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Purified rat lens aldose reductase

Polyol production *in vitro* and its inhibition by aldose reductase inhibitors

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The production of polyols *in vitro* by highly purified aldose reductase (EC 1.1.1.21) was monitored by g.l.c. In the presence of NADPH aldose reductase reduced glucose, galactose and xylose to the respective polyols sorbitol, galactitol and xylitol. The rates of formation of these polyols closely mirrored the K_m values for the substrates obtained from kinetic measurements that monitored the rate of disappearance of NADPH. No polyol production occurred in the absence of purified aldose reductase, and analysis by g.l.c. revealed only the presence of unchanged monosaccharides. Addition of the aldose reductase inhibitor sorbinil to purified rat lens aldose reductase incubated with xylose in the presence of NADPH resulted in decreased xylitol production. However, aldose reductase inhibitors produced no effect in altering the rate of Nitro Blue Tetrazolium formation from either glucose or xylose, indicating that the observed inhibition *in vitro* does not result from a free-radical-scavenger effect.

INTRODUCTION

The metabolic conversion of glucose into fructose through the sorbitol pathway was first described by Hers (1956, 1957) to occur in the seminal vesicles and placenta. In the first step of this pathway glucose was shown to be reduced to the sugar alcohol sorbitol by the enzyme aldose reductase (EC 1.1.1.21). Shortly thereafter van Heyningen (1959) observed in both animal feeding experiments and lens homogenate incubations that aldose reductase, utilizing NADPH, could convert glucose, xylose and galactose into their respective sugar alcohols sorbitol, xylitol and galactitol in the rat lens. In addition, she reported the presence of sorbitol in the human diabetic lens. These initial observations led to the development of the Polyol Hypothesis of sugar cataract development (Kinoshita, 1974), which states that the aldose reductase-initiated accumulation of polyols produces a hyperosmotic effect, which results in lens fibre swelling and eventual cataract formation. This hypothesis has been verified by studies with numerous animal models and by the development of potent aldose reductase inhibitors (Kinoshita *et al.*, 1983). Numerous studies also suggest that the aldose reductase-initiated accumulation of polyols is involved in the pathogenesis of other ocular and systemic diabetic complications (Kador *et al.*, 1985*b*). This has spurred great interest in the development of potent non-toxic inhibitors of aldose reductase.

Despite the overwhelming evidence for the involvement of aldose reductase-initiated polyol accumulation in the pathogenesis of diabetic complications, some reports have questioned both the biochemical importance of lens polyol formation and the mechanism through which polyols are formed by aldose reductase (Crabbe, 1984; Wolff *et al.*, 1984; Wolff & Crabbe, 1985). These questions are largely based on the inability of these investigators to demonstrate *in vitro* the production of polyols by purified lens aldose reductase (Crabbe, 1984). It is suggested that the spectrophotometric determination of NADPH utilization as an index of aldose reductase activity is open to question because of an artifact caused by spontaneous monosaccharide oxidation in which free radicals that can oxidize NADPH to $NADP^+$ are generated by the formation of an enediol. By this mechanism, the monosaccharide is oxidized to an α-dicarbonyl instead of being reduced to a polyol (Wolff *et al.*, 1984). It is also proposed that aldose reductase inhibitors depend on an antioxidant mechanism rather than inhibition of aldose reductase as such.

These reports that aldose reductase activity is an artifact have prompted us to demonstrate the NADPH-dependent production of polyols from aldose sugars *in vitro* by highly purified rat lens aldose reductase. Moreover, the structure–activity relationships of aldose reductase inhibitors are not consistent with a radical-scavenging mechanism, and in this paper we report the lack of activity of a selection of potent aldose reductase inhibitors in a system sensitive to superoxide dismutase.

MATERIALS AND METHODS

Chemicals

Unless otherwise stated, all chemicals were of reagent-grade quality. $(NH_4)_2SO_4$ (enzyme grade) was obtained from Bethesda Research Laboratories, Rockville, MD, U.S.A. NADPH and $NADP^+$ were obtained from Boehringer Mannheim Biochemicals, Indianapolis, IN, U.S.A. Isocitrate, isocitrate dehydrogenase, 0.3 M-$ZnSO_4$ and 0.3 M-$Ba(OH)_2$ were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Matrex Gel Orange A was obtained from Amicon Corp., Danvers, MA, U.S.A. *NO*-Bis(trimethylsilyl)trifluoroacetamide containing 1% chlorotrimethylsilane was purchased from Regis Chemical Co., Morton Grove, IL, U.S.A. The SE 30 Chromosorb g.l.c. column was obtained from Supelco, Bellefonte, PA, U.S.A. Nitro Blue Tetrazolium (grade III crystalline) was purchased from Sigma Chemical Co., Poole, Dorset, U.K.

