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Aldehydes and dicarbonyls in non-enzymic glycosylation of proteins

We agree with Harding & Beswick (1988) that the Amadori product (*N*-substituted 1-amino-1-deoxyfructose), as described by Hodge (1953), appears to be one of the early products of the non-enzymic glycosylation of proteins *in vitro*. There is however an additional early pathway, which we refer to as 'autooxidative glycosylation', which has not previously been recognized, perhaps because it forms an adduct which is not readily distinguishable from the Amadori product (Wolff & Dean, 1987). In this autooxidative pathway, α -ketoaldehydes are formed by the transition-metal-catalysed autooxidation of glucose enediol, and attach to protein. Part of our evidence for this lies in the ability of the metal chelator diethylenetriaminepenta-acetic acid (DETAPAC) to inhibit glucose autooxidation and simultaneously diminish the attachment of glucose to protein. However, at high concentrations of glucose (for instance, greater than 100 mM, which far exceeds pathological levels) the importance of autooxidative glycosylation is substantially reduced (Wolff & Dean, 1987; Figs. 5 and 8).

The criticisms raised by Harding and Beswick were anticipated and discussed in our original paper. First, they express surprise that addition of copper does not enhance glucose attachment to protein. Our system already contains concentrations of adventitious catalytic transition metal in excess of the low amounts of autooxidation-susceptible enediol present, as shown earlier for simple monosaccharide autooxidation (Wolff *et al.*, 1984). Addition of further transition metal cannot stimulate an already transition metal-saturated system. The inclusion of DETAPAC, however, decreases the catalytic potential of the adventitious transition metal (Figs. 1 and 3 of Wolff & Dean, 1987) and inhibits glycosylation (Fig. 3 of Wolff & Dean, 1987) and ketoaldehyde formation from glucose (Fig. 1 of Wolff & Dean, 1987). Transition metal-DETAPAC chelates retain some catalytic activity towards enediol oxidation (Martell, 1980) and this is exemplified by their ability to stimulate ketoaldehyde formation from glucose in dose-dependent fashion (Fig. 1 of Wolff & Dean, 1987).

Second, the specificity of the Girard T reagent for detection of α -ketoaldehydes is well supported by the

original methodological studies (Mitchel & Birnboim, 1977). In addition, our previous work showed a parallelism between levels of α -ketoaldehydes detected by the Girard T reagent, and by the specific glyoxalase assay (Thornalley *et al.*, 1984; Wolff *et al.*, 1984) and this was confirmed for glucose (S. P. Wolff & R. T. Dean, unpublished work) prior to using the more convenient Girard T reagent for routine studies (Wolff & Dean, 1987).

Third, Harding and Beswick discuss the work of Ahmed *et al.* (1986), showing the transition-metal-catalysed oxidative cleavage of *N*-formyl-fructosyl-lysine (which yields erythronic acid and carboxymethyl-lysine), as a model for the later reactions of the Amadori product. If these reactions were a quantitatively dominant contribution to glycosylation under our conditions of physiological glucose concentration, then DETAPAC would increase the amount of label attached to protein (by avoiding the loss of two-thirds of the label as erythronic acid), since we were using uniformly labelled glucose (Wolff & Dean, 1987). In fact, DETAPAC caused a decrease in attached label.

Fourth, Harding and Beswick point out that the rate of protein attachment of the non-autooxidizable sugar, 2-deoxyglucose, is similar to that of glucose, in the presence of sodium cyanoborohydride (Beswick & Harding, 1985). This rate is dictated by the rate of Schiff base aldimine formation and its trapping by the reducing agent (Jentoft & Dearborn, 1979), and not by the rate of the autooxidative or Amadori routes. This was confirmed by their model studies with glucose and glycine which showed the formation of only the primary aldimine under their experimental conditions (Beswick & Harding, 1985). They could detect the formation of neither the accepted Amadori structure, nor our proposed ketoaldehyde adduct. The experiment could thus not distinguish between the latter possibilities. Similarly, our data indicate that in 20 days of incubation of 2.8 mM-glucose (Beswick & Harding, 1986), less than 0.5% of the glucose will be converted into autooxidation products, and it is thus not surprising that they failed to detect them using gas chromatography in conjunction with mass spectrometry. They also failed to detect the mannose and fructose formed from glucose enediol by the Lobry-de-Bruyn-van Ekenstein transposition. Nevertheless, as we showed, ketoaldehyde can be detected in these incubations by using specific reagents. Our calculations show that the amounts formed are in the range consistent for some contribution to total protein-bound monosaccharide (Wolff & Dean, 1987).

Finally, McLaughlin *et al.* (1980) have shown, contrary to the arguments of Harding and Beswick, that α -ketoaldehydes are able to react with both lysine and arginine in proteins. Furthermore, at glucose concentrations nearer the physiological [i.e. 250 mM (Hull *et al.*, 1986), as opposed to 1 M in earlier work (Eble *et al.*, 1983)], arginine is lost from protein during glycosylation (Hull *et al.*, 1986).

Harding and Beswick are concerned about our suggestion that free radicals are involved in the early glycosylation reactions, although they accept that they play a role in the later ones. Whatever the chronology of glycosylative free radical involvement, the design of therapeutic agents will have to take it into account (Wolff, 1987). For example, in a study with implications for cataract, limited non-enzymic fragmentation of lens

crystallins occurred during their exposure to autoxidizable sugar (Beswick & Harding, 1987), consistent with the known reactions of free radicals with proteins (Wolff & Dean, 1986; Wolff *et al.*, 1986).

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