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PROTEIN CROSS-LINKING IN THE MAILLARD REACTION

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in the discipline of Biology

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ABSTRACT

Post-Amadori formation of Maillard products was studied during *in vitro* sugar-free ageing of pre-glycated and native proteins. Glucose was used as the glycating sugar throughout.

Protein cross-links and fluorophores formed during sugar-free ageing of pre-glycated lysozyme with native β-lactoglobulin. Fluorescence development was associated with a glycation threshold; this was not observed for cross-linking. Similar results had been reported with fructose in the same system (Liggins and Furth, 1996).

Glycation-dependent cross-linking between lysozyme and HSA was demonstrated biochemically. Cross-linking of glycated HSA to native rat tail tendon collagen was demonstrated by X-ray diffraction. Glycated rat tail tendon formed collagen/collagen cross-links more readily than cross-linking with native HSA. Formation of mixed protein cross-links involving HSA *in vitro* demonstrates the potential for glycation-dependent cross-linking of serum proteins to structural protein *in vivo*.

The periodate assay, used to detect putative Amadori product, was found to detect a broader spectrum of compounds. This comprised glycation-dependent and glycation-independent components. Formation of the larger, glycation-dependent fraction of periodate positive material (PPM) was associated with a threshold effect. The smaller, glycation-independent fraction formed from native and glycated

proteins during sugar-free ageing. Glycation-dependent PPM was initially protein-bound, but became susceptible to dialysis during sugar-free ageing. This may have included small dicarbonyl compounds. PPM formation was inhibited by aminoguanidine.

Inhibitor studies showed that aminoguanidine reduced glycation-dependent cross-linking and fluorescence when present together with free sugar. This inhibitory effect continued during sugar-free ageing of pre-glycated lysozyme and native β-lactoglobulin after removal of both free sugar and free inhibitor. Aminoguanidine present without free sugar unexpectedly enhanced the formation of fluorescent and cross-linked products on both native and glycated protein, particularly in the presence of lipid. This was attributed either to pro-oxidant properties of aminoguanidine, or to slower reactions of aminoguanidine with protein-bound monocarbonyl groups after depletion of free and protein-bound dicarbonyl groups. It is not clear whether the cross-linked and fluorescent products enhanced by aminoguanidine are Maillard products or different compounds with similar characteristics. The results raise concerns regarding the use of aminoguanidine *in vivo* under conditions in which low sugar concentrations may occur.

ABBREVIATIONS

AG	aminoguanidine
AGE	advanced glycation end-product
AGE-BSA	bovine serum albumin, modified by advanced glycation end-products
ALE	advanced lipid oxidation end-product
apoB	apoprotein B
arg	arginine
β-2-m	β_2 microglobulin
βLG / BLG	β-lactoglobulin
BSA	bovine serum albumin
CEL	N(ε)-(carboxyethyl)lysine
CML	N(ε) -(carboxymethyl)lysine
3DG-AIm	3-deoxyglucosone/arginine imidazolone
DCCT	Diabetes Control and Complications Trial Research Group
DOLD	3-deoxyglucosone-lysine dimer (imidazolium cross-link)
DRA	dialysis-associated amyloidosis
DTPA	diethylene triamine pentaacetic acid
EDTA	ethylene diamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ESRD	end-stage renal disease
ex/em	excitation/emission wavelengths (fluorescence)
FFI	(2-furoyl)-4-(5)-(2-furanyl)-1H-imidazole
fFL	N(α)-formyl-N(ε)-fructoselysine
GOLD	glyoxal-lysine dimer (imidazolium cross-link)
his	histidine

HNE	4-hydroxynonenal
HSA	human serum albumin
LDL	low-density lipoprotein
lys	lysine
Lyz	lysozyme
MDA	malondialdehyde
MOLD	methylglyoxal-lysine dimer (imidazolium cross-link)
ox-LDL	oxidised low-density lipoprotein
PLP	pyridoxal-5-phosphate
PPM	periodate positive material
PUFA	polyunsaturated fatty acids
RNase	ribonuclease
RTT	rat tail tendon
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
UKPDS	United Kingdom Prospective Diabetes Study Group
v/v	volume/volume
w/v	weight/volume

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CHAPTER 1

PROTEIN CROSS-LINKING IN THE MAILLARD REACTION: A LITERATURE REVIEW

1.1 INTRODUCTION

The Maillard reaction may be explained in simple terms as the non-enzymatic reaction of reducing sugars with proteins. However, recent research increasingly shows this view to be a gross over-simplification. Rather than a single reaction or even a linear reaction sequence, the term refers to a complex cascade of consecutive and parallel steps. It leads ultimately to the accumulation of coloured, fluorescent and cross-linked proteins, known collectively as advanced glycation end-products (AGEs), also referred to as late Maillard products.

The reaction series has extensive implications for both food science and medicine. It is responsible for many of the pleasant flavours in food and in this context is a desirable occurrence. It is important - and entirely undesirable – in the context of human health since sugars, particularly glucose, in the bloodstream are in intimate and prolonged contact with long-lived proteins such as collagens. Since the formation of late Maillard products or AGEs is essentially irreversible, such proteins may accumulate modifications over time, with detrimental effects on protein conformation and function.

Protein turnover counteracts the accumulation to some extent, but cannot cancel it entirely. This process is thought to underlie some of the complications of diabetes and ageing (Wells-Knecht *et al.*, 1996; Furth, 1997). It has also been implicated in a growing number of other conditions including cataract (Beswick and Harding, 1987), atherosclerosis (Bucala *et al.*, 1994), uraemia (Niwa *et al.*, 1996), dialysis-related amyloidosis (Miyata and Maeda, 1996), Alzheimer's disease (Smith *et al.*, 1994) and osteoarthritis (TeKoppele *et al.*, 1998). The mechanisms underlying the Maillard reaction as it pertains to human health, and particularly to diabetic complications, is the focus of this study.

The initial reaction of the Maillard sequence is between an electronegative carbonyl group, as found in glucose in its straight chain form, and an electropositive amino group on protein. The latter may be either the N-terminal amino group or one in a side chain, typically the ε-amino group of lysine. The first product formed is an unstable aldimine or Schiff base. A wide range of reactions is possible following its formation. The traditional view is that the Schiff base undergoes a minimally reversible rearrangement over time to form a ketoamine, the Amadori product, which then reacts further to form largely uncharacterised late Maillard products (AGEs). However, recent evidence indicates the possibility of alternative fates for the Schiff base and it is becoming increasingly clear that the neat characterisation of protein plus sugar leading to Schiff base, leading to Amadori product, is probably no longer applicable (reviewed by Furth, 1997). What can be said is that there appear to be 'early' and 'late' Maillard reactions (Menzel *et al.*, 1997), although the assignment of specific reactions to either category is presently ambiguous. In general, early reactions, including formation of Amadori product, are dependent on the presence of free sugar or its derivatives and

are not necessarily dependent on the presence of oxygen (Fu *et al.*, 1994). Late reactions are independent of free sugar (Brighton and Furth, 1989), but are dependent on oxygen (Baynes, 1991; Fu *et al.*, 1994). The importance of carbonyl compounds, particularly dicarbonyls, in the Maillard reaction has received increasing attention. These may arise from autoxidation of sugars (Fu *et al.*, 1994; Wells-Knecht *et al.*, 1995a), from metal-catalysed oxidative processes (Baynes, 1994) and other reactions on early Maillard products (Glomb and Monnier, 1995; Zyzack *et al.*, 1995), or from free radicals such as peroxides arising from oxidation of protein or protein-associated lipid (Dean *et al.*, 1997). Oxidation and glycation (in the sense of directly sugar-dependent reactions) are closely interrelated. They share certain intermediates and may give rise to similar end-products, *e.g.* cross-linked, carboxymethylated or fragmented proteins (Fu *et al.*, 1996; Traverso *et al.*, 1997). It is therefore of little surprise that either process may enhance the other (Menzel *et al.*, 1997; Traverso *et al.*, 1997), and that separating them *in vivo* and even *in vitro* appears increasingly difficult and, indeed, artificial.

Although much effort has been invested in identifying late Maillard products chemically, only relatively few structures have been characterised to date and these represent but a small fraction of the total (Wells-Knecht *et al.*, 1996). Of these, the most is known about N(ε)-(carboxymethyl)lysine (CML) and pentosidine. Both have been shown to increase with age in various tissues and are increased in diabetics relative to age-matched controls (Dyer *et al.*, 1993; Schleicher *et al.*, 1997). The CML adduct on protein is stable, non-fluorescent and does not form cross-links, but does introduce charge changes on CML-modified proteins. Pentosidine is a fluorescent protein cross-link, also known to increase in tissues with age and diabetes or uraemia (Sell *et al.*,

1991, 1992). It remains to be clearly established whether these and other chemically characterised Maillard structures are causally related to diabetic pathology or whether they are simply markers of the underlying pathological process (Wells-Knecht *et al.*, 1996). Until such relationships can be clarified, less specific end-points may serve as useful indicators of functional impairment. As an example, protein cross-linking causes marked structural changes and may be linked *in vivo* with stiffening of collagen (Bailey and Kent, 1989) and entrapment of serum proteins (Michael and Brown, 1981; Brownlee *et al.*, 1986). This study concentrates on the 'functional' end-point of protein cross-linking, while bearing in mind the chemical nature both of possible cross-linking agents and of the cross-links themselves.

A wide variety of compounds have been tested for their ability to intervene in the Maillard reaction, and thereby prevent diabetic complications and possibly other conditions associated with accumulation of Maillard products. One which is presently nearing the end of clinical trials is the hydrazine compound, aminoguanidine. It has been shown to ameliorate diabetic complications in animal models (Jerums *et al.*, 1994) and to prevent accumulation of Maillard products in sugar/protein systems *in vitro* (Edelstein and Brownlee, 1992). However, its mechanism of action remains unclear. Aminoguanidine contains both guanidium and hydrazine moieties, leading to complex and as yet incompletely characterised interactions with Maillard products. Initially it was thought to block the carbonyl group on Amadori product, thereby preventing post-Amadori reactions (Brownlee *et al.*, 1986b). Subsequent studies have indicated that dicarbonyl compounds arising from the Amadori product or other early Maillard intermediates are more likely sites for its inhibitory action (Glomb and Monnier, 1995; Hirsch *et al.*, 1995a; Wells-Knecht *et al.*, 1995a). It also scavenges carbonyl products

of lipid oxidation (Requena *et al.*, 1992; Al-Abed and Bucala, 1997), and has been demonstrated to interfere in oxidation of both glycated and non-glycated lipoprotein, showing both pro-oxidant and classical antioxidant activity (Picard *et al.*, 1992; Philis-Tsimikas *et al.*, 1995; Skamarauskas *et al.*, 1996; Giardino *et al.*, 1998). Its *in vivo* targets may be expected to involve products from both sugar-dependent and oxygen-dependent Maillard reactions, and from oxidative reactions unrelated to the Maillard reaction. In this review hypotheses regarding the mode of action of aminoguanidine are evaluated in the light of present concepts of the Maillard reaction. Its roles in both glycation and oxidation are discussed and reasons for caution at its pharmaceutical application are highlighted.

Most studies of the Maillard reaction and of its inhibition by aminoguanidine have utilised experimental systems in which sugar and protein are present together throughout the study, simulating extreme and continuous hyperglycaemia *in vivo*. This approach holds a number of drawbacks. Firstly, it makes it difficult to investigate earlier sugar-dependent processes separately from later sugar-independent ones, and obscures the effect of aminoguanidine inhibition on early relative to later Maillard reactions. Secondly, and perhaps more importantly, it is a system of dubious physiological relevance. Poorly controlled diabetes – a proven risk factor for the development of complications in both type 1 and type 2 diabetes (DCCT, 1993; UKPDS, 1998) – is more likely to be characterised by fluctuating glycaemic status, having periods of high blood glucose interspersed with periods of near-normal blood glucose and even hypoglycaemia. A more relevant system would allow proteins to be subjected to brief exposure to elevated glucose, followed by evaluation of changes during subsequent sugar-free ageing. This is the approach adopted in the present

investigation, and the relatively few available studies of this nature are emphasised in this review.

The issues to be addressed in the course of this overview of recent thinking about the Maillard reaction are therefore as follows:

i) formation of Maillard products, with emphasis on protein cross-linking and candidate cross-linking agents (section 1.2);

ii) effects of aminoguanidine on the Maillard reaction, with reference to its impact on both glycation and oxidative processes (section 1.3); and

iii) Maillard reactions of pre-glycated proteins, and the impact of aminoguanidine thereon, in the absence of free sugar (section 1.4).

The hypotheses underlying this investigation are outlined in section 1.5.

It is difficult to standardise terminology completely, since authors tend to use collective terms for products of the Maillard reaction slightly differently. As far as possible, this review will adhere to the following definitions. *Maillard reaction* refers to the entire group of amine-carbonyl interactions between proteins, lipoproteins, lipids, reducing sugars and oxidative processes which results in the accumulation of irreversible protein modifications. *Glycation* refers to purely sugar-dependent steps in the Maillard reaction, occurring early in the process and excluding the role of oxidative reactions. *Advanced glycation end-products (AGEs)* is a term which is gradually being replaced in

the literature by *Maillard products*. Both refer to compounds formed on protein as long-term products of the Maillard reaction, but the latter term recognises that these products may arise independently of glucose, *e.g.* by oxidative reactions, and that they may be precursors to even later products rather than themselves being end-products. Where possible, Maillard products will be used instead of AGEs, but since many authors still refer to AGEs it is not always possible to maintain complete consistency in this.

1.2 GLYCATION AND OXIDATION IN THE MAILLARD REACTION

The Schiff base was defined as the first unstable product of glycation and the Amadori product as the first stable product (Hodge, 1953). At the same time a large number of intermediate compounds were described which form more slowly, but which arise prior to the formation of cross-linked, coloured and fluorescent late Maillard products. These intermediates include deoxyosones, furfurals, reductones, and low molecular weight carbonyl and dicarbonyl compounds (Hodge, 1953; Ledl and Schleicher, 1990). Many may be formed by either oxidative or non-oxidative pathways. Since oxidative stress is often cited alongside glycaemic stress as a factor in the development of complications in both ageing and diabetes, the relative contributions of glycation and oxidation to the overall effects of the Maillard reaction are vigorously debated in the research literature. The main arguments have been reviewed by Baynes (1996). He identified three prevailing theories: *glycation, autoxidative glycosylation* and *glycoxidation*. While all recognise oxidation as important in the formation of Maillard products, they differ in the sequence assigned to sugar-dependent and oxidative reactions. The three hypotheses

are discussed in the following section. The major pathways to formation of late Maillard products are shown schematically in Fig. 3.1. (This and all other figures are presented at the end of the chapter.)

1.2.1 Theories of oxidation in the Maillard reaction

In the *glycation* scheme, oxidation reactions occur subsequent to the reaction of sugar with protein. According to Hayashi and Namiki (1986), oxidative and free radical reactions may occur very soon after the initial reaction of glucose with protein, leading to fragmentation of the Schiff base prior to formation of the Amadori product (dubbed the Namiki pathway). The Schiff base fragmentation products yield reactive intermediates which contribute to the formation of Maillard products. Other authors see oxidation as occurring only later in the Maillard reaction. Hunt *et al.* (1993) argue that glycation of albumin *in vitro* precedes oxidation and cleavage of the protein. Support for glycation as a separate event preceding oxidation, derives from the observation that formation of reactive dicarbonyl deoxyglucosones *in vitro* can be explained by rearrangement and dehydration reactions of Amadori compounds without invoking oxidative chemistry (Feather, 1981; Ledl and Schleicher, 1990). Of the deoxyglucosones studied in model systems, 3-deoxyglucosone has been detected in human plasma and urine (Wells-Knecht *et al.*, 1994), indicating that this compound is of significance *in vivo*. Browning reactions of protein (*i.e.* formation of late Maillard products) by dicarbonyl sugars and sugar derivatives (3-deoxyglucosone, glyoxal, methylglyoxal, dehydroascorbate) has been shown in numerous studies to proceed rapidly in the absence of oxygen. However, browning of protein by hexoses such as

glucose proceeds very slowly under anaerobic conditions. It is this observation which lead to the proposal of oxidative involvement at the earliest stages of the Maillard reaction.

The *autoxidative glycosylation* theory proposes that the reaction of free sugars with oxygen to yield reactive intermediates, referred to as sugar autoxidation, precedes reaction with proteins. For instance, under oxidative conditions glucose decomposes to triose 'reductones' and then glyoxal (Baynes, 1996). Wolff and Dean (1987) observed that the combination of oxygen and transition metals greatly enhanced the reaction of glucose with proteins, leading to the hypothesis that metal-catalysed oxidation of glucose by molecular oxygen is necessary before modification of protein can occur, *i.e.* glucose autoxidation products – rather than glucose itself - interact with protein. Further support for autoxidative glycosylation was provided by Chace *et al.* (1991) and Fu *et al.* (1992, 1994), who demonstrated that autoxidative conditions were necessary for cross-linking and browning of collagen by glucose, and hence that oxidation of glucose itself was the rate-limiting step. Wells-Knecht *et al.* (1995a) identified arabinose and the important dicarbonyl glyoxal as products of autoxidation of glucose. When ascorbate is used as the glycating species instead of glucose, it well-established that oxidation to dehydroascorbate is required before browning reactions on protein can occur (Ortwerth and Olsen, 1988). Thus there is a strong case to be argued for the oxidative generation of dicarbonyl compounds from sugars at the earliest stages of the Maillard reaction.

Glycoxidation, a term coined by Baynes and co-workers, is seen as being the nett effect of glycation (exclusively sugar-dependent) and oxidative reactions, irrespective of

the order in which they occur. This approach recognises that it is difficult to determine which of glycation or oxidation occurs first, and sees oxidation acting as a 'fixative' of glycation damage to proteins (Baynes, 1991). Since the Amadori product is more readily oxidised than glucose, the autoxidation products of both may contribute to the formation of later products of Maillard reaction. Wells-Knecht *et al.* (1995b) have demonstrated that oxidation of the Amadori product and glucose proceeds simultaneously during *in vitro* incubation of protein and sugar, the relative rates of oxidation varying with the concentration of glucose, phosphate and metal ions. A further attraction of the glycoxidation approach is that it recognises that oxidative species, such as hydroperoxy radicals, hypochlorous acid and peroxynitrite, arising from routes such as lipid oxidation or other metabolic processes, are likely to contribute to the formation of Maillard products *in vivo*. In this, glycoxidation provides a link with the free radical theory of ageing (Baynes, 1996). By providing a link between glycaemic stress and oxidative damage, this correlates invitingly with the view of diabetes as an accelerated ageing of tissue proteins and other molecules.

1.2.2 Importance of dicarbonyl intermediates in the Maillard reaction

Irrespective of the sequence in which glycation and oxidation occur in the Maillard reaction, it has been firmly established that dicarbonyl compounds are important intermediates in both oxidative and non-oxidative pathways (Glomb and Monnier, 1995; Wells-Knecht *et al.*, 1995a, b; Zyzack *et al.*, 1995; Thornalley *et al.*, 1999). It appears to be the dicarbonyl grouping which is of importance, since a variety of dicarbonyl sugars and their derivatives are involved. The compounds presently receiving the

greatest attention as Maillard intermediates are 3-deoxyglucosone, glyoxal and methylglyoxal.

The degree to which oxidation is essential to generate the reactive dicarbonyl grouping depends on the carbohydrate glycating species. Deoxyglucosones are associated with non-oxidative formation of Maillard products by glucose, while glyoxal requires oxidative conditions for its formation from glucose or from glycated protein (Baynes, 1996; Thornalley *et al.*, 1999). Oxygen is a rate-limiting requirement for dicarbonyl formation from ascorbate, being necessary for its conversion to dehydroascorbate. On the other hand, pentoses and tetroses react at comparable rates in the presence or absence of oxygen. Glyoxal is formed as a common intermediate upon autoxidation of glucose, fructose and arabinose. Indeed, fructose reacts more rapidly in the Maillard reaction than glucose because it is more rapidly oxidised to glyoxal (Baynes, 1996).

Baynes and Thorpe (1999) have commented that diabetic complications may be indicative not of oxidative stress, but of carbonyl stress due to the generation of reactive carbonyl compounds arising from the interaction of glycation and oxidation chemistry. This may be seen as an elaboration of Baynes' concept of glycoxidative stress, which sees formation of Maillard products as the result of the combination of oxidative and glycaemic stress (Baynes, 1996). It links, too, with increasing evidence that dicarbonyl chemistry is a critical factor in the development of complications of uraemia (Miyata *et al.*, 1999).

1.2.3 Lipoprotein oxidation and the Maillard reaction

Fluorescent cross-links are known to form between lipid oxidation products and proteins, analogous to the formation of fluorescent products in the Maillard reaction (Dean *et al.*, 1997). Hunt *et al.* (1990) and others have described promotion of lipid peroxidation reactions by glycation of lipoprotein. Traverso *et al.* (1997) demonstrated that glycation and oxidation of lipid-containing bovine serum albumin resulted in similar and additive increases in fluorescence, loss of free sulphydryl groups and increases in protein-associated carbonyls. Glycation increased oxidation-mediated structural damage. Such reports have lead to speculation that glycation and associated oxidative reactions on protein may enhance oxidation of lipoprotein, a risk factor for vascular disease in diabetes and other diseases (Bucala *et al.*, 1993; Menzel *et al.*, 1997).

