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Glucose autoxidation and protein modification

The potential role of 'autoxidative glycosylation' in diabetes

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Monosaccharide autoxidation (a transition metal-catalysed process that generates H_2O_2 and ketoaldehydes) appears to contribute to protein modification by glucose *in vitro*. The metal-chelating agent diethylenetriaminepenta-acetic acid (DETAPAC), which inhibits glucose autoxidation, also reduces the covalent attachment of glucose to bovine serum albumin. A maximal 45% inhibition of covalent attachment was observed, but this varied with glucose and DETAPAC concentrations in a complex fashion, suggesting at least two modes of attachment. The extent of inhibition of the metal-catalysed pathway correlated with the extent of inhibition of glycosylation-associated chromo- and fluorophore development. DETAPAC also inhibited tryptophan fluorescence quenching associated with glycosylation. Conversely, ketoaldehydes analogous to those produced by glucose autoxidation, but generated by ^{60}Co irradiation, bound avidly to albumin and accelerated browning reactions. It is therefore suggested that a component of protein glycosylation is dependent upon glucose autoxidation and subsequent covalent attachment of ketoaldehydes. The process of glucose autoxidation, or ketoaldehydes derived therefrom, appear to be important in chromophoric and fluorophoric alterations. It is noted, consistent with these observations, that the chemical evidence for the currently accepted 'Amadori' product derived from the reaction of glucose with protein amino groups is consistent also with the structure expected for the attachment of a glucose-derived ketoaldehyde to protein. The concept of 'autoxidative glycosylation' is briefly discussed in relation to oxidative stress in diabetes mellitus.

INTRODUCTION

Hyperglycaemia, the primary clinical manifestation of diabetes, is associated with development of certain of the diabetic complications (Pirart, 1978; Brownlee & Cerami, 1981). Various cytotoxic roles for glucose have therefore been proposed, including the slow nonenzymic glycosylation of proteins (Brownlee & Cerami, 1981). This produces new protein-bound chromo- and fluorophores and may lead to protein conformational and functional alterations (Brownlee *et al.*, 1984; Harding, 1985). The significance of the process to human diabetes is not clear but recently the extent of tissue browning (measured by collagen-associated fluorescence in skin biopsies) has been correlated with the incidence and severity of several complications in human diabetics (Monnier *et al.*, 1986).

Little attention has been given to the reducing properties of monosaccharides in relation to diabetes. In addition to direct glycosylation reactions, monosaccharides can enolize and thereby reduce molecular oxygen under physiological conditions, yielding α-ketoaldehydes, H_2O_2 and free radical intermediates (Scheme 1) (Wolff *et al.*, 1984; Thornalley *et al.*, 1984). The occurrence of this process *in vivo* could contribute to the elevated levels of plasma peroxides found in diabetics (particularly those with complications) (Nishigaki *et al.*, 1981; Sato *et al.*, 1979) and may contribute to protein modification reactions performed with glucose *in vitro* (Wolff, 1986). Such studies frequently involve the long-term incubation of protein with glucose followed by investigation of structural or functional alterations caused by such treatment (Harding, 1985). As ketoaldehydes analogous to those derived from autoxidation can bind to lysine groups of proteins and form novel chromophores (McLaughlin *et al.*, 1980; Gascoyne, 1980) it is conceivable that glucose autoxidation might contribute to monosaccharide attachment, chromophore development and protein oxidative damage. In that case, the extent of nonenzymic glycosylation could be a reflection of cumulative oxidative stress. The present study was therefore undertaken to assess the contribution of glucose autoxidation to protein glycosylation and associated protein chromophoric and fluorophoric alterations.

METHODS

Glycosylation studies *in vitro*

Bovine serum albumin (BSA; Boehringer Mannheim; Fraction V) was incubated at a concentration of 10 mg/ml (150 μM) with glucose (Aldrich; Gold) containing 1–2.5 μCi of [U-^{14}C]glucose/ml (Amersham) under sterile conditions. Purchased batches of [U-^{14}C]-glucose were diluted into an excess of unlabelled glucose and treated with activated charcoal. Such solutions contained no substances able to react with Girard-T reagent or thiobarbituric acid, prior to incubation at 37 °C, and the absolute incorporation of radioactive

Abbreviations used: DETAPAC, diethylenetriaminepenta-acetic acid; BSA, bovine serum albumin.

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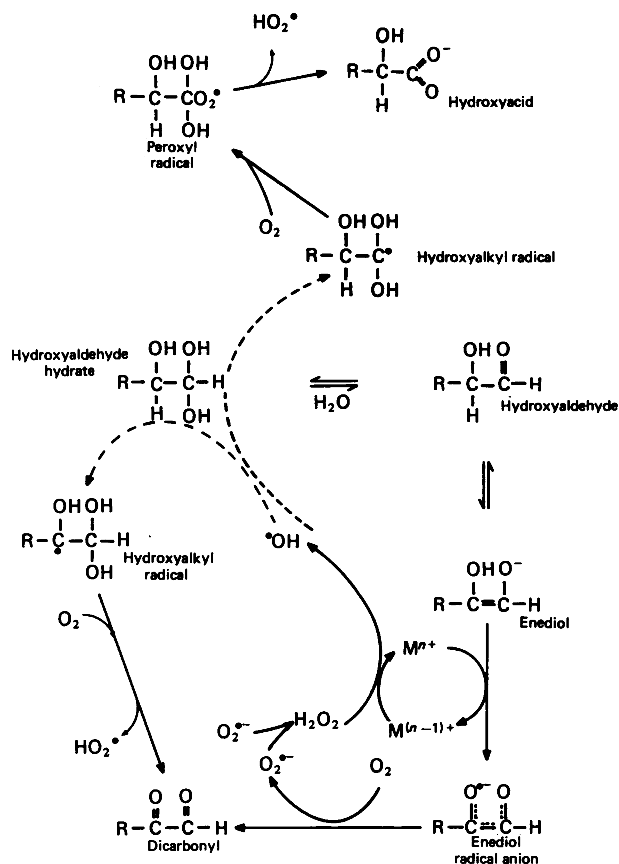


