on carp retinal ganglion cells: non-interaction of substance P and acetylcholine. Physiologist 23:68, 1980.

12. Gesteland RC, Howland B, Lettvin JY, and Pitts WH: Comments on microelectrodes. Proc Inst Radio Eng 47:1856, 1959.

13. Sastry BR: Effects of substance P, acetylcholine and stimulation of habenula on rat interpeduncular neuronal activity. Brain Res 144:404, 1978.

14. Guyenet PG and Aghajanian GK: ACh, substance P, and met-enkephalin in locus coeruleus: pharmacological evidence for independent sites of action. Eur J Pharmacol 53:319, 1979.

13C-Nuclear magnetic resonance studies of sugar cataractogenesis in the single intact rabbit lens.

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In this report we demonstrate that ^{13}C-nuclear magnetic resonance spectroscopy may be used to monitor the sorbitol and glycolytic pathways in a single, intact, crystalline lens of the rabbit, thereby providing a new, noninvasive method for the study of sugar cataractogenesis. This approach is particularly useful for the measurement of intralenticular metabolic fluxes in single lenses under various conditions, at a time resolution previously unavailable in lens biochemistry. (Invest Ophthalmol Vis Sci 22:808-811, 1982.)

Exposure of the crystalline lens from several species to elevated glucose levels in vivo or in vitro leads to rapid opacification, the so-called "sugar" cataract. That the sorbitol pathway plays a fundamental role in sugar cataract formation in animals has been established by decades of careful, reproducible experimentation.[1] The sorbitol pathway is also suspected to be an important factor in the approximately five-fold greater incidence of cataracts occurring in humans with diabetes mellitus.[2] Recent publications, however, indicate that the susceptibility of the human lens to sorbitol accumulation differs from the animal lens in several significant aspects.[3, 4] In fact, one recent report[5] suggests that the sorbitol pathway may actually protect the lens against osmotic stress derived from rapid fluctuations in blood–aqueous humor glucose concentrations. It is clear that the response of the human lens to high-glucose stress must be carefully quantitated, and not merely assumed to be similar to that of animal lenses. The scarcity of normal human lenses for experimentation and the atypical

features of plentiful cataractous lenses make such study extremely difficult. This report describes a relatively new, noninvasive technique, ^{13}C-nuclear magnetic resonance (NMR) spectroscopy, for the study of the sorbitol pathway in the intact crystalline lens. Because relatively few lenses are required to gain extensive metabolic information and because the time response of a single intact lens to high-glucose stress may be followed by this technique, it offers an improved means by which to study the role of the sorbitol pathway in human cataractogenesis.

This accessory pathway of glucose metabolism is comprised of two enzymes: aldose reductase, with nicotinamide-adenine dinucleotide phosphate as a cofactor, reduces glucose to sorbitol; polyol dehydrogenase, with reduced nicotinamide-adenine dinucleotide as cofactor, oxidizes sorbitol to fructose. When exposed to high glucose the lens accumulates sorbitol, and since this polyol is impermeable, its accumulation raises the cytoplasmic osmolarity. As water enters, the lens swells and ultimately opacifies.