There is, as yet, little direct evidence to support Maillard reaction-associated enhancement of lipid oxidation *in vivo.* However, results from *in vitro* studies support the probability of such a relationship. Low density lipoprotein (LDL) from diabetic patients has been shown to be more susceptible to oxidation *in vitro.* This susceptibility was found to correlate with the extent of glycation of the protein (Bowie *et al.*, 1993). Similar results have been reported by Bucala *et al.* (1993). These authors found that *in vitro* glycation of LDL promoted formation of Maillard products and peroxidation of LDL lipids. In addition, Maillard products (AGEs) were increased on LDL isolated from the serum of diabetic patients, as was AGE-LDL in patients with end stage renal disease (Bucala *et al.*, 1993). Since both these patient groups are at increased risk of vascular disease, these results suggest correlations among AGE-modified LDL, LDL oxidation and atherogenic risk. Similarly, Menzel *et al.* (1997) have reported that oxidised LDL is

more susceptible to modification by Maillard processes *in vitro,* and Maillard-modified LDL is more strongly oxidised than native LDL. However, Baynes (1996) points out the importance of separating glycation of LDL from formation of late Maillard products, since the conversion from glycated protein to late Maillard products on protein and associated lipid peroxidation depend on many factors not related to glycaemic status. These include the inherent oxidisability of LDL lipids, the level of antioxidant vitamins present in LDL and overall oxidative stress.

In much the same way as Maillard products appear to enhance lipid oxidation, there are several possible routes by which lipid oxidation reactions can contribute to Maillard product formation. Under oxidative stress (to which Maillard reactions may contribute through generation of reactive carbonyl compounds), polyunsaturated fatty acids (PUFA) of LDL undergo peroxidation reactions to yield hydro- and endo-peroxides. Subsequent fragmentation reactions produce a broad range of reactive intermediates, including alkanals, alkenals, hydroxyaldehydes, hydroxyalkenals (*e.g.* 4-hydroxynonenal, HNE), malondialdehyde (MDA) and other carbonyl compounds. HNE and MDA each account for approximately 20% of the aldehydes formed during *in vitro* oxidation of LDL, and both yield stable adducts with lysine residues of apoprotein B (apoB) of LDL. This creates the oxidised-LDL (ox-LDL) epitopes which are recognised by scavenger macrophages. Receptor-mediated uptake of ox-LDL results in the formation of fat-filled macrophages, known as foam cells, which are associated with atherosclerotic lesions (Esterbauer *et al.*, 1992; Requena *et al.*, 1997a).

Oxygen-centred free radicals which are formed during lipid peroxidation - including peroxy, hydroxy, alkoxy and perhydroxyl (hydrated superoxide) radicals - can autoxidise

free sugars, fragment glycation-derived Schiff base or oxidise Amadori and other glycation/glycoxidation products to contribute to the formation later Maillard products (Baynes, 1996; Dean *et al.*, 1997). Reactive aldehydes such as HNE and MDA form another point of interaction between PUFA oxidation and the Maillard reaction. Both form adducts with lysine which do not persist in their initial configuration, but may mature to other structures (Requena *et al.*, 1997a). MDA can form a Schiff base with lysine through either end of the MDA molecule (Requena *et al.*, 1997a) or it can form a dihydropyridine (Slatter *et al.*, 1998). Both structures are able to cross-link proteins and to promote Maillard product formation. HNE forms Michael addition adducts with lysine (a thioether linkage of protein-SH and HNE), which can also mature to protein cross-links. For example, HNE forms both intra- and intermolecular cross-links with glyceraldehyde-3-phosphate dehydrogenase, thereby inactivating the enzyme (Dean *et al.*, 1997). Other carbonyl products of PUFA oxidation may also contribute to the Maillard reaction.

Glyoxal and methylglyoxal have been detected among lipid oxidation products *in vitro* (Degenhardt *et al.*, 1998a). Both are important intermediates in the Maillard reaction. Glyoxal is a known precursor of the Maillard product carboxymethyllysine (CML), which has been detected in ox-LDL in diabetic and normoglycaemic end stage renal disease (ESRD) patients (Fu *et al.*, 1996). Since CML has been shown to be a major antigenic AGE (Reddy *et al.*, 1995), it is also possible that CML-modified LDL is implicated in the recognition of ox-LDL by scavenger macrophages.

Schiff bases on protein can thus form from carbonyl compounds originating from either Maillard processes or lipid oxidation. These compounds comprise lipid-derived

aldehydes, sugars (native or autoxidised), and amino-acid derived aldehydes arising from protein oxidation mediated by either oxygen-centred free radicals or metal catalysed oxidation. Metal catalysed oxidation is also important in the Maillard reaction, at least *in vitro*, and it appears to play a significant role in systems which contain lipid hydro-peroxides. For some lipid-derived carbonyls, such as HNE, Michael addition may be a more significant route to protein modification than Schiff base formation. In the case of dialdehydes (such as MDA) or of α-dicarbonyls (such as glyoxal), Schiff base formation and Michael addition could occur simultaneously, thereby cross-linking lysine residues on either the same or different protein molecules (Dean *et al.*, 1997).

In membranes, which contain both lipid and protein in close proximity, interaction between protein and lipid oxidation may be expected to occur (Dean *et al.*, 1997). In analogous fashion, Maillard reactions in membranes may be expected to affect both lipid and protein. Bucala *et al.* (1994) argued that lipids such as phosphatidylethanolamine should in principle be modified through the amine group to form Maillard products. In support of this, peptides modified with Maillard products were found to react with LDL to increase immunogenic Maillard products in both the lipid and protein components of LDL. Requena *et al.* (1997b) subsequently isolated Maillard products from both the protein and lipid fractions of red blood cell membranes. It is thus likely that Maillard reaction products and lipid- or protein-derived oxidation products may form on both lipid and protein under oxidative conditions. Since many intermediates (MDA, HNE, glyoxal) can participate in both oxidative and Maillard processes, it can be expected that the reactions and their late products would be extremely difficult to separate *in vivo*.

1.2.4 Late Maillard products / Advanced glycation end-products (AGEs)

The formation of late Maillard products by the various routes described in the preceding sections is summarised in Fig. 3.1.

The Diabetes Control and Complications Trial clearly established a link between hyperglycaemia and glycation of proteins (measured as glycated haemoglobin, HbA1c), and similarly links between hyperglycaemia and diabetic complications have been well documented (DCCT, 1993; UKPDS, 1998). This suggests there is also a link between protein glycation and diabetic complications. However, glycated proteins (*i.e.* proteins modified with early products of the Maillard reaction) are continuously removed by a combination of processes and rarely represent more than 1% of lysine residues on proteins. There is little evidence that early Maillard products themselves can be causally linked to the development of diabetic complications (Wells-Knecht *et al.*, 1996). Late Maillard products (AGEs) arise from early glycation products on protein, but also involve many other factors in their formation, most importantly oxidation. Their formation is irreversible and they are resistant to degradation, therefore they tend to accumulate on long-lived proteins. While there is still little direct evidence linking such products causally with diabetic complications, there is a large and growing body of evidence correlating levels of various late Maillard products with the extent of complications (Wells-Knecht *et al.*, 1996; Wolff, 1996; Degenhardt *et al.*, 1998b). This focus on the role of the accumulation of irreversible chemical modifications of, particularly, long-lived proteins as a potential cause of diabetic complications and other diseases has been dubbed the 'advanced glycation end-product hypothesis' (Bucala and Cerami, 1992; Vlassara *et al.*, 1994). As an example of experimental evidence

which lends support to this hypothesis, Van Boekkel *et al.* (1996) reported that glycation of the major lens protein, α-crystallin, did not decrease its chaperone-like activity in protecting enzymes from inactivation while late Maillard products, including protein cross-links, did. An alternative view of the role of Maillard products in disease is to view their presence as biomarkers of cumulative protein damage, rather than as a direct cause of pathology. The observation that some young diabetics develop complications while older diabetics with similar levels of late Maillard products do not, coupled with the known age-related accumulation of Maillard products on long-lived proteins, lead Wells-Knecht *et al.* (1996) to speculate that age-corrected levels of Maillard products may be more significant than absolute levels at any age in predicting or precipitating diabetic complications.

Numerous authors have demonstrated the presence of Maillard products on long-lived proteins such as lens crystallins (Reddy *et al.*, 1995; Degenhardt *et al.*, 1998b; Brinkman Frye *et al.*, 1998) and tissue collagens (Sell and Monnier, 1989; Sell *et al.*, 1992; Dyer *et al.*, 1993; Degenhardt *et al.*, 1998b). In addition, Maillard products have been detected by chemical or immunological methods on plasma proteins (Makita *et al.*, 1992; Degenhardt *et al.*, 1998b), lipoproteins (Bucala *et al.*, 1994; Lyons and Johnson, 1994) and intracellular proteins (Giardino *et al.*, 1994). Of those identified to date, carboxymethyllysine and pentosidine have been reproducibly demonstrated to increase with age in tissue proteins and to accumulate at an accelerated rate in diabetes (Sell *et al.*, 1992; Dyer *et al.*, 1993). However, the number of known structures still represents only a fraction of Maillard products formed *in vivo*. Knowledge of chemical structures is urgently needed in order to elucidate the relationship between Maillard products and pathology. There is a growing literature

base describing immunological detection of late Maillard products (AGEs) using polyclonal antibodies raised against proteins modified *in vitro* by high sugar concentrations and long incubations under oxidative conditions (referred to in the literature as anti-AGE antibodies). Several leading authors have expressed reservations regarding such studies since the nature of epitopes detected by these antibodies remains uncertain (Baynes, 1996; Cohen, 1996; Wells-Knecht *et al.*, 1996; Wolff, 1996; Furth , 1997). It is noteworthy that, in recent studies using immunological detection of Maillard products, increasing effort has been expended to characterise the specific epitope recognised by anti-AGE antibodies (Niwa *et al.*, 1997a, b, c; Shamsi *et al.*, 1998; Hammes *et al.*, 1999).

In the paragraphs to follow, a number of well-established Maillard compounds - carboxymethyllysine, pentosidine, pyrraline and crosslines - are discussed, and some recently proposed structures are described.

N(ε)–(carboxymethyl)lysine

N(ε)-(carboxymethyl)lysine (carboxymethyllysine, CML) is a non-cross-linking, non-fluorescent, chemically stable Maillard product, usually formed at the ε-amino group of lysine residues on protein. It is thought to impact on protein structure and function by causing a change in charge at the modified lysine residue. Age-corrected CML levels are reported to show two-fold enhancement in skin collagen in diabetes (Dyer *et al.*, 1993) and to correlate positively with occurrence of retinopathy and nephropathy (McCance *et al.*, 1993). CML has been shown to be the major epitope recognised in

tissue-proteins by antibodies raised to uncharacterised Maillard products, so-called anti-AGE antibodies (Reddy *et al.*, 1995).

CML, and probably pentosidine, can form from oxidation of Amadori product on glycated protein (Glomb and Monnier, 1995). Transition metal-catalysed oxidation of Amadori product results in the release of erythronic acid and formation of CML (Ahmed *et al.*, 1986; Baynes, 1991).

It is also formed *in vitro* by reaction of glyoxal with lysine, hence as a product of glucose autoxidation (Glomb and Monnier, 1995). Glyoxal, along with the pentosidine precursor arabinose, is produced by autoxidation of glucose (Wells-Knecht *et al.*, 1995a). A Cannizzaro mechanism has been proposed for the autoxidative formation of CML. Glyoxal is the common product of oxidation of a variety of carbohydrates, including aldoses, ketoses and ascorbate (Wells-Knecht *et al.*, 1996). It has also been detected in the products of lipid oxidation (Esterbauer *et al.*, 1992; Fu *et al.,* 1996). This provides strong circumstantial evidence for involvement of glyoxal in a general mechanism for the formation of CML.

Once the oxidatively-derived precursors of CML are present, formation proceeds at similar rates in the presence or absence of oxygen. The same is true for pentosidine (Wells-Knecht *et al.*, 1996). It has been estimated that in *in vitro* incubations of glucose with an amine, 50% of CML is formed from glyoxal or glycolaldehyde, with the remaining 50% formed via oxidative cleavage of Amadori product (Glomb and Monnier, 1995; Glomb and Nagaraj, 1998). CML is also formed from ascorbic acid (Dunn *et al.*, 1990), providing at least three possible routes for its formation *in vivo*.

Pentosidine

Pentosidine is a highly fluorescent cross-linking compound, characterised by a imidazo(4,5,6)pyridinium ring derived from arginine, lysine and a pentose (Sell and Monnier, 1989). It occurs in tissue proteins at less than 1% of the concentration of CML and, unlike CML, is not a major determinant for anti-AGE antibodies (Wells-Knecht *et al.*, 1996). Pentosidine levels increase with age and with diabetes, correlate positively with nephropathy and retinopathy, and are thought to account for up to 40% of new fluorophores in lens proteins (Sell *et al.*, 1992). Pentosidine has been detected in aged and cataractous lens (Nagaraj *et al.*, 1991; Sell *et al.*, 1991), and in lens proteins of diabetic dogs with poor glycaemic control (Nagaraj *et al.*, 1996). Concentrations in plasma protein increases 2.5-fold in diabetes and 23-fold in uraemia associated with ESRD (Odetti *et al.*, 1992). Since uraemia is not typically associated with hyperglycaemia, this latter increase is likely to be related to factors other than glycation. It has been suggested that uraemia-associated Maillard products may be related to reactions of ascorbate, possibly increased ascorbate oxidation as a result of elevated levels of decompartmentalised copper ion (Wolff, 1996). Alternatively, Bucala *et al.* (1994) have suggested that impaired clearance of products of protein catabolism in ESRD results in an accumulation of peptides modified with Maillard products in the plasma. However, recently Miyata *et al.* (1999) have refuted both these arguments and have suggested that the complications of uraemia and ESRD are associated with a carbonyl stress similar to that proposed to be operating in diabetes (Baynes and Thorpe, 1999).

Glucose, fructose or ascorbate can serve as the glycating species in pentosidine formation. Metal-catalysed oxidation and decarboxylation reactions are thought to be involved (Grandhee and Monnier, 1990; Dyer *et al.*, 1991a), as probably are deoxy-pentose intermediates (Wells-Knecht *et al.*, 1996). The reactive pentose may be produced from a hexose by sugar autoxidation, *e.g.* arabinose from glucose (Wells-Knecht *et al.*, 1995a). As with CML, oxidative reactions of ascorbate are also thought to contribute to formation of pentosidine. The reaction of ascorbate with lens crystallins is thought to be a major contributor to cataract formation. As a consequence of failure of the glutathione and ascorbate reductase defence systems, ascorbate is oxidised to dehydroascorbate and 2,3-diketogulonate. This gives rise to xylosone by decarboxylation, from which in turn pentosidine can form.

Because of the role of metal- and oxygen-dependent oxidation reactions in pentosidine formation, it has been suggested that pentosidine provides an indirect measure of the rate of these reactions occurring *in vivo* (Sinclair *et al.*, 1991). The same argument can be applied to CML (Wolff, 1996).

Pyrraline

Pyrraline has been detected in plasma proteins and tissue collagens (Hayase *et al.*, 1989; Potero-Otin *et al.*, 1995), and in the neurofibrillary tangles and senile plaques characteristic of Alzheimer's disease (Smith *et al.*, 1994). It is increased in plasma proteins in diabetes, but does not appear to increase in collagen with age (Wells-

Knecht *et al.*, 1996). Pyrraline probably forms by reaction of protein with 3-deoxyglucosone and can dimerise via an ether linkage to form protein cross-links (Smith *et al.*, 1994; Portero-Otin *et al.*, 1995). Removal of monomeric pyrraline by dimerisation may account for the observation of steady-state pyrraline concentrations in collagen.

Crosslines

This Maillard product has been isolated from model systems incubating N(ε)-(acetyl)lysine with glucose (Nakamura *et al.*, 1992). The fluorescence spectrum maximum of crosslines (ex/em 370/440 nm) correlate well with those of aged lens proteins and collagens. It has been detected immunohistochemically in renal basement membrane and appears to increase in diabetes (Wells-Knecht *et al.*, 1996).

Other Maillard products / AGEs

Since the emergence of the central role played by α-dicarbonyl compounds in the formation of Maillard products, a number of new structures have been proposed, based on studies using model systems with purified dicarbonyl compounds. Several groups have since also reported their detection in tissue proteins, either by chemical or by immunological techniques.

N(ε)-(carboxyethyl)lysine (CEL)

One of the recently proposed structures, N(ε)-(2-carboxyethyl)lysine, is the methylglyoxal homologue of CML. It was produced from reaction of methylglyoxal and N(α)-blocked lysine, and has also been detected in model systems of methylglyoxal and protein. Unlike CML, it is not formed by oxidative decomposition of Amadori product. It appears to arise as a result of direct reaction of methylglyoxal with protein, possibly by a Canizzaro mechanism analogous to that proposed for the reaction of lysine and glyoxal to yield CML (Wells-Knecht *et al.*, 1996).

Argpyrimidine

A novel fluorescent pyrimidine was isolated from reaction of methylglyoxal with N(α)-t-BOC-arginine. It was identified as N(δ)-(5-hydroxy-4,6-dimethylpyrimidine-2-yl)-L-ornithine. This product, dubbed argpyrimidine, was also detected in incubations of higher sugars with proteins. It appears to be formed through an intermediate 3-hydroxypentane-2,4-dione and is suggested to contribute to enhanced protein fluorescence in diabetic tissues (Shipanova *et al.*, 1997; Glomb and Nagaraj, 1998).

Imidazolones

A significant observation to emerge from recent investigations is that dicarbonyl sugars, such as glyoxal and methylglyoxal, react preferentially with arginine rather than with lysine residues in proteins (Wells-Knecht *et al.*, 1996). The guanidino group of arginine

reacts with a dicarbonyl grouping, such as glyoxal, to give a hydroxyimidazolinone as its only product. Acid hydrolysis converts this to an imidazolone, in which form the resultant compound may be quantified (Glomb and Nagaraj, 1998). Imidazolones have only recently been detected as products of the reaction of dicarbonyls with proteins, partly because they exist as mixtures of isomers in acid hydrolysates and partly because they are readily oxidised to dehydroimidazolones which are unstable to acid hydrolysis. However, several Maillard products proposed recently are based on this structure (Wells Knecht *et al.*, 1996). The imidazolone adduct of 3-deoxyglucosone to the guanidino group of arginine (3DG-AIm) is one such compound (Konishi *et al.*, 1994), which is not fluorescent. Glomb and Nagaraj (1998) describe a fluorescent imidazolone adduct formed by reaction of glyoxal with arginine. A protein-bound imidazolone resulting from reaction of methylglyoxal with arginine residues has also been proposed (Henle *et al.*, 1994). The stability of such adducts has not been established under physiological conditions. The reaction may be reversible, or the initial adduct may mature to form protein cross-links (Wells-Knecht *et al.*, 1996). Indeed, Lederer *et al.* (1998) have demonstrated cross-linked products, based on the imidazolone structure reported by Henle *et al.* (1994), in model reactions of methylglyoxal and butylamine with creatinine or α-N-acetyl-L-arginine. Products were isolated in reasonable yield and in 1:1:1 ratio of the compounds participating in the reaction. The authors claim to have demonstrated a mechanism for cross-linking of primary amines to guanidine derivatives which might be of relevance to lysine/arginine cross-linking in the Maillard reaction *in vivo*.

Imidazolium salts (GOLD, MOLD, DOLD)

Baynes' group identified two imidazolium salt cross-links, originally detected in reactions of glyoxal and methylglyoxal with N(ε)-(hippuryl)lysine at physiological pH. These have been dubbed, respectively, glyoxal-lysine dimer (GOLD) and methylglyoxal-lysine dimer (MOLD). Yields are comparable to those of CML and CEL formed under similar conditions and proposed mechanisms – Cannizzaro rearrangement and elimination reactions – are also similar (Wells-Knecht *et al.*, 1996). Their formation involves a diimine intermediate, as proposed by Glomb and Monnier (1995). Both of these imidazolium cross-links lack fluorescence, corresponding with the suggestion that major protein cross-links are likely to be colourless and have no fluorescence (Dyer *et al.*, 1991b; Wells-Knecht *et al.*, 1996). They show a similar core structure to (2-furoyl)-4(5)-(2-furanyl)-1H-imidazole (FFI), a proposed Maillard product subsequently shown to be an artefact of sample preparation (Horiuchi *et al.*, 1988). Antibodies raised against FFI may therefore be expected to cross-react with GOLD and MOLD in tissue proteins (Wells-Knecht *et al.*, 1996). Formation of an analogous cross-link with 3-deoxyglucosone and hippuryllysine (dubbed 3-deoxyglucosone lysine dimer, DOLD) has recently been reported by Skovsted *et al.* (1998).

Advanced lipid oxidation end-products (ALEs)

Recognition of the contribution of non-glycation-associated oxidation products, particularly from PUFA oxidation, to the formation of Maillard products has lead to the identification of lysine-derived cross-links which can be expected to contribute to the pool of Maillard products *in vivo*. However, in these advanced lipid oxidation end-

products (ALEs), carbonyls of lipid origin form the cross-linking species. Requena *et al.* (1997a) provided the first chemical characterisation of MDA and HNE adducts to the ε-amino group of lysine residues in LDL. Their results indicated that MDA forms both mono- and di-Schiff base adducts, of which the di-Schiff base forms a lysine-MDA-lysine iminopropene cross-link. MDA adducts were detected as their acid hydrolysis products: lysine-MDA as 3-[N(ε)-lysino] propan-1-ol, and lysine-MDA-lysine as 1,3-di[N(ε)-lysino] propane. Both were detected in *in vitro* incubations of RNase with MDA and in LDL isolated from healthy volunteers. HNE was found to react with lysine residues primarily via a Michael addition reaction, detected as the acid hydrolysis product, 3-[N(ε)-lysino]-4-hydroxy nonan-1-ol. This was found in RNase treated *in vitro* with HNE, but not in native LDL. Slatter *et al.* (1998) contested that the cross-linking Schiff base adduct of MDA proposed by Requena *et al.* (1997a) was too unstable in solution to form the major reaction product with lysine residues. These authors suggest instead the formation of a more stable dihydropyridine derivative, arguing that this forms Schiff base-derived cross-links which are more energetically favourable, hence more stable and possessed of greater potential to modify long-lived proteins irreversibly.