Scheme 1. Monosaccharide autoxidation

Compounds with the α-hydroxyaldehyde structure enolize and reduce transition metal and oxygen sequentially, forming α-ketoaldehydes as major products. H_2O_2, formed by superoxide ($O_2^{\cdot-}$) dismutation, regenerates the catalytic metal oxidation state and produces hydroxyl radical ($^{\cdot}OH$). The latter reactive species is scavenged giving rise to α-hydroxyacids and further ketoaldehydes.

material into protein (mol/mol) was consistently much lower than that reported by Trueb *et al.* (1980) in batches of radioactive glucose shown to contain protein-reactive contaminants. The buffer employed was 100 mM-potassium phosphate buffer, pH 7.4, unless otherwise indicated. Reagents such as $NaCNBH_3$ (Aldrich) and DETAPAC (Aldrich) were included in some incubations and all reagents were titrated to the appropriate pH before mixing. Incubations did not fluctuate by more than 0.2 pH units up to 3 weeks. At selected time intervals 1 ml aliquots were withdrawn from incubation mixtures (aseptically where appropriate), precipitated by addition of 200 μl of 30% (w/v) trichloroacetic acid and centrifuged at 10000 *g* for 3 min. The resultant pellet was washed twice in 5% trichloroacetic acid by resuspension, vortex-mixing and centrifugation, redissolved in 200 mM-potassium phosphate, pH 7.4, by incubation at 37 °C for 15 h, and reprecipitated with 5% trichloroacetic acid. The supernatant was retained. The pellet was washed once more in 5% trichloroacetic acid, redissolved in formic acid at room temperature, and counted down to 3% error. Very similar results were obtained if the pellet was redissolved in 200 mM-NaOH prior to counting or if exhaustive dialysis was used for removal of non-bound or easily dissociable glucose from protein. Similar results were also obtained if DETAPAC was used as supplied or recrystallized. The amount of radioactivity present in the supernatant after the second precipitation was less than 1.5% of that present in the pellet derived therefrom, thus demonstrating the efficacy of the washing procedure. Extending the potassium phosphate buffer incubation time from 15 to 36 h caused the proportion of supernatant radioactivity to increase to 2% of that found in the pellet. This is consistent with the very slow reversibility of dialysis-stable glycosylation products (Mortensen & Christophersen, 1983) but is too slow to introduce significant variation in our system. There was little increase in measurable protein in the trichloroacetic acid-soluble fraction over the time course of the experiments, and it did not vary with different glycosylation conditions. Exact conditions for each experiment are described in the legends to Figures. Data were calculated as the means ± S.D. of three or four determinations and the coefficient of variation within each set was less than 4%.

Fluorescence and absorbance measurements and glucose oxidation

Fluorescence and absorbance measurements were obtained on Perkin–Elmer LS3 and 555 machines, respectively. α-Ketoaldehyde concentrations were estimated by adduct formation with the Girard T reagent, trimethylaminoacetohydrazide chloride (Sigma, but recrystallized from hot ethanol) using the pH 9.2 method described by Mitchel & Birnboim (1977). We assumed an absorption coefficient for the D-*arabino*-hexosulose adduct of 1.88×10^4 at 325 nm [that found for the glyoxal adduct (Mitchel & Birnboim, 1977)] and little contribution from the β-, γ- or other dicarbonyls anticipated from radiation studies of glucose oxidation (Schuchmann & von Sonntag, 1977). H_2O_2 was estimated by the horseradish peroxidase-catalysed oxidation of 4-aminoantipyrine/phenol described by Frew *et al.* (1983). Glucose oxidation products were generated by irradiation of a 250 mM solution of glucose (containing trace [^{14}C]glucose) in air-saturated potassium phosphate buffer (10 mM, pH 7.4) in the 2000 Ci cobalt source at Brunel (for further details see Wolff & Dean, 1986). The yield of D-*arabino*-hexosulose was calculated from the reported *G* value (Schuchmann & von Sonntag, 1977) and corresponded well with the value obtained using the Girard T reagent and the absorption coefficient assumed.