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Purification of rat lens aldose reductase

Aldose reductase was purified from rat lens by affinity chromatography as previously described but with a modified phosphate buffer, namely 20 mM-sodium phosphate buffer, pH 7.5, containing 7 mM-2-mercaptoethanol, 0.5 mM-EDTA and 10% (v/v) glycerol (Herrmann *et al.*, 1983; Shiono *et al.*, 1986). Batches of 800 lenses were homogenized in 600 ml of buffer, centrifuged at 12000 *g* and fractionated with $(NH_4)_2SO_4$. The precipitate of the 40–70%-saturation fraction was collected by centrifugation at 12000 *g*, dialysed against the same buffer and applied to a 2.5 cm × 45 cm Bio-Rad column containing Amicon Matrex Gel Orange A. The column was washed with 450 ml of buffer, and the the enzyme was eluted with buffer containing 0.1 mM-NADPH. Fractions containing aldose reductase activity were concentrated with an Amicon column concentrator containing a YM-10 membrane. The enzyme had an approximate specific activity of 3.5 units (μmol/min)/mg of protein as determined with glyceraldehyde and appeared as a single band of M_r approx. 38000 on SDS/polyacrylamide-gel electrophoresis (Fig. 1).

Incubation in the presence of an NADPH-generating system

Sealed 7 ml glass vials containing purified aldose reductase (approx. 1.05 units), 10 mM-aldose sugar, 10 mM-NADPH, 1 mM-$NADP^+$, 1 mM-DL-isocitrate, 2 mM-$MgCl_2$, isocitrate dehydrogenase (13.8 units) and 0.1 M-sodium phosphate buffer, pH 7.8, in 2 ml final volume were placed in a 37 °C incubator. After overnight incubation (14 h) the solution was treated with 1 ml each of 0.3 M-$Ba(OH)_2$ and 0.3 M-$ZnSO_4$. The supernatant obtained by centrifugation was freeze-dried and treated in pyridine with bis(trimethylsilyl)trifluoroacetamide containing 1% chlorotrimethylsilane.

Incubation in the absence of an NADPH-generating system

Sealed 7 ml glass vials containing in a total 1 ml volume purified aldose reductase (approx. 0.5 unit), 10 mM-xylose, 10 mM-NADPH and 0.1 M-sodium phosphate buffer, pH 6.2, were placed in a 37 °C incubator. After overnight incubation (14 h) the solution was treated with 0.5 ml each of 0.3 M-$Ba(OH)_2$ and 0.3 M-$ZnSO_4$ and then processed as above.

G.l.c.

G.l.c. with an SE 30 Chromosorb glass column at 150 °C and helium flow under 0.4 MPa (58 lbf/in²) pressure was conducted on a Varian model 3700 gas chromatograph equipped with a Vista CDS-401 data system. The retention times of the aldose sugars and their corresponding polyols were determined through chromatography of appropriate standards.

Evaluation of aldose reductase inhibitors against generated free radicals

The reduction of Nitro Blue Tetrazolium to formazan by aldopentoses was monitored spectrophotometrically in an adaption of the method of Kashimura *et al.* (1982). The reaction mixture consisted of 0.15 mM-Nitro Blue Tetrazolium and 0.1 mM-EDTA (dipotassium salt) in 3 ml of 0.05 M-sodium bicarbonate buffer, pH 10.2, and an aldopentose, generally D(+)-xylose (10 mg/ml). The rate of formazan production was monitored spectrophotometrically at 560 nm with a Pye–Unicam SP.8200 instrument.