Detection in tissue proteins

Initial reports of the *in vivo* occurrence of the novel Maillard products described above have recently begun to appear in the literature. CEL has been found to increase in lens protein and skin collagen with age, and in skin collagen with diabetes (Degenhardt *et al.*, 1998b). The imidazolium cross-links GOLD and MOLD have been found to be

elevated in lens protein with age and cataract, in skin collagen with diabetes, and in plasma proteins with uraemia (Brinkman Frye *et al.*, 1998; Degenhardt *et al.*, 1998b; Chellan and Nagaraj, 1999). Degenhardt *et al.* (1998b) propose that CML, CEL, GOLD and MOLD together represent the major Maillard modification of tissue proteins.

In addition to these products, Paul *et al.* (1998) demonstrated the presence of imidazolone adducts on tendon collagen incubated with ribose. Methyl-glyoxal derived imidazolone adducts on matrix collagen were also found to interfere with cell-matrix interactions in tissue culture (Paul *et al.*, 1999).

The above authors identified dicarbonyl-derived Maillard products by chemical analysis. A number of investigators have also used well-characterised antibodies to detect specific Maillard products on tissue proteins. Niwa and co-workers produced monoclonal antibodies to 3-deoxyglucosone-derived imidazolone. These were used to detect imidazolone adducts in kidneys of streptozotocin-diabetic rats, kidneys and aortas of diabetic patients, and on amyloid tissues from patients with dialysis-related amyloidosis (Niwa *et al.*, 1997a, b, c). Shamsi *et al.* (1998) used immunological methods to demonstrate the presence of methylglyoxal-derived products in serum proteins and corneal collagen. Antibodies raised to methylglyoxal-modified RNase A were demonstrated to recognise proteins modified by glucose, fructose, glyceraldehyde, glyoxal, ascorbate and ascorbate oxidation products. Specific structures recognised were imidazolysine (a lysine-lysine protein cross-link), a glyoxal-derived lysine-lysine cross-link and argpyrimidine (a fluorescent arginine modification). Such characterisation of specific antibodies *in vitro,* and demonstration of their reactivity *in vivo,* adds to chemical evidence for the occurrence of dicarbonyl-mediated

protein modification both *in vivo* and *in vitro*. These reports provide far stronger evidence for Maillard products in tissues than do uncharacterised 'anti-AGE' antibodies used in earlier studies, and still utilised by some workers.

1.2.5 Protein cross-linking

The preceding discussion has concerned itself with the roles of glycation and oxidation in the Maillard reaction, and with known and postulated structures of late Maillard products (AGEs). Since Maillard cross-linking reactions form the focus of this thesis, it is appropriate at this point to consolidate those aspects pertaining specifically to protein cross-linking.

General mechanisms

In principle, nonenzymatic reactions of sugar and proteins can lead to protein cross-linking by both non-oxidative and oxidative routes. However, experimental evidence indicates that this only occurs to significant extent under oxidative conditions (Fu *et al.*, 1992, 1994). Such non-oxidative cross-linking as does happen can be explained by reactions of 3-deoxyglucosone and methylglyoxal arising from degradation of Amadori product (Degenhardt *et al.*, 1998b), while the Amadori product itself has a free carbonyl group available for further reaction. Possible oxidative mechanisms for formation of cross-links are various, but generally involve dicarbonyl oxidation products of glucose or of the Amadori product. Among the most widely recognised are glyoxal,

methylglyoxal and 3-deoxyglucosone (Thornalley *et al.*, 1999). Autoxidation of ascorbate to dehydroascorbate yields a further potential cross-linking agent. Oxygen-centred free radicals arising from protein or lipid oxidation have also been implicated. Aldehyde products of lipid oxidation – notably MDA and HNE – have been identified as potential cross-linking agents, acting through lysine residues (Requena *et al.*, 1997a). Glyoxal and methylglyoxal are also produced (Dyer *et al.*; 1991b) as minor products during lipid oxidation. Lipid oxidation reactions are enhanced on lipoproteins modified with Maillard products, and Maillard reactions are enhanced on oxidised lipoproteins *in vitro* (Bucala *et al.*, 1994; Traverso *et al.*, 1997; Menzel *et al.*, 1997).

Cross-linking reactions occur primarily at lysine or arginine residues on protein. Lysine has been widely studied as a site for cross-link formation, but glyoxal and methylglyoxal have recently been found to react preferentially with arginine (Wells-Knecht *et al.*, 1996). Among known Maillard products, pentosidine is a cross-link formed from lysine, arginine and a pentose. Crosslines is another known cross-link structure, while pyrraline may mature to form cross-links. Among recently proposed structures, the compounds GOLD, MOLD and DOLD are dimers formed at lysine residues by glyoxal, methylglyoxal and 3-deoxyglucosone, respectively (Wells-Knecht *et al.*, 1996; Skovsted *et al.*, 1998). Bailey *et al.* (1995) described a non-fluorescent cross-linking compound, dubbed NFC-1, which is formed in the incubation of ribose with collagen. Lederer *et al.* (1998) described cross-links, based on the imidazolone structure, which can form between arginine and lysine residues in the presence of methylglyoxal. Protein cross-links derived from reaction of malondialdehyde with lysine residues on protein have been demonstrated and structures postulated (Requena *et al.*, 1997a; Slatter *et al.*, 1998).

Prabhakaram and Ortwerth (1994) developed a radiolabelling assay for investigating the cross-linking potential of a number of glycating sugars and amino acids. The glucose metabolites, glyceraldehyde and dihydroxyacetone, were found to cross-link twice as effectively as erythrose and threose, and eight times more effectively than ribose. The dicarbonyl sugars 3-deoxyglucosone and xylosone were at least as effective cross-linkers as ribose, as were the oxidation products of ascorbic acid. Little or no cross-linking was achieved with glucose, fructose or galactose, or with their phosphorylated derivatives. The rate of dimerisation of several amino acids was evaluated by following the incorporation of C-14 lysine into polylysine, polyarginine and polyhistidine. With threose as the glycating species, lys-lys cross-links were formed preferentially, followed by lys-arg and then lys-his. Glycation with dehydroascorbic acid resulted in preferential formation of lys-arg cross-links, followed by lys-his and then lys-lys. This difference in cross-linking potential of amino acids with the two glycating compounds suggests different cross-linking mechanisms.

Protein cross-links are late products of the Maillard reaction. From either the glycation or glycoxidation perspectives (although questionably from that of autoxidative glycosylation), it may therefore be expected that cross-links can form from glycated proteins in the absence of free sugars. Eble *et al.* (1983) were among the first authors to demonstrate this *in vitro*. Incubation of RNase A with glucose resulted in formation of RNase dimer and trimer. When glucose was removed from RNase by dialysis and the glycated protein re-incubated in the absence of glucose, polymers continued to form. Liggins and Furth (1996) demonstrated dimerisation of briefly glycated lysozyme (prepared by incubation of lysozyme with fructose for 0.5 to 12 hours) with unglycated

β-lactoglobulin, thereby showing that cross-linking also occurs in relatively lightly glycated protein and requires only one of the cross-linked proteins to have had prior exposure to glycating sugar. The physiological relevance of these observation was earlier demonstrated by Brighton and Furth (1989) who reported that thiobarbituric-acid-reactive-substances continued to increase on plasma protein of a patient admitted to hospital with acute hyperglycaemia for some time after plasma glucose levels returned to near normal levels. Sajithlal *et al.* (1998) demonstrated that sugar-free incubation of rat tail tendon collagen with heavily AGE-modified bovine serum albumin (BSA) increased cross-linking of collagen. Studies on cross-linking and other Maillard end-points conducted in the absence of free sugar are reviewed in detail in a later section.

There is an extensive literature base supporting the nonenzymatic formation of protein cross-links under conditions of glycaemic and/or oxidative stress. Studies have concentrated particularly on long-lived structural proteins such as collagens and lens proteins, but cross-linking of several non-structural proteins with a more rapid turnover in living systems has also been demonstrated. Studies of non-structural proteins are briefly reviewed below. This is followed by a slightly more detailed overview of Maillard cross-linking in collagen, as an illustration of the impact of the Maillard reaction on a structural protein. The use of biophysical techniques in the investigation of Maillard cross-linking of collagen is highlighted since the present study includes the use of X-ray diffraction to investigate glycation-associated cross-linking of collagen.

Cross-linking of non-structural proteins as a consequence of Maillard reactions

A number of non-structural proteins have been shown to undergo Maillard cross-linking. Pentosidine has been detected by immunostaining in the senile plaques and neurofibrillary tangles characteristic of brain tissue in Alzheimer's disease (Smith *et al.*, 1994). Immunoglobulin G incubated *in vitro* for 22 days with physiological glucose concentrations (55.5mM) formed high molecular weight polymers on SDS-PAGE. This was accompanied by an increase in Maillard-associated fluorescence and in browning products with absorbance at 350nm (Dolhofer-Bliesener and Gerbitz, 1990). Aggregation of β-2-microglobulin (β-2-m) is involved in dialysis-related amyloidosis (DRA), a significant complication of dialysis in uraemia. Modification of β-2-m isolated from patients is consistent with AGE formation, especially formation of pentosidine (Miyata *et al.*, 1994, 1996). Levels of 3-deoxyglucosone have been found to be markedly elevated in uraemic patients. Dimerisation of β-2-m occurs upon incubation with 3-deoxyglucosone *in vitro*, and the same β-2-m dimer was isolated from amyloid tissue of a patient with DRA (Niwa *et al.*, 1996). Imidazolone adducts derived from 3-deoxyglucosone have been detected immunologucally in uraemic serum and β-2-m amyloid deposits (Niwa *et al*, 1997c). Since amyloidosis is associated with inflammation and an increase in oxidative stress, rather than with increased glycaemic stress, it may be difficult to separate non-enzymatic protein cross-links of glycaemic or of oxidative origin *in vivo*.

Cross-linking of structural protein as a consequence of Maillard reactions: Collagen

In mature collagen, post-translation modifications include enzymatic attachment of carbohydrates, and enzymatic (lysyl oxidase) cross-linking via lysine and hydroxylysine residues to form intra- and intermolecular cross-links in collagen fibres (Bailey *et al*, 1974; Eyre *et al.*, 1984; Stryer, 1981). Collagen cross-links also form non-enzymatically via sugar-dependent Maillard reactions to form advanced Maillard products. This has been demonstrated by a number of authors (Monnier and Cerami, 1981; Tanaka *et al.*, 1988a; Bailey and Kent, 1989; Chace *et al.*, 1991; Bailey *et al.*, 1995), and such adducts have been shown to increase on collagen with age and disease (Reiser, 1991; Dyer *et al.*, 1993; Degenhardt *et al.*, 1998b). Some authors have argued that the Maillard reaction on collagen is not necessarily associated with marked additional cross-linking of collagen to itself, but rather with effects on the maturation of cross-links or with a few additional cross-links which are very specifically located (Brennan, 1989).

Collagen modification by Maillard processes both *in vitro* and *in vivo* results in, among other effects, decreased susceptibility to collagenase and protease digestion, decreased solubility of older collagen, abnormal intermolecular cross-link formation, and disturbances of ligand binding and of intra- and intermolecular association (Cohen, 1996). Incubation of Type I collagen with glucose in the presence of transition metal ions leads to formation of insoluble collagen aggregates (Chace *et al.*, 1991). Glycated collagen chains have been observed to dimerise by sugar-derived cross-links, and glycation leads to expansion of collagen molecular packing (Tanaka *et al.*, 1988a, b).

Incubation of rat tail tendon collagen with glucose resulted in increased maximum resistance of the tendon to mechanical stress, covalent attachment of glucose to collagen and increased Maillard fluorescence. These changes were reduced by introducing an alternative source of free amine groups, *e.g.* Tris buffer, into the incubation system (Andreassen *et al.*, 1988).

Living systems are characterised by interactions among many different proteins and other molecules. In such systems it may be expected that Maillard reactions on collagens and other structural components of the extracellular matrix disturb interactions required for normal functioning. This has indeed been either observed or postulated in a number of contexts. An *in vivo* study comparing 14 diabetic and 14 non-diabetic patients with coronary artery disease during artery by-pass surgery indicated that Maillard-associated modification of vessel wall proteins was an important factor in the decrease of arterial elasticity in diabetic patients (Airaksinen *et al.*, 1993). This observation was consistent with increased cross-linking of arterial wall proteins. It has also been speculated that Maillard cross-linking may contribute to the entrapment of serum proteins by nerve, kidney and other tissues (Miller and Michael, 1976; Michael and Brown, 1981; Brownlee *et al.*, 1986a). This could occur by cross-linking of native serum proteins to glycated structural collagens in blood vessels and basement membranes, or by cross-linking of native collagens to glycated serum proteins. Isolated glomerular basement membrane can be cross-linked with glutaraldehyde *in vitro*, causing increases in membrane permeability to protein and membrane thickening. This was attributed at least in part to increased cross-linking of basement membrane proteins (Walton *et al.*, 1992). Incubation of isolated pig glomerular basement membrane with fructose or glucose lead to cross-linking and associated

increases in permeability to water, myoglobin and glycated serum albumin. The changes in permeability were judged to be glycoxidative in nature since they were inhibited by ethylene diamine tetraacetic acid (EDTA), diethylene triamine pentaacetic acid (DTPA) and aminoguanidine. Oxidation with hypochlorite also increased glomerular basement membrane permeability (Cochrane *et al.*, 1997). Glycation (and subsequent Maillard reactions) of basement membrane collagen has been shown to disrupt self-assembly and interactions with other membrane components (Tarsio *et al.*, 1987; Tsilibary *et al.*, 1988), and to interfere with cell-matrix interactions (Paul *et al.*, 1999).

Biophysical approaches to investigating Maillard cross-linking in collagen

Biophysical methods have been less extensively applied to studying the Maillard reaction than have biochemical approaches. Kent *et al.* (1985) investigated the biomechanical properties of glycated collagen and reported these to be significantly altered by glycation. Tanaka *et al.* (1988b) investigated the structure of glycated collagen by X-ray diffraction and reported that glycation increased the intermolecular spacing of collagen *in vitro*. This was thought to be due to the formation of intermolecular cross-links in the overlap zone of the collagen fibril, which act to push the molecules apart. It is difficult to understand how a very few additional cross-links in one small region along the collagen molecule could have such a marked effect. It appears more likely that increased distance between collagen molecules would reflect changes occurring at several sites along the length of the molecule, thus it is possible that only those cross-links appearing in the regions of closest collagen packing were detected by this technique. Changes in the lateral packing of skin with diabetes have

also been reported by James *et al.* (1991), using X-ray diffraction. Malik *et al.* (1992) investigated intermolecular spacing of corneal and scleral collagen by X-ray diffraction, and found the cross-sectional area of collagen in both tissues to increase with age. It was proposed that this was associated with increased Maillard cross-linking - a possibility borne out by biochemical assays. Bai *et al.* (1992) incubated rat tail tendon in ribose and reported decreased solubility and increased fluorescent cross-links. Diameters of collagen fibrils were increased and became increasingly irregular. Fusion of fibrils was also observed.

The location of sugar binding sites along the collagen fibre (along the collagen axis, as opposed to lateral associations between fibres) has not been extensively investigated. It has been reported that collagen glycation occurs preferentially at hydroxylysine residues (rather than at lysine residues, as has been reported for other proteins). It was suggested that the presence of the hydroxyl group may enhance the nucleophilicity of the ε-amino group, thereby facilitating the formation of the Schiff base (Perejda *et al.*, 1984; Le Pape *et al.*, 1984). Hadley *et al.* (1998) monitored uptake of the electron-optical stain phosphotungstic acid and demonstrated that glycation of type I collagen with fructose changed the charge distribution along the collagen fibril. Loss of positive surface charge at specific sites along the D-period was observed, indicating involvement of lysine and arginine residues in Maillard reactions on collagen. Since the loss of positive charge showed noticeable specificity, not all lysine and arginine residues were consumed. Wess *et al.* (1990) used neutron diffraction to identify the sites of enzymatic cross-links in non-diabetic tendon collagen. Attempts at comparing diabetic and control tendon collagen by the same method showed little evidence of non-reducible cross-links associated with the Maillard reaction (Wess *et al.*, 1993).

However, the authors pointed out that such structures could possibly be detected by X-ray diffraction.

It is interesting to note that the animal model used in the study of Wee *et al.* (1993) was a spontaneously diabetic insulin-dependent rat. This contrasts with most animal models used in glycation studies, which do not attempt any regulation of blood sugar. Indeed, the authors point out that their results differ from those Brennan (1989), which used streptozotocin-dibaetic rats and a biochemical approach to address the same question. This latter study showed only small changes in glycation-derived cross-linking, but did indicate changes in the level of naturally occurring cross-links. Wess *et al.* (1993, 1996) argue that their model is more likely to model the effects of hyperglycaemia in insulin-controlled diabetes than is the study of Brennan (1989), a view which is supported in this review and in the aims of this study. However, the differences in experimental methods between the two studies rather clouds the comparison.

1.3 INHIBITION OF THE MAILLARD REACTION BY AMINOGUANIDINE

A number of compounds have been identified which inhibit advanced Maillard reactions *in vivo*. Measures of inhibition have included reduced levels of glycoxidation products (CML, pentosidine), of browning products and of collagen cross-linking. Most of the inhibitors investigated to date have some antioxidant activity and include chelators (DTPA, phytic acid, penicillamine), reducing agents (dithiothreitol, glutathione, penicillamine, lipoic acid) and free radical scavengers (thiourea, tiron, aspirin,

salicylate) (Baynes, 1996). The best known classes of Maillard inhibitors and their modes of action are summarised in Table 3.1 Fig. 3.2 elaborates on the schematic representation of Maillard product formation given in Fig 3.1, indicating where the major classes of inhibitors act.

The inhibitor which has arguably received the greatest attention in recent years is aminoguanidine. This compound combines guanidium and hydrazine moieties, giving it mixed functionality. It has been shown to block Maillard reactions, although the mechanism by which this occurs remains unclear. Despite this uncertainty, it is nearing completion of clinical trials as a possible therapeutic agent for the amelioration of diabetic complications (Brownlee, 1999; Friedman, 1999).

1.3.1. Structural and functional studies in animal models

Amelioration of certain secondary complications of diabetes by aminoguanidine has been demonstrated in a number of animal models, although results have not always been consistent across studies. The original study on the inhibitory effects of aminoguanidine demonstrated prevention of arterial wall collagen cross-linking and decrease in collagen fluorescence in alloxan-diabetic rats (Brownlee *et al.*, 1986b). Subsequent studies with the same or similar systems have reported variable results (Soulis *et al.*, 1988; Reiser *et al.*, 1993; Nyengaard *et al.*, 1997). It has been suggested that these differences may relate to the use of different extraction and assay methods (Nyengaard *et al.*, 1997).

A number of animal studies have been summarised by Jerums *et al.* (1994), covering effects in the extracellular matrix, kidney, vasculature, eye and nerves. Aminoguanidine has been shown to reduce diabetes-associated increases in collagen stability in diabetic rats treated with aminoguanidine for 120 days (Oxlund and Andreassen, 1992), and to improve reduced erythrocyte deformability in alloxan diabetic rabbits (Brown *et al.*, 1993). In a comprehensive 6 month study in streptozotocin-diabetic rats, Nyengaard *et al.* (1997) found that aminoguanidine prevented increased collagen stability in kidney and reduced collagen pentosidine levels in aorta. However, these authors also found that aminoguanidine had little impact on collagen fluorescence or actually increased fluorescence relative to that in untreated diabetic animals in several tissues (aorta, kidney, skin). Soulis-Liporata *et al.* (1991) found that aminoguanidine did not decrease fluorescence in unfractionated kidney, although fluorescence in extracts of glomeruli and renal tubules was decreased relative to untreated diabetic rats.

Nephropathy is one of the most common diabetic lesions, associated with both high morbidity and high mortality. The efficacy of aminoguanidine in limiting aspects thereof is of particular interest. Tissue fluorescence of renal basement membrane was reduced by 7 months aminoguanidine treatment in streptozotocin-diabetic mice, although no structural and functional correlates were determined (Nicholls and Mandel, 1989). Albuminuria, glomerular fluorescence and increase in fractional mesangial volume were shown to be prevented by 32 weeks treatment in streptozotocin-diabetic rats, but glomerular basement membrane thickening was not reduced (Soulis-Liporata *et al.*, 1991). A similar lack of response to aminoguanidine treatment was reported for glomerular basement membrane thickening by Nyengaard *et al.* (1997). These authors

also found that aminoguanidine did not prevent increase in the mesangial volume in diabetic rats. This is the structural parameter which correlates best with reduced glomerular filtration rate in diabetic nephropathy in humans. However, Ellis and Good (1991) found that glomerular basement membrane thickening was prevented by aminoguanidine in a different rat strain. Albuminuria was decreased by aminoguanidine treatment in the diabetic hypertensive rat (Edelstein and Brownlee, 1992). In a 10 week study of proteinuria in diabetic rats, aminoguanidine reduced excretion of albumin and other high molecular weight proteins without influencing total proteinuria (Itakura *et al.*, 1991). A 6 month study of aminoguanidine treatment in streptozotocin-diabetic rats also showed a decrease in albuminuria in treated rats (Nyengaard *et al.*, 1997). Degenhardt *et al.* (1999) reported that aminoguanidine inhibited development of albuminuria in streptozotocin-diabetic rats by 60%. However, pentosidine and CML in diabetic rat skin collagen were not reduced, indicating that the effect on albuminuria was not the consequence of inhibition of Maillard product formation. This may explain some of the apparently conflicting results reported for aminoguanidine in nephropathy by other authors.

