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Low apparent aldose reductase activity produced by monosaccharide autoxidation

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Low apparent aldose reductase activity, as measured by NADPH oxidation, can be produced by the spontaneous autoxidation of monosaccharides. NADPH is oxidized to metabolically active $NADP^+$ in a solution of autoxidizing DL-glyceraldehyde at rates of up to 15×10^{-4} A_{340}/min. The close parallelism between the effects of buffer salt type and concentration, monosaccharide structure and temperature activation on autoxidation and NADPH oxidation imply that autoxidation is a prerequisite for the NADPH oxidation, probably via the hydroperoxy radical. Nucleotide-binding proteins enhanced NADPH oxidation induced by DL-glyceraldehyde, up to 10.6-fold with glucose-6-phosphate dehydrogenase. Glutathione reductase-catalysed NADPH oxidation in the presence of autoxidizing monosaccharide showed many characteristics of the aldose reductase reaction. Aldose reductase inhibitors acted as antioxidants in inhibiting this NADPH oxidation. These results indicate that low apparent aldose reductase activities may be due to artifacts of monosaccharide autoxidation, and could provide an explanation for the non-linear steady-state kinetics observed with DL-glyceraldehyde and aldose reductase.

Aldose reductase (EC 1.1.1.21) from bovine lens catalyses the oxidation of NADPH in the presence of a broad range of simple monosaccharides *in vitro* and requires the spontaneously formed open-chain anomer of glucose, the presumed physiological substrate, for activity (Inagaki *et al.*, 1982; Doughty & Conrad, 1982; Halder & Crabbe, 1984). The purified homogeneous protein exhibits Michaelis–Menten kinetics and a ternary-complex mechanism with D-glucose as substrate (Halder & Crabbe, 1984), but the rate equation is at least 2:2 in degree with DL-glyceraldehyde (Halder & Crabbe, 1984), and it has been suggested that the enzyme obeys a random mechanism with this substrate (Doughty & Conrad, 1982). We have previously shown that simple monosaccharides, including glyceraldehyde, are able to autoxidize, via the enediol tautomer, generating cytotoxic dicarbonyl compounds and H_2O_2, as well as superoxide, hydroxyl and carbon-centred free radicals (Wolff *et al.*, 1984; Thornalley *et al.*, 1984*a*,*b*). In the present paper we demonstrate that low apparent aldose reductase activity, as measured *in vitro* by NADPH oxidation, can be produced as an artifact of this spontaneous process. This suggests that the non-linear kinetics observed with DL-glyceraldehyde may be due to chemical effects derived from the autoxidation, rather than to a random enzyme mechanism, and urges caution in the interpretation of initial-rate data derived from NADPH oxidation with autoxidizable substrates when low rates of activity are being measured.

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Materials and methods

Materials

Monosaccharides and nicotinamide nucleotides were obtained from sources described previously (Wolff *et al.*, 1984; Thornalley *et al.*, 1984*a*,*b*; Halder & Crabbe, 1984). Homogeneous bovine lens aldose reductase (0.2 unit/mg as assayed with DL-glyceraldehyde) was obtained as described previously (Halder & Crabbe, 1984). Homogeneous human erythrocyte superoxide dismutase (3300 units/mg) was generously given by Dr. J. V. Bannister. All other enzymes and metalloproteins were obtained from Sigma Chemical Co. Hexamethyldisilazane, trichloromethylsilane, reduced

glutathione and Girard's T reagent were obtained from Sigma Chemical Co. The chelating reagent diethylenetriaminepenta-acetic acid was obtained from Aldrich Chemical Co. Aldose reductase inhibitors were kindly supplied by I.C.I. Pharmaceuticals Ltd. and Pfizer Research Ltd. All other materials were of the highest grade obtainable.