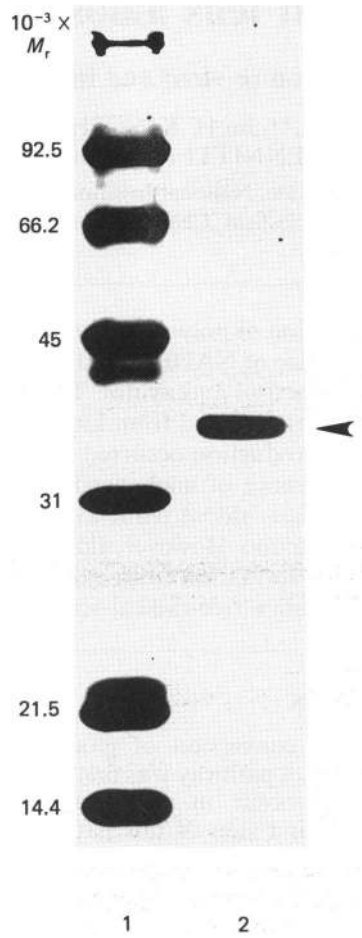
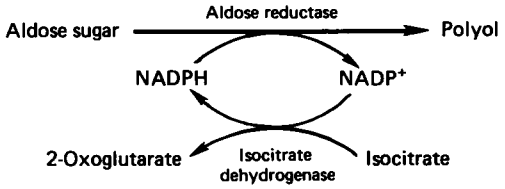


Fig. 1. SDS/polyacrylamide-gel electrophoresis of purified rat lens aldose reductase

For experimental details see the text. Lane 1, M_r markers; lane 2, rat lens aldose reductase.

RESULTS

In order to demonstrate the production of polyols *in vitro* by rat lens aldose reductase, the highly purified enzyme was initially added to a sealed system capable of generating NADPH from $NADP^+$. This system consisted of isocitrate dehydrogenase and its substrate isocitric acid in phosphate buffer at the pH optimum of isocitrate dehydrogenase (pH 7.8) and containing $MgCl_2$. Overnight (14 h) incubation at 37 °C of aldose reductase with 10 mM-xylose, -galactose or -glucose and 10 mM-NADPH in this sealed system resulted in the production of the corresponding polyol xylitol, galactitol or sorbitol (Scheme 1). G.l.c. analysis indicated nearly 100% conversion of xylose into xylitol, 45% conversion of galactose into galactitol and 25% conversion of glucose into sorbitol (Fig. 2). Only the starting aldose and corresponding polyol product peaks could be detected in the chromatograms. No conversion of xylose into xylitol was observed in the absence of aldose reductase compared with 19.7 ± 0.3 μmol in the complete incubation mixture. Omitting the $NADP^+$ from the reaction mixture did not decrease xylitol production



Scheme 1. Scheme illustrating conversion of aldose sugar into polyol by rat lens aldose reductase in the presence of isocitrate dehydrogenase

(19.5±0.5 μmol), whereas omitting NADPH resulted in a 60% decrease in xylitol production to 8.76±0.1 μmol, suggesting that polyol production could also be demonstrated in the absence of the NADPH-regenerative system.

That polyol production *in vitro* by homogeneous aldose reductase proceeded in the absence of the NADPH-generating system was confirmed by studies in which the purified aldose reductase was incubated overnight (14 h) at 37 °C with 10 mM-xylose and 10 mM-NADPH in phosphate buffer, pH 6.2, the pH optimum of aldose reductase. Xylitol (5.3±0.5 μmol) was formed in the complete system, but no xylitol production was observed in the absence of aldose reductase. Moreover, addition of 0.1 mM of the aldose reductase inhibitor sorbinil [(*S*)-6-fluorospirochroman-4,5′-imidazolidine-2′,4′-dione] to the vials containing enzyme resulted in a 85% decrease in xylitol production (to 0.8±0.1 μmol). Incubation in the absence of 10 mM-NADPH also resulted in an 81% decrease in xylitol production (to 1.0±0.1 μmol). The residual xylitol production could have utilized the NADPH associated with the enzyme from the final purification step, which was estimated to be 5 μM. No attempt was made to remove NADPH, since this can decrease the stability of the enzyme.