Aminoguanidine was shown to reduce diabetes-related vascular permeability, although there remains some controversy regarding the mechanism by which this occurs (Williamson *et al.*, 1993; Nyengaard *et al.*, 1997). Jerums *et al.* (1994) reported reduction of retinal and uveal (anterior and posterior) albumin clearances by aminoguanidine. In contrast, when Nyengaard *et al.* (1997) investigated albumin permeation in a range of tissues (kidney, ocular tissues, sciatic nerve, skin and aorta), uvea was found to be the only one in which aminoguanidine attenuated increased albumin permeation. Several aspects of the development of diabetic retinopathy were

shown to be reduced by aminoguanidine therapy (Hammes *et al.*, 1991), but cataract formation was not reduced by aminoguanidine after 12 weeks in streptozotocin-induced diabetes (Panagiotopoulos *et al.*, 1993a). It is possible that this may relate to the extent to which aminoguanidine penetrates the lens.

Nerve blood flow and conduction velocity were improved by aminoguanidine treatment in streptozotocin-diabetic rats, and an increase in albumin vascular permeability in sciatic nerve was shown to be prevented by aminoguanidine (25 or 50 mg/kg body weight) in studies for up to 24 weeks (Kihara *et al.*, 1991). This contrasts with the results of Nyengaard *et al.* (1997), who in a longer term study (6 months) found no decrease in sciatic nerve albumin permeation upon treatment of diabetic rats with aminoguanidine (50 mg/kg body weight). These authors also argued that alterations in blood flow after induction of diabetes in rat models was a temporary phenomenon. Although ameliorated by aminoguanidine, this was probably mediated through nitric oxide synthase inhibition, not through any effect on Maillard processes. Yagihashi *et al.* (1992) reported that long-term aminoguanidine therapy inhibited accumulation of fluorescent AGEs in diabetic nerves and that this was associated with improvements in myelinated fibre size and degree of axonal atrophy. In another study, diabetes-associated defects in sciatic motor nerve conduction velocity and saphenous sensory nerve conduction velocity were ameliorated by 8 weeks aminoguanidine therapy, probably via a vascular mechanism (Cameron *et al.*, 1992).

Aminoguanidine has also been indicated as potentially useful in atherosclerosis, both in the presence and absence of diabetes. Bucala *et al.* (1994) have suggested that reaction of Maillard products with plasma lipoproteins may prevent their recognition by

LDL receptors. In support of this hypothesis, studies with human diabetic patients claimed that aminoguanidine at mean plasma levels of 10μg/ml (oral dose of 1200mg/day) decreased circulating LDL levels by 28%. Unfortunately, the authors failed to correct the reported impact of aminoguanidine for placebo effects, which would lower this to nearer 10%. Aminoguanidine was also demonstrated to reduce atherosclerotic plaque formation in the non-diabetic cholesterol-fed rabbit (Panagiotopoulos *et al.*, 1993b).

The effects of aminoguanidine have been shown to be related to duration of treatment, with the best results obtained for treatment throughout a 16 week study period in diabetic rats. Initiation of treatment during either the first or the last eight weeks of a the study yielded similar results which were intermediate between those for animals treated throughout the sixteen weeks and for no treatment (Soulis *et al.*, 1996). Nyengaard *et al.* (1997) further showed that results with longer duration of aminoguanidine treatment (6 months) differed in several aspects from those in studies of shorter duration.

A problem with *in vivo* studies in diabetic animals is that the complexity of biochemical changes occurring with diabetes makes it difficult to demonstrate conclusively that observed effects of aminoguanidine are related specifically to its impact on Maillard reactions. Comparison of the effects of aminoguanidine on renal tissue fluorescence and albuminuria with those of antioxidants and an aldose reductase inhibitor on the same parameters showed that only aminoguanidine had a significant effect, indicating that aminoguanidine is most likely to act via effects on Maillard processes rather than through inhibition of oxidative stress or aldose reductase activity (Jerums *et al.*, 1994).

Vlassara *et al.* (1992) incubated rat or rabbit serum albumin with sugar to yield serum albumin modified by late Maillard products, termed AGE-serum albumin. This was administered to non-diabetic rats and rabbits over a number of weeks. The authors described accumulation of immunoreactive Maillard products (AGEs) in aortic tissue, increased vascular permeability, increased mononuclear cell migratory activity in tissues and impairment of the vasodilatory response, relative to control animals. All effects were substantially reduced by simultaneous administration of aminoguanidine. A similar normoglycaemic animal experimental system was used to demonstrate up-regulation of gene expression for extracellular matrix components associated with late Maillard products (Yang *et al.*, 1994). Glomerular volume increases, basement membrane thickening, glomerulosclerosis, proteinuria and albuminuria were associated with longer term (several months) exposure to slightly elevated AGE-serum albumin levels (Vlassara *et al.*, 1994). Again, reported effects were eliminated or reduced by aminoguanidine. These results were interpreted as strong evidence that the observed changes were directly associated with AGE exposure and that the effect of aminoguanidine was mediated by direct interference with advanced Maillard reactions. A disadvantage of these studies is that the chemical identity of immunogenic AGEs/Maillard products was not investigated and therefore cannot be confirmed as physiologically relevant.

Some authors argue that the renal damage reported by Vlassara *et al.* (1994) was not typical of diabetic nephropathy, being more representative of non-specific glomerular injury (Cohen, 1996; Nyengaard *et al.*, 1997). Nyengaard *et al.* (1997) further suggest that the reported effect of aminoguanidine is better explained by inhibition of inducible nitric oxide synthase than by inhibition of Maillard reactions. These authors concluded

that the sometimes variable results reported for studies on the effect of aminoguanidine are indicative of a multifactorial pathogenesis of diabetic complications, only some of which factors are susceptible to aminoguanidine inhibition. These, it is proposed, are formation of glycation products and inhibition of nitric oxide synthase.

1.3.2 *In vitro* mechanistic studies

While animal studies may indicate the ability of aminoguanidine to ameliorate aspects of diabetic complications, the complexity of natural systems precludes in depth evaluation of the interaction of aminoguanidine and Maillard products *in vivo*. *In vitro* studies are necessary to elucidate the mechanism of action of aminoguanidine.

Initially, aminoguanidine was thought to act by blocking the free reactive carbonyl group on the Amadori product, preventing it from further participation in advanced Maillard reactions, including protein cross-linking (Brownlee *et al.*, 1986b). This theory received some support from studies by Lewis and Harding (1990) which demonstrated aminoguanidine binding to lens protein modified with Maillard products. However, a noticeable though lesser non-specific binding of aminoguanidine to native protein was also observed. More recent published work questions Amadori carbonyl binding as the major mechanism for aminoguanidine inhibition of glycation. Requena *et al.* (1993) showed that while aminoguanidine does bind to Amadori product, this accounts for only a limited proportion of the inhibition of the development of Maillard-associated fluorescence. Liggins and Furth (1997) reported that aminoguanidine inhibited fluorescence development at low concentrations while blocking of protein-bound

carbonyl groups, as measured by a dinitrophenyl hydrazine assay, was considerably less effective. Such results indicate clearly that aminoguanidine action cannot be explained simply in terms of Amadori carbonyl blocking.

An alternative suggestion was that aminoguanidine inhibits the Maillard reaction by competing with glucose for protein binding sites or by reacting with free glucose. Competition for protein binding sites seems unlikely. Lewis and Harding (1990) showed that aminoguanidine was able to prevent sugar binding to non-glycated protein without affecting carbamylation by cyanate. Since the latter process occurs through the same lysine residues as sugar binding, inhibition probably did not occur by competition with glucose.

Direct reaction with free sugar similarly seems an unsatisfactory explanation, since examples abound of experimental systems in which aminoguanidine inhibition of glycation has been reported despite sugar concentrations in considerable excess over those of aminoguanidine. Lewis and Harding (1990) demonstrated effective inhibition of glycation of solubilised lens protein by glucose-6-phosphate even when the sugar was in ten-fold excess over aminoguanidine. However, there is evidence of low molecular weight products formed in reaction systems containing glucose, aminoguanidine and protein. Khatami *et al.* (1988) detected a new low molecular weight coloured and radioactive compound when aminoguanidine was incubated with labelled glucose. Requena *et al.* (1993) also reported formation of a new low molecular weight chromophore during glycation in the presence of aminoguanidine. In neither case was the compound identified, but Khatami *et al.* (1988) interpreted their results as indicating a direct reaction between glucose and aminoguanidine and suggested that

this may contribute significantly to aminoguanidine inhibition of advanced glycation reactions. By contrast, Hirsch *et al.* (1995b) have shown that direct reaction between glucose and aminoguanidine occurs extrememly slowly.

Both Khatami *et al.* (1988) and Lewis and Harding (1990) suggested that scavenging of the reactive carbonyl (acyclic) form of the sugar plays a role in aminoguanidine action. Harding (1991) re-iterated the importance of relative rates of sugar acyclisation and aminoguanidine reaction. Such an explanation seems problematic on the grounds of chemical principles since the concentration of acyclic sugar should be maintained by equilibrium between cyclic and acyclic forms. However, Feather (1994) pointed out that free sugar carbonyls react very slowly with aminoguanidine, indicating a slow rate of acyclisation. This would seem consistent with an explanation of steady-state Schiff base sequestration of acyclic sugar carbonyls, as proposed by Booth *et al.* (1996, 1997) on the basis of observations that aminoguanidine inhibition of immunoreactive AGEs decreased at later stages of the Maillard reaction. Liggins *et al.* (1998) suggest that only carbonyl groups not involved in ring closure are available for reaction with aminoguanidine. This would imply that glucose itself is not able to react significantly, but dicarbonyl derivatives of glucose or of the Amadori product would be able to react. Since such compounds would represent only a small fraction of the glucose concentration, this offers a more satisfactory explanation than direct reaction with free glucose. Feather and Mossine (1998) reported that model Amadori products bearing one sugar moiety showed similar rates of acyclisation in solution to the parent sugar. However, model compounds bearing two sugar moieties (*e.g.* difructoselysine) contained significant amounts of the acyclic form in solution and were highly reactive.

Such compounds, if significant in physiological systems, might be expected to affect reactivity in the Maillard reaction and towards aminoguanidine.

Most current opinion on the mechanism of inhibition by aminoguanidine favours an interaction between aminoguanidine and highly reactive small dicarbonyl compounds resulting from decomposition of glucose, Schiff base or Amadori product. The importance of dicarbonyl intermediates in the Maillard reaction was highlighted earlier, with 3-deoxyglucosone, glyoxal and methylglyoxal receiving particular mention. Aminoguanidine is thought to react directly and irreversibly with such dicarbonyls, thereby removing them from the Maillard reaction. A role for 3-deoxyglucosone was supported by the observation that 3-deoxyglucosone caused fluorescence development on BSA much more effectively than does glucose, but that much less fluorescence develops if the 3-deoxyglucosone is pre-incubated with aminoguanidine (Oimoni and Igaki, 1989; Oimoni *et al.*, 1989). Similar conclusions were reached by Hayase *et al.* (1991), comparing the effects of aminoguanidine and semicarbazide on polymerisation of lysozyme as a consequence of Maillard reactions. A comparable mechanism was also invoked to explain aminoguanidine inhibition of Maillard product formation without evidence of aminoguanidine binding to glycated RNase peptides (Edelstein and Brownlee, 1992). Reiser *et al.* (1993) reported that radiolabelled aminoguanidine did not bind to Amadori product on collagen *in vitro*, but to unglycated amino acids. This altered both non-enzymatic Maillard and normal enzymatic cross-linking. *In vivo* studies indicated that aminoguanidine affected both non-enzymatic and enzymatic cross-linking, with differences observed among tissues. Requena *et al.* (1993) observed that aminoguanidine did not reduce the yield of Amadori product on either RNase or BSA, as might be expected if the Amadori product were the primary site of

aminoguanidine reaction. They refuted Amadori carbonyl blocking as the primary mechanism of aminoguanidine action and suggested that their results – including formation of small yellow chromophores - could be explained by interaction of aminoguanidine with small reactive intermediate compounds.

A possible chemical basis for the interaction between aminoguanidine and dicarbonyl intermediates was suggested by Hirsch *et al.* (1991) and elaborated by Hirsch and Feather (1994). The latter showed that aminoguanidine reacts rapidly and completely with sugar-derived dicarbonyl intermediates, including 3-deoxyglucosone, to form corresponding hydrazones and then stable 3-amino-1,2,4-triazine derivatives which are unable to participate further in Maillard reactions. Chen and Cerami (1993) also isolated a triazine derivative and a bishydrazone derivative of a dicarbonyl intermediate from reaction of a model Amadori product with aminoguanidine. Further support for dicarbonyl trapping by aminoguanidine came from isolation of the triazine derivatives of glyoxal, methylglyoxal and 1-deoxyglucosone (but not of 3-deoxyglucosone) from incubation of aminoguanidine with a model Amadori product and with glycated BSA under physiological conditions (Glomb *et al.*, 1994). This was extended by Araki *et al.* (1998), who incubated aminoguanidine with glucose, with glucose and protein, and with glycated protein. They analysed triazines formed under these conditions and in plasma from aminoguanidine-treated diabetic rats. Both *in vitro* and *in vivo*, the triazine derivatives of 3-deoxyglucosone and methylglyoxal were the major products detected, thereby strongly supporting a role for sequestration of dicarbonyl compounds in the inhibitory action of aminoguanidine.

Since authors such as Requena *et al.* (1993) have demonstrated that Amadori product concentrations are not reduced by aminoguanidine, the inhibitor must act at a point distal of this intermediate or by a pathway independent of Amadori product. On the other hand, Booth *et al.* (1996, 1997) showed that aminoguanidine inhibition is less effective in late Maillard reactions. Present understanding indicates that aminoguanidine inhibits by trapping reactive dicarbonyl compounds, particularly if the resulting product can be stabilised as a triazine. This appears to point to aminoguanidine acting as a scavenger of reactive dicarbonyls arising during 'intermediate' Maillard reactions, occurring after (or alternative to) Amadori product formation, but before the appearance of cross-linked and fluorescent late Maillard products.

1.3.3 Aminoguanidine and lipoprotein oxidation

Lipid oxidation yields reactive aldehyde products which are capable of modifying lysine residues in a similar manner to Maillard-derived dicarbonyls, *e.g.* modification of lysine residues of apoB of LDL. The similarity between these processes lead to the suggestion that aminoguanidine might inhibit protein modification by aldehydes arising from lipid oxidation, such as HNE and MDA. Since both Maillard reactions and modification of LDL apoB by lipid oxidation products contribute to recognition of ox-LDL by scavenger receptors, it was hypothesised that aminoguanidine may hold potential as an anti-atherogenic agent (Picard *et al.*, 1992). This was echoed by Bucala *et al.* (1994), who established that aminoguanidine decreased circulating levels of ox-LDL *in vivo*.

Investigations by Picard *et al.* (1992) established that aminoguanidine did indeed inhibit modification of LDL apoB lysine residues by reactive aldehydes. These findings were supported by Requena *et al.* (1992) who found that aminoguanidine inhibited several indicators of oxidation in copper (II)-catalysed LDL oxidation and in BSA incubated with MDA. Picard *et al.* (1992) also reported that aminoguanidine increased the lag time for conjugated diene formation (a measure of total lipid oxidation), although it did not affect the plateau value. This is similar to the observation of Booth *et al.* (1996, 1997) regarding the Maillard reaction, *viz.* that aminoguanidine delayed formation of antigenic AGEs, but did not significantly reduce final concentrations thereof. In model systems containing aminoguanidine and either HNE or MDA, aminoguanidine was shown to be an efficient scavenger of these lipid-derived aldehydes (Al-Abed and Bucala, 1997). Aminoguanidine also competed effectively with HNE for binding to the nucleophilic amino acids, cysteine, histidine and lysine. Taken together, these results appear to support a role for aminoguanidine inhibition of protein modification by lipid oxidation products. The efficacy of aminoguanidine as an anti-atherogenic was, however, questionable when compared to ascorbate (Scaccini *et al.*, 1994). Whereas ascorbate inhibited oxidative modification of LDL by >95% for all parameters measured, aminoguanidine inhibition was partial at best. These authors also pointed out that the aminoguanidine concentration in the study (20mM) was high relative to physiologically relevant levels while ascorbate was used at realistic concentration. They also noted that aminoguanidine appeared to form reversible associations with some products of lipid oxidation, causing interference in some assays.

A mixed role of aminoguanidine in oxidative reactions was reported by Philis-Tsimikas *et al.* (1995). Concentrations of aminoguanidine in the range 0.05–1mM inhibited copper(II)-catalysed modification of LDL by two mechanisms: inhibition of lipid peroxidation (as measured by loss of PUFA and by formation of conjugated dienes), and trapping of reactive breakdown products of lipid peroxidation. Furthermore, there existed a low aminoguanidine concentration (generally near 0.01mM) which promoted lipid oxidation and subsequent protein modification. The aminoguanidine concentration at which this occurred varied with the pre-existing degree of oxidation of the LDL, a parameter which was found to be extremely variable. The pro-oxidant activity of aminoguanidine was increased in LDL with elevated lipid hydroperoxide content (*e.g.* as a result of protein ageing), and was decreased by pre-treatment of LDL with an anti-oxidant such as vitamin E. The authors proposed a mechanism by which these apparently contradictory effects might be mediated. They suggest that aminoguanidine makes a nucleophilic attack on a PUFA carbon containing a peroxy radical. This leads to the formation of a transient, or possibly stable, adduct of aminoguanidine with fatty acid. Subsequent liberation of a protonated superoxide anion-like radical (also known as a perhydroxyl radical) promotes lipid peroxidation at low aminoguanidine concentrations. However, in the presence of a relative excess of aminoguanidine, the released radicals are removed by reaction with aminoguanidine, possibly forming a nitrone-like compound. This can decompose to a variety of products, possibly including nitric oxide. It was proposed that long-term aminoguanidine therapy should perhaps be combined with antioxidant therapy to counter the potential for increased oxidation.

Skamarauskas *et al.* (1996) investigated the pro-oxidant activity of aminoguanidine on glycated lipoprotein in the absence of sugar. BSA was glycated in the presence of

DTPA, then re-incubated in the absence of glucose and presence of aminoguanidine (varying concentrations up to 50mM, mostly 10mM) and copper(II) (100μM). In this system, aminoguanidine generated oxidants similar in reactivity to hydroxyl radicals and fragmented the protein, both effects increasing with copper(II) concentration. Aminoguanidine with copper increased dicarbonyl generation from BSA, and increased the release of protein-bound sugar-derived carbon. Although aminoguanidine decreased fluorescence generation from pre-glycated BSA, it increased fluorescence on unglycated BSA in control treatments. The authors propose that aminoguanidine hydrolyses to hydrazine which generates oxidants. Among the effects of these oxidants on glycated proteins is an increase in protein fragmentation and in degradation of protein-bound glucose adducts. This would serve to increase the susceptibility of Maillard-modified protein to proteolysis, thereby facilitating turnover of damaged protein. However, the pro-oxidant activity by which aminoguanidine is thought to aid in removal of Maillard-damaged protein was acknowledged as also being potentially detrimental. The anti-oxidant activity reported by Philis-Tsimikas *et al.* (1995) in copper(II)-catalysed oxidation of LDL was not observed with pre-glycated BSA.

1.3.4 A cautionary note

The continuing uncertainty surrounding the mechanism by which aminoguanidine inhibits the formation of AGEs would appear to be a cause for concern in a compound undergoing clinical testing as a possible therapeutic agent. Ou and Wolff (1993) report that acute and chronic toxicity trials of aminoguanidine indicated very low toxicity.

However, several authors have expressed doubts about possible biochemical interactions.

Khatami (1990) showed that aminoguanidine can bind to pyridoxal and pyridoxal phosphate, the biologically active form of vitamin B6. Pyridoxylation is an important regulatory mechanism in the action of aldose reductase. Reaction of pyridoxal/ pyridoxal phosphate with aminoguanidine may result in inactive complexes of this compound, with possible effects on aldose reductase and subsequent sorbitol accumulation. Defects in the sorbitol pathway have been implicated in certain diabetic complications.

Ou and Wolff (1993, 1994) show that aminoguanidine incubated alone generates hydrogen peroxide by metal-catalysed oxidation, and increases hydrogen peroxide generation from glucose. Hyperglycaemia increases hydrogen peroxide production in erythrocytes and this may be exacerbated by aminoguanidine, possibly leading to increase in oxidative stress. Aminoguanidine has also been reported to have pro-oxidant activity in two studies in sugar-free incubation systems (Philis-Tsimikas *et al.*, 1995; Skamarauskas *et al.*, 1996). It has been argued that hydrogen peroxide generation from glucose is negligible when compared with that generated during metabolic processes such as respiration. However, when viewed in the light of observations that aminoguanidine inhibits catalase (Ou and Wolff, 1993), which degrades hydrogen peroxide, this finding may be of significance *in vivo*.