Methods

Product analysis by t.l.c. was as described previously (Thornalley *et al.*, 1984*b*). Enzymic assays for determination of glyceraldehyde, hydroxypyruvate and α-oxo aldehydes were as described previously (Thornalley *et al.*, 1984*b*). Production of metabolically active $NADP^+$ from NADPH in solutions of autoxidizing glyceraldehyde was measured by using yeast glucose-6-phosphate dehydrogenase. NADPH (1mM) was incubated with glyceraldehyde (50mM) in 50mM-potassium phosphate buffer, pH6.2, in a total volume of 1ml until the absorbance at 340nm fell to zero. Of this reaction mixture 10% was added to an assay mixture containing glucose-6-phosphate dehydrogenase (2 units/ml), glucose 6-phosphate (10mM) and $MgCl_2$ (0.1mM) in 100mM-Tris/HCl buffer, pH7.4, until a constant absorbance at 340nm was obtained. A molar absorption coefficient of $6.22 \times 10^3 M^{-1} \cdot cm^{-1}$ was used for NADPH at 340nm. The control contained NADPH in the absence of glyceraldehyde. One unit of activity is defined as that amount of enzyme catalysing the oxidation of 1μmol of substrate/min. Enhancement of NADPH in the presence of nucleotide-binding proteins was determined as follows: enzyme (equivalent to 0.25μM nucleotide-binding site concentration), or 5mg of bovine serum albumin/ml, was incubated with NADPH (250μM) and DL-glyceraldehyde (50mM) in 100mM-potassium phosphate buffer, pH6.2, at 37°C in a total volume of 1ml, and the fall in absorbance was monitored at 340nm relative to a control without enzyme but with all other reagents. Data presented are the means of two determinations. All other methods were as described previously (Thornalley *et al.*, 1984*b*; Halder & Crabbe, 1984). Where values are presented showing the mean of two or more determinations, results were within ±10% of one another.

Results and discussion

Oxidation of NADPH in autoxidizing glyceraldehyde solutions

Incubation of NADPH (100μM) with DL-glyceraldehyde (50mM) produced spectral changes consistent with the oxidation of NADPH to $NADP^+$, together with the concomitant production of hydroxypyruvaldehyde. NADPH (1mM) incubated with DL-glyceraldehyde (50mM) in 50mM-potassium phosphate buffer, pH6.2, until the absorbance at 340nm had fallen to zero produced 624±151μM (mean±S.D., $n=4$) metabolically active $NADP^+$. The remaining NADPH was destroyed by hydration. The rate of hydroxypyruvaldehyde production from glyceraldehyde (50mM) in 50mM-potassium phosphate buffer, pH6.2, was 9.28±0.43μM/min and 8.62±0.72μM/min in the presence and in the absence of NADPH (1mM) respectively. Table 1 shows the effect of modifiers of glyceraldehyde-induced NADPH oxidation at 5mM- and 50mM-DL-glyceraldehyde, relative to an NADPH hydration control. Hydration of reduced nicotinamide nucleotides at acid pH results in bleaching of absorbance at 340nm (Miksic & Brown, 1978). The glyceraldehyde-induced NADPH oxidation exhibited rectangular hyperbolic behaviour, with a maximum rate of $15 \times 10^{-4} A_{340}$/min at 80mM-DL-glyceraldehyde, and did not show first-order proportionality between NADPH oxidation and glyceraldehyde

Table 1. *Factors influencing the oxidation of NADPH by* DL-*glyceraldehyde*

Reaction mixtures contained 100μM-NADPH and DL-glyceraldehyde (50mM or 5mM) in 100mM-potassium phosphate buffer, pH6.2. Other additions were made as indicated. All incubations were at 37°C. The 100% initial rate was $1.6 \times 10^{-3} A_{340}$/min with 50mM-DL-glyceraldehyde and $4.1 \times 10^{-4} A_{340}$/min with 5mM-DL-glyceraldehyde. Data presented are means for four determinations. Abbreviations: DETAPAC, diethylenetriaminepenta-acetic acid; SOD, superoxide dismutase; N.D., not detected.

