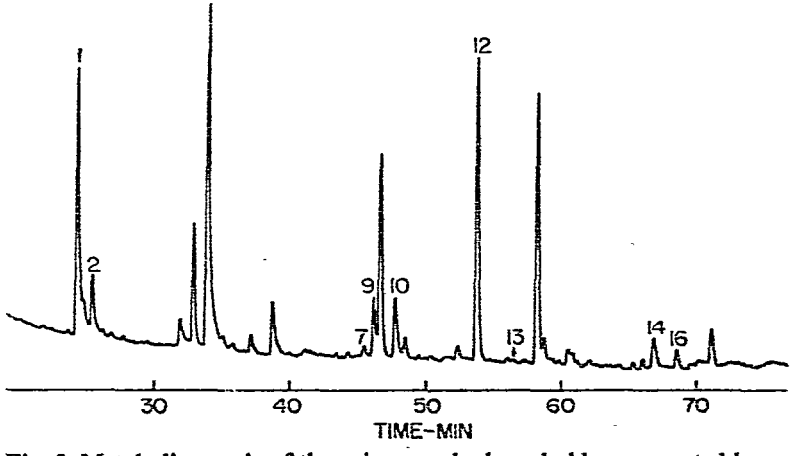

Fig. 5. Metabolic proule of the urinary polyols and aldoses excreted by a controlled diabetic patient from whom senile cataractous lens 1 was obtained. Separation was carried out on a 60-m SE-30 open-tubular glass capillary column temperature programmed 1°/min from 150°. For further conditions see text. 1 = Erythritol; 2 = threitol; 7 = ribitol; 9 = arabinitol; 10 = xylitol; 12 = glucose; 13 = galactose; 14 = *myo*-inositol; 16 = glucitol.

Excretion values for glucose, *myo*-inositol and glucitol in this sample were 56, 6, and 4 mg/g creatinine, respectively. The excretion values for sample LU11, also from a controlled diabetic with senile cataract, are similar except for elevated mannitol excretion.

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