Tasaka *et al.* (1994) reported that isolated pancreatic islet cells showed impaired glucose-stimulated insulin release at elevated glucose (>16.7mM) and aminoguanidine

(>1.8mM) concentrations. They suggested this effect may be due to aminoguanidine toxicity to pancreatic islet cells.

Aminoguanidine has been reported to be a weak tumour promoter (Oturai *et al.*, 1994). Work by Nyengaard *et al.* (1997) lead these authors to suggest the effect may be to favour growth of tumours rather than induction of additional tumours. Either possibility would be highly undesirable in a drug intended for long-term prophylactic use.

In the light of these reservations, it is clear that a better understanding of the mechanism of action of aminoguanidine is needed.

1.4 MAILLARD REACTION OF GLYCATED PROTEINS IN THE ABSENCE OF FREE SUGAR

Most of the many *in vitro* studies on the Maillard reaction and its inhibition by aminoguanidine and other compounds have been conducted by incubating proteins with high concentrations of sugars for long periods, or on chemically synthesised model compounds. While such experimental design ensures sufficient quantities of the product of interest, they are difficult to relate to physiological conditions of hyperglycaemic episodes (glucose concentrations unlikely to exceed 50mM) interspersed with periods of near-normoglycaemia and possibly even hypoglycaemia. Very few *in vitro* studies have examined the fate of proteins exposed briefly to elevated sugar concentrations and then incubated in the absence of free sugar.

Studies on re-incubation of pre-glycated proteins in the absence of free sugar are summarised below with respect to the different Maillard end-points and inhibitors reviewed in the previous sections.

1.4.1 Cross-linking

One of the earliest reports to emphasise the importance of considering physiologically-relevant conditions in experimental design was that of Eble *et al.* (1983). Studies on cross-linking of RNase incubated with high glucose concentrations at elevated temperature were supplemented by investigations under physiologically relevant conditions, and by others in which glycated RNase was re-incubated in the absence of glucose. Initial incubations with glucose at physiological concentrations showed that the rate of RNase cross-linking depended markedly on glucose in the concentration range 5-25mM. Glucose-free incubations of RNase modified with either 3 or 8 moles glucose per mole RNase showed that cross-linking to form RNase dimers and trimers continued for 2-3 weeks after removal of free sugar. Glycated RNase incubated with native RNase similarly formed polymers, while glycated RNase incubated with free lysine or α-blocked lysine formed RNase-lysine adducts. The authors concluded that the primary Maillard cross-linking mechanism under physiological conditions involved reaction of glycated amino acid residues with unmodified amino acid residues on the same or other proteins. While condensation products of two glycated amino acids had been detected by other investigators from *in vitro* Maillard reaction systems, it was unnecessary to invoke such compounds to explain Maillard cross-linking *in vivo*.

McPherson *et al.* (1988) reported that sugar-free re-incubation of RNase, pre-glycated with fructose or glucose for 7 days, enhanced cross-linking relative to protein incubated continuously in the presence of sugar. The continuation of cross-linking in the absence of free sugar resembled that reported by Eble *et al.* (1983). Enhancement of cross-linking in the absence of free sugar was ascribed to elimination of the competition for reactive sites on the protein which occurred in the presence of sugar.

Cross-linking of glycated protein to unglycated protein was further investigated by Liggins and Furth (1996), using two globular proteins. Lysozyme was glycated with fructose to different degrees, free sugar removed by dialysis and fructated lysozyme re-incubated with native β-lactoglobulin in the absence of sugar. Cross-linking was detected as the formation of a lysozyme/β-lactoglobulin heterodimer band on SDS-PAGE. Strong heterodimer formation was observed with lysozyme pre-glycated with 0.5M fructose for 4 hours at 37°C. However, even very mild pre-glycating conditions resulted in formation of some heterodimer. Some β-lactoglobulin homodimer also formed in sugar-free re-incubations with pre-glycated lysozyme, indicating that a cross-linking agent originating from glycated lysozyme was able to induce cross-linking in a different native protein in solution. The demonstrated ability of mildly glycated protein to form dimers with glycated protein suggests a plausible mechanism by which glycated serum proteins may cross-link with structural proteins *in vivo*.

Sajithlal *et al.* (1998) demonstrated that incubation of native rat tail tendon collagen with heavily glycated and aged BSA (AGE-BSA, incubated with glucose for 3 months and in the absence of glucose for a further 3 months) resulted in enhanced cross-

linking of collagen, measured as increased resistance to chemical and enzymatic digestion. Cross-linking was boosted by AGE-BSA in a concentration-dependent manner. It was found to be independent of free radical or oxidative reactions, possibly because the heavily modified BSA already carried the products of such reactions. This was interpreted as strong evidence of small, reactive dicarbonyl cross-linking compounds originating from AGE-BSA. Immunogenic AGEs were also found to have increased on collagen incubated with AGE-BSA. The disadvantage of this observation lies in the antigen used to raise antibodies for the ELISA technique by which AGEs were detected. This comprised highly modified RNase (3 months incubation in 0.5M glucose), which is unlikely to be relevant to the products of far milder glycation which occur *in vivo* . The authors did not investigate the possibility of cross-linking between the Maillard-modified BSA and native collagen. However, the results strongly re-inforce the observation of Liggins and Furth (1996), *viz.* that glycation of one protein can cause cross-linking in native protein in the absence of free sugar.

1.4.2 Fluorescence

McPherson *et al.* (1988), in the study described above, observed that fluorescence development continued on pre-glycated RNase incubated in the absence of sugar. However, they noted that cross-linking appeared to precede fluorescence, since fluorescence continued to increase when cross-linking stopped.

The development of fluorescence by BSA pre-glycated with glucose for 3 days and then allowed to age under sugar-free conditions for 10 days was investigated by Le

Guen *et al.* (1992). While agreeing that glycation promoted the development of fluorescence, they refuted arguments that this was associated solely with sugar-derived products. Their results particularly indicated a role for metal-catalysed oxidation and reactive oxygen species in the development of fluorescence on BSA. Thus, it was proposed that amino acid oxidation products, advanced Maillard products and lipid peroxidation products all contributed to the sugar-independent development of fluorescence during ageing of pre-glycated lipoprotein.

Liggins and Furth (1996) simultaneously monitored Maillard fluorescence and cross-linking during sugar-free ageing of pre-glycated and native protein. They showed that cross-linking developed under milder glycating exposures than fluorescence. This suggests that a sizeable proportion of protein cross-links are not fluorescent, and that fluorescence measurements may underestimate the formation of advanced Maillard products (AGEs), particularly under mild glycating conditions. This resembles the results of McPherson *et al.* (1988), which indicated that cross-linking preceded fluorescence development in late Maillard reactions.

Sajithlal *et al.* (1998) reported that incubation of AGE-modified BSA with native rat tail tendon collagen enhanced fluorescence of collagen, as was also observed for collagen cross-linking.

1.4.3 Specific Maillard products

Fu *et al.* (1992) conducted a wide-ranging study into the relative contributions of glucose and oxygen to Maillard reactions. A part of this study investigated the effect of extended pre-incubation with sugar on subsequent levels of early Maillard products during sugar-free re-incubation. Rat tail tendon collagen was incubated for 5 weeks with 0.25M glucose, then re-incubated for a further 5 weeks in the absence of glucose. A modest, but statistically significant, decrease in collagen-bound fructoselysine was observed, which the authors attributed to reversibility of the initial glycation reaction. Only small changes were noted in levels of CML, pentosidine and collagen-linked fluorescence during incubation without glucose, none of which was affected by aminoguanidine. It is possible that few changes were observed because the long initial incubation period allowed formation of late Maillard products to proceed virtually to completion in the presence of glucose.

Zyzack *et al.* (1995) compared the decomposition under sugar-free conditions of the model Amadori compound N(α)-formyl-N(ε)-fructoselysine (fFL) and of fructoselysine on glycated collagen. The latter was prepared by incubating rat tail tendon collagen with glucose for 4 weeks at 37°C under antioxidative conditions. Since phosphate ion promotes Amadori product decomposition and later Maillard reactions - probably by acting as an acid-base catalyst - the effect of varying phosphate concentration was also evaluated. Similar products were detected from both Amadori products (fFL and glycated collagen) at high and at low phosphate concentrations, indicating that the catalytic effect of phosphate did not affect product distribution. Sugar-free decomposition of the model compound fFL yielded mannose, glucose and tetroses,

with lesser amounts of hexoses and pentoses. The only dicarbonyl product detected was 3-deoxyglucosone, and this was found at extremely low concentrations. The authors do not exclude the generation of other 2- or 3-carbon sugars, but point out that these would probably have been lost during sample preparation. Collagen-associated fructoselysine yielded similar products as fFL and exhibited a similar half-life, approximately 75 days in 10mM phosphate. The authors offer an important reminder that the decomposition products detected are not uniquely of Maillard origin and may arise from both chemical and metabolic processes *in vivo*. It is this which makes studies of the fate of Amadori product in the presence of free sugar or *in vivo* extremely difficult to interpret.

The occurrence and distribution of the Maillard product, CML, was investigated by Schleicher *et al.* (1997) in a wide-ranging investigation. A small part of the study concerned itself with the *in vitro* formation of CML on human serum albumin (HSA) by routes other than glucose autoxidation, and with its inhibition. HSA was glycated for 7 days under anaerobic conditions in order to exclude CML formation or other post-Amadori reactions. When free glucose was removed by dialysis under argon and glycated protein re-incubated under aerobic conditions, CML was produced in a time-dependent manner, indicating that glucose autoxidation products were not necessary for CML formation.

1.4.4 Maillard inhibitors

McPherson *et al.* (1988) included penicillamine in re-incubations of pre-glycated RNase, and found this inhibitor reduced the rate of fluorescence development. Cross-linking was initially reversed, but subsequently resumed. The authors proposed that penicillamine acted by binding to the sugar moiety to prevent further Schiff base-mediated cross-linking.

Le Guen *et al.* (1992), investigating fluorescence development during sugar-free ageing of pre-glycated BSA, found that this was inhibited by a number of anti-oxidants (catalase, thiourea, mannitol, desferrioxamine), although fluorescence was anomalously boosted by superoxide dismutase. Guanidine and aminoguanidine at high concentration (200mg/ml) also inhibited fluorescence. Aminoguanidine was more effective at inhibiting fluorescence generation from non-glycated BSA than from glycated BSA. However, at the concentration used it is difficult to determine whether the effects observed were associated with protein denaturation, delipidation or direct interference with Maillard chemistry.

Liggins and Furth (1996) demonstrated that formation of heterodimers of pre-glycated lysozyme and native β-lactoglobulin was inhibited by anti-oxidants and by aminoguanidine. This indicated that cross-linking in their system involved transition metal-catalysed oxidation and aminoguanidine-sensitive carbonyls. Since dimerisation of native β-lactoglobulin was enhanced even though β-lactoglobulin was not pre-incubated with sugar (but was incubated in the presence of pre-glycated lysozyme), the

carbonyl cross-link mediators inhibited by aminoguanidine may be small diffusable compounds such as 3-deoxyglucosone.

Skamarauskas *et al.* (1996) investigated the pro-oxidant activity of aminoguanidine. These authors showed that aminoguanidine alone generated oxidants (measured as benzoate hydroxylation) in the presence of copper (II) and absence of protein. They also investigated the effect of copper-(II)-catalysed oxidation and aminoguanidine on glycated BSA in the absence of free sugar (already partially reviewed). Glycated BSA was prepared by incubation with glucose for 2 weeks in the presence of the free metal ion chelator, DTPA. These conditions could be expected to promote accumulation of early Maillard products. The authors reported that their glycated BSA generated oxidants in the presence of copper (II), while native BSA did not. However, when aminoguanidine and copper (II) were added, oxidant generation was greater from native than from glycated BSA. Copper (II) enhanced fragmentation of glycated BSA to a greater extent than of native BSA, while aminoguanidine and copper (II) together fragmented both native and glycated BSA. Since oxidant generation by aminoguanidine was less in glycated BSA and fragmentation greater, both relative to native BSA, the authors suggested that oxidant formation and protein fragmentation were mutually exclusive processes, and possibly that aminoguanidine specificity for glycated protein was related to alterations in antioxidant properties. Aminoguanidine increased the release of glucose-derived carbon from glycated BSA in a copper (II)-dependent manner under sugar-free conditions. Copper (II) itself showed a similar, but less marked, effect. Aminoguanidine increased generation of fluorophores on native BSA, and this was enhanced by copper (II). Fluorescence of glycated BSA was decreased under the same conditions. Copper (II) alone caused a slight increase in

fluorescence of both native and glycated BSA. Aminoguanidine and copper (II) together enhanced dicarbonyl generation from native BSA and produced approximately twice this level of dicarbonyls from glycated BSA. On the basis of these observations, Skamarauskas *et al.* (1996) proposed that aminoguanidine action represents a combination of protein oxidation and protein fragmentation, with aminoguanidine-promoted oxidation as the source of slight increases in fluorescence and dicarbonyl generation on native BSA. They suggest that aminoguanidine may undergo initial rapid reaction with dicarbonyls and show slower pro-oxidant activity dependent on transition metal ions. The pro-oxidant activity of aminoguanidine would be difficult to demonstrate in experimental systems in which the glycating sugar is present throughout, since oxidants are also generated from sugar under oxidative conditions.

Booth *et al.* (1996, 1997) emphasised the drawbacks of inhibitor studies carried out in the presence of glycating sugar, pointing out that it is extremely difficult to interpret whether inhibition involves the free sugar, oxidative production of intermediates from free sugar or Schiff base, or carbonyls arising from Amadori product intermediates. These authors investigated the inhibitory effects of aminoguanidine and a number of vitamin B1 and B6 derivatives, both in the presence of free sugar and in a sugar-free 'interrupted glycation' procedure involving pre-glycation of RNase A or BSA with ribose for 24 hours. Under the latter conditions, ribose generated a reactive intermediate (not isolated) which rapidly formed advanced Maillard products during sugar-free re-incubation (considered to be representative of post-Amadori reactions). Similar reactive intermediates could not be isolated when glucose was the glycating sugar. Late Maillard products were detected immunologically, using antibodies raised against

proteins incubated for 60-90 days with glucose. Results for uninterrupted incubation with glucose and ribose indicated that aminoguanidine tended to reduce the rate of formation of advanced Maillard products, but had little effect on final levels thereof. Those vitamin B1 (thiamine pyrophosphate) and B6 (pyridoxamine) derivatives which showed inhibitory properties either reduced final levels of immunogenic Maillard products or reduced both final levels and formation rates. Aminoguanidine added only to post-Amadori stages had little inhibitory effect, while vitamin B derivatives showed stronger inhibition. The authors concluded that aminoguanidine acts at early stages of the Maillard reaction. They suggested that, in addition to recognised dicarbonyl scavenging activity, aminoguanidine inhibits the reactive open chain form of sugars or sugar-protein binding. However, some doubt must remain about the significance of their results *in vivo* since ribose appears to form different intermediates from glucose, and since the nature of the products detected by the antibodies used is not known.

Schleicher *et al.* (1997), in a study described previously, included a range of inhibitors in glucose-free incubation of glycated HSA. Aminoguanidine was among the less effective inhibitors of post-Amadori Maillard reactions, although inhibition was greater than that reported by Booth *et al.* (1997). Lipoic acid and aminoguanidine inhibited CML formation from glycated HSA by 30%, while catalase and superoxide dismutase inhibited it by 50%, vitamin E by 70-89% and desoxiferamine by 100%.

Sajithlal *et al.* (1988) investigated the effects of a number of inhibitors on cross-linking of native rat tail tendon collagen. They reported that free radical scavengers (catalase, mannitol) and a metal chelator (EDTA) inhibited cross-linking in the presence of glucose, but had no effect on cross-linking mediated by heavily AGE-modified BSA in

the absence of glucose. Aminoguanidine (which blocks carbonyls) and aspirin (which blocks free amines) inhibited cross-linking in both systems. This was interpreted as indicating that free radical and oxidative reactions were not involved in cross-linking mediated by AGE-BSA, but amine-carbonyl interactions were involved.

1.4.5 Contribution of sugar-free studies to knowledge of the Maillard reaction

Taken together, studies involving ageing of pre-glycated proteins in the absence of free sugar clearly demonstrate the ability of early Maillard products on protein, notably Amadori product, to form a variety of advanced Maillard products under oxidative conditions. Reactive intermediates arising from late Maillard products may also mediate cross-linking of native protein. Autoxidation products of free sugar or reversibly bound sugar (Schiff base) were not essential to this process. Comparison of the effects of various inhibitors on post-Amadori Maillard reactions in the absence of sugar indicate that aminoguanidine is among the less effective inhibitors of late Maillard reactions and, furthermore, that it appears to have pro-oxidant activity. All are observations which would have been either difficult or impossible to achieve in the presence of sugar.

1.5 AIMS OF THIS THESIS

The preceding sections have reviewed current thinking regarding mechanisms underlying the formation of advanced Maillard products, notably cross-linked proteins.

Late Maillard products are thought to be involved in the pathogenesis of diabetic secondary complications and a variety of other conditions, through a complex interaction of increased levels of reducing sugars and localised pro-oxidative conditions. Few published studies have considered specifically post-Amadori reactions of glycated proteins. Particularly, there are insufficient studies on proteins which have been exposed to glucose for short periods, comparable to the duration of a hyperglycaemic event.

This thesis aimed predominantly to investigate the post-Amadori fate of proteins subjected to short initial glycation with glucose, particularly cross-linking of glycated protein to unglycated protein. It is a question of considerable physiological relevance in the light of the fluctuating blood glucose levels which typify moderately- and poorly-controlled diabetes. For example, circulating short-lived proteins, such as serum albumin, may be glycated during a hyperglycaemic event and may subsequently cross-link to unglycated long-lived structural proteins, such as collagen. Alternatively, glycated collagen, which is not readily regenerated through protein turnover, may entrap unglycated freshly synthesised serum albumin. Both reactions can potentially occur under normoglycaemic conditions some time after the hyperglycaemic event which resulted in the initial glycation. To support this postulate, this investigation aimed to demonstrate cross-linking between glucated (glycated with glucose) and native protein in the absence of free glucose.

Aminoguanidine has received much attention in recent years as a potential therapeutic agent to counteract diabetic complications. Studies on its mechanism of action indicate it to be complex and impingeing on both glycation and oxidation reactions. However,

few investigations have attempted to compare its ability to inhibit specific Maillard endpoints when introduced at different stages of the Maillard process, nor has its effect on glycated or oxidised protein in the absence of free sugar been thoroughly assessed. The present study aimed to address these questions by looking at the effect of aminoguanidine on both glycated and native protein in the absence of free sugar.

Specifically, protein cross-linking was investigated biochemically using the model system developed by Liggins and Furth (1996), in which pre-glycated lysozyme was re-incubated with native β-lactoglobulin in the absence of free sugar. Formation of protein dimers was followed by SDS-PAGE, early glycation products were estimated using a sodium periodate oxidation assay (Ahmed and Furth, 1991), and non-specific late Maillard products were monitored by following changes in protein fluorescence. Biophysical techniques were evaluated as a means of investigating the fate of larger proteins likely to be involved in physiologically relevant cross-link formation. Rat tail tendon collagen and HSA were chosen as representative structural and serum proteins, respectively. X-ray diffraction was used to investigate changes in high level organisation of collagen incubated with HSA under various glycating regimes.

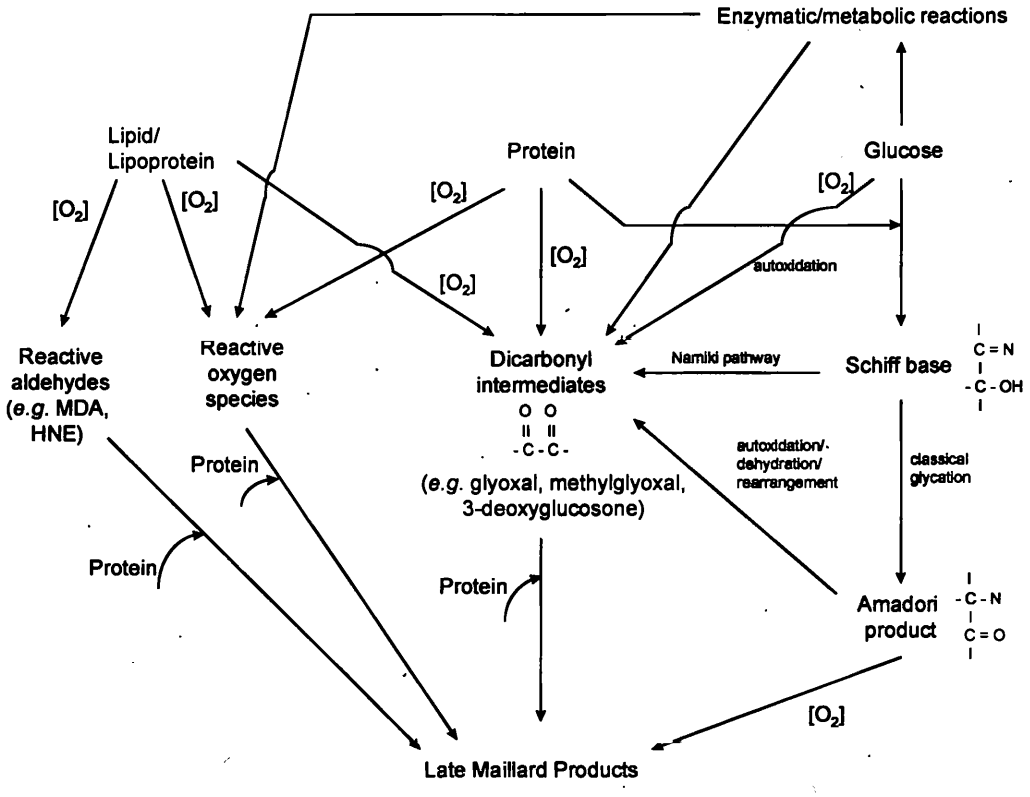


Fig. 1:1

Schematic representation of pathways to the formation of late Maillard products.