Additions	Relative initial rate (%)	
	Glyceraldehyde (50mM)	Glyceraldehyde (5mM)
None	100	100
N_2 saturation	34.6	6.5
DETAPAC (1mM)	33.7	N.D.
SOD (330 units/ml)	19.5	2.4
Catalase (240 units/ml)	72.0	4.8
$MnCl_2$ (0.1mM)	(9.5-fold)	114.7
2-Methylpropan-2-ol (1M)	136	N.D.

concentration. The DL-glyceraldehyde concentration that gave half this maximal velocity (i.e. the $[S]_{0.5}$ value) was 18mM. This $[S]_{0.5}$ value was lowered on the addition of NADPH-binding proteins, typically to 30μM in the presence of aldose reductase. The results from these experiments, for example the inhibition with superoxide dismutase, indicates that NADPH oxidation in the presence of DL-glyceraldehyde and potassium phosphate buffer is a complex free-radical-mediated process. At 50mM-glyceraldehyde, superoxide appears to mediate NADPH oxidation. Mn^{2+} has a similar stimulatory effect upon the superoxide-mediated oxidation of NADPH by leucocyte granules (Patriarcha *et al.*, 1975), and it has been demonstrated that Mn^{2+} is able to form an MnO_2^+ complex with superoxide (Pick-Kaplan & Rabani, 1976; Bielski & Chan, 1978). At the lower glyceraldehyde concentration, NADPH oxidation is probably mediated by autoxidation-derived oxidants, such as hydroxyl radicals, in addition to superoxide. NADPH appears to compete with glyceraldehyde for radical species in an analogous fashion to competition with the spin adduct 5,5'-dimethyl-1-pyrroline *N*-oxide (Wolff *et al.*, 1984; Thornalley *et al.*, 1984*b*). This is consistent with the lack of direct proportionality between the rate of NADPH oxidation and glyceraldehyde concentration. Table 2 illustrates the effect of various buffer salts on glyceraldehyde-stimulated NADPH oxidation, and compares this with their effect on cytochrome *c* reduction and O_2 uptake. Table 3 shows the ability of various monosaccharides to oxidize NADPH and reduce cytochrome *c*. Cytochrome *c* reduction and NADPH oxidation by glyceraldehyde showed parallel temperature–activation curves. The pH–activity profile of glyceraldehyde-induced NADPH oxidation and glyceraldehyde enolization (measured by I_2 uptake) is shown in Fig. 1(*a*). The increasing rate of NADPH oxidation with decreasing pH contrasts with the decreasing rate of enolization towards lower pH values, suggesting that NADPH oxidation is mediated by an oxidizing species of low pK_a produced by the autoxidation, and implicates the hydroperoxy radical ($HO_2^{\bullet}$, conjugate acid of superoxide) as the oxidant. A similar conclusion has been drawn for the reaction of NADH with superoxide generated by the flash photolysis of H_2O_2 (Nadezhdin & Dunford, 1979). A mechanism for the oxidation of NADPH by autoxidizing glyceraldehyde is shown in Scheme 1.

Glyceraldehyde-stimulated oxidation of NADPH in the presence of nucleotide-binding proteins

Bielski & Chan (1973, 1978) demonstrated the chain oxidation of NADH by superoxide in an enzyme–nucleotide complex. By analogy, nucleotide-binding proteins may be expected to enhance the oxidation of NADPH induced by autoxidizing glyceraldehyde. Nucleotide-binding proteins enhanced NADPH oxidation induced by glyceraldehyde as follows: bovine lens aldose reductase (NADPH-binding), 4-fold; glutathione reductase (NADPH-binding), 4.8-fold; isocitrate dehydrogenase (NADPH-binding), 5.2-fold; glucose-6-phosphate dehydrogenase (NADPH-binding), 10.6-fold; glyceraldehyde-3-phosphate dehydrogenase (NADH-binding), 3.6-fold. There was no significant enhancement when bovine serum albu-