RESULTS

Glucose autoxidation: dependence upon transition metal

Monosaccharide autoxidation is a metal-catalysed process which can be inhibited by metal chelating agents and accelerated by phosphate anions, probably because of acid/base catalysis of aldehyde dehydration/enolization (Wolff *et al.*, 1984; Thornalley *et al.*, 1984). The accumulation of ketoaldehyde within a phosphate-buffered glucose solution increases with increasing glucose concentration, but is a slow process. Only about 10 μM-ketoaldehyde accumulates in a 25 mM glucose solution after 72 h (Fig. 1). The addition of Cu^{2+} (10 or 100 μM) does not accelerate ketoaldehyde formation but DETAPAC causes almost complete inhibition, at least in the absence of added transition metal (Fig. 1). The production of ketoaldehyde from solutions of glucose

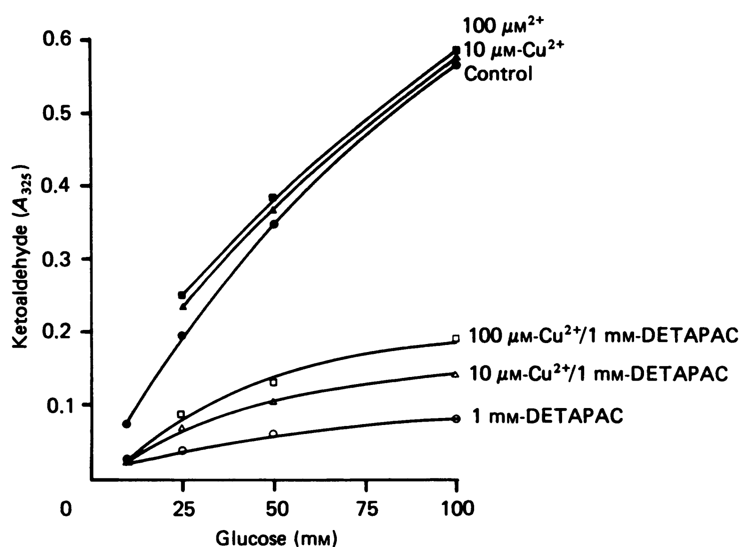


Fig 1. Metal catalysis of glucose-derived ketoaldehyde formation

Glucose solutions were incubated in 100 mM-potassium phosphate, pH 7.4, at 37 °C under sterile conditions for 4 days. Aliquots were withdrawn for reaction with Girard T reagent. Data are the means of three determinations. S.D. values are omitted for clarity in this and other Figures, but lay within 4% of the mean.

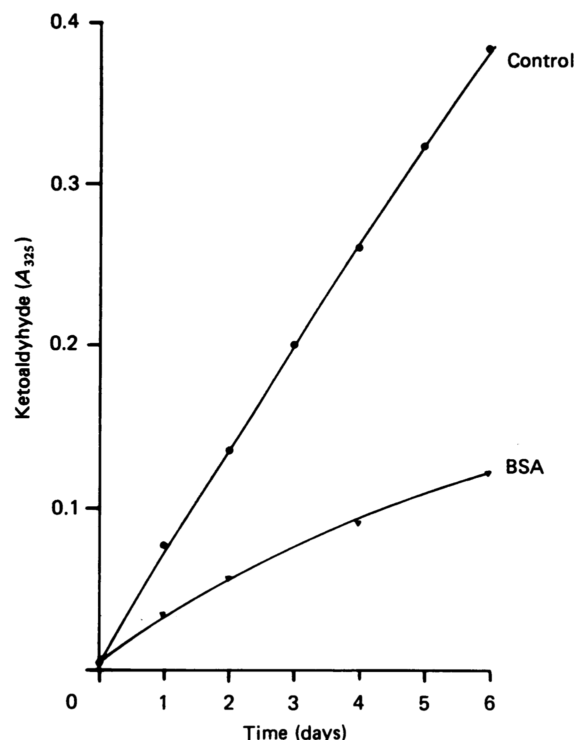


Fig. 2. Bovine serum albumin decreases the concentration of detectable ketoaldehydes

Glucose (25 mM) was incubated in the presence and absence of BSA (5 mg/ml) in 100 mM-potassium phosphate buffer, pH 7.4, for 6 days at 37 °C.

and DETAPAC on addition of Cu^{2+} indicates that complexes of Cu^{2+} and DETAPAC retain some catalytic activity (Fig. 1), as also observed for the analogous situation of ascorbate oxidation (Martell, 1980). H_2O_2 can be detected in incubated solutions of glucose, but not at concentrations stoichiometric with detected keto-aldehyde. For example, in an incubated solution of 25 mM-glucose containing 25 μM-ketoaldehyde, only 5 μM-H_2O_2 could be measured, presumably because H_2O_2 is both generated and consumed in the autoxidative process, as also observed for glyceraldehyde autoxidation (Wolff *et al.*, 1984; Thornalley *et al.*, 1984).

A metal-catalysed process contributes to protein–glucose binding

The idea that glucose autoxidation contributes to protein glycosylation is supported by the finding that BSA lowers the concentration of ketoaldehyde detectable in an incubated glucose solution (Fig. 2) and that DETAPAC inhibits the covalent attachment of trichloroacetic acid-precipitable label to BSA (Fig. 3). Conversely, if the glucose solution contains a small amount of hexosulose (about 50 μM, generated by irradiation) the initial rate of attachment is substantially greater but soon returns to that seen with unirradiated glucose (Fig. 3). The addition of Cu^{2+} produces no increase in extent of attachment, and this suggests that, like monosaccharide autoxidation, the component which appears to require metal catalysis is saturated with respect to transition metal. This component may reflect covalent attachment of hexosulose produced by glucose autoxidation. After 6 days incubation of 25 mM-glucose with 150 μM-BSA, the concentration of trichloroacetic acid-precipitable glucose is approx. 110 μM, and in the range expected for some contribution from autoxidation products. The extent of monosaccharide attachment increases with phosphate concentration, and phosphate also catalyses the generation of ketoaldehyde from glucose in a parallel fashion (Fig. 4). Fig. 3 also shows the extent of glucose attachment in the presence of 25 mM-$NaCNBH_3$. This reagent selectively reduces Schiff's bases and thus provides an indication of total Schiff base formation during the time course of the experiment.