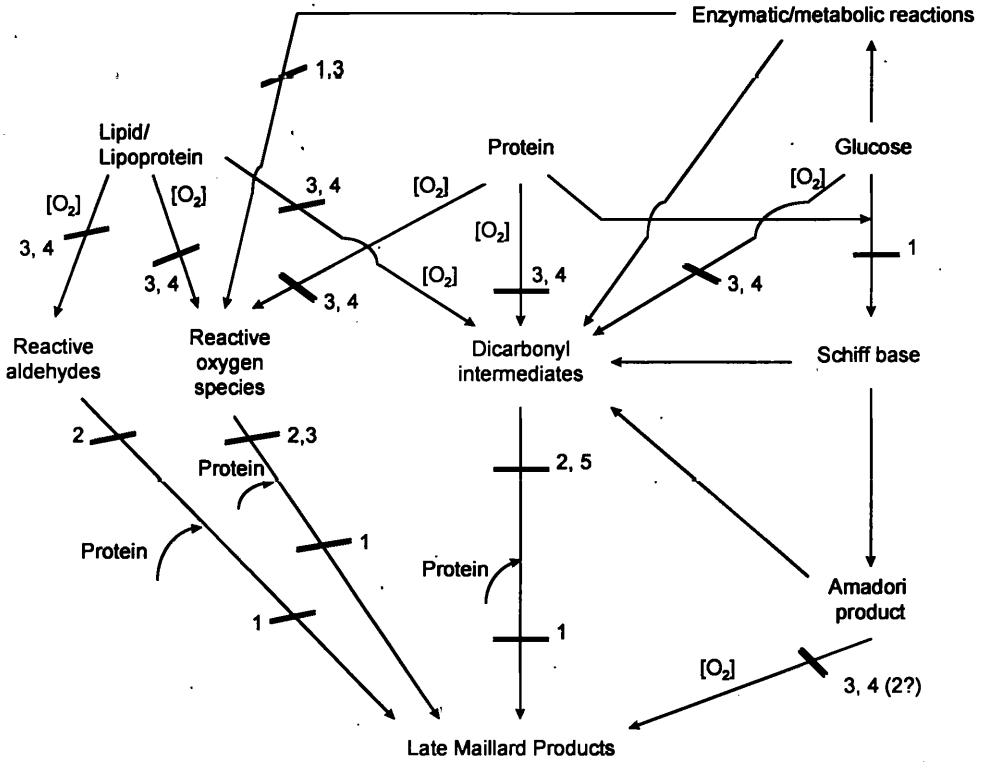


Fig. 1.2

Schematic representation of pathways to the formation of late Maillard products, showing the sites of action of several classes of inhibitors of the Maillard reaction.

1 Acetylating agents

2 Aminoguanidine

3 Anti-oxidants

4 Metal chelators

5 Dicarbonyl breaking thiazoliums

Table 1.1

Summary of inhibitors of the Maillard reaction and their modes of action.

Inhibitor	Mode of action
Aspirin, non-steroidal anti-inflammatory drugs	- Acetylation of amino groups on proteins - Interaction with metabolic and enzymatic reactions to reduce reactive oxygen species
Aminoguanidine	- Scavenging of reactive intermediates (i) dicarbonyl intermediates of Maillard reaction, oxidation and metabolic reaction (ii) reactive aldehyde products of lipid oxidation (iii) reactive oxygen species produced during lipoprotein oxidation Of the above, (i) is the best documented. - Pro-oxidant activity has been demonstrated in sugar-free systems
Anti-oxidants	Trapping of reactive oxygen species arising during Maillard reaction, lipid and protein oxidation, and metabolic reactions
Metal chelators	Chelation of transition metals which can catalyse oxidation reactions
Dicarbonyl breakers (thiazoliums)	Inactivation/destruction of reactive dicarbonyl intermediates from various sources, including Maillard reaction
Inhibitors of nitric oxide synthase	Nitric oxide (NO) mediates smooth muscle and vasodilatory effects typical of early diabetic changes. NO synthase inhibitors prevent these.

CHAPTER 2

MATERIALS AND METHODS

2.1 INTRODUCTION

An experimental system developed for investigating cross-linking of glycated protein to native protein in the absence of free glycating sugar (Liggins, 1996; Liggins and Furth, 1996) was used as the basis of all biochemical investigations in this thesis. Three assays, described below, were employed to investigate the Maillard reaction biochemically. Early glycation products was estimated using a sodium periodate microassay (Ahmed and Furth, 1991; Ahmed, 1992). Fluorescence is a classical indicator of the development of Maillard products and was used in this capacity. Protein cross-linking was followed on polyacrylamide gels (Liggins and Furth, 1996). General methods are described in the following sections. Variations specific to the investigations reported in each chapter are given at the beginning of the relevant chapter.

2.2 PROTEIN GLYCATION AND SUGAR-FREE RE-INCUBATION

Lysozyme (10mg/ml) was incubated for varying periods (24 hours to 5 days) at 37°C in the presence of varying concentrations of glucose in 0.1M phosphate buffer (pH 7.4) containing 3mM sodium azide as anti-microbial agent. Free glucose was then removed by exhaustive dialysis for 4 days against phosphate buffer at 4°C. The pre-glycated lysozyme so formed was re-incubated with native ß-lactoglobulin

(total protein concentration 10mg/ml, approximately equal concentrations of each protein) in phosphate buffer in the absence of glucose for three weeks. Aliquots were withdrawn at initiation of the re-incubation and again after 1, 2 and 3 weeks re-incubation. These were stored frozen at -20°C until analysed.

Parallel treatments that resembled the above, but which excluded glucose, were also performed. This permitted comparison of changes occurring in native and in pre-glycated proteins during prolonged sugar-free incubation.

Hen eggwhite lysozyme and 98% pure ß-lactoglobulin were purchased from Sigma. All other chemicals were from Aldrich, BDH (Poole, UK), or Sigma. Protein concentration was determined after both the first and second incubations by a bicinchoninic acid assay kit (Pierce).

Some treatments involved slight alterations or additions to the basic protocol outlined above, *eg.* addition of inhibitors or substitution of HSA for β-lactoglobulin. These changes and the underlying rationale are described in the relevant chapters.

2.3 PERIODATE ASSAY

Fructose calibration standards from 0 to 60nM were prepared in the same buffer as the sample, generally phosphate buffer. Duplicate 60µl aliquots of sample (approximately 10mg/ml protein) or standard were pipetted into 1.5ml micro-centrifuge tubes. To this was added 30µl hydrochloric acid (0.1M), followed by 30µl sodium periodate (0.05M). The mixture was vortexed and allowed to react at room temperature for 30 minutes. The reaction was stopped by placing the tubes on ice

and adding 30µl pre-cooled sodium hydroxide (0.7M), followed by 30µl pre-cooled zinc sulphate (15% w/v). This mixture was vortexed and centrifuged at 14 000g at 4°C for 10 minutes. The bulk of the resultant supernatant (100µl) was removed to a microplate and 200µl formaldehyde reaction reagent (10ml ammonium acetate, 3.3M, and 46µl acetyl acetone) was added. The microplate was covered, gently agitated to ensure mixing, and incubated for 1 hour at 37°C. Absorbance was measured at 405nm on a Bio-Tech microplate reader (model EL311, Luminar Technology, Hampshire, UK).

2.4 FLUORESCENCE

Samples were diluted to approximately 1mg/ml protein, using phosphate buffer prepared with ultrapure distilled water (Elgastat Option 3 high quality water purifier). Fluorescence (ex/em 350/420 nm) was measured on a Perkin Elmer LS50B spectrometer.

2.5 SDS-PAGE

Protein cross-linking was followed by standard polyacrylamide gel electrophoresis under denaturing conditions (SDS-PAGE; Laemmli, 1970). Gels were stained with a colloidal Coomassie stain, reported to have significantly increased sensitivity relative to other Coomassie blue staining methods. The stain comprised 0.1% (w/v) CBB G-250 in 2% (v/v) phosphoric acid, 10% (w/v) ammonium sulphate and 20% (v/v) methanol (Dunn, 1993). After overnight staining, gels were washed briefly with 25% (v/v) methanol and fixed with two overnight changes of 20-25% (w/v)

ammonium sulphate before photographing using Polaroid film (No. 53 or No. 665). Stained gels were scanned in an LKB Ultrascan XL laser densitometer to quantify the percentage of the total stain associated with each protein band.

2.6 STATISTICAL ANALYSIS

Most treatments were carried out in duplicate. Data are presented as the mean of the two replicates, and in graphical presentation, the minimum and maximum deviation from the mean are indicated by error bars. Data manipulation was carried out using Microsoft Excel (Windows 95, Version 7.0a). In comparing treatments, differences were considered significant if the error bars did not overlap. While it is recognised that this represents a crude evaluation of variability and of the significance of differences, the small number of replicates rendered more sophisticated statistical tests unsuitable. Better replication of experimental treatments, and consequently more rigorous statistical analysis, would have been desirable. However, this proved difficult to achieve within the time constraints of this study.

2.7 X-RAY DIFFRACTION STUDIES

The experimental design and methods used for investigation of glycation-mediated collagen cross-linking by X-ray diffraction are specific to Chap. 6 and are therefore described at the beginning of that chapter.

CHAPTER 3

PROTEIN CROSS-LINKING UNDER VARYING GLYCATING CONDITIONS

3.1 INTRODUCTION

The need for investigations into the fate of sugar-modified proteins in the absence of free sugar has been explored in Chap. 1. The model system used here was developed with the aim of combining the experimental advantages of using small, soluble proteins (to facilitate detection of Maillard products, particularly electrophoretic detection of cross-linked protein) with experimental conditions likely to be of importance physiologically (short glycating exposures, combinations of pre-glycated and native proteins, and of lipid-free and lipid-associated proteins). The system has proved useful in investigating fructose as the glycating sugar (Liggins, 1996; Liggins and Furth, 1996) and is extended here to glucose. This sugar is physiologically more prevalent, but is a less potent glycating agent than fructose. Longer incubation periods were therefore required, with the associated complications of decreased yield of cross-linked products and increased opportunity for interference from glycation-independent reactions, including oxidation.

The formation and detection of a lysozyme/β-lactoglobulin heterodimer during sugar-free re-incubation of pre-glycated lysozyme and native β-lactoglobulin was described by Liggins and Furth (1996). It is characterised by the appearance of a new protein band on SDS-PAGE, corresponding in relative molecular mass to the

sum of lysozyme (M_r 14k) and β-lactoglobulin (M_r 18k). The advantage of monitoring heterodimer cross-linking is that this dimer can arise only from reaction of glycated lysozyme after addition of β-lactoglobulin during re-incubation. It is therefore unequivocally linked to reactions of glycated lysozyme. Monomeric lysozyme and β-lactoglobulin are subject to some *in vivo* glycation and/or oxidation prior to isolation, therefore some homodimer of each protein is present initially. This applies particularly to β-lactoglobulin which is isolated from bovine milk and is exposed to lactose *in vivo*. Solutions of β-lactoglobulin are also thought to exist naturally as mixtures of monomer and homodimer (Aymard *et al.*, 1996). Furthermore, unlike lysozyme, β-lactoglobulin contains 1 mole lipid per mole of protein and is therefore susceptible to lipid oxidation. These effects should be small in comparison to those brought about by experimental glycation.

Increase in fluorescence is one of the defining features of the Maillard reaction, and fluorescence has long been used to follow formation of Maillard products under experimental conditions. The functional groupings responsible for fluorescence remain poorly characterised. However, it provides a ready means of comparison among published studies using a variety of other measures of Maillard products.

The development of fluorescent and cross-linked Maillard products was examined under a range of glycating conditions. A question of interest was whether threshold glycating conditions could be identified which needed to be exceeded for cross-linking to occur.

One of the objectives of the project was to investigate whether the cross-linking behaviour of the model proteins lysozyme and ß-lactoglobulin could be repeated with proteins likely to be glycated *in vivo*. Serum proteins are an obvious choice

since their glycation status reflects the concentration of glucose in blood, especially during episodes of hyperglycaemia in diabetes. For this reason, the above system was also used to investigate cross-linking of lysozyme and HSA.

3.2 MATERIALS AND METHODS

3.2.1 Choice of buffer

Decreasing concentrations of phosphate buffer were tested to seek a concentration at which interference from the buffer system could be minimised. A number of alternative buffer systems were evaluated for their suitability as a medium for glycation experiments. Changes in pH and fluorescence were monitored over one week in incubations of HSA (10 mg/ml) with 0.5M glucose at 37°C.

In addition to phosphate buffer (sodium phosphate), the following additional buffers were tested as follows:

- Phosphate-buffered saline was included to examine the effect of non-buffering salts, such as would be present *in vivo*.
- A bicarbonate buffer was tested since this is the most important buffering system under physiological conditions and has been used previously (Liggins, 1996).
- A bicarbonate-based buffer with a physiologically-representative concentration of phosphate (approximately 2mM) was tested as an example of a relatively simple system which mimicked physiological buffering.
- HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesylphonic acid) buffer served as a non-ionic buffer comparison and was expected to yield low rates

of glycation, as has been found for organic and cationic buffers in other studies (Watkins *et al.*, 1987).

Buffer concentration was 0.1M, except for phosphate (0.01M to 0.1M). Sodium azide was included in buffers at 3mM as an anti-bacterial agent.

3.2.2 Cross-linking and fluorescence of two model proteins under varying glycation conditions

Lysozyme and ß-lactoglobulin were used as model proteins in accordance with Liggins and Furth (1996), with cross-linking conditions as described in Chap. 2. Lysozyme was pre-incubated with glucose, followed by dialysis to remove free glucose and sugar-free re-incubation in the presence of native ß-lactoglobulin.

The following pre-glycation treatments were investigated:

- 0.5M glucose, 37°C, 5 days
- 0.5M glucose, 37°C, 24 hours
- 0.05M glucose, 37°C, 24 hours
- 'Minimal glycation' conditions, achieved by dialysing a control sample (*i.e.* pre-incubation in the absence of glucose) together with glycated samples. This resulted in the exposure of the dialysed control to low concentrations of glucose in the dialysing buffer at low temperatures. It is estimated that the glycation conditions corresponded to approximately 5mM glucose at 4°C over 8-16 hours.

A completely sugar-free control was run alongside all glycation treatments. This was also dialysed to ensure that all treatments were exposed to the same experimental conditions. However, no contact between glycated and control samples was allowed during dialysis.

Glycation was followed by assaying for PPM (reported in Chapter 5), measuring fluorescence (ex/em 350/420 nm), and subjecting samples to SDS-PAGE on 12.5% or 15% gels to protein cross-linking.

3.2.3 Cross-linking of lysozyme and serum protein

Pre-glycated lysozyme (incubated for 5 days at 37°C in the presence of 0.5M glucose) was re-incubated with native HSA in the absence of free sugar for 3 weeks. The glycation time was chosen on the basis of results obtained using increasing glycation exposure with the lysozyme/β-lactoglobulin model system. Low purity (96-99%) and high purity (>99%) preparations of native HSA were compared, since previous work had indicated that high purity HSA preparations occasionally yielded anomalous results (Cochrane, 1995).

Similarly, HSA (>99%) was pre-glycated by incubation with 0.5M glucose for 24 hours at 37°C, and then re-incubated with native lysozyme under sugar-free conditions for three weeks. A shorter pre-glycation time was chosen because results emerging from inhibitor studies with the lysozyme/β-lactoglobulin model system (reported in Chapter 4) indicated that the lipid-associated protein, β-lactoglobulin, was susceptible to a number of interfering reactions during prolonged incubation. HSA acts as a carrier of free fatty acids in physiological systems,

therefore preparations of the proteins have a lipid component (Saifer and Goldman, 1961). Since it was desirable to limit the effects of the initial incubation to glycation alone, as far as possible, the duration of the pre-glycating incubation was reduced from 5 days to 24 hours.

Glycated samples and non-glycated controls were subjected to SDS-PAGE on 7.5% and 12.5% gels.

3.3 RESULTS

All figures referred to in the text below are presented at the end of the chapter.

3.3.1 Choice of buffer

Most glycation studies to date have been conducted in phosphate buffer. However, it has been widely reported that phosphate buffer itself may catalyse glycation, as a result both of the action of phosphate ions and of oxidative reactions catalysed by metal contaminants (Watkins *et al.*, 1987; Fu *et al.*, 1992; Smith and Thornalley, 1992; Zyzak *et al.*, 1995; Tessier and Birlouez-Aragon, 1998; Thornalley *et al.*, 1999). Since it would be preferable to avoid involvement of the buffer in the reaction being studied, a range of phosphate concentrations and of alternative buffers were evaluated.

None of the alternative buffers tested offered a marked advantage over phosphate buffer, either in terms of buffering capacity or in terms of reducing the autocatalytic function of the buffer without impeding the Maillard reaction (results not shown). It

was therefore decided to continue using phosphate buffer, since it is utilised in most published work. A concentration of 0.1M was selected since buffering capacity at lower concentrations was occasionally variable.

3.3.2 Cross-linking and fluorescence of two model proteins under varying glycation conditions

Development of fluorescent and cross-linked Maillard products during sugar-free re-incubation, following increasingly intensive pre-glycation, is depicted in Fig. 3.1 - 3.6. Note that, whereas results for most pre-glycation treatments represent the mean of two replicates, control (sugar-free) treatments have been pooled where possible to give a larger data set.

Fluorescence

The development of Maillard-associated fluorescence, during sugar-free re-incubation of proteins subjected to the pre-glycation regimes outlined, is shown in Fig. 3.1. No change was observed in minimal glycation and 24hr / 0.05M glucose pre-glycation treatments, relative to sugar-free controls. Both these treatments and the control gave a small increase in fluorescence over three weeks sugar-free ageing. A small increase relative to sugar-free controls was noted for the 24hr / 0.5M glucose pre-glycation treatment, appearing to reach a plateau after two weeks. However, this was overshadowed by the very strong fluorescence that developed after 5 days initial glycation in 0.5M glucose. This continued to develop throughout the re-incubation period in an approximately linear manner, fluorescence

after three weeks being roughly an order of magnitude greater than that in sugar-free controls.

Cross-linking

Fig. 3.2 presents an example of a SDS-PAGE gel of pre-glycated lysozyme (5 days, 37°C, 0.5M glucose) re-incubated with native β-lactoglobulin in the absence of sugar for three weeks. This represents the most intensive pre-glycation of lysozyme. Sugar-free controls at re-incubation weeks 0 through 3 are shown to the left of the M_r marker (lanes 1-4, molecular weight marker in lane 5). Sugar-free re-incubations (weeks 0 through 3) of pre-glycated lysozyme with native β-lactoglobulin are shown to the right of the marker (lanes 6-9). The strongest bands in all lanes are the lysozyme (M_r 14k) and β-lactoglobulin (M_r 18k) monomers. The lysozyme/β-lactoglobulin heterodimer band appears in pre-glycated samples at week 1 between bands corresponding to the lysozyme and β-lactoglobulin homodimers, and becomes progressively more intense with increasing incubation time (lanes 7-9). This indicates the formation of a new protein heterodimer of M_r 32k, associated only with pre-glycation of one of the model proteins. It forms within one week after glycation, and continues forming throughout sugar-free ageing. Lysozyme homodimer (M_r 28k) appeared enhanced after glycation (lane 6-9), indicating that additional cross-linking of lysozyme occurred during glycation (lane 6), and that dimerisation of pre-glycated protein continued during sugar-free ageing (lane 7-9). The β-lactoglobulin homodimer (M_r 36k) appeared to decrease in intensity with time in both sugar-free controls and pre-glycated proteins.

Similar gels were obtained for all glycation regimes tested, although Fig. 3.2 showed the clearest evidence of protein dimerisation during sugar-free ageing. Cross-linking over the three week re-incubation period was quantified by scanning laser densitometry of SDS-PAGE gels. Combined results from all pre-glycation regimes tested are shown in Fig. 3.3 (lysozyme/β-lactoglobulin heterodimer), Fig. 3.4 (lysozyme homodimer) and Fig. 3.5 (β-lactoglobulin homodimer).

Lysozyme / β-lactoglobulin heterodimer

No heterodimer was present at the start of re-incubation. After three weeks re-incubation, all four glycation pre-treatments led to noticeable dimerisation, the amount of heterodimer increasing with both glucose concentration and time in the initial glycating medium (Fig. 3.3). Results showed considerable variability as a consequence both of the low quantities of heterodimer and of inaccuracies inherent in gel staining and scanning by laser densitometry. It was therefore not possible to determine whether differences among the three mildest glycation regimes were consistently significant, although a trend is suggested. However, heterodimer levels in the 5 days / 0.5M glucose pre-glycation treatment were clearly greater than for the other treatments. Here, heterodimer was clearly visible after one week sugar-free ageing and showed little further increase, possibly indicating saturation of cross-linking sites. Heterodimer in the 24hr / 0.5M glucose pre-glycation treatment was clearly visible from the second week of sugar-free ageing and was still increasing by the end of the re-incubation period. Initial glycation for 24 hr with 0.05M glucose gave variable results in the first two weeks of sugar-free ageing, with heterodimer clearly present by the third week. Minimal glycation yielded traces of heterodimer at week 2, increasing by week 3.