Table 2. *Dependence of buffer salt type on NADPH oxidation, cytochrome c reduction and O_2 uptake by* DL-*glyceraldehyde*

All reaction mixtures contained 50mM-DL-glyceraldehyde and 100mM buffer. Other conditions were: for NADPH oxidation, 100μM-NADPH, pH6.8; for cytochrome *c* reduction, 25μM-cytochrome *c*, pH7.2; for O_2 uptake, 178μM-O_2 (initial), pH7.4. All incubations were at 37°C. The 100% rate was 1.0×10^{-3} A_{340}/min for NADPH oxidation and 8.5×10^{-2} A_{550}/min for cytochrome *c* reduction. Data presented are means for four determinations. —, Not determined.

Buffer	Relative rate (%) NADPH oxidation	Cytochrome *c* reduction	O_2 uptake
Phosphate	100	100	100
Imidazole	50.1	44.3	—
Triethanolamine	32.9	33.8	—
Hepes	26.5	33.4	31.0
Tris/HCl	12.1	16.5	2.2

Table 3. *NADPH oxidation and ferricytochrome c reduction by various monosaccharide species*

All reaction mixtures contained 50mM monosaccharide and 100mM-potassium phosphate buffer. Other conditions were: for NADPH oxidation, 250μM-NADPH, pH6.2; for cytochrome *c* oxidation, 25μM-cytochrome *c*, pH7.4. All incubations were at 37°C. The 100% rate was 1.6×10^{-3} A_{340}/min for NADPH oxidation and 8.5×10^{-2} A_{550}/min for cytochrome *c* reduction. Data presented are means for three determinations.

Monosaccharide	Relative rate (%) NADPH oxidation	Cytochrome reduction
DL-Glyceraldehyde	100	100
Dihydroxyacetone	113	115
Hydroxypyruvate	102	107
Glycolaldehyde	79	72
Erythrose	51	41
D-Xylose	37	28
D-Ribose	26	16
D-Glucose	5	2

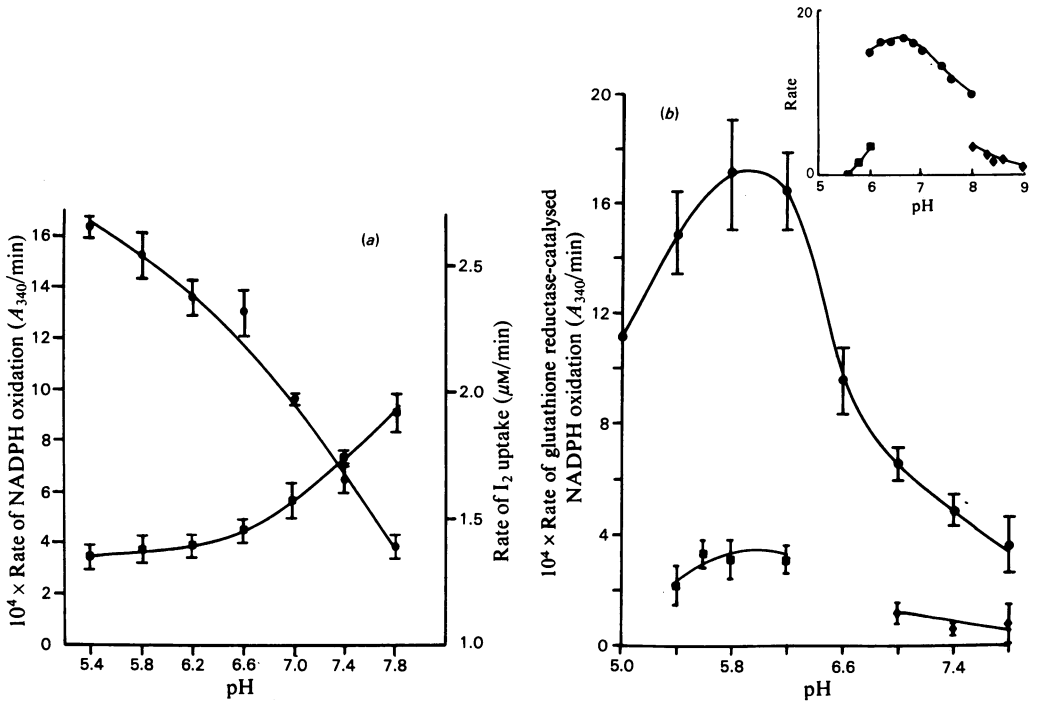
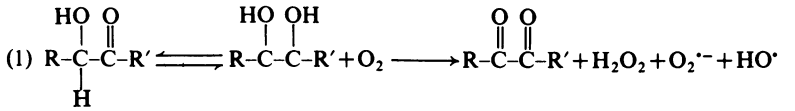