Materials and methods. The lenses used for this study were obtained from New Zealand white rabbits weighing 0.7 kg. The rabbits were sacrificed by air embolus and the eyes were enucleated. The globe was entered posteriorly by way of a cruciate incision; the sclera was peeled back and cut, the vitreous was stripped off, and the zonules were cut. The lens was carefully removed with a plastic scoop and placed in a 10 mm NRM tube fitted with a Teflon stage to position the lens at the proper height. TC-199/bicarbonate incubation medium (290 ± 3 mOsm/L) containing supplemental glucose as previously described[6] was used. The incubation medium contained 30% ^2H_2O. The lens and the incubation medium were maintained under an atmosphere of 5% CO_2 and 95% air at 37° C and 100% humidity. The NMR tube also contained an eccentrically placed capillary tube filled with ^{13}C-enriched methanol, which served as an external standard. In the experiments described here, one lens was subjected to 35.5 mM glucose 90% enriched with ^{13}C at the C-1 position (obtained from Merck, Sharpe & Dohme) for a total of 48 hr. The contralateral lens was subjected to a normal (5.5 mM) glucose load, which was similarly enriched with ^{13}C, for 24 hr and was then subjected to a high-glucose load (35.5 mM) during the ensuing 24 hr. For spectral accumulation, the enriched medium was removed and the lens was washed with the corresponding unenriched medium and incubated in unenriched medium for the duration
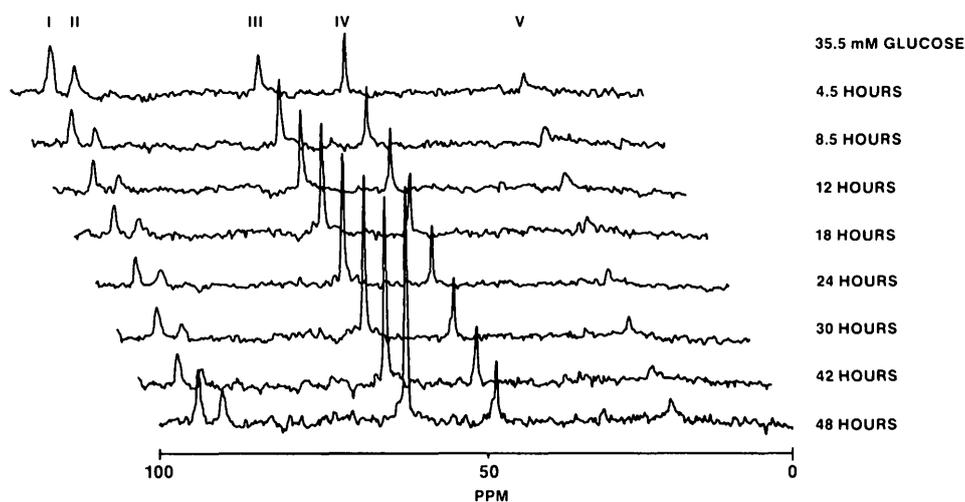
Fig. 1. This series of ^{13}C-NMR spectra represents the metabolism of a single rabbit lens over a 48 hr period in high-glucose medium (35.5 mM). The glucose was enriched at the C-1 position. The metabolite resonances are labeled as follows: *I*, glucose C-1 (β-anomer); *II*, glucose C-1 (α-anomer); *III*, Sorbitol C-1; *IV*, methanol external standard; *V*, lactate. Each spectrum represents 1200 accumulations.
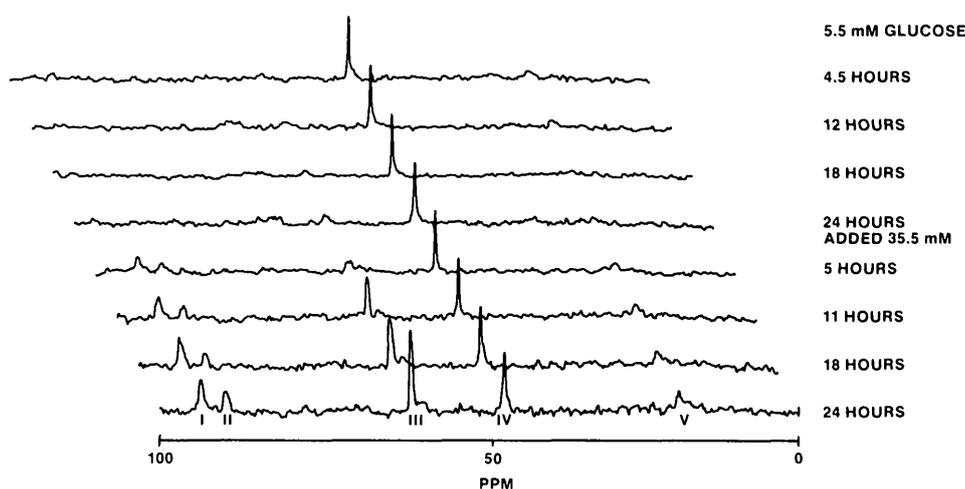
Fig. 2. This series of ^{13}C-NMR spectra represents the metabolism of a single rabbit lens in low-glucose (normal) medium (5.5 mM) for 24 hr, followed by exposure to a high-glucose medium (35 mM) for the subsequent 24 hr. The assignment of resonances is as in Fig. 1.

of the NMR measurement. ^{13}C-NMR spectra were obtained with a JEOL FX-100 spectrometer operating in the Fourier Transform mode at 25 MHz, with a ^{2}H lock and proton decoupling. Typically 45 degree radio frequency pulses were used at a repetition rate of 2 pulses/sec. The sample was nonspinning, and the sample temperature was maintained at 37° C. Each spectrum was obtained in approximately 10 min.

