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Rat lens aldose reductase and polyol production

The paper by Kador *et al.* (1986) describes a g.l.c. method to indicate qualitatively the formation of some polyols from sugars, and shows that at pH 10.2 some aldose reductase inhibitors do not affect superoxide scavenging by Nitro Blue Tetrazolium. Kador *et al.* (1986) suggest that we question the biochemical importance of lens polyol formation and the mechanism of its formation because of our inability to demonstrate, *in vitro*, the production of polyols by purified lens aldose reductase. In fact a number of investigators have raised these and related questions in the past (see Harding & Crabbe, 1984, for a review). Our paper on monosaccharide autoxidation as it relates to aldose reductase (Wolff & Crabbe, 1985) actually concerns a different issue: the use of spectrophotometric determination of NADPH consumption as an index of low levels of aldose reductase activity. Their paper still does not answer the question we raise about the relevance of monitoring 'aldose reductase' activity by only following NADPH oxidation in the presence of aldoses *in vitro*. This is particularly relevant to the lens and cataract in diabetes as the human lens contains far lower levels of 'aldose reductase' activity than the rat lens (see, e.g., Halder & Crabbe, 1984). Where low levels of enzyme activity are found, aldose reductase may be an artefact of monosaccharide autoxidation.

The original experiments by Hers (1956) and van Heyningen (1959) had used t.l.c. to show that tissue extracts could catalyse the conversion of glucose to sorbitol, galactose to dulcitol and sorbitol to fructose. It is interesting that basic investigations into the chemistry and stoichiometry of these reactions are only now being undertaken, stimulated by steady-state kinetic experiments and studies on monosaccharide autoxidation (Wolff *et al.*, 1984; Thornalley *et al.*, 1984a,b; Wolff & Crabbe, 1985) nearly 30 years after the original reports. In the meantime, studies on the rat lens gave rise to the polyol hypothesis for the pathogenesis of cataract, and later other diabetic complications, in humans (Kinoshita, 1974).

Since aldose reductase activity is normally assayed by the co-incubation of tissue homogenates, aldoses (usually DL-glyceraldehyde, which autoxidizes rapidly) and NADPH, it was reasonable to speculate that NADPH consumption could result from free-radical-mediated oxidation. Peroxyl and other free radicals are known for their ability to oxidize reduced nucleotides, particularly in the presence of nucleotide-binding proteins (Bielski & Chan, 1978). We observed that the NADPH-oxidizing activity was not linked to polyol production using homogenates or purified enzyme from bovine lens; i.e., that polyol production was not coupled to NADPH oxidation. We used bovine lens as this has similar aldose

reductase and polyol dehydrogenase activity levels to the human lens, and is thus a better model for the human case than the rat lens. We then demonstrated that the steady-state properties of aldose reductase could be reproduced *in vitro*, owing to autoxidation-derived species causing oxidation of the NADPH (Wolff & Crabbe, 1985). We were able to mimic levels of aldose reductase activity *in vitro* consistent with those of bovine and human lenses by using other nucleotide-binding enzymes. Aldose reductase inhibitors inhibited such non-reductive oxidation, a finding consistent with the known inhibitory activity of flavonoid aldose reductase inhibitors (which give 50% inhibition of aldose reductase activity at $\geq 10^{-8}$ M) upon lipid peroxidation (Porter, 1980). This highlighted pitfalls in the standard spectrophotometric assay for aldose reductase, and suggested that other methods should be used to demonstrate the presence of the enzyme. In addition, it has been shown, by ourselves and others, that lens aldose reductase has k_{cat}/K_m values for glucose about 10^9 -fold lower than the diffusion-controlled encounter frequency (Tanimoto *et al.*, 1984; Crabbe *et al.*, 1986a), and that rate enhancements by homogeneous lens aldose reductase are not significantly higher than with other NADPH-binding proteins (Wolff & Crabbe, 1985; Crabbe *et al.*, 1986b). There is also a lack of absolute enzyme stereospecificity for many sugars (see Harding & Crabbe, 1984, for review).

Although Kador *et al.* (1986) have used g.l.c. to measure enzyme activity, there are problems associated with the Nelson–Somogyi method used for sample preparation. This method, used prior to h.p.l.c. sugar analysis, gave rise to apparent loss of glucose (up to 80% loss of the α anomer) and an artefactual peak in the sugar alcohol region (Petchey & Crabbe, 1984; Lloyd & Crabbe, 1985; Petchey, 1985). It would be interesting to see whether this was also observed for xylose. For this reason we always use a centrifugation method for our sample preparation prior to analysis (Petchey & Crabbe, 1984).