Fig. 1. *pH profiles for apparent aldose reductase activities*

(*a*) pH profiles of NADPH oxidation (●) and I_2 uptake (■) by autoxidizing glyceraldehyde. Reaction mixtures contained NADPH (100 μM) or KI (50 mM), I_2 (30 μM) and DL-glyceraldehyde in 100 mM-potassium phosphate buffer. Initial rates are means ± S.D. for four determinations. (*b*) pH profile of DL-glyceraldehyde-stimulated NADPH oxidation in the presence of glutathione reductase. The reaction mixture contained glutathione reductase (1 unit/ml, defined as in the Materials and methods section), NADPH (100 μM) and DL-glyceraldehyde (50 mM) in 100 mM-buffer (○, potassium phosphate; ■, citrate; ◆, Tris/HCl) at 37°C in a total volume of 1 ml. The inset shows the pH profile of bovine lens aldose reductase taken from Crabbe & Halder (1979), with rate units as in Fig. 1(*b*).



(1) $R\text{-}CH(OH)\text{-}C(=O)\text{-}R' \rightleftharpoons R\text{-}C(OH)=C(OH)\text{-}R' + O_2 \longrightarrow R\text{-}C(=O)\text{-}C(=O)\text{-}R' + H_2O_2 + O_2^{\cdot -} + HO^{\cdot}$

$k_1 = 5 \times 10^{-5}\ M^{-1} \cdot s^{-1}$ (P_i) for glyceraldehyde at 37°C at pH 7.4

(2) $O_2^{\cdot -} + H^+ \rightleftharpoons HO_2^{\cdot}$
$pK_a = 4.88$

(3) $NADPH + HO_2^{\cdot} \longrightarrow NADP^{\cdot} + H_2O_2$
$k_3 = 1.8 \times 10^5\ M^{-1} \cdot s^{-1}$ for NADH (Nadezhdin & Dunford, 1979)

(4) $NADP^{\cdot} + O_2 \longrightarrow NADP^+ + O_2^{\cdot -}$
$k_4 = 2 \times 10^9\ M^{-1} \cdot s^{-1}$ for $NAD^{\cdot}$

(5) $X + O_2^{\cdot -} \longrightarrow$ Products

General termination reaction limiting the number of NADPH molecules oxidized per autoxidation-derived superoxide molecule

Scheme 1. *Oxidation of NADPH by autoxidizing monosaccharide*

min was used. The homogeneous bovine lens aldose reductase used in these studies showed typical steady-state kinetic properties (see Harding & Crabbe, 1984), but in incubations of up to 4 h showed no production of sorbitol from glucose, judged by g.l.c. and h.p.l.c. analysis. Indeed, no homogeneous preparation of lens aldose reductase has been shown to catalyse the production of sorbitol from glucose (see Crabbe, 1984). The enhancement studies were performed with a constant amount of NADPH-binding capacity (0.25 μM nucleotide-binding site concentration) in

