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21. A choice between 50 μl of sucrose solution ($10^{-1}M$) in distilled water and 50 μl of **1** ($10^{-2}M$) in the same sucrose solution was given to 12 groups of 50 ants each, isolated in petri dishes. The number of ants feeding on each solution was counted 3 minutes after the beginning of the experiment. After this delay, the ants recovered from the disturbance. The distribution of ants on both solutions was analyzed for significance by the Walsh test. About half of the ants (52%, 12 replications pooled) fed on the solution of **1** ($P >> 0.05$, not significant).
22. Drops of 5 μl of either pure water, sucrose ($10^{-1}M$) solution in water, or solution of **1** ($10^{-2}M$) in the sucrose solution were given to three groups of 50 ants isolated in petri dishes until they were satiated. Care was taken to give about the same amount of liquid to each of the three groups. Each day the dead ants were counted and the live ants were refed. After 3 days, more than 50% mortality was reached in the group fed with **1**. At this stage, the ants had ingested a mean of 2.7 μl of the solution per ant—that is, about 7 μg of **1** per ant. The mortality in the group fed with **1** was significantly higher ($P < 0.001$, χ^2 test corrected for continuity) than that in those fed with either pure water or the sugared solution. Mortality in these two control groups did not differ significantly ($P > 0.7$).
23. The concentration was estimated on the basis of the following figures: volume of secretion, 0.29 μl per beetle (*9*); the secretion of 1200 beetles yielded 16 mg of **1**; and molecular weight 256.
24. We thank R. Ottinger for the ^1H and ^{13}C NMR spectra, C. Moulard for the mass spectra, and J. André for the amino acid analysis.

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Study of Aldose Reductase Inhibition in Intact Lenses by ^{13}C Nuclear Magnetic Resonance Spectroscopy

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Carbon-13 nuclear magnetic resonance spectroscopy has been used in the study of glucose metabolism, specifically aldose reductase inhibition, in intact rabbit lenses maintained in organ culture. This technique provides an effective method of screening potential inhibitors of aldose reductase under conditions that more closely approximate in vivo conditions than do earlier methods. The aspirin substitutes acetaminophen and ibuprofen were studied as aldose reductase inhibitors and were found to be effective in reducing sorbitol accumulation in lenses exposed to high glucose stress. Results of this work with various inhibitors of aldose reductase are discussed in terms of lens metabolism and implications regarding diabetic complications such as cataract formation.

CATARACT FORMATION IS ONE OF many secondary complications associated with diabetes mellitus. It has been estimated that in the United States alone over 10 million people suffer from diabetes and over 75,000 diabetic cataract extractions are performed each year (*1*). Cataracts are five times as likely to develop in a diabetic as in a nondiabetic of the same age and sex (*2*). Unfortunately, neither the disorder, diabetes mellitus, nor the causes of cataract formation are well understood. Glucose metabolism in the lens, via the sorbitol pathway and the enzyme aldose reductase (E.C. 1.1.1.21), appears to be directly related to the higher incidence of cataracts in diabetics. In this investigation we used a nondestructive technique, ^{13}C nuclear magnetic resonance (NMR) spectroscopy, to study glucose metabolism, glycolysis, and the sorbitol pathway in intact rabbit lenses and also the effectiveness of various aldose reductase inhibitors.

The normal physiological role of the sorbitol pathway remains unknown. Under the conditions of high plasma-sugar concentrations encountered in diabetics, hexokinase apparently becomes saturated and excess glucose is converted to its sugar alcohol, sorbitol, by aldose reductase with reduced nicotinamide adenine dinucleotide phosphate (NADPH) acting as the cofactor. In the second reaction of the sorbitol pathway, sorbitol is oxidized to its keto sugar, fructose, by polyol dehydrogenase with nicotinamide adenine dinucleotide (NAD^+) as the cofactor. Sorbitol is not readily metabolized and does not penetrate cell membranes easily. Once formed, sorbitol is trapped intracellularly. Numerous animal studies (*3, 4*) and limited research with human lenses (*5*) have suggested that the accumulation of sorbitol initiates a sequence of osmotic changes that leads to the formation of cataracts. In order to maintain osmotic equilibrium as sorbitol accumulates, water is drawn into the lens fibers, which causes swelling. Eventually the lens fibers rupture, and a lenticular opacity is observed. It has been suggested that osmotic effects are the causative factor in cataract formation under high glucose stress. Sorbitol pathway activity has various effects on lens metabolism that may be important in cataract development. Recently, research has suggested that a primary effect of sorbitol pathway activity is altered redox ratios of pyridine nucleotides (*6*). Strong evidence supporting the importance of the sorbitol pathway in cataract formation has come from the discovery of various compounds that are effective in blocking sorbitol formation and in delaying or preventing resulting cataractogenic changes (*7*). Moreover, the enzyme aldose reductase and high sorbitol levels have been found in other tissues involved in such secondary diabetic complications as retinopathy, nephropathy, and neuropathy (*8, 9*). The development of an orally active inhibitor of aldose reductase effective in human tissues could lead to a pharmacological approach for the treatment or even prevention of diabetic complications such as cataract formation.