Metal-catalysed and metal-independent glycosylation?

The binding of glucose to BSA and its inhibition by DETAPAC has a peculiar dependence upon glucose and DETAPAC concentrations. As the concentration of glucose is increased, the component which can be inhibited by DETAPAC (at a constant concentration) decreases (Fig. 5), perhaps because glucose is able to form complexes with transition metals from which chelating agents can only remove metal slowly (Aruoma *et al.*, 1987). Conversely, at a fixed glucose concentration, varying the concentration of DETAPAC causes varying degrees of inhibition of the binding (Fig. 6). DETAPAC at 1 mM caused less inhibition (33%) than did 100 μM-DETAPAC (45%). The reasons for this biphasic effect are not clear but it may be taken as evidence for at least two routes of glucose attachment, one dependent upon trace metals, and one which is metal-independent but subject to acid-base catalysis. Thus, at the lower concentrations DETAPAC sequesters metal and inhibits the 'autoxidative' pathway; at higher concentrations inhibition of the former pathway may be concealed by acid-base catalysis of the 'non-autoxidative' route. Consistent with this idea is the observation that

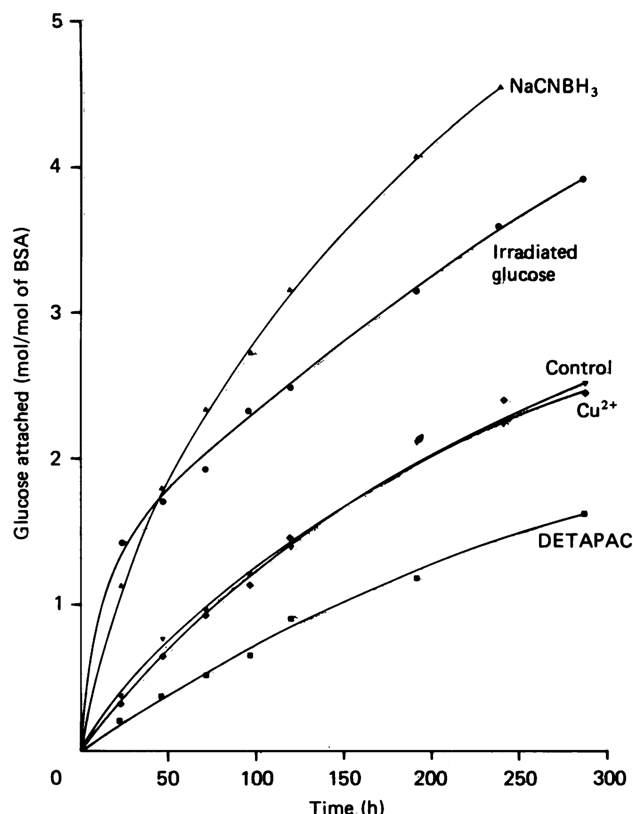


Fig. 3. Modification of glycosylation rates by DETAPAC, $NaCNBH_3$ and glucose oxidation

Glucose (25 mM) was incubated with BSA (10 mg/ml) in 100 mM-potassium phosphate buffer, pH 7.4. Trichloroacetic acid-precipitable radioactivity was measured as described in the Methods section. Concentration of $NaCNBH_3$ was 25 mM; DETAPAC, 500 μM; Cu^{2+}, 10 μM; ketoaldehyde produced by irradiation of glucose, 50 μM.

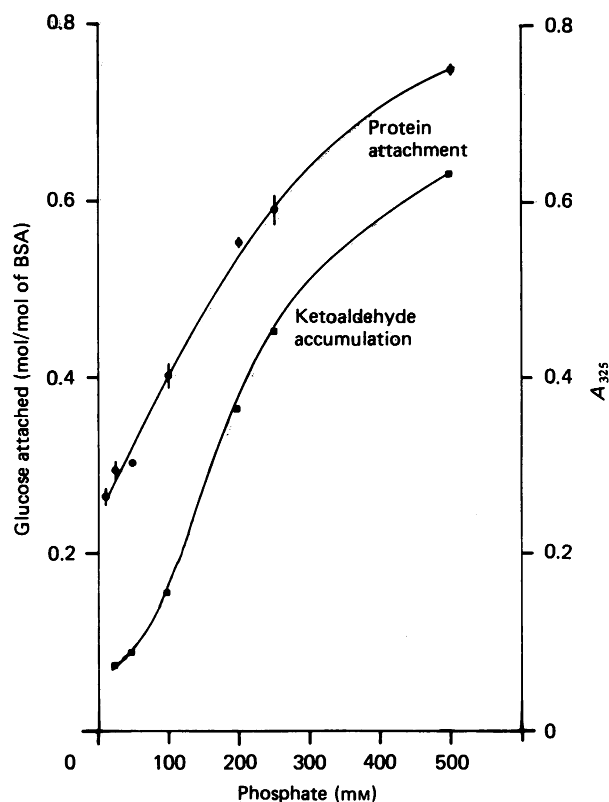


Fig. 4. Parallelism between effects of phosphate concentration upon ketoaldehyde formation and glycosylation

Glucose (25 mM) was incubated in the presence and absence of BSA (10 mg/ml) for 48 h at pH 7.4.

carboxylate groups, in the form of acetate, slightly accelerated binding at concentrations equal to the concentration of carboxylate provided by DETAPAC (Fig. 6). The less affine transition metal chelating agent EDTA also produced inhibition of monosaccharide attachment, but less than that seen with DETAPAC at the same concentration (Fig. 6).

