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Changes in *myo*-inositol permeability in the lens due to cataractous conditions

In previous studies[1,2] of lens phospholipid metabolism, a decreased $^{32}P_i$ incorporation into phosphatidylinositol was found in galactose and diabetic cataracts. On the other hand, the incorporation of [^{14}C]*myo*-inositol into phosphatidylinositol in the cataractous lenses was accelerated. A possible explanation of this apparent contradiction was suggested by the observation that the *myo*-inositol content was lowered in senile cataractous lenses[3] and that this substance was excreted in increased amounts in the urine of diabetic[4] persons. This could mean that in sugar cataract, *myo*-inositol was lost from the lens to a considerable extent. The purpose of the present study was to investigate this conclusion by determining the concentration, uptake rate and efflux rate of *myo*-inositol in sugar cataracts.

The content of the various hexitols was determined in lenses of diabetic and galactosemic female Wistar rats (60 g body weight). The animals were fed either R.M.H. diet (rat-mouse-hamster diet, Hope Farms, Woerden, The Netherlands) containing 22.4 % protein, 6.5 % fat, 4.2 % fiber, 51 % carbohydrates, 10.5 % moisture, 5.4 % minerals, and vitamins, or a 40 % galactose diet prepared by mixing proportional amounts of purified D-galactose (E. Merck, Germany) and ground R.M.H. diet.

Lenses taken after 6 days of galactose diet invariably showed a small equatorial ring of opacities. Other animals received two subcutaneous injections, 24 h apart, of 147 mg/kg alloxantetrahydrate[5]. Blood glucose was determined by glucose oxidase with Dextrostix (Laboratoires Ames, France) and with hexokinase–glucose-6-phosphate dehydrogenase (Biochemica Test Combination, Boehringer, Germany). A glucose level over 250 mg/100 ml was taken as "diabetic". Lenses were extracted by homogenization in water at room temperature during 10 min and proteins were precipitated with $Ba(OH)_2$ and $ZnSO_4$. After centrifugation aliquots of the supernatant were evaporated *in vacuo*. Hexitols were analyzed in the residue by gas chromatography after conversion to their trimethylsilyl ethers[6]. The same derivatives were prepared from galactitol, sorbitol and *myo*-inositol ("für die Bakteriologie", E. Merck, Germany) and the obtained compounds were used as standards (purity > 99 %). Total lens protein was measured after homogenization in 10 % trichloroacetic acid as described previously[7].

The uptake of [2-^{3}H]*myo*-inositol (0.25 mC/0.035 mg, New England Nuclear Corp., Boston) in normal lenses was measured after preincubation in media containing 30 mM fructose (control), glucose or galactose, followed by incubation with the active material as is described in Table II. In some cases 1 mM ouabain (E. Merck, Germany) was present. The purity of this substance, calculated on dry weight basis, was 95–105 %. The purity of the [2-^{3}H]*myo*-inositol was tested as follows. Paper chromatography in ethyl acetate–pyridine–water (60:50:40, by vol.) yielded one spot, which contained 99 % of the activity. Gas chromatography of the trimethylsilyl ether, as described elsewhere in this paper, yielded only one peak. Incubations were performed under an atmosphere of 90 % N_2, 5 % CO_2 and 5 % O_2 at 37° (pH 7.4). Freezing-point osmolality was determined as described previously[8].

The *myo*-inositol efflux from rat lenses was determined after preincubation with [2-^{3}H]*myo*-inositol in the same media as used in the uptake experiments. These lenses

were thereafter incubated in media of the same composition as in the preincubation period, except that active *myo*-inositol had been replaced by 10 mM inactive *myo*-inositol in order to prevent re-uptake of the active material. Hexitols were extracted as described and radioactivity was measured after various intervals in aliquots of the extract in a scintillation counter as described previously for plasma[1]. The water content of lenses was determined according to a known technique[7].

TABLE I

THE CONTENT OF VARIOUS HEXITOLS IN LENSES OF DIABETIC AND GALACTOSEMIC RATS

Hexitols were determined by gas–liquid chromatography after conversion to trimethylsilyl ethers. The column (10 ft × $^1/_8$ inch) was packed with 1 % UCW 98 on 80–100 mesh Diatoport S (Applied Science Laboratories, State College, Pa.)*. The gas flow rate was 12 ml/min at the inlet pressure of 70 lbs·inch^{-2} and the oven temperature was 185°. Results are expressed as μmoles/g protein. Each value is the mean of two duplicate determinations in two groups of 8 lenses. The standard deviation of the mean in duplicate measurements was 0.5 for *myo*-inositol and 4 for the other values.