Multinuclear magnetic resonance spectroscopy (^1H, ^{31}P, and ^{13}C) has been used in the study of the lens (*6, 10–13*). Use of ^{13}C NMR spectroscopy has been limited to the study of lenses exposed to various levels of glucose stress (*11, 12*) and to the determination of polyol pathway flux rates (*6, 13*). We now report that ^{13}C NMR spectroscopy provides an excellent means of studying the activity of pharmacological agents, such as aldose reductase inhibitors. The incubation of intact rabbit lenses in a high-concentration [1-^{13}C]glucose medium with and without inhibitors has been studied; with this procedure it is possible to study the utilization of glucose in the lens as changes occur. By monitoring the distribution of the ^{13}C it is possible to follow glycolytic and sorbitol pathway activities. The ^{13}C NMR spectrum

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obtained from a single intact rabbit lens after 15 hours of incubation in the presence of a high concentration of labeled glucose is shown in Fig. 1a. Resonances for the C-1 of the β and α anomers of glucose were observed at 97 and 93 parts per million (ppm), respectively. The resonance for lactate, the end product of glycolysis, was found at 21 ppm. No glycolytic intermediates were of sufficient concentration to be observed in any of our experiments. Earlier NMR investigations have also failed to detect glycolytic intermediates. The products of the sorbitol pathway, sorbitol and fructose, gave rise to resonances at 63 and 65 ppm, respectively. As expected, a large sorbitol resonance was observed for the lens exposed to high glucose stress (33.5 m*M*), mimicking the diabetic situation. Analysis of the medium used for incubation by ^{13}C NMR spectroscopy showed that only glucose and lactate were present at detectable levels. Lactate is able to permeate lens membranes, and therefore its presence in the incubation medium is expected. No sorbitol was found in the medium, which is consistent with its being trapped intracellularly. When a lens was exposed to normal glucose conditions (5.5 m*M*) for 15 hours, only glucose and a small amount of lactate were detected by ^{13}C NMR spectroscopy.

The ^{13}C NMR spectrum observed for a single, intact rabbit lens after incubation in the presence of a high concentration of labeled glucose with $1 \times 10^{-8}M$ sulindac is shown in Fig. 1b. The antirheumatic drug sulindac is a potent inhibitor of aldose reductase (*12*). Addition of this inhibitor to incubation medium substantially reduced the sorbitol resonance, corresponding to more than 65% inhibition of aldose reductase. At a concentration of $1 \times 10^{-4}M$, sulindac was more than 80% effective. For a series of compounds (Table 1) we have determined the following relative abilities to inhibit aldose reductase: tolrestat ≥ sorbinil > sulindac ≫ ibuprofen > acetaminophen ≅ aspirin. As expected, inclusion of an aldose reductase inhibitor in the high-concentration glucose medium resulted in decreased swelling of the lens and delayed changes in lens clarity (*5, 7*). Of the compounds investigated, sorbinil and tolrestat appear to be the most potent aldose reductase inhibitors in intact rabbit lens tissue with inhibitions of ~65 and 75%, respectively. Both tolrestat and sorbinil at a $1 \times 10^{-12}M$ concentration prevented lens swelling and loss of transparency under our experimental conditions. However, less potent inhibitors of aldose reductase could also be important therapeutically since factors other than inhibitory activity (such as tissue absorbency, toxicity, and side effects) are important considerations for overall drug effectiveness. Various drugs to treat rheumatoid arthritis, notably aspirin, would appear to be quite promising as anticataract treatments (*14*). Both acetaminophen and ibuprofen substantially reduce sorbitol accumulation under our experimental conditions and appear to be more potent inhibitors of aldose reductase than aspirin.