Protein absorbance and fluorescence changes

The effects of glucose binding, and its manipulation by chelating agents, were examined with respect to chromophoric and fluorophoric properties of the protein. Incubation of BSA with glucose leads to a characteristic 'browning' (caused by a tail into the visible of an absorbance shoulder) and an increase in absorbance at 280 nm (Fig. 7). DETAPAC decreased the extent of development of this colour (Fig. 7), and this was proportional to the extent of inhibition of monosaccharide attachment at various concentrations of glucose (Fig. 8). For example, in the presence of 500 mM-glucose, 1 mM-DETAPAC caused an 14% inhibition of binding (Fig. 8), and a 38% inhibition of colour development (as measured at 320 nm), whereas with 25 mM-glucose, 1 mM-DETAPAC inhibited attachment by 30% and associated colour development by 60% (Fig. 8). The DETAPAC-inhibitable (metal-catalysed)

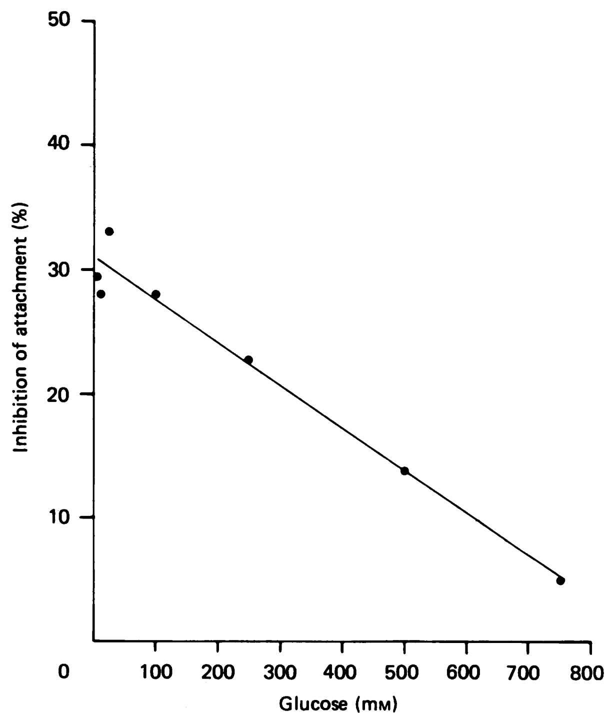


Fig. 5. Inhibition of glycosylation by DETAPAC (1 mM) at different concentrations of glucose

Inhibition of attachment decreases with increasing concentration of glucose with 1 mM-DETAPAC. See Fig. 6.

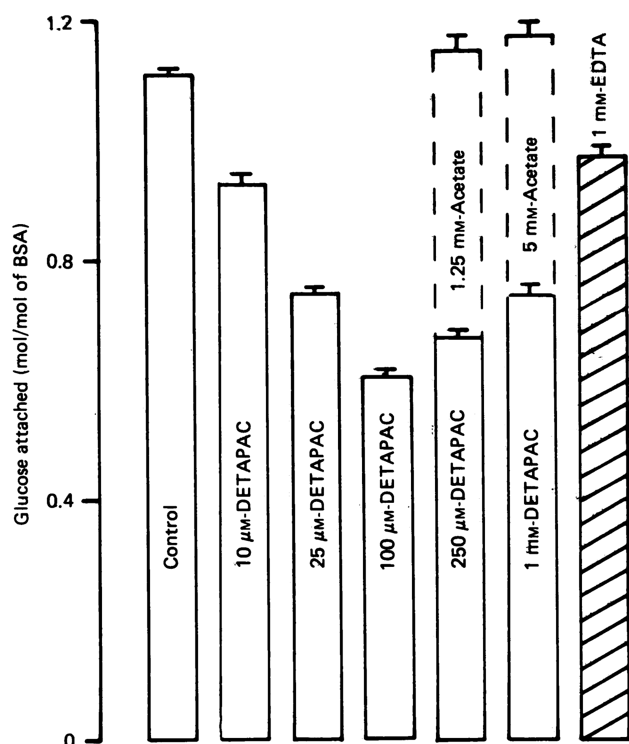


Fig. 6. Anomalous effect of DETAPAC concentration upon inhibition of attachment

BSA (10 mg/ml) was incubated for 4 days with 25 mM-glucose in 100 mM-potassium phosphate buffer. Broken bars indicate the efffect of equivalent concentrations of acetate at the DETAPAC concentration beneath.