It is not clear why Kador *et al.* (1986) used xylose in their incubation media. Xylose cataract is not a model for human diabetic cataract. In a study using ^{13}C -n.m.r. with whole human lenses, Cousins *et al.* (1985) failed to demonstrate sorbitol production from glucose, or galactitol production from galactose. We still await the clear quantitative stoichiometric demonstration, under physiological conditions, without preincubation of substrate, of sorbitol production from glucose as NADPH is oxidized to NADP^+ by homogeneous lens aldose reductase. This relationship should be proportional to the amount of homogeneous aldose reductase, and should not occur in the presence of other NADPH-binding proteins. Until these demonstrations have been made beyond all reasonable doubt, the coupling of polyol formation to NADPH oxidation by a single enzyme, aldose reductase, must remain in doubt.

If the authors' contention that the rate of polyol formation closely mirrored the kinetic parameters relating to NADPH oxidation and sorbinil inhibition was correct, there should have been 56 μmol of xylitol formed/24 h per lens rather than the 11.5 μmol found (relative to glucose), and 65.6 μmol of xylitol formed in the authors' incubation rather than 19.7 μmol . Incubation mixtures containing 0.5 i.u. of aldose reductase should produce about 0.5 μmol of xylitol/min (the relative maximal velocity with xylose is 91% that of DL-glyceraldehyde). This would be about 30 μmol of xylitol/h in their incubations, and yet overnight incubation only gave 19.7 μmol . With this amount of enzyme, the xylose should have disappeared totally in under 3 h. This also suggests an uncoupling of polyol formation and NADPH oxidation.

It is not clear why 100 μM -sorbinil only produced an 85% reduction in xylitol formation, as this concentration should inhibit activity by 100%; 0.1 μM -sorbinil has been shown by many authors to inhibit aldose reductase activity by 50%. And yet the authors only used 10 μM concentrations in their pH 10.2 Nitro Blue Tetrazolium experiments. Wolff & Crabbe (1985) showed that aldose reductase inhibitors at 10 μM lowered DL-glyceraldehyde-stimulated NADPH oxidation, while 1 μM concentrations were sufficient if an NADPH-binding protein was present. The differences in sensitivity to aldose reductase inhibitors between the whole animal, the tissue and the purified protein suggest uncoupling between NADPH oxidation and polyol formation, together with one or more alternative modes of action of the inhibitors themselves.

We have suggested that these agents may have some antioxidant effect and that this could contribute to their effects *in vivo* (Wolff & Crabbe, 1985). This is based on the ability of these drugs to interfere with processes we consider to be mediated by free radicals. The inability of Kador and colleagues to demonstrate interference of Nitro Blue Tetrazolium reduction by monosaccharides using these agents at high pH does not contradict that view, but merely shows that they have used an inappropriate assay. We have shown that pH influences the course of monosaccharide autoxidation; above pH 8.6 there is a preferential production of hydroxyl radicals relative to carbon-centred radicals (Wolff *et al.*, 1984; Thornalley *et al.*, 1984a). Incubation mixtures containing monosaccharides and NADPH are complex systems generating a variety of free radical species, not merely superoxide radical anions (Wolff & Crabbe, 1985; Wolff & Dean, 1987). A role for aldose reductase inhibitors other than that of inhibiting aldose reductase is supported by their ability to inhibit naphthalene cataract in the rat (Wegener *et al.*, 1986).

Most support for the polyol hypothesis and the role of aldose reductase inhibitors stems from studies in the rat, although even there firm biochemical evidence is lacking (Reddy *et al.*, 1976; Harding & Crabbe, 1984; Crabbe, 1987). It is very hard to understand the pathology solely in terms of an 'osmotic effect' sucking water into the lens (Kinoshita, 1974). Such uncertainties cannot be eliminated by demonstrating that a protein in the rat lens is

required for the conversion of xylose to xylitol, nor by misrepresentation of criticism. For the alleviation of cataract and other diabetic complications, alternative chemotherapeutic strategies, relating to antioxidant therapy and the prevention of glucose-derived crosslinks (Cerami & Crabbe, 1986) should be seriously considered.

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