Sugar-free controls showed occasional faint traces of heterodimer by week 3 which were not consistently detectable by laser densitometry. Protein cross-linking can occur at very slow rates as a result of oxidative modifications of protein or associated lipid, even in the absence of free sugar. For example, MDA, generated oxidatively from lipid, is a demonstrated cross-linking agent (Requena *et al.*, 1997a; Slatter *et al.*, 1998). Hence it is reasonable to expect occasional traces of cross-linked protein even in sugar-free control treatments, especially where proteins have been incubated in air for extended periods.

Lysozyme homodimer

Surprisingly, although lysozyme was the protein subjected to initial glycation, only the most intensive glycation conditions (5 days / 0.5M glucose) resulted in significant increase in levels of lysozyme homodimer above levels in sugar-free controls (Fig. 3.4). Here, the enhanced homodimer was present at the beginning of the sugar-free re-incubation period and did not increase further. This again suggests saturation of cross-linking sites. The remaining milder treatments, including the sugar-free control, gave some increase in lysozyme homodimer during sugar-free ageing. The differences were small and variable, making it unclear whether they were significant. Since any increase in homodimer over time occurred similarly in sugar-free and the milder glycation treatments, this effect appears to be independent of sugar and was probably a consequence of oxidation during sugar-free ageing.

β-lactoglobulin homodimer

As expected, mean levels of β-lactoglobulin homodimer were similar among treatments at the beginning of the re-incubation, irrespective of pre-glycation treatment (Fig. 3.5). There was large variability between replicates, which made it difficult to discern differences among treatments. This variability decreased during re-incubation, but remained enough to obscure differences among pre-treatments, even after three weeks. A surprising trend observed for all treatments, including sugar-free controls, was that levels of β-lactoglobulin homodimer decreased throughout the re-incubation period. This may have been a consequence of formation of higher oligomers and subsequent protein precipitation. However, results showed little change if precipitated protein was removed by centrifugation prior to SDS-PAGE, or if samples were vortexed to resuspend (and thereby include) any protein precipitate before removing aliquots for electrophoresis. On examining SDS-PAGE gels, samples from later re-incubation weeks also did not show notable deposits of insoluble protein at the tops of gels. It is more probable, therefore, that the decrease in β-lactoglobulin homodimer levels reflects protein fragmentation. The decrease appears somewhat more rapid and marked in treatments representing more intensive pre-glycation conditions, but it is not clear whether this difference was significant.

Relative rates of formation of fluorescent and cross-linked products

Fig. 3.6 compares mean levels of fluorescent Maillard products and lysozyme/β-lactoglobulin heterodimer, both after three weeks sugar-free ageing of pre-glycated lysozyme with native β-lactoglobulin. There was little change in fluorescence in the

minimal glycation and 24hr / 0.05M glucose pre-glycation treatments, and only small increases over 3 weeks in the 24hr / 0.5M glucose pre-glycation. Fluorescence increased by almost an order of magnitude when the initial glycation period was increased from 24 hours to 5 days. In contrast, heterodimer formation rose gradually as the glucose exposure of lysozyme increased.

The delay in formation of fluorescent Maillard products indicates a threshold condition which must be exceeded for significant formation of fluorophores. The rise in fluorescence was associated initially with glucose concentration (increase from 0.05M glucose to 0.5M glucose, both for 24 hours), but the greatest increase was associated with the duration of pre-glycation (from 24 hours to 5 days, both at 0.5M glucose). The observed threshold may indicate the need for accumulation of precursors in a sugar-dependent oxidation reaction, since oxidative changes are usually indicated by a time lag (Picard *et al.*, 1992). The same threshold effect was not observed for formation of cross-linked Maillard products. This indicates either that fluorescent and cross-linked Maillard products are formed by significantly different routes, or perhaps that cross-linked products may mature to fluorescent structures. These possibilities were not investigated further in the present investigation.

3.3.3 Cross-linking of lysozyme and serum protein

Fig. 3.7 and 3.8 show representative SDS-PAGE gels (7.5%) from re-incubations of pre-glycated lysozyme with native HSA, and of pre-glycated HSA with native lysozyme. Fig. 3.9 and 3.10 show the same treatment combinations on 12.5%

SDS-PAGE gels. The theoretical M_r of lysozyme/HSA oligomers and M_r of new bands appearing on gels are summarised in Table 3.1.

SDS-PAGE showed the development of two new bands, appearing at molecular weights higher than those of either lysozyme or HSA, in re-incubations both of pre-glycated lysozyme with native HSA (Fig. 3.7, 3.9) and of pre-glycated HSA with native lysozyme (Fig. 3.8, 3.10). The same bands were observed in re-incubation of pre-glycated lysozyme with both high and low purity HSA preparations. Results shown here represent the higher purity HSA. One band (M_r 91.5k) had a molecular mass approximately equally to the sum of one HSA and two lysozyme molecules (Table 3.1). This was observed on both 7.5% and 12.5% gels for both treatments (pre-glycation of lysozyme and pre-glycation of HSA). A second new band (M_r 80.4k), a little less consistently observed, occurred at molecular mass corresponding approximately to one HSA and one lysozyme (Table 3.1). This band was observed on 12.5% gels for both pre-glycation treatments, but on 7.5% gels only with pre-glycated HSA - possibly because the 7.5% gels did not always resolve it from the broad HSA monomer band sufficiently. A preliminary gel (7.5%) run for the pre-glycated lysozyme treatment also showed a faint band corresponding in molecular weight to one HSA and three lysozyme molecules (result not shown), but this was not consistently observed in subsequent gel runs.

It may be noted that putative HSA/lysozyme oligomers were observed in both pre-glycated treatments and sugar-free controls, but were clearly enhanced by pre-glycation of either protein. This differs from results obtained with the lysozyme/β-lactoglobulin system, for which sugar-free controls showed only trace amounts of lysozyme/β-lactoglobulin heterodimer after three weeks sugar-free ageing. The difference could reflect the influence of the lipid component of HSA, a relatively

higher degree of *in vivo* glycation of the HSA preparation used, or a combination of these factors.

On 12.5% SDS-PAGE gels (Fig 3.9, 3.10), all treatments produced a noticeable band corresponding to lysozyme homodimer (M_r 28. 2k - 29.2k). With pre-glycated lysozyme, this band was enhanced relative to sugar-free controls, continuing to increase during sugar-free re-incubation. With pre-glycated HSA, the lysozyme homodimer band did not show consistent differences relative to sugar-free controls. This discrepancy could reflect the greater availability of glycated monomeric lysozyme for cross-linking during sugar-free ageing in the former experiment. In the latter, the concentration of glycated lysozyme would be extremely low, being derived only from *in vivo* glycation of the protein preparation.

Interestingly, there is some evidence of smearing and enhanced lower molecular weight bands (in the region M_r 45k - 66k) in longer re-incubations of glycated HSA with native lysozyme (Fig. 3.8, lane 9 *vs* lane 8). This may be indicative of protein fragmentation, as was suggested for β-lactoglobulin in the previous section. The same effect was not observed in co-incubations of native HSA with pre-glycated lysozyme, which again raises questions regarding the role of protein-associated lipid in ageing reactions of pre-glycated proteins.

3.4 DISCUSSION

Cross-linking of pre-glycated protein to a different native protein during sugar-free ageing has been demonstrated here with two different combinations of model proteins. In each case, one protein was lipid-free (lysozyme), while the other had

an associated lipid component (β-lactoglobulin or HSA). Similar oligomers formed in the lysozyme/HSA system, irrespective of which protein was pre-glycated.

The formation of lysozyme/HSA oligomers demonstrates that cross-linking reactions do occur between pre-glycated lysozyme and native HSA during sugar-free ageing *in vitro*, and similarly between pre-glycated HSA and native lysozyme. This suggests that, *in vivo,* serum proteins could cross-link with other (possibly non-serum) proteins, provided at least one of the cross-linking proteins has been previously exposed to elevated glucose concentrations.

Cross-linking of glycated protein to form homo-oligomers is well-documented in the literature. Eble *et al.* (1983) were among the first to demonstrate this, using RNase A as the model protein. This study also demonstrated formation of RNase polymers from glycated RNase re-incubated in the absence of sugar. Sakurai *et al.* (1984) demonstrated cross-linking of HSA incubated with glucose for 8 days to form oligomers up to the hexamer. Such oligomers were not observed with HSA under the milder pre-glycating conditions of this study. Prabhakaram and Ortwerth (1994) demonstrated cross-linking of lysozyme - also used in the present study - with a number of glycating sugars, using a radiolabelling technique and with sugar present throughout. These authors found cross-linking with glucose to be minimal when compared with other, more reactive sugars.

Little information is available on glycation-mediated cross-linking between different proteins. Considering the heterogenous nature of *in vivo* systems, this is likely to be the more relevant scenario physiologically and would also help to explain processes such as entrapment of serum proteins by glycated basement membrane proteins (Miller and Michael, 1976; Michael and Brown, 1981; Brownlee *et al.*, 1986a).

Liggins and Furth (1996) first described the model system used in this study. It was used to investigate the cross-linking of native β-lactoglobulin to lysozyme pre-glycated with fructose. They reported formation of a lysozyme/β-lactoglobulin heterodimer, as observed here with glucose, during sugar-free re-incubation following a range of fructating conditions. Comparison of heterodimer formation under fructating conditions (Liggins and Furth, 1996) and under the glucating conditions investigated here, demonstrates clearly the extent to which fructose is the more effective glycating sugar. Liggins and Furth (1996) reported a staining intensity of heterodimer of between 5% and 6% of total stain after 12 hours initial fructation and 1 week re-incubation. Similar levels of heterodimer were reached only after 5 days glucation (glucose at the same concentration as fructose) and 3 weeks sugar-free re-incubation.

Studying the formation of Maillard products from glucose under physiological conditions is difficult because it is a far less potent glycating agent than smaller sugars or sugar-derived intermediates (such as glucosones and α-dicarbonyls). Results obtained here show that while pre-glycation of proteins with glucose does carry disadvantages in terms of convenience of experimentation and yield of Maillard products, it is still feasible to detect glucose-associated changes occurring during sugar-free ageing. Furthermore, cross-linking was detectable within one week of glycation, even under relatively mild glycating conditions. This supports the contention of Thornalley *et al.* (1999) that advanced Maillard products may form quite soon after glycation and after relatively mild elevations of glucose concentration. The authors describe these effects as being mediated by α-dicarbonyls (including glyoxal, methylglyoxal and 3-deoxyglucosone) generated from degradation of glucose and of early glycation products.

While the present study did not aim to identify reactive intermediates arising from glycated proteins which may have acted as cross-linking agents, the results do suggest certain conclusions. Cross-linked and fluorescent products were low or absent immediately after glycation. Dialysis at this point would have removed free small dicarbonyl compounds. However, re-incubation of glycated protein with native proteins in the absence of free sugar (as a source of such cross-linking compounds) resulted in the formation of late Maillard products. Therefore, a significant proportion of the dicarbonyl glycation intermediates thought to be responsible for formation of cross-linked and fluorescent products of the Maillard reaction must initially have been protein-bound with sufficient stability to survive extensive dialysis. This corresponds with reports that Maillard intermediates such as 3-deoxyglucosone, glyoxal and methylglyoxal are generated from glycation products on protein as well as from the glycating sugar itself (Wells-Knecht *et al.*, 1995b; Zyzack *et al.*, 1995; Thornalley *et al.*, 1999).

Comparison of the relative formation of fluorescent and cross-linked Maillard products under increasingly intensive glycating conditions (Fig 3.6) showed that these differed. McPherson *et al.* (1988) and Liggins and Furth (1996) similarly found differences between fluorescence development and cross-linking during sugar-free ageing. The range of glycation (glucation) treatments investigated here was less extensive than the range of fructation durations used by Liggins and Furth (1996), and the present study combined both sugar concentration and glycating duration to achieve intensive glucating exposures. Despite this, a similar trend is indicated. In results reported here, protein cross-linking - which arguably has greater functional implications than protein fluorescence - occurred at lower glucose concentrations and shorter glycating exposures than did increases in protein fluorescence. Liggins and Furth (1996) reported that fluorescence developed with lysozyme which had been fructated for at least 3 hours, whereas cross-linked

protein products could be detected with lysozyme which had been fructated for only 30 min. Thus, the possibility of under-estimating Maillard product formation when using fluorescence as the primary estimator exists equally for glucose and fructose. This emphasises the need for measurement of multiple end-points in studies of processes as complex in nature as the Maillard reaction.

The difference between fluorescence development and protein cross-linking during sugar-free ageing of pre-glycated proteins suggests these end-points arise, at least in part, by different routes. Dyer *et al.* (1991b) and Wells-Knecht *et al.* (1996) have argued that many protein cross-links are not fluorescent. Le Guen *et al.* (1992) reported that fluorescence of glycated proteins - particularly those with associated lipid - arises mainly from the action of hydrogen peroxide, metal-catalysed oxidation and hydroxyl radicals. This strongly oxidant-mediated route to fluorescence fits with the longer pre-glycation time required for formation of fluorophores than for cross-links. By contrast, the major protein cross-linking agents implicated in glycation are thought to be α-dicarbonyls such as glyoxal and methylglyoxal (Brinkman Frye *et al.*, 1998; Degenhardt *et al.*, 1998b; Odani *et al.*, 1998), with the addition of carbonyl lipid oxidation products such as MDA (Requena *et al.*, 1997a; Slatter *et al.*, 1998) for lipid-associated proteins. Dicarbonyl compounds form rapidly by autoxidation of sugars and lipids, and by degradation of early glycation products on protein, suggesting that protein cross-linking can occur after exposure to relatively mild glycating conditions and is not as late a Maillard end-point as previously thought (Thornalley *et al.*, 1999). A hypothesis of this nature corresponds well with the results observed in this study.

Liggins and Furth (1996) reported enhanced cross-linking of native β-lactoglobulin during sugar-free ageing in the presence of fructated lysozyme. They proposed

that this was mediated by a 'diffusable cross-linking agent' originating from fructated lysozyme. This observation was not replicated in the present investigation when glucose was used as the glycating sugar. While β-lactoglobulin homodimers were detected, these were not enhanced by exposure of native β-lactoglobulin to glycated lysozyme. Indeed, there appeared to be some decrease in levels of β-lactoglobulin homodimers. Similarly, incubation of native lysozyme with pre-glycated HSA did not enhance lysozyme dimerisation. The difference may reflect the shorter pre-incubation times necessary to achieve significant levels of protein modification in the presence of fructose as compared with glucose. Periodate assay of early glycation products (reported in Chap. 5) strongly indicated that these are initially protein-bound, but are released (either in original form or as derivatives) during subsequent ageing. It is possible that, under the conditions used by Liggins and Furth (1996), the diffusable agent which promoted cross-linking of native β-lactoglobulin was still substantially protein-bound (and therefore stable to dialysis) after their shorter glycation periods and was released as a free diffusable compound during subsequent re-incubation. By contrast, the longer pre-incubation periods required for protein modification by glucose may have meant that this agent was already predominantly present in the diffusable form at the end of the glycation period and was therefore removed from the experimental system during dialysis to remove free sugar.

An alternative view is suggested by results reported by Sajithlal *et al.* (1998). They incubated heavily AGE-modified BSA with native collagen in the absence of free sugar, and observed enhanced cross-linking of collagen. This suggests that AGEs on BSA acted as a source of diffusable cross-linking agents, similar to observations reported by Liggins and Furth (1996) for fructose, but with protein which had been incubated with glucose for much longer periods and on which late Maillard reactions

had been allowed to proceed prior to co-incubation with collagen. Thus it is possible that glucose-modified proteins begin to give rise to diffusable cross-linking agents considerably later and slower than fructose-modified proteins. This suggests that - although diffusable glycation-derived products did arise during sugar-free ageing (Chap. 5) - cross-linking compounds were possibly not generated at sufficient concentration to mediate dimerisation of a different native protein in solution under the conditions of the present study.

While the reactions of protein-associated lipid were not specifically targeted in this investigation, the inclusion of a lipid-associated protein in the model system did indicate that lipid contributes to the ageing reactions of glycated proteins. This is in accordance with observations in various lipoprotein systems, in which the presence of lipid has been associated with enhanced fluorescence (Le Guen *et al.*, 1992; Cochrane and Furth, 1993) and protein fragmentation (Hunt *et al.*, 1993). Fragmentation in particular has been associated with oxidation of the glycated protein (Coussons *et al.*, 1997). However, enhanced fragmentation of glycated lipid-associated protein has not been consistently observed by all investigators (Ahmed and Furth, 1990; Cochrane, 1995). In particular, Cochrane (1995) observed fragmentation of glycated HSA only in the presence of glucose, with no significant enhancement of fragmentation by HSA-associated lipid during sugar-free incubation.

3.5 SUMMARY

- Cross-linked lysozyme/ß-lactoglobulin heterodimer was formed during sugar-free re-incubation of pre-glucated proteins with native proteins, as has been previously demonstrated for fructose.

- Formation of late Maillard products continued after removal of free sugar. This indicated that at least a proportion of early precursors to the late Maillard products appear to be protein-bound with sufficient stability to survive prolonged dialysis.

- Heterodimer formation was observed during sugar-free re-incubation after all pre-glycation regimes investigated, with no clear evidence of a threshold effect. Fluorescent products increased markedly only under intensive pre-glycation. This indicates that a threshold condition was associated with fluorophore formation.

- New protein bands were formed during sugar-free re-incubation of pre-glycated lysozyme with native HSA, and of pre-glycated HSA with native lysozyme. These bands corresponded in molecular weight to oligomers of one HSA molecule with either one or two lysozyme molecules. The potential for cross-linking reactions between serum proteins with other (potentially non-serum) proteins as a consequence of brief glycation is therefore confirmed.

- Protein-associated lipid may influence the susceptibility of lipoproteins to cross-linking and to fragmentation.

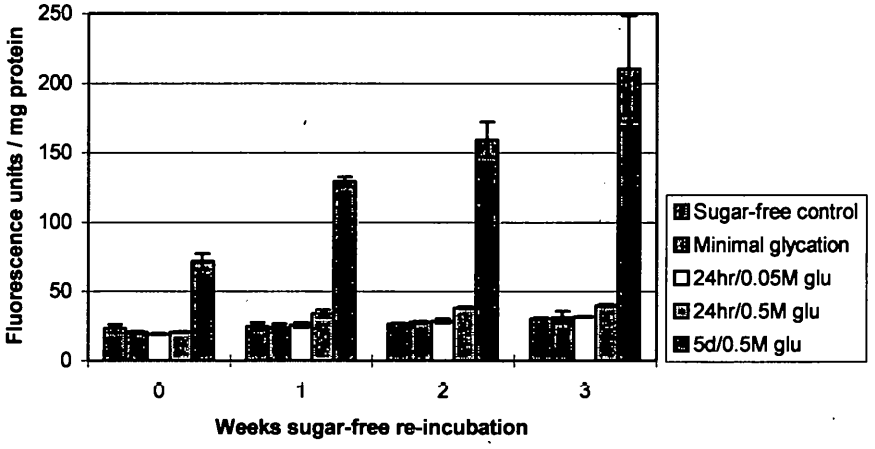


Fig. 3.1

Fluorescence development (ex/em 350/420nm) in co-incubations of pre-glycated lysozyme and native β-lactoglobulin during 3 weeks sugar-free ageing. Lysozyme was exposed to a range of pre-glycating conditions.

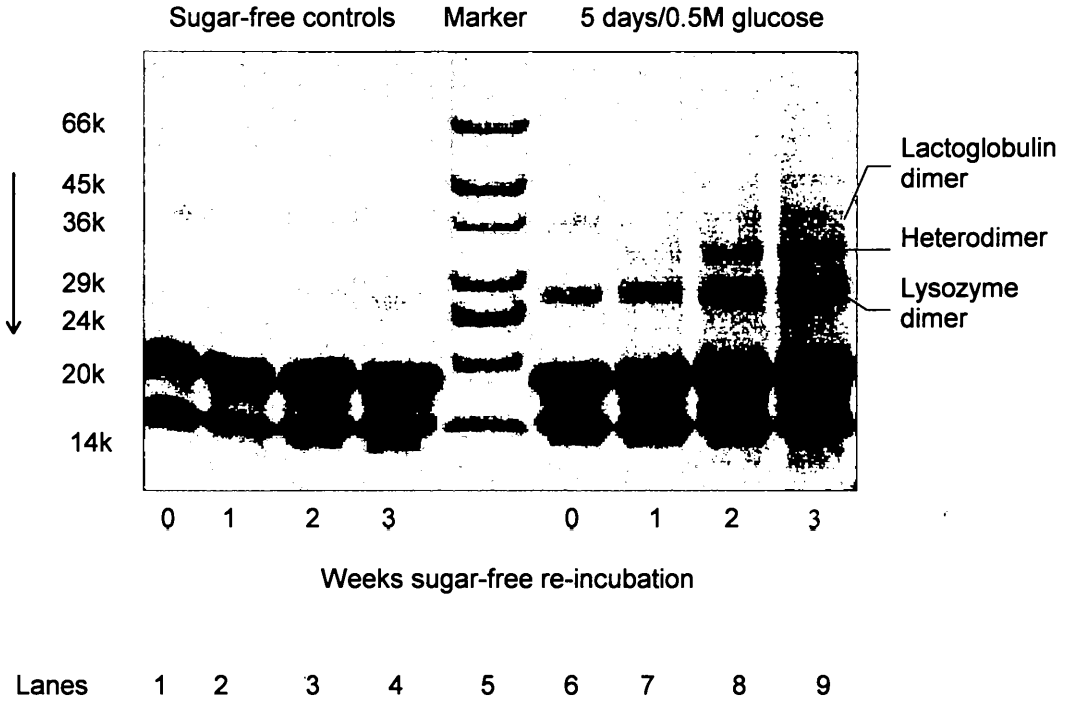


Fig. 3.2

Cross-linking of pre-glycated lysozyme (glycated for 5 days in 0.5M glucose at 37°C) and native β-lactoglobulin during 3 weeks sugar-free ageing.