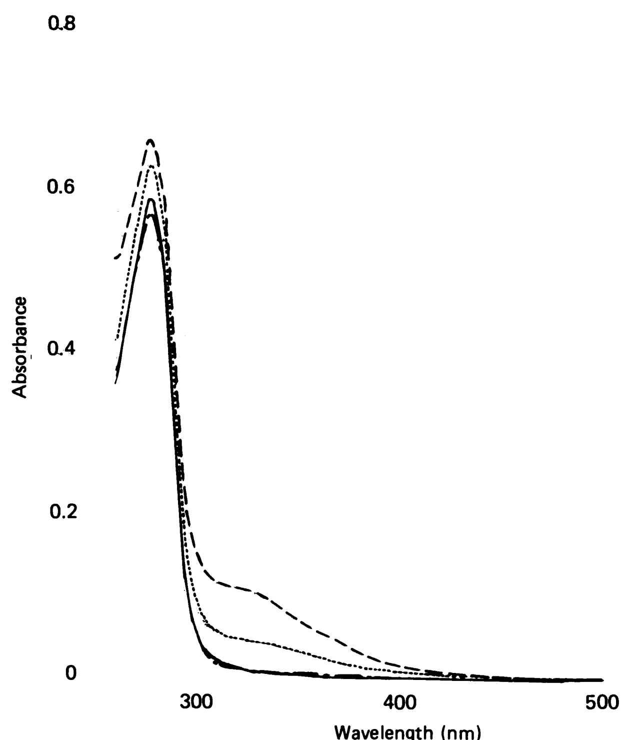


Fig. 7. Modification of glycosylation-associated chromophore development

BSA (10 mg/ml) was incubated with 25 mM-glucose for 15 days and dialysed before scanning. ----, Protein incubated with glucose alone; ······, protein incubated with glucose and DETAPAC (1 mM); —·—·—, protein incubated with glucose and $NaCNBH_3$; ——, control incubated protein.

component of glycosylation appears to be responsible, at least in part, for chromophore development. Incubation of BSA with glucose containing ketoaldehyde produced by irradiation accelerated browning relative to non-irradiated glucose (results not shown). In contrast, despite the acceleration of glycosylation, no new chromophores developed in the presence of $NaCNBH_3$, demonstrating that chromophore development requires Schiff bases (Fig. 7). EDTA also modified glycosylation-associated chromophore development (results not shown).

Similar trends were observed in the case of protein fluorescence changes. Incubation of glucose with protein leads to the development of a new fluorophore (excitation maximum 350 nm; emission maximum 415 nm) and this is decreased by DETAPAC (Fig. 9). EDTA also causes some inhibition of development of this fluorophore. The almost total inhibition seen with $NaCNBH_3$ again suggests an important role for Schiffs bases (Fig. 9).

Incubation of glucose with protein is known to cause tryptophan fluorescence quenching and this has been ascribed to conformational change and consequent changes in the tryptophan microenvironment (Shaklai *et al.*, 1984). Incubation of 25 mM-glucose with BSA for 20 days leads to a substantial decrease in fluorescence at 350 nm (excitation 275 nm) but this was largely prevented if DETAPAC was included in the incubation mixture. EDTA produced a smaller, but significant, inhibition of quenching (Fig. 10). Conformational change is thus

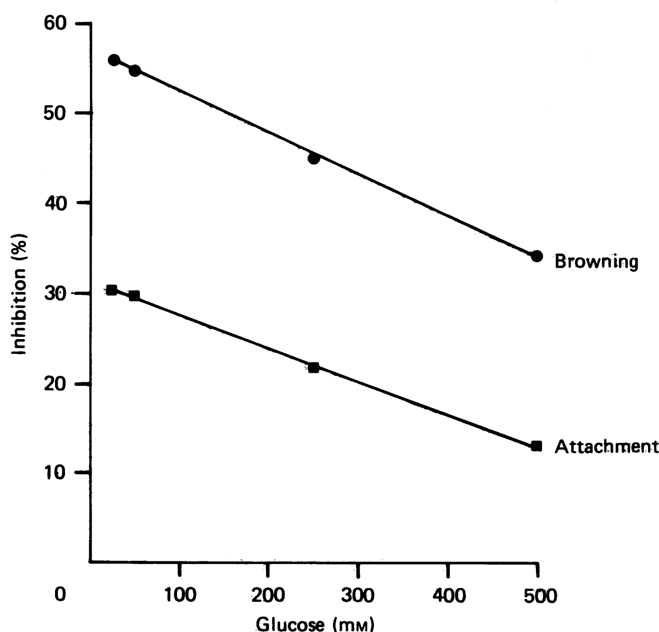


Fig. 8. Parallelism between inhibition (by 1 mM-DETAPAC) of glycosylation and browning

Incorporation of label from 25 mM-glucose into trichloroacetic acid-precipitable protein was measured as described in the Methods section and the legend to Fig. 3. Browning was measured by using the absorption at 320 nm.

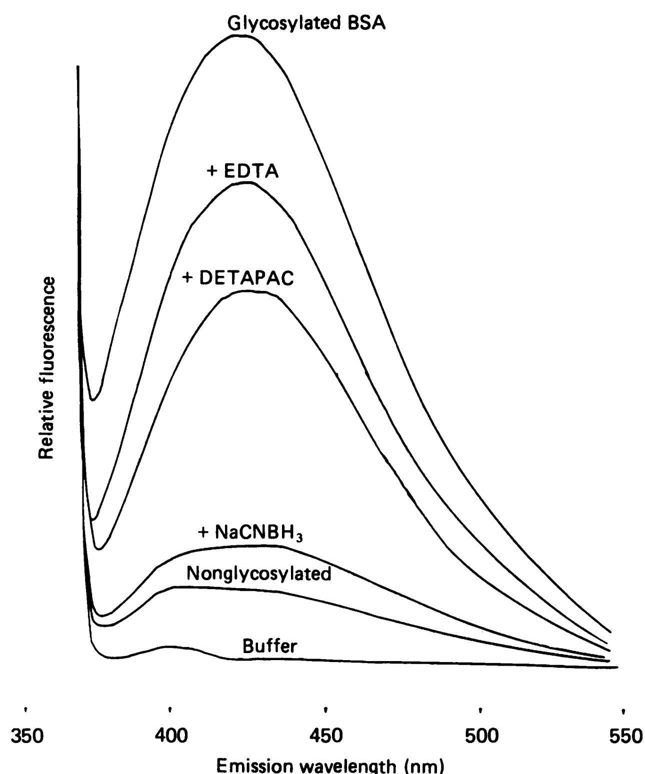


Fig. 9. Effect of chelating agents and $NaCNBH_3$ upon fluorescence development

DETAPAC and EDTA, 1 mM; $NaCNBH_3$, 25 mM; BSA, 10 mg/ml; glucose, 25 mM; potassium phosphate, pH 7.4, 100 mM; 37 °C for 15 days. Excitation wavelength 350 nm.