Results and discussion. Fig. 1 shows ^{13}C-NMR spectra obtained over a 48 hr period from a single lens subjected to a high-glucose load. A total of four lenses were studied, each yielding spectra similar to those in Fig. 1. After 4.5 hr of incubation, several major metabolites were clearly evident, i.e., glucose, sorbitol, and lactate. Throughout the 48 hr the sorbitol levels continued to rise while the lactate levels remained essentially the same. (Lactate
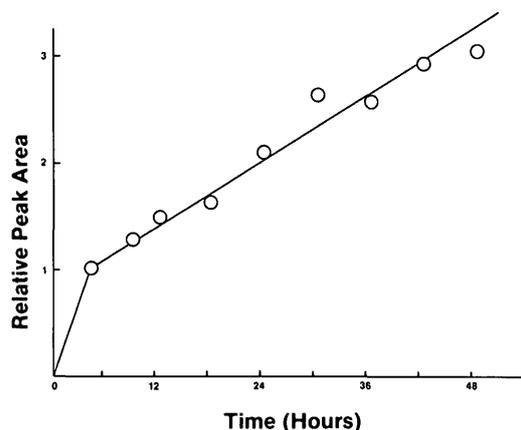
Fig. 3. Time-dependent accumulation of ^{13}C-enriched sorbitol by a single intact rabbit lens in a high-glucose medium (35.5 mM). Each point was obtained by integration of the sorbitol resonance and normalized to the area of the external reference standard. The linearity of the sorbitol accumulation is in agreement with the results of in vitro studies.[6]
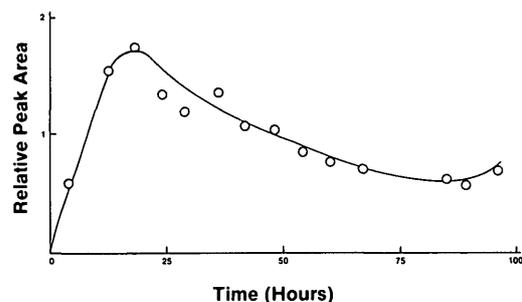

Fig. 4. Time-dependent accumulation and subsequent depletion of ^{13}C-enriched sorbitol in a single intact rabbit lens subjected to various glucose concentrations. The lens was subjected to a high-glucose stress (35.5 mM) for 12 hr, followed by a return to normal glucose level (5.5 mM) for the ensuing 73 hr, whereupon a high-glucose load was reinstated. Each point was obtained as described in Fig. 3.

is able to permeate lens membranes and diffuses into the incubation medium.) The lenticular glucose concentration after an initial increase and slight decrease remained constant. These findings are in excellent accord with those of previous studies using invasive methods.[6] By contrast, the glucose and sorbitol levels for the contralateral lens

were below those required for detection during the first 24 hr of exposure to normal glucose levels, as shown in Fig. 2. However, there was evidence of lactate formation, but at a reduced level. When this lens was subjected to a high-glucose stress, once again glucose and sorbitol were clearly observed, as well as an enhanced production of lactate.

To more clearly examine the relative increases in sorbitol concentration, the sorbitol C-1 resonances (see Fig. 1) were integrated and normalized to the integrated area of the external reference standard. The relative areas as a function of time are shown in Fig. 3. The linear increase in sorbitol concentration during the first 48 hr of incubation under a 35.5 mM glucose concentration is consistent with the results of previous studies.[6] Although NMR detects only a threefold increase in lens sorbitol, compared with the nearly sixfold increase noted in an earlier report,[6] only the sorbitol with ^{13}C incorporated is detected. In unenriched media, absolute concentrations of lens metabolites cannot be determined solely by NMR. Gas chromatographic/ultraviolet spectroscopic methods have been used to assess the total amount of sorbitol synthesized during an incubation.[6]