Results of our ^{13}C NMR studies of aldose reductase inhibitors are in general agreement with earlier studies of these inhibitors performed under diverse experimental conditions with the use of classical biochemical methods (*7, 15*). Aldose reductase inhibition is known to be species-, tissue-, and substrate-dependent (*15*). Although the current work has been done in vitro, it is expected to more accurately reflect the in vivo potencies of the various inhibitors since intact, functioning tissue was analyzed. Previous studies have generally used invasive, destructive techniques. Aldose reductase is difficult to purify and is unstable in a purified form (*15, 16*). Also, it has been reported that the effectiveness of inhibitors varies depending on the degree of enzyme purification (*9*). The use of ^{13}C NMR spectroscopy provides a method of studying aldose reductase inhibition under conditions more closely simulating the situation in diabetics.

Concomitant with the decrease in sorbitol observed, increases in the glucose and lactate resonances were observed by ^{13}C NMR spectroscopy when an aldose reductase inhibitor was included in the incubation medium (Fig. 1). A variety of structurally diverse inhibitors of aldose reductase have been studied with similar results. When inhibitors were included in the high-concentration glucose medium, the glucose resonances remained essentially the same or increased slightly (less than 10% change), while the lactate resonance increased (2 to 30% change). There are several possible interpretations of the observed changes in metabolite levels as a result of inhibition. The increase in the levels of glucose and lactate in the lens may result from the utilization of less glucose through the sorbitol pathway. It

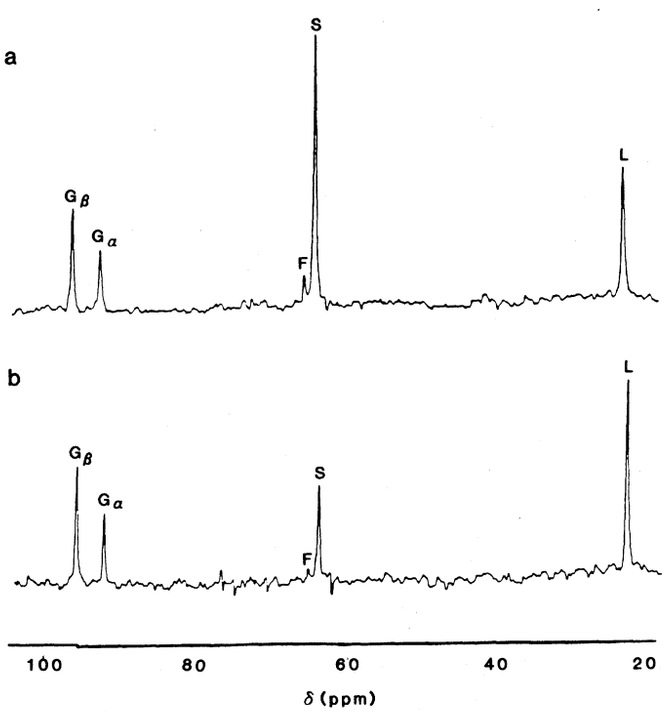

Fig. 1. (a) The ^{13}C NMR spectrum observed for a single, intact rabbit lens after incubation in a 35.5 m*M* [1-^{13}C]glucose medium for 15 hours. (b) The ^{13}C NMR spectrum observed for a single intact rabbit lens after incubation for 15 hours in a 35.5 m*M* [1-^{13}C]glucose medium containing $1 \times 10^{-8}M$ sulindac. The [1-^{13}C]glucose used was 99% enriched. The resonances are as follows: G_β, C-1 β-glucose; G_α, C-1 α-glucose; F, C-1 fructose; S, C-1 sorbitol; and L, C-1 lactate. Resonances were assigned on the basis of previously reported chemical shifts and the addition of known metabolites to the medium. New Zealand White rabbits weighing approximately 0.7 to 1.0 kg were killed by sodium pentobarbital injections. Lenses were removed by a posterior approach and incubated at 37°C under an atmosphere of 5% CO_2 and 95% air in the standard TC-199 incubation medium used for rabbit lenses (*4*) with and without inhibitors. Each lens was incubated in 2 ml of medium for 15 hours under sterile conditions. To determine the spectra, we replaced the labeled glucose medium with identical unlabeled medium and positioned the lens in the center of the coil on a glass stage in a 10-mm NMR tube. By means of a Bruker WP-200 NMR spectrometer, we measured ^{13}C spectra at 50.32 MHz, with a pulse width of 25 μsec (90°), 16×10^3 data points, a sweep width of 10,000 Hz, an acquisition time of 0.82 second, a repetition time of 1.83 seconds, deuterium locking, and bi-level broadband proton decoupling to avoid excessive heating of the sample. Spectra were acquired under nonspinning conditions at 37°C. Each spectrum was obtained in approximately 2 hours (4000 scans). The C-1 β-glucose resonance was used as an internal chemical shift reference; ^{13}C-labeled methanol was used as a secondary external reference.