Days of high blood aldose concentration	*Galactose-fed rats*		*Diabetic rats*	
	Myo-inositol	*Galactitol*	*Myo-inositol*	*Sorbitol*
0	6.4	0	7.0	5
3	1.7	107	4.5	85
6	0.3	164	1.9	147
10	—	—	0.8	152

* UCW 98 showed the same separation characteristics as SE 52 (ref. 7). Since sorbitol and galactitol had the same retention times, it was assumed that galactitol accumulated in galactose cataracts and sorbitol in diabetic cataracts.

Table I shows a decrease in lens *myo*-inositol content in galactosemic rats together with a rapid increase in galactitol content. After 6 days of the galactose diet about 95 % of the *myo*-inositol had disappeared from the lens. The galactitol content after 6 days corresponded with values previously determined[9]. Similar effects could be demonstrated in lenses from diabetic animals for *myo*-inositol and sorbitol, although the changes developed more slowly. A decrease of the lens *myo*-inositol content was reported by PIRIE[3] in diabetic human subjects and in rabbits with X-ray cataracts. In the latter case, as in sugar cataracts, there was an increase in water content of the lens.

KINOSHITA, MEROLA AND HAYMAN[7] found that the increased hydration was primarily responsible for the loss in efficiency of the amino acid-concentrating mechanism. These results, combined with our data for lens, led us to an investigation of the uptake of *myo*-inositol by the lens.

From the results in Table II it appears that the *myo*-inositol-concentrating mechanism is inhibited by the presence of high glucose or galactose levels. When 1 mM ouabain was present the *myo*-inositol uptake decreased by 41 % in the control medium, by 42 % in the 30 mM glucose and by 26 % in the 30 mM galactose medium. This inhibition suggests that *myo*-inositol accumulation by the lens is coupled to the ouabain-sensitive cation pump. The inhibition of *myo*-inositol uptake in the presence of high glucose or galactose levels was similar to the inhibition of amino acid transport under those conditions[7]. In the 30 mM galactose medium the active uptake of *myo*-

TABLE II

[^{3}H]*myo*-INOSITOL UPTAKE BY RAT LENSES EXPOSED TO HIGH ALDOSE CONCENTRATIONS

Media were prepared as follows: 9 vol. Krebs-Ringer bicarbonate were mixed with 1 vol. of either 300 mM glucose, galactose or fructose. In addition to 30 mM of these sugars, 5 mM glucose was present in all cases. A hypotonic medium was prepared by decreasing the NaCl concentration in the control medium. In experiments with osmotically compensated media, increasing amounts of sorbitol (30, 60, 90 and 120 mM) were added[8]. Four lenses were preincubated in 10 ml of each medium. Media were changed after 4, 8, 12 and 21 h. After 21 h 2 lenses were incubated for 2 h in 2 ml of each medium, to which 5 μC [2-^{3}H]*myo*-inositol was added. The results are the averages ± S.E. from 3 experiments in duplicate.

Medium	*Tonicity (mosM)*	*10^3 Counts/min per ml lens water*
Fructose (control)	304	94.1 ± 5.5
Glucose	304	38.4 ± 2.4
Galactose	304	23.8 ± 2.2
Galactose (hypertonic medium)	334–424	92.7 ± 5.6
Hypotonic medium	275	75.6 ± 5.1

inositol could be maintained by gradually increasing the tonicity of the medium by addition of sorbitol, as first described by KINOSHITA, MEROLA AND HAYMAN[7] for amino acid uptake. Lowering the tonicity to 275 mosM also decreased the uptake of *myo*-inositol. Consequently the inositol accumulation was not primarily inhibited by the presence of galactitol or sorbitol as such, but by the osmotic swelling resulting from their presence in high concentration.