A different experiment was performed to assess the response of the lens to a temporary high-glucose load followed by a return to normal levels. The rationale behind this experiment is the fact that insulin-dependent diabetics experience fluctuating glucose levels. The results are shown in Fig. 4. After 12 hr at a glucose concentration of 35.5 mM, sorbitol (as well as glucose) in the lens reached the expected levels. However, after 6 hr, following a reduction to normal glucose level (5.5 mM), the sorbitol levels were then observed to drop at a decreasing rate over the subsequent 67 hr. Finally, the lens was once again exposed to high-glucose conditions, and the sorbitol level increased, but to a lower point than during the initial 18 hr of lens incubation. The contralateral lens from the same rabbit was kept at 5.5 mM glucose for the duration of the experiment as a control. In this lens, glucose and sorbitol remained at levels below detection by NMR, but lactate was observed at low levels. This experiment confirms an earlier report[5] in which fluctuations in ambient glucose occurred more rapidly than diffusion across the lens membrane. That 6 hr were needed to restore intralenticular glucose levels to those outside the lens suggests that glucose itself may be a source of significant osmotic stress in the lens.

The experiments described have been repeated

several times with similar results. We have reported only the results obtained from the study of four lenses from two rabbits to emphasize the ability of this technique to obtain a great deal of information from a minimum amount of tissue. Very recently, analogous ^{31}P-NMR measurements on the intact lens have been reported,[7] thus affording a complementary approach to the study of lenticular metabolism. Similar studies are also underway in this laboratory.

REFERENCES

1. Kinoshita JH: Mechanisms initiating cataract formation. Proctor Lecture. INVEST OPHTHALMOL 13:713, 1974.
2. Chylack LT Jr: Sugar cataracts—possibly the beginning of medical anticataract therapy. *In* Mechanisms of Cataract Formation in the Human Lens, Duncan G, editor. London, 1980, Academic Press, Inc. Ltd., p. 237.
3. Jedziniak JA, Chylack LT Jr, Cheng HM, Gillis MK, and Tung WH: The sorbitol pathway in the human lens. I, Aldose reductase and polyol dehydrogenase. INVEST OPHTHALMOL VIS SCI 20:314, 1981.
4. Kador PF, Kinoshita JH, Tung WH, Chylack LT Jr: Differences in the susceptibility of various aldose reductases to inhibition. II. INVEST OPHTHALMOL VIS SCI 19:980, 1980.
5. Chylack LT Jr, Cheng HM, Gillis MK, Kalustian AA, and Tung WH: The sorbitol pathway in the human lens: aldose reductase. INVEST OPHTHALMOL VIS SCI 19(ARVO Suppl.):11, 1980.
6. Chylack LT Jr and Kinoshita JH: A biochemical evaluation of cataract induced in high glucose medium. INVEST OPHTHALMOL 8:401, 1969.
7. Greiner JV, Kopp SJ, Sanders DR, and Glonek T: Organophosphates of the crystalline lens: a nuclear magnetic resonance spectroscopic study. INVEST OPHTHALMOL VIS SCI 21:700, 1981.

Transmitted light microscopy of the unstained endothelial cell layer of excised human corneal tissue.

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We describe a transmission light microscopic system for evaluation of unstained corneal endothelium of corneoscleral buttons. To investigate the nature of the morphologic details seen with this system, we developed an experimental relocation technique by which identical areas of endothelial cells could be viewed before and after vital staining. (INVEST OPHTHALMOL VIS SCI 22: 811-818, 1982.)

Examination of the unstained endothelium of excised human[1] corneas is currently possible with the specular microscope. Cell density measurements and morphometry have been used as indirect measures of the corneal vitality of this layer, as observed with the specular microscope. In the laboratory, specular microscopy and serial pachometry have provided measures of the functional capacity of the endothelium being observed. We have used transmitted light microscopy[2] to examine the unstained endothelial layer of excised human corneoscleral buttons.

In standardization experiments, microscopic features observed in this layer were investigated and defined further by vital staining[3] and observation of the same areas after staining by means of a relocating technique. The only other published method of examination of the same area of endothelium before and after staining is that described by Sherrard,[4] who used specular microscopy and subsequent silver staining of the outlines and a novel relocating technique to study the relationship between endothelial features and their subsequent stained outlines. No reports to date have described the use of transmitted light microscopy on the unstained corneal endothelial layer and subsequent examination of the same areas of endothelium by vital staining techniques.

Material and methods. The corneas used for this experiment were human corneoscleral buttons obtained by the Medical Eye Bank, Inc., but found unsuitable for transplantation use because of elapsed time between the donor's death and enucleation or between death and intended use, or because of the cause of death. The donor age and sex, the cause of death, and the length of time from death to enucleation and removal of cornea were all known. The corneas in all cases were