could be reasoned that, when the sorbitol pathway is partially inhibited, excess glucose is metabolized through the glycolytic pathway, resulting in an increased level of lactate in the lens. However, it is generally assumed that the glycolytic pathway is saturated when aldose reductase becomes activated (*17*). The observed increase in the lactate resonance cannot be attributed to lactate synthesis through the pentose phosphate pathway, since lactate produced in that manner would not carry a ^{13}C label when [1-^{13}C]glucose is utilized. Fructose may act as an important secondary energy source in the lens (*13, 18*). Fructose produced from sorbitol would enter into glycolysis and produce ^{13}C-labeled lactate. It seems unlikely that this could account for the increased lactate levels observed with inhibition. When an aldose reductase inhibitor was included in the incubation medium, sorbitol production, and therefore fructose production, was reduced.

In addition, it is possible that the observed increases in the glucose and lactate resonances with inhibition of aldose reductase are a result of osmotic effects; there might be no actual increase in lactate synthesis. The high lactate level could be caused by retention of lactate and glucose to offset the osmotic pressure from a high glucose level in the medium. To study this possibility, we analyzed the ^{13}C-labeled incubation media from experiments with and without inhibitors by ^{13}C NMR spectroscopy. The media obtained after incubation of a lens in the absence of added reductase inhibitor was found to contain more lactate than that obtained after incubation with an inhibitor. This result supports the interpretation of increased lactate retention in the lens being the result of an osmotic effect. It has been proposed that the normal role of the sorbitol pathway is the production of sorbitol and fructose to counteract the osmotic pressure from high glucose levels in the blood and aqueous fluids (*13, 19*). Our results are consistent with this theory, although there may be other possible explanations.

Regardless of its normal physiological role, the sorbitol pathway is certainly an important glucose-utilizing pathway within the lens. When the lens was exposed to 35.5 m*M* glucose, sorbitol pathway activity accounted for over one-third of the total glucose turnover (*6*). It has been suggested that a significant effect of high aldose reductase activity is the alteration of pyridine-nucleotide redox ratios that results from the high sorbitol pathway activity, rather than the accumulation of sorbitol and the resulting osmotic changes (*6, 20*). If the increased lactate level observed upon inhibition of aldose reductase results from osmotic effects, as is indicated in this investigation, the significance of the osmotic pressure due to sorbitol accumulation is again brought into question. Effects on other metabolic pathways in the lens that make use of pyridine nucleotide cofactors could be the critical factor in cataract formation. Glutathione reductase is present in the lens in very high concentrations and is known to protect the lens from oxidative damage (*21*). The cofactor for the glutathione reductase reaction is NADPH, the same cofactor used by aldose reductase. High sorbitol pathway activity could affect the lens by altering the availability of NADPH to glutathione reductase. Clearly, the various pathways within the lens are highly interrelated.

NMR spectroscopy has tremendous potential as a noninvasive probe of biological systems such as the lens. With the continuing development of imaging and spectroscopic techniques, it is possible that NMR spectroscopy, notably ^{13}C, ^{31}P, and ^{1}H, will be used with humans to monitor metabolite patterns in the lens in order to detect early, precataractogenic changes. It is hoped that a better understanding of the various metabolic pathways within the lens will lead to the development of effective methods for treatment or even prevention of diabetic cataract formation. NMR spectroscopy may be important in the development of such therapeutic agents, notably aldose reductase inhibitors, and as a means of monitoring in vivo the condition of the lens and treatment effectiveness.

Table 1. Summary of inhibitory activity for various compounds that block sorbitol production in intact rabbit lenses. Inhibition was calculated by comparing integrated sorbitol peak areas in the absence and presence of an inhibitor. An average of two or more experiments (at least two rabbits and, in most cases, three or four rabbits) are shown for each drug. Results are reproducible (±2.5%) provided incubation (for example, media preparation and temperature control) and spectral acquisition conditions (for example, positioning of the lens in the tube, magnet shim, and temperature control) are maintained precisely.

Compound	Concentration (*M*)	Inhibition (%)
Sulindac	1×10^{-4}	82
	1×10^{-8}	68
Sorbinil	1×10^{-4}	90
	1×10^{-12}	64
Tolrestat	1×10^{-4}	96
	1×10^{-12}	74
Aspirin	1×10^{-4}	28
Acetaminophen	1×10^{-4}	30
Ibuprofen	1×10^{-4}	45

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