To illustrate that selective aldose reductase inhibitors assert their pharmacological effects through inhibition of the enzyme aldose reductase rather than non-specific free-radical scavenging, a series of potent aldose reductase inhibitors were evaluated for their ability to react with superoxide ions generated by either glucose or xylose at pH 10.2. Free-radical production was monitored spectrophotometrically by measuring the appearance of Formazan Blue, which is formed upon reaction of the generated superoxide ions with Nitro Blue Tetrazolium. The rate of Nitro Blue Tetrazolium production at pH 10.2 was directly proportional to the concentration of glucose present over the range 10–220 mM, and 111 mM-glucose was used in the standard reaction mixtures. Superoxide ions were more easily generated from xylose than from glucose, although the rate of reduction of Nitro Blue Tetrazolium was no longer proportional to xylose at concentrations above 50 mM-xylose. With 66 mM-xylose, however, the rate of Formazan Blue formation was directly proportional to the concentrations of Nitro Blue Tetrazolium present in the reaction mixture, and thus this was used as the standard system.

Addition of 10 μM of sorbinil, Statil [4(3)-bromo-2-fluorobenzyl-4-oxo-3*H*-phthalazin-1-ylacetic acid], tolrestat {*N*-methyl-*N*-[(5-trifluoromethyl-6-methoxy-

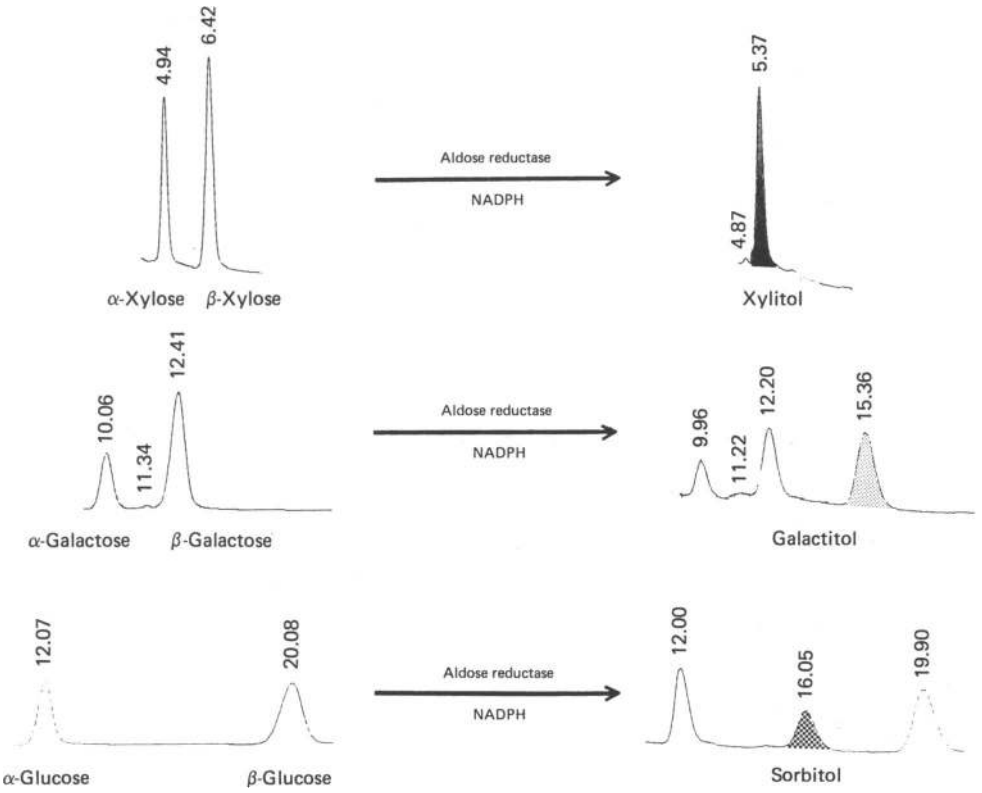


Fig. 2. Composite of g.l.c. results illustrating the formation of polyols from xylose, galactose or glucose *in vitro* by rat lens aldose reductase

For experimental details see the text.

Table 1. Effect of aldose reductase inhibitors on superoxide ions generated *in vitro*

Free-radical production by sugars at pH 10.2 was followed spectrophotometrically by monitoring the formation of Formazan Blue by superoxide ions fropm Nitro Blue Tetrazolium. Abbreviation: SOD, superoxide dismutase.