Concomitant with the decreased uptake of *myo*-inositol there was an increased efflux of *myo*-inositol from lenses incubated in the presence of high galactose concentrations (Fig. 1). The increased efflux could again be prevented by osmotic compen-

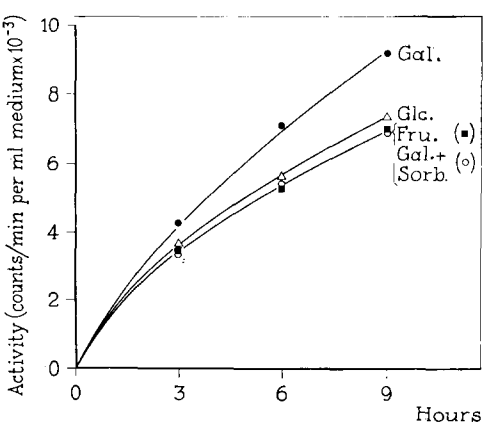

Fig. 1. Efflux of [2-^{3}H]*myo*-inositol from rat lens incubated in media containing high aldose concentrations (see Table II). Twelve lenses, weighing 31.5 ± 1.5 mg, were preincubated for 16 h in 6 ml Krebs-Ringer bicarbonate (pH 7.4) containing 5 mM glucose and 10 μC [2-^{3}H]*myo*-inositol. Thereafter groups of 3 lenses were incubated in 2 ml medium containing 10 mM inactive *myo*-inositol. Media were changed after 3 and 6 h. The osmotically compensated media were prepared by adding sorbitol to the 30 mM galactose medium in the following amounts: from 0 to 3 h 30 mM, from 3 to 6 h 53 mM, from 6 to 9 h 75 mM sorbitol. Sorb. = sorbitol.

sation. The smaller efflux increase in the 30 mM glucose medium corresponds to slower loss of *myo*-inositol from lenses of diabetic rats (Table I).

These observations show that in sugar cataracts the osmotic swelling due to hexitol accumulation leads to a reduced uptake and an increased efflux of *myo*-inositol, resulting in a low *myo*-inositol level in the lens.

When this work was concluded an abstract from the work of COTLIER[11] was published. His results are in agreement with our findings concerning the accumulation of *myo*-inositol by the normal lens and its inhibition by ouabain.

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1 R. M. BROEKHUYSE AND J. H. VEERKAMP, *Biochim. Biophys. Acta*, 152 (1968) 316.
2 R. M. BROEKHUYSE, in preparation.
3 A. PIRIE, *Exptl. Eye Res.*, 3 (1964) 124.
4 W. H. DAUGHADAY AND J. LARNER, *J. Clin. Invest.*, 33 (1954) 326.
5 A. GELLHORN AND W. BENJAMIN, *Biochim. Biophys. Acta*, 84 (1964) 167.
6 C. C. SWEELEY, R. BENTLEY, M. MAKITA AND W. W. WELLS, *J. Am. Chem. Soc.*, 85 (1963) 2497.
7 J. H. KINOSHITA, L. O. MEROLA AND S. HAYMAN, *J. Biol. Chem.*, 240 (1965) 310.
8 R. L. BOUWMAN, H. V. TRANTHAM AND P. A. CAULFIELD, *J. Lab. Clin. Med.*, 43 (1954) 310.
9 R. QUAN-MA AND W. W. WELLS, *Biochem., Biophys. Res. Commun.*, 20 (1965) 486.
10 M. A. STEWART, W. R. SHERMAN, M. R. KURIEN, G. I. MOONSAMMY AND M. WISGERHOF, *J. Neurochem.*, 14 (1967) 1057.
11 E. COTLIER, *Invest. Ophthalmol.*, 7 (1968) 118.

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Isolation of a membrane fraction enriched in nerve-end membranes from rat brain by zonal centrifugation

Nerve-end particles (synaptosomes) from brain homogenates have been isolated by a combination of differential centrifugation and isopycnic banding[1–3]. Centrifugation of a crude mitochondrial fraction from rat brain in a titanium B-XV zonal centrifuge[4] yields two major bands, one of which has a prominent shoulder (isotonic sucrose profile, Fig. 1). The first zone (banding in 23 % sucrose (w/w)) consists of myelin and membrane fragments. Electron microscopy shows the second band at 35 % sucrose (w/w) to contain mostly nerve-end particles and a few free mitochondria. The shoulder at 38–41 % sucrose is a quite homogeneous population of free mitochondria.

Recently, nerve-end membranes (*e.g.*, plasma membranes originating from nerve endings) have been separated from an osmotically shocked crude mitochondrial fraction on discontinuous sucrose gradients[5]. When the crude brain mitochondrial fraction is subjected to osmotic shock and resolved on a linear sucrose gradient in a