each experiment. The enhancement showed a linear relationship with concentration of glutathione reductase over a 5-fold enzyme concentration range. The pH–activity profile of the DL-glyceraldehyde-stimulated NADPH oxidation in the presence of glutathione reductase is shown in Fig. 1(*b*), and is very similar to that found for bovine lens aldose reductase (shown in the insert), with decreased initial velocities in citrate and Tris/HCl buffers. The addition of NADPH-binding protein to an incubation mixture of glyceraldehyde (or another autoxidizable substrate) and NADPH is commonly used for the assay of aldose reductase activity (Jedziniak & Kinoshita, 1971; Halder & Crabbe, 1984). *p*-Nitrobenzaldehyde, one of the best substrates of aldose reductase, is also capable of autoxidation (Swern, 1970). The initial rates of NADPH oxidation obtained from the autoxidation are within an order of magnitude of the initial rates obtained with homogeneous preparations of aldose reductase. The pH–activity profile of glutathione reductase-enhanced NADPH oxidation induced by glyceraldehyde was identical with that for bovine lens aldose reductase (Crabbe & Halder, 1979; Halder & Crabbe, 1984), with a pH optimum near 6.2 and diminished activity in Tris/HCl and imidazole/HCl buffers. NADPH hydration was not altered by the addition of NADPH-binding proteins, judged by control experiments, indicating that the proteins stimulated the formation of metabolically active $NADP^+$. The binding of the reduced nucleotide to the protein may enhance its ability to react with peroxy radicals produced by the autoxidizing glyceraldehyde, so producing $NADP^+$, in an analogous fashion to the rate enhancement seen for NADH oxidation by superoxide in the presence of lactate dehydrogenase in pulse-radiolysis studies, by lowering the energy required for proton abstraction (Bielski & Chan, 1973). Table 4 shows the effects of various additives, buffer systems and monosaccharides on the rate of glutathione reductase-enhanced NADPH oxidation. The influence of the modifiers parallels the effects observed with unenhanced glyceraldehyde-induced NADPH oxidation. Thus the observed NADPH oxidation in the presence of nucleotide-binding protein is mediated by the oxidizing products of glyceraldehyde autoxidation. The close parallelism between the characteristics of glutathione reductase-catalysed NADPH oxidation in the presence of autoxidizing monosaccharide and aldose reductase activity indicates that low initial rates of apparent aldose reductase activity may be due to monosaccharide autoxidation. Thus superoxide and other radical species derived from the autoxidation oxidize NADPH in a chain oxidation catalysed by the nucleotide-binding protein. This may explain why bovine lens aldose reductase, and a similar protein from human erythrocytes (Crabbe *et al.*, 1981), exhibit non-linear steady-state kinetics with increasing concentrations of DL-glyceraldehyde in potassium phosphate buffer.

Influence of aldose reductase inhibitors on glyceraldehyde-induced NADPH oxidation

Table 5 shows that the aldose reductase inhibitors sorbinil [D-6-fluorospiro(chroman-4,4′-imidazolidine)-2′,4′-dione], quercitrin and ICI 105552 [1-(3,4-dichlorobenzyl)-1,2-dihydro-3-methyl-2-oxoquinol-4-ylacetic acid] were able to inhibit simple glyceraldehyde-induced NADPH oxidation and glutathione reductase-enhanced NADPH oxidation in a parallel manner. The concentrations of inhibitors used were up to 10^3-fold more concentrated than those reported to inhibit authentic aldose reductases. The aldose reductase inhibitors do not, however, inhibit the underlying glyceraldehyde autoxidation. Indeed, there was a slight

Table 4. *Factors influencing the glutathione reductase-catalysed oxidation of NADPH in the presence of glyceraldehyde*
All reaction mixtures contained 50mM monosaccharide, 100μM-NADPH and 5 units of glutathione reductase/ml in 100mM buffer, pH6.2. Other additions were made where indicated. All incubations were at 37°C. The 100% rate was 72.36×10^{-4} A_{340}/min. Data presented are means for four determinations. Abbreviations: DETAPAC, diethylenetriaminepenta-acetic acid; SOD, superoxide dismutase.

