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The possible contribution of glucose autoxidation to protein modification of diabetes

The non-enzymic reaction of glucose with proteins is widely recognized and is thought to be important in the aetiology of the long-term complications of diabetes (Harding, 1985). The initial reaction appears to be the formation of a Schiff base between protein amino groups and the open-chain form of glucose, followed by an Amadori rearrangement to form a more stable adduct, although that is slowly changed to a variety of other products by the Maillard or browning reactions. It is important to know the mechanism of non-enzymic glycosylation, because different views on the initial reaction could lead to different approaches to therapy to prevent diabetic complications. Wolff & Dean (1987) have again raised the possibility that α-ketoaldehydes formed from glucose by autoxidation are responsible for a significant proportion of the initial reaction of non-enzymic glycosylation of proteins. They have not identified such adducts in modified proteins, nor indeed in model systems after incubation of glucose with amino acids. Their case depends largely on the decrease in the amount of radiolabel bound to serum albumin during incubation with [U-^{14}C]glucose brought about by diethylenetriaminepenta-acetic acid (DETAPAC). They assume that the only action of DETAPAC is to inhibit the metal-catalysed autoxidation of glucose. Interestingly binding was not increased by addition of 'Cu^{2+}'. DETAPAC inhibits the formation of Girard-T positive material, taken to be α-ketoaldehydes. The authors do not demonstrate that the Girard-T method they use is specific, nor is such a demonstration to be found in the quoted description of the method. There is nothing in the paper that is not consistent with an alternative proposal that DETAPAC is inhibiting a reaction that *follows* the original formation of a Schiff base and then Amadori product from glucose and protein. It is well-known that a complex series of reactions can follow from the Amadori product, reactions involved in the browning of food (Hodge, 1953; Mauron, 1981). Some of these proceed by free-radical mechanisms and may well be catalysed by metals. Incubation of a model Amadori product, N^{α}-formyl-N^{ϵ}-fructosyl-lysine, in 0.2 M-phosphate at physiological pH and temperature resulted in the formation of carboxymethyl-lysine and erythronic acid (Ahmed *et al.*, 1986). This reaction appeared to be a free-radical-mediated reaction and showed certain similarities to the processes studied by Wolff & Dean (1987): both were strongly dependent on phosphate concentration, both require oxygen and both are inhibited by DETAPAC. Ahmed *et al.* (1986) are clearly studying post-Amadori reactions and identify the products unequivocally, whereas Wolff & Dean (1987) assume that they are studying the initial reactions of glucose but do not attempt to identify the adducts. In addition to their model experiments Ahmed *et al.* (1986) also identified carboxymethyl-lysine in lens protein from a human diabetic, indicating that their reactions occur *in vivo* after initial reaction of glucose as a Schiff base that undergoes Amadori rearrangement.

In a previous contribution to the debate on what exactly reacts with proteins during non-enzymic glycosylation, we conducted experiments to distinguish between the reaction of glucose as an aldehyde and its reaction only after autoxidation to a ketoaldehyde (Beswick & Harding, 1985, 1986). First, we showed that the rate of binding of 2-deoxyglucose, a sugar that cannot autoxidize by Wolff's mechanisms, is similar to that of glucose. Secondly, we isolated the adduct from the reaction of glucose with glycine and used proton n.m.r. and fast-atom-bombardment mass spectroscopy to show that it was the product of glucose reacting as the open-chain aldehyde, not the product from a ketoaldehyde (Beswick & Harding, 1985). Furthermore, incubation of glucose alone for up to 20 days produces no new products detectable using gas chromatography in conjunction with mass spectroscopy (Beswick & Harding, 1986). If glucose autoxidized at a significant rate by the mechanism proposed by Wolff & Dean (1987), that is via an enediol, formation of mannose and fructose would be expected following the well-known Lobry-de-Bruyn–van Ekenstein transposition in addition to the dicarbonyl compound. We concluded that it is the open-chain aldehyde form of glucose that reacts with proteins, not an autoxidation product. If α-dicarbonyls played a role in the modification of protein by glucose the specificity of α-dicarbonyl reactions would make arginine the most likely amino acid to be attacked (Yankeelov, 1972). Indeed 3-deoxyglucosone, a 1,2-dicarbonyl compound derived from glucose, reacts with arginine in bovine serum albumin and other proteins in preference to lysine under physiological conditions, whereas glucose itself reacts more extensively with lysine (Kato *et al.*, 1987). However, no loss of arginine was seen in ribonuclease incubated with glucose until most of the lysine had disappeared (Eble *et al.*, 1983). If an arginine adduct were formed it would be relatively easy to detect.

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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 'autoxidative 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 autoxidative pathway, α-ketoaldehydes are formed by the transition-metal-catalysed autoxidation 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 autoxidation 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 autoxidative 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 autoxidation-susceptible enediol present, as shown earlier for simple monosaccharide autoxidation (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-autoxidizable 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 autoxidative 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 condtions (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 autoxidation 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