Superoxide source	Compound (10 μM)	Mean effect (% of control rate)
Xylose	SOD (50 units/ml)	41
	Statil	101
	Tolrestat	101
	Sorbinil	99
	Quercetin	105
Glucose	SOD (50 units/ml)	48
	Statil	101
	Tolrestat	103
	Sorbinil	105
	Quercetin	95

1-naphthalenyl)thioxomethyl]glycine} or quercetin [2-(3′,4′-dihydroxyphenyl)-3,5,7-trihydroxy-4-oxo-4 *H*-chromen] produced no effect in altering the rate of Nitro Blue Tetrazolium formation from either glucose or xylose, but the presence of superoxide dismutase (50 units/ml) decreased Nitro Blue Tetrazolium formation by 48% with glucose and by 41% with xylose (Table 1).

An unphysiological pH 10.2 was selected for these studies as the optimum for free-radical generation by sugars. At pH 7.0 no Formazan Blue formation can be detected from either xylose or glucose incubated in the presence of Nitro Blue Tetrazolium. With the autoxidation of ascorbate as a source of free radicals at pH 7.0, Nitro Blue Tetrazolium is converted into Formazan Blue, and this reaction is sensitive to inhibition by superoxide dismutase but not by aldose reductase inhibitors (D. J. Mirrlees, unpublished work).

DISCUSSION

Recent reports (see the Introduction) have questioned both the role of aldose reductase in polyol production and the mechanisms through which aldose reductase might be involved in the onset of diabetic complications. Based initially on the inability to demonstrate polyol production *in vitro* in the presence of purified bovine lens aldose reductase, these reports suggest that the enzymic activity attributed to aldose reductase is the artifact result of monosaccharide autoxidation generating peroxy radicals, which in turn oxidize NADPH to $NADP^+$. Aldose reductase inhibitors are claimed to exert their biological effects through their ability to serve as free-radical scavengers rather than as specific inhibitors of aldose reductase.

In the present study enzymically active aldose reductase was purified to apparent homogeneity from the rat lens and incubated overnight in the presence of aldose substrate and NADPH with or without the presence of an NADPH-generating system. Incubation of glucose, galactose and xylose and aldose reductase in the presence of the NADPH-generating system resulted in the respective formation of sorbitol, galactitol and xylitol. The rate of formation of these polyols closely mirrors the K_m and relative $V_{max.}$ values for these substrates obtained from kinetic studies by monitoring the rate of disappearance of NADPH as concentrations of substrate are varied (Table 2). This rate of formation also mirrors the formation of these respective polyols in intact rabbit lenses cultured *in vitro* in TC-199 medium (Table 2). Polyol formation was also observed at pH 6.2 and 7.8, indicating that polyol production can occur over a wide pH range. No polyol production occurred in the absence of purified aldose reductase, and analysis by g.l.c. revealed only the presence of unchanged monosaccharides.

These results demonstrate that polyols are produced *in vitro* by purified aldose reductase and verify van Heyningen's (1959) original observation that xylitol, galactitol and sorbitol are produced *in vitro* by rat lens homogenates. A stoichiometric production of sorbitol (determined by g.l.c.) and NADPH utilization (determined spectrophotometrically) has also been demonstrated with semi-purified bovine lens aldose reductase in open cuvettes (Stribling & Brittain, 1986). Additional evidence for enzymic involvement in this process is demonstrated by the fact that only D-xylose is converted into xylitol whereas L-xylose remains unchanged (Obazawa *et al.*, 1974). This observation is not consistent

Table 2. Comparison of the *in vitro* properties of purified rat lens aldose reductase

Aldose reductase was incubated for 14 h at 37 °C in sealed vials containing 10 mM-aldose, 10 mM-NADPH, 1 mM-$NADP^+$, 1 mM-DL-isocitrate, 2 mM-$MgCl_2$, isocitrate dehydrogenase (13.8 units) and 0.1 M-phosphate buffer, pH 7.8.

Substrate	Incubation (μmol of polyol formed)	Kinetics* K_m (mM)	Kinetics* $V_{max.}$ (% of $V_{max.}$ for glyceraldehyde)	Lens polyol (μmol/lens in 24 h)	Lens swelling (mg of water/lens in 24 h†)
Xylose	19.7±0.3	14	69	11.5	45
Galactose	9.1±0.6	83	47	7.0	19
Glucose	4.5±0.1	204	48	4.0	12

* Kinetic analyses were performed on the PROPHET computer system as previously described (Kador *et al.*, 1981).