Monosaccharide	Buffer	Addition	Relative rate (%)
Glyceraldehyde	Phosphate	None	100
Dihydroxyacetone	Phosphate	None	126
Glycolaldehyde	Phosphate	None	88
D-Xylose	Phosphate	None	52
D-Glucose	Phosphate	None	11
Glyceraldehyde	Imidazole	None	63
Glyceraldehyde	Hepes	None	38
Glyceraldehyde	Tris/HCl	None	25
Glyceraldehyde	Phosphate	SOD (330 units/ml)	11
Glyceraldehyde	Phosphate	DETAPAC (1mM)	24
Glyceraldehyde	Phosphate	Catalase (240 units/ml)	56

Table 5. *Inhibitors of glyceraldehyde-stimulated NADPH oxidation*

Reaction mixtures contained the following: for (*a*) 50mM-glyceraldehyde and 100μM-NADPH in 100mM-potassium phosphate buffer, pH6.2; for (*b*), 5mM-glyceraldehyde, 100μM-NADPH and 5 units of glutathione reductase/ml in 100mM-potassium phosphate buffer, pH6.2. Other additions were made as indicated. All incubations were at 37°C. The 100% initial rate was 1.6×10^{-3} A_{340}/min for (*a*) and 7.2×10^{-3} A_{340}/min for (*b*). Data are means for four determinations.

Addition	Relative initial rate (%)
(*a*) Basal glyceraldehyde-induced NADPH oxidation	
None	100
Sorbinil (10μM)	56
Quercitrin (10μM)	65
ICI 105552 (10μM)	47
DETAPAC (1mM)	29
DETAPAC+sorbinil	18
(*b*) Glutathione reductase-catalysed NADPH oxidation	
None	100
Sorbinil (1μM)	49
ICI 105552 (1μM)	54
Quercitrin (1μM)	72

increase (10%) in O_2 consumption and hydroxypyruvaldehyde production in the presence of sorbinil or ICI 105552. Thus the ability of aldose reductase inhibitors to block low initial rates of NADPH oxidation in the presence of autoxidizable substrate is related to their ability, as antioxidants, to react with oxidizing species derived from the autoxidation. Reaction with peroxy radicals, specifically superoxide, for example, would increase the rate of autoxidation by preventing back-reduction of the oxyenediol radical by superoxide. This mechanism was proposed to account for the ability of superoxide dismutase to increase the concentration of radicals spin-trapped in autoxidizing solutions of glyceraldehyde (Wolff *et al.*, 1984; Thornalley *et al.*, 1984*b*). Reaction of superoxide or hydroxyl radicals with the aldose reductase inhibitors would prevent secondary NADPH oxidation. Antioxidant mechanism may involve the formation of radicals more stable than the intermediates of radical-mediated chain oxidations or chelation of pro-oxidant metal (Porter, 1980). Interestingly, the flavonoids are effective inhibitors of aldose reductase activity *in vitro* (Varma & Kinoshita, 1976), and are also good antioxidants in lipid-peroxidation systems (Porter, 1980), and it has been suggested that aldose reductase inhibitors act, at least in part, as 'free-radical scavengers', sparing lenticular reduced glutathione (Stevens *et al.*, 1978). The aldose reductase inhibitor sorbinil, which can penetrate into human aqueous humour and lens *in vivo* in 3h (Crabbe *et al.*, 1985), rapidly restores concentrations of reduced glutathione in lenses of diabetic rats (Gonzalez *et al.*, 1983). We suggest that monosaccharide autoxidation may be implicated in the development of diabetic complications in tissues where damage is thought to be associated with low aldose reductase activity.

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