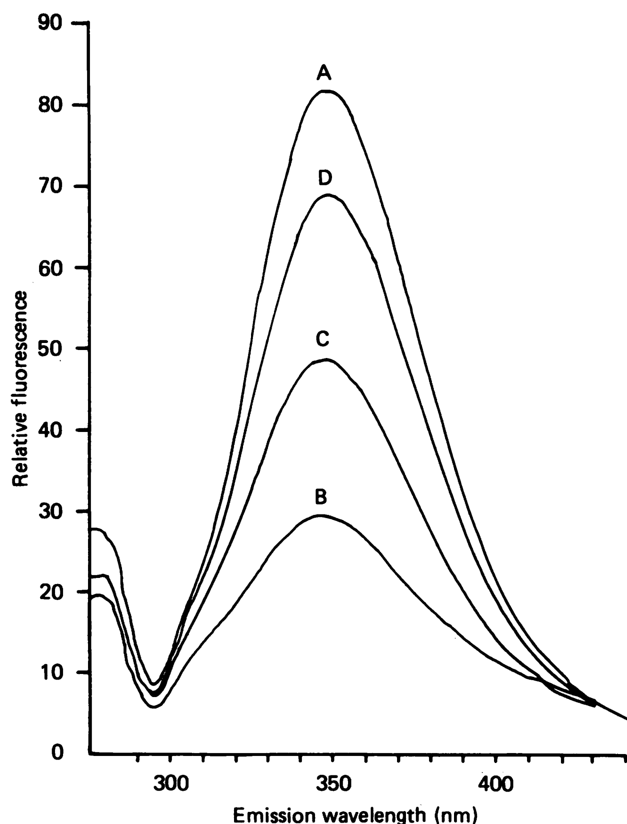


Fig. 10. Inhibition of glycosylation-induced fluorescence quenching by chelating agents

Excitation wavelength 275 nm; other conditions as for Fig. 9. A, control; B, glucose; C, glucose+EDTA; D, glucose+DETAPAC.

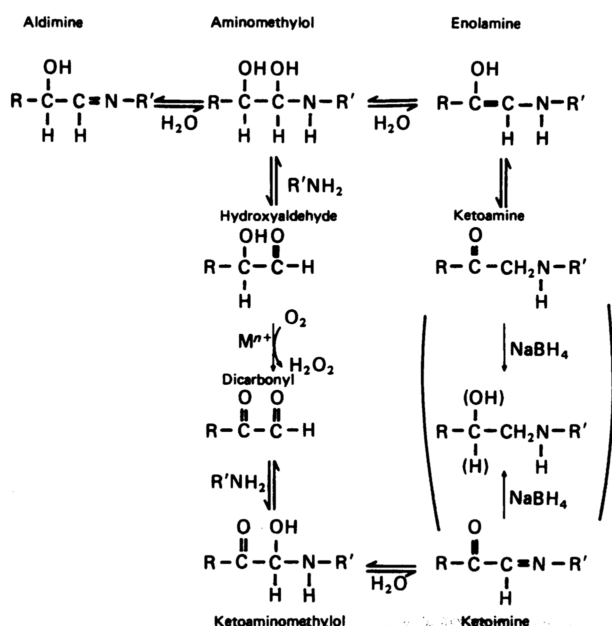
associated with metal-catalysed reactions. $NaCNBH_3$ also prevented tryptophan fluorescence quenching (results not shown).

DISCUSSION

Non-enzymic glycosylation and subsequent browning reactions have been implicated in pathogenesis related to hyperglycaemia in diabetes and in ageing (Editorial, 1986; Harding, 1985). Macromolecular conformation changes, altered receptor recognition and crosslinking reactions have been proposed (Brownlee *et al.*, 1984) and there is considerable interest in the nature of the fluorescent products generated by glycosylation reactions. One such product, the fluorescent chromophore 2-(2-furoyl)-4(5)-(2-furanyl)-1*H*-imidazole, has recently been isolated after acid hydrolysis of albumin heavily glycosylated *in vitro* (Pongor *et al.*, 1984). Our studies indicate that glucose autoxidation, or related metal-catalysed autoxidative processes, contribute to glycosylation-related protein chromophore development.

Glucose autoxidizes *in vitro* and generates H_2O_2 and ketoaldehyde compounds. The formation of ketoaldehyde is inhibited by DETAPAC (indicating the catalytic importance of transition metals), although the provision of exogenous Cu^{2+} produces no further increase. In the case of glyceraldehyde autoxidation the lack of acceleration caused by added transition metal was ascribed to the very low concentration of available (autoxidizable) enediol relative to adventitious transition metal (Wolff *et al.*, 1984; Thornalley *et al.*, 1984). Similar considerations appear to apply in the case of glucose.