† Data from rabbit lenses cultured for 24 h in TC-199 medium containing 30 mM-xylose, -galactose or -glucose (Obazawa *et al.*, 1974).

with the process of non-enzymic autoxidation of monosaccharides, which should use either the D or the L form of the sugar. Moreover, addition of antibodies raised against purified aldose reductase to the purified enzyme system can also block the utilization of NADPH.

Aldose reductase activities are extremely low in the mouse lens, and mice do not develop sugar cataracts. In contrast, octadon degus develop cataracts rapidly even when these rodents are only mildly diabetic. Examination of the lenses of octadon degus has revaled that they possess higher aldose reductase activity than found in any other species. These results are also consistent with the observation that cataracts and other animal models of diabetic complications develop more rapidly in galactosaemic than diabetic animals (Kador & Kinoshita, 1984). In each case the tissues can be shown to contain increased concentrations of polyols, yet the monosaccharide oxidation thesis does not predict the formation of polyols.

Further evidence for the enzymic involvement of aldose reductase-associated polyol formation in diabetic complications comes from inhibitor studies. Addition of 0.1 mM-sorbinil to purified rat lens aldose reductase incubated with xylose in the presence of NADPH results in an 85% decrease in xylitol production (Table 2). Addition of the aldose reductase inhibitors sorbinil, Statil, tolrestat and quercetin at concentrations that produce greater than 50% inhibition of lens aldose reductase *in vitro* produced no effect in altering the rate of Nitro Blue Tetrazolium formation from either glucose or xylose, indicating that the observed inhibition *in vitro* does not result from a free-radical-scavenger effect (Table 1). One criticism of the Nitro Blue Tetrazolium system, however, is that a pH of 10.2 is necessary for the optimal generation of free radicals. No measurable rate of free-radical formation can be observed at pH 8.2 or below. This is a reflection of the absence of free-radical formation from either xylose or glucose, because in the presence of ascorbate at pH 7.0 Formazan Blue is formed from Nitro Blue Tetrazolium. This reaction is superoxide dismutase-sensitive but is not affected by aldose reductase inhibitors, further substantiating the conclusion that free radicals do not play an essential role in the oxidation of NADPH in the presence of xylose or glucose at physiological pH or in the mechanism of aldose reductase inhibitors.

Long-chain fatty acids were the first known inhibitors of aldose reductase. Of these octanoic acid, a compound virtually devoid of redox potential, was the first promising inhibitor to produce 59% inhibition at 0.1 mM against bovine lens aldose reductase (Hayman & Kinoshita, 1965). This led to the development of tetramethyleneglutaric acid and its analogues as inhibitors. These inhibitors prevented cataract formation *in vitro* by suppressing polyol formation. Tetramethyleneglutaric acid and pentamethyleneglutaric acid displayed equal inhibitory potencies against calf lens aldose reductase, inhibiting 60% in the 0.1 mM range; however, addition of an α-methyl group to the pentamethylene ring diminished inhibitory potency to 17% at 0.1 mM (Jedziniak & Kinoshita, 1971). Such a decrease in inhibition is consistent with steric interaction between the inhibitor and enzyme inhibitor site, but not with a change in redox potential of this cycloalkyl diacid. Also consistent with the inhibitor–enzyme concept are the various structure–activity relationships, which indicate specific steric and nucleophilic acceptor requirements (Kador & Sharpless, 1983; Kador *et al.*, 1985*a*).

Studies performed *in vivo* on diabetic rats with the aldose reductase inhibitors sorbinil and its analogue 6-fluoro-2-methylspirochroman-4,5′-imidazolidine-2′,4′-dione (M 79175) also indicate that these inhibitors contain little, if any, antioxidant activity, as judged by their effects on lipoperoxide formation (Kaydoya *et al.*, 1983). Examination of the lenses of inhibitor-treated rats indicated no significant decreases in the activities of either peroxidase or glutathione reductase.

In conclusion, we have presented evidence that purified aldose reductase in the presence of NADPH and substrate can produce polyols. This production is consistent within all previously observed findings obtained *in vitro* and *in vivo*, indicating that this is an enzyme-catalysed reaction. Moreover, we find no evidence that aldose reductase inhibitors act as antioxidants but exert their effects by direct inhibition of aldose reductase.

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