A proportion of the monosaccharide attachment to BSA in the presence of glucose is inhibited by DETAPAC. There appears to be a coincidence between this component and the formation of chromo- and fluorophores. Like monosaccharide autoxidation, covalent attachment is not accelerated by added Cu^{2+}. These results, taken together with the ability of glucose-derived ketoaldehyde to bind to BSA and accelerate browning reactions, suggest that products derived from glucose autoxidation are responsible for a substantial part of monosaccharide attachment to protein and the associated browning and fluorescence. The ketoaldehyde compounds themselves, or oxidants derived from glucose autoxidation, may be responsible for the decrease in tryptophan fluorescence. Peroxide and free radicals are known to cause protein conformational change and altered fluorescence (Wolff & Dean, 1986; Wolff *et al.*, 1986). In this context it is interesting that little protein fluorescence or absorbance change was observed after glycosylation in the presence of $NaCNBH_3$. This reducing agent accelerates attachment and probably acts as an antioxidant, protecting the protein, and thus dissociates incorporation of monosaccharide *per se* from protein damage associated with the exposure of macromolecules to autoxidizable monosaccharide, at least under these conditions.



Scheme 2. Initial reactions of monosaccharides in the presence of protein

The glycosylation of amino groups by glucose aldehyde (see Scheme 2) proceeds via aminomethylol formation followed by dehydration reactions yielding a primary Schiff's base and, after enolization, the 'Amadori product' (Hodge, 1955). The Amadori structure is proposed to be a major stable glycosylation product *in vivo* (Cerami, 1987). Critical evidence for this is provided by the treatment of protein glycosylated *in vivo* with the less selective reducing agent $NaBH_4$. This traps protein-bound monosaccharide as glucitol and mannitol amine conjugates, consistent with the ketoamine structure (Bookchin & Gallop, 1968), but also consistent with the structure expected for the Schiff's base produced from a ketoaldehyde and amino group (Scheme 2). Beswick & Harding (1985) tried to distinguish between the latter possibilities using a model system consisting of glycine, glucose and $NaCNBH_3$ coupled with mass spectrometry but, under their experimental conditions, could find evidence only for formation of the glucose aldehyde Schiff's base. They found little evidence of molecular fragments of size/charge ratio consistent with the putative dicarbonyl adduct nor Amadori rearrangement product. Attempts to detect hexosulose in a solution of autoxidizing glucose by g.l.c.–m.s. of tetramethylsilyl esters were also unsuccessful (Beswick & Harding, 1986), presumably because of the low concentrations present. Nevertheless, ketoaldehydes are produced slowly and at concentrations in the range necessary for contribution to total protein-bound monosaccharide.

Glucose autoxidation may thus contribute to stable protein-bound adducts. This or a related metal-catalysed process, such as further autoxidation of protein-bound dicarbonyls, appears to contribute to browning and associated tryptophan fluorescence quenching. It has been observed previously that the ketoaminomethylol intermediates initially formed on reaction of dicarbonyls with protein (see Scheme 2) are able to enolize to the enediolamine and autoxidize in analogous fashion to the parent monosaccharides (Gascoyne, 1980). These reactions occur rapidly at physiological pH (S. P. Wolff & R. T. Dean, unpublished work), in contrast with the autoxidation of enolamines which is reported to take place only above pH 10 (Johnson *et al.*, 1983).

The major primary product of the reaction of monosaccharides with protein amino groups is the aldimine (Scheme 2). This is also formed from monosaccharides such as 2-deoxyglucose, which cannot enolize, nor form aminomethylol intermediates capable of undergoing 1,2-dehydration to enolamines and hence to Amadori products. $NaCNBH_3$ selectively reduces aldimines and produces similar rates of incorporation of glucose and 2-deoxyglucose into protein (Beswick & Harding, 1985). In the absence of this reducing agent 2-deoxyglucose produces no dialysis-stable glycosylation products (Mortensen & Christophersen, 1983).

The relative contributions of glucose autoxidation and the Amadori rearrangement to glycosylation *in vivo* may differ from those observed in these experiments *in vitro*. Here, inhibition of binding by chelating agents was used as an index of the glucose autoxidative component. This approach requires the use of metal chelators to sequester the trace amounts of metal required for catalysis of autoxidation. It may be the case, however, that copper bound to high-affinity sites on BSA cannot be removed by chelating agents and may continue to catalyse enediol oxidation (Marx & Chevion, 1986). Cu^{2+} complexed with DETAPAC is still catalytic in ascorbate oxidation, although less efficiently than free transition metal (Martell, 1980). Furthermore, DETAPAC, despite high affinity, can only remove transition metals from monosaccharide slowly (Aruoma *et al.*, 1987) and this may explain the lesser degree of inhibition of glycosylation by DETAPAC in the case of very high, but physiologically irrelevant, levels of glucose. DETAPAC may therefore be only partially successful in preventing autoxidation at high concentrations of glucose and in the presence of protein, leading to an underestimate of the metal-catalysed glycosylation pathway. The 45% inhibition of covalent attachment observed with 25 mM-glucose and 100 μM-DETAPAC suggests that at least 45% of glucose attaches via the autoxidative route.

Protein–monosaccharide interactions have been extensively researched in relation to both food chemistry, and the pathogenesis of the diabetic complications, but are still poorly understood. The major difficulty is the isolation of modified amino acids from protein without the use of powerful reducing agents (to stabilize adducts) nor extremes of pH, which may cause the formation of unphysiological products. Modification of such reactions *in vitro*, despite some complications, may thus provide useful chemical insight and if 'autoxidative glycosylation occurs *in vivo*, then monosaccharide autoxidation may be a contributory factor to the increased oxidative stress observed in diabetes mellitus (Wolff, 1987).

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