Lanes 1-4 Sugar-free controls, *i.e.* lysozyme initially incubated without glucose

Lanes 6-9 Pre-glycated lysozyme

Native β-lactoglobulin present throughout ageing

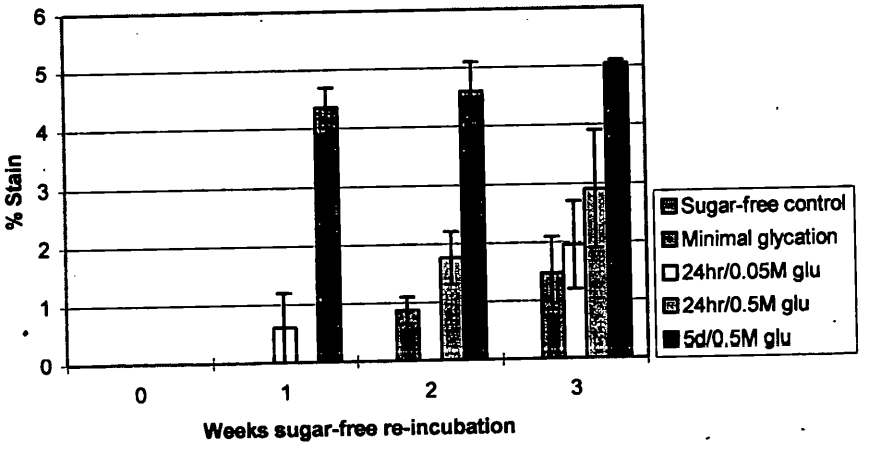


Fig. 3.3

Formation of lysozyme/β-lactoglobulin heterodimer in co-incubations of pre-glycated lysozyme and native β-lactoglobulin during 3 weeks sugar-free ageing. Lysozyme was exposed to a range of pre-glycating conditions.

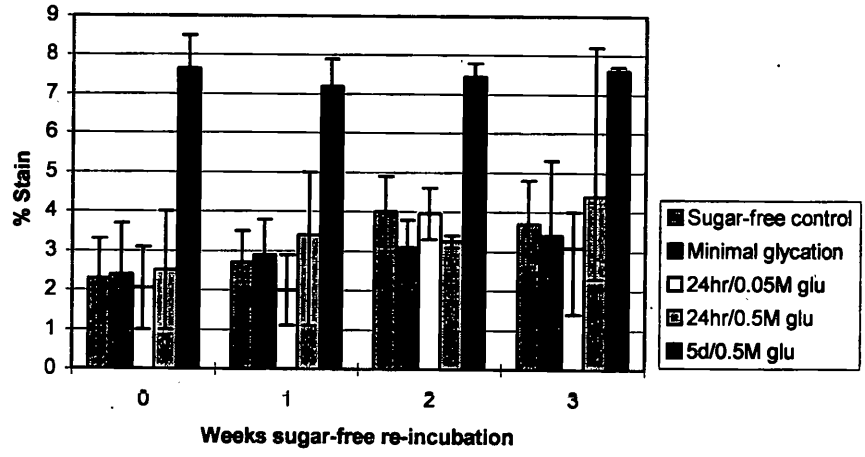


Fig. 3.4

Formation of lysozyme homodimer in co-incubations of pre-glycated lysozyme and native β-lactoglobulin during 3 weeks sugar-free ageing. Lysozyme was exposed to a range of pre-glycating conditions.

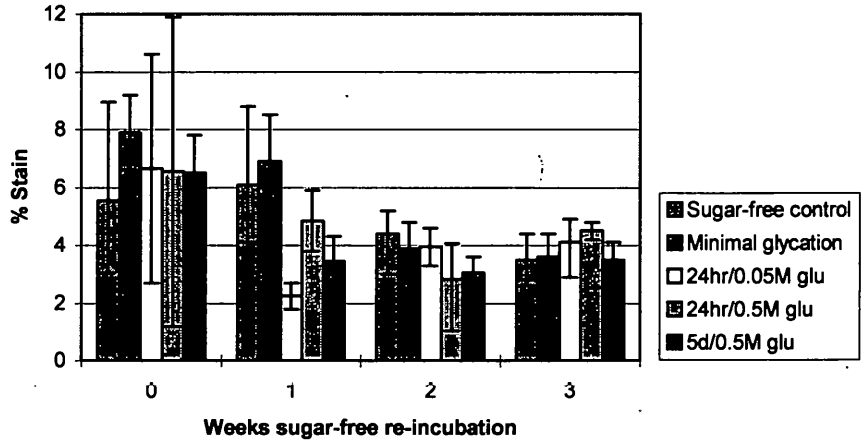


Fig. 3.5

Formation of β-lactoglobulin homodimer in co-incubations of pre-glycated lysozyme and native β-lactoglobulin during 3 weeks sugar-free ageing. Lysozyme was exposed to a range of pre-glycating conditions.

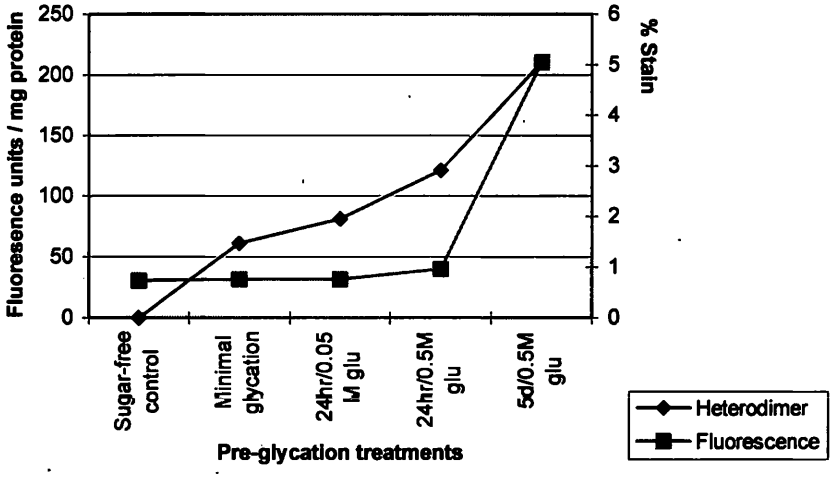


Fig. 3.6

Comparison of mean fluorescence and mean heterodimer staining intensity in co-incubations of pre-glycated lysozyme and β-lactoglobulin after 3 weeks sugar-free ageing. Lysozyme was exposed to a range of pre-glycating conditions.

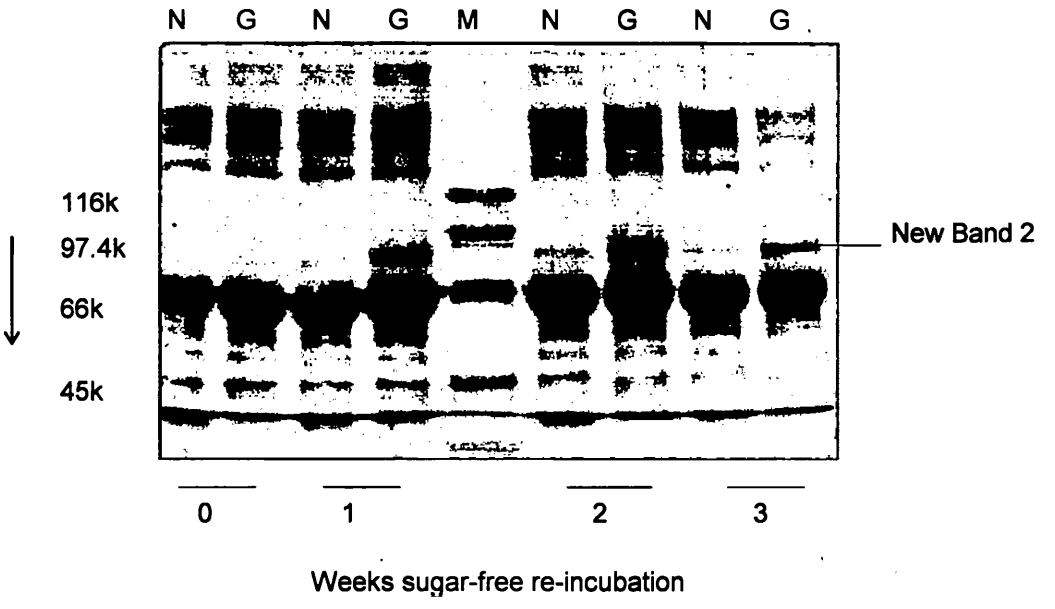


Fig. 3.7

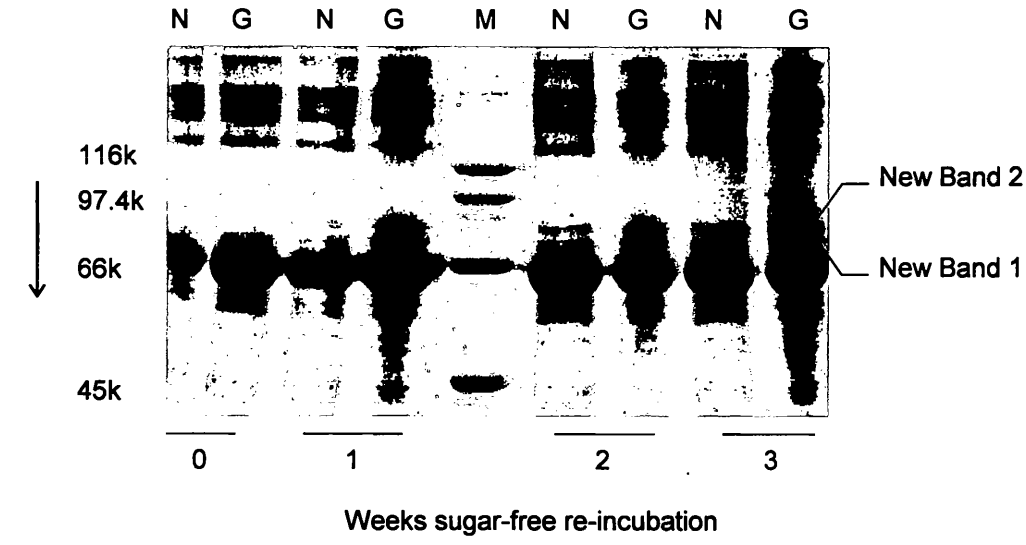
Cross-linking of pre-glycated lysozyme and native HSA during 3 weeks sugar-free ageing, as shown on 7.5% SDS-PAGE gels.

M Marker

N Native protein (lysozyme initially incubated without glucose)

G Pre-glycated lysozyme

Native HSA present in all N and G



Lanes 1 2 3 4 5 6 7 8 9

Fig. 3.8

Cross-linking of native lysozyme and pre-glycated HSA during 3 weeks sugar-free ageing, as shown on 7.5% SDS-PAGE gels.

M Marker

N Native protein (HSA initially incubated without glucose)

G Pre-glycated HSA

Native lysozyme present in all N and G

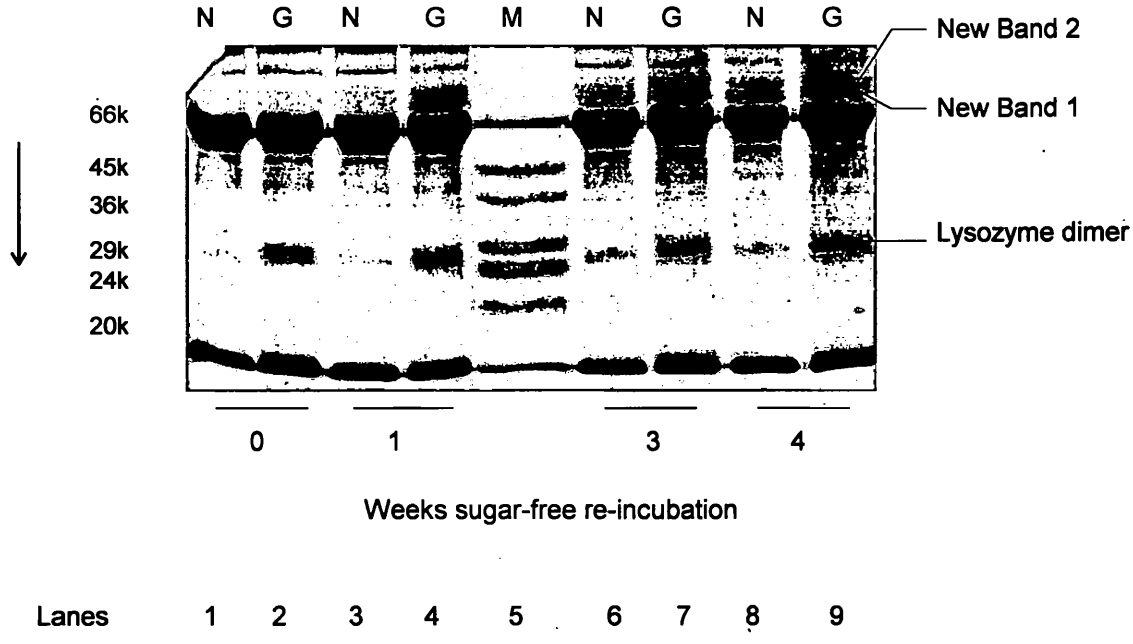


Fig. 3.9

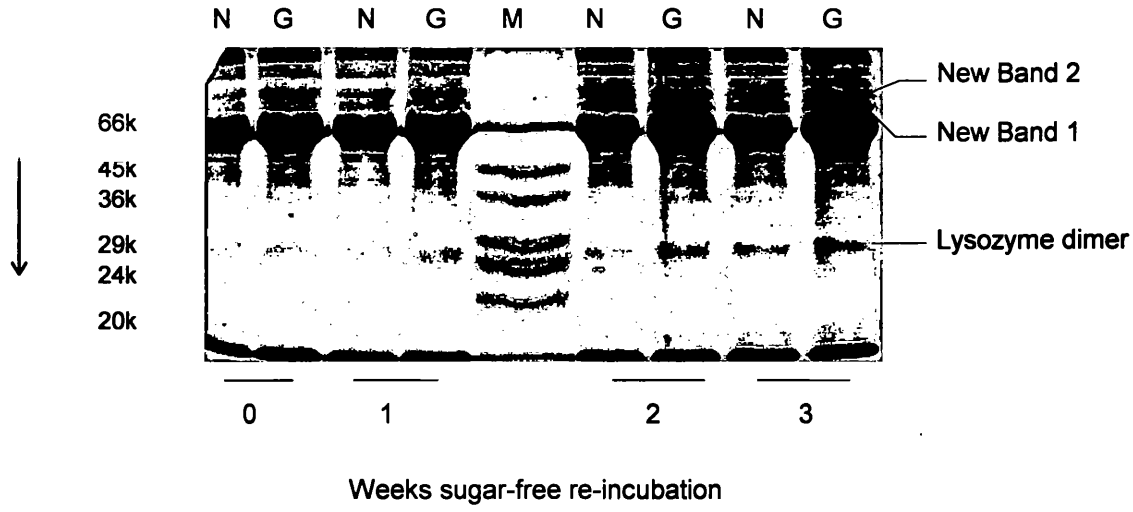
Cross-linking of pre-glycated lysozyme and native HSA during 3 weeks sugar-free ageing, as shown on 12.5% SDS-PAGE gels.

M Marker

N Native protein (lysozyme initially glycated without glucose)

G Pre-glycated lysozyme

Native HSA present in all N and G



Lanes	1	2	3	4	5	6	7	8	9

Fig. 3.10

Cross-linking of native lysozyme and pre-glycated HSA during 3 weeks sugar-free ageing, as shown on 12.5% SDS-PAGE gels.

M Marker

N Native protein (HSA initially glycated without glucose)

G Pre-glycated HSA

Native lysozyme present in all N and G

Table 3.1

Theoretical and estimated relative molecular mass (M_r) of lysozyme/HSA oligomers.

Theoretical M_r of lysozyme/HSA oligomers		
Lysozyme		14k
HSA		66k
HSA + Lysozyme		80k
HSA + 2(Lysozyme)		94k
Estimated M_r of cross-linked products from HSA/lysozyme pre-glycation treatments (cf. Fig. 3.7 - 3.10)		
New Band 1	Mean	80.4k
	Range	79.4k - 81.3k
New Band 2	Mean	91.5k
	Range	87.1k - 97.7k

CHAPTER 4

INHIBITORS OF THE MAILLARD REACTION

4.1 INTRODUCTION

The aim of studies reported in this chapter was to investigate the effect of inhibitors of post-Amadori reactions - predominantly aminoguanidine - on the formation of fluorescent and cross-linked Maillard products.

An initial trial compared the effects of aminoguanidine with those of the free metal chelator, diethylene triamine pentaacetic acid (DTPA). This was expanded to more comprehensive experiments investigating the effect of aminoguanidine when present during glycation or during later Maillard reactions.

The incorporation of aminoguanidine in the first glycating incubation (protocol i) provided a model for the effect of this inhibitor on the sugar-dependent early reactions of glycation. The parallel sugar-free incubation with aminoguanidine provided insight into the effect of brief pre-exposure to aminoguanidine on normoglycaemic ageing of native protein.

The incorporation of aminoguanidine in the sugar-free re-incubation (protocol ii) served as a model for its effect on later sugar-independent Maillard reactions. A parallel

sugar-free incubation modelled the effect of longer term aminoguanidine exposure during normoglycaemic protein ageing.

The glucose concentration in the glycating incubation was selected on the basis of results reported in Chap. 3. Under pre-glycating conditions of 0.5M glucose for 24 hours, measurable cross-linking was detected. A marked increase in cross-linked and fluorescent products occurred when the glycation period was increased to 5 days. However, under these conditions there are more likely to be late Maillard products formed both during glycation and subsequent sugar-free re-incubation. Therefore the glycating regime used in the experiments reported here was 0.5M glucose for 24 hours at 37°C.

Aminoguanidine concentrations were selected to produce an aminoguanidine/glucose ratio comparable to that in clinical trials of aminoguanidine. Bucala *et al.* (1994) reported a mean plasma concentration of approximately 0.1mM aminoguanidine. Maximum blood glucose concentrations in hyperglycaemia are unlikely to exceed 50mM, yielding an approximately 500-fold excess of glucose over aminoguanidine. Since the glucose concentration in the glycating medium was 500mM, a corresponding aminoguanidine concentration of 1mM was selected. Since low aminoguanidine concentrations have been reported to be associated with pro-oxidant activity (Philis-Tsimikas *et al.*, 1995; Skamarauskas *et al.*, 1996), a higher concentration (25mM) was also tested.

4.2 METHODS

4.2.1 Preliminary trial with DTPA and aminoguanidine

In an early pilot experiment, the inhibitors DTPA and aminoguanidine (bicarbonate salt) were included in glycation reaction mixtures. The intention was to boost levels of early glycation products to provide additional intermediates for later Maillard reactions during sugar-free re-incubation. Lysozyme (10mg/ml) was incubated for 24 hours in the presence of 0.5M glucose, with or without either aminoguanidine (25mM) or DTPA (1mM). Free sugar and inhibitors were removed by dialysis. The glycated lysozyme was re-incubated with native ß-lactoglobulin (total protein concentration 10mg/ml) for one week. Pre-glycated treatments were conducted in duplicate. Fluorescence and cross-linking were monitored as described in Chapter 2.

No significant cross-linked products were detected after one week, therefore samples were re-incubated for a further two weeks and monitored again for cross-linking. Fluorescence was not measured again due to insufficient sample volumes.

4.2.2 Aminoguanidine present in either first (glycating) or second (sugar-free) incubation

The experimental system described in Chap. 2 for investigating cross-linking of glycated protein to native protein in the absence of free sugar was used. Aminoguanidine was included in either the first incubation in the presence of free sugar

(protocol i) or the second sugar-free incubation (protocol ii). All incubations were carried out in duplicate.

In protocol (i), lysozyme (10mg/ml) was incubated for 24 hours at 37°C in the presence of 0.5M glucose in 0.1M phosphate buffer (pH 7.4), with or without aminoguanidine at 1mM or 25mM. Free glucose and aminoguanidine were then removed by exhaustive dialysis for 4 days against phosphate buffer at 4°C. The glycated lysozyme so formed was re-incubated with native ß-lactoglobulin (approximately equal concentrations; total protein concentration 10mg/ml) in phosphate buffer in the absence of both glucose and aminoguanidine for three weeks. Aliquots were withdrawn at 0, 1, 2 and 3 weeks and were stored frozen at -20°C until analysed. Native proteins were investigated by parallel treatments that resembled the above, but excluded glucose.

In protocol (ii), lysozyme (10mg/ml) was incubated for 24 hours at 37°C in the presence of 0.5M glucose in 0.1M phosphate buffer (pH 7.4). Free glucose was removed by exhaustive dialysis against phosphate buffer at 4°C. The glycated lysozyme was re-incubated with native ß-lactoglobulin (total protein concentration 10mg/ml), with or without addition of aminoguanidine (1mM or 25mM), for 3 weeks. Again, corresponding glucose-free treatments were carried out. Aliquots were withdrawn at 0, 1, 2 and 3 weeks and were stored frozen at -20°C. At the end of the re-incubation, all aliquots for protocol (ii) were dialysed again, to remove aminoguanidine prior to analysis.

4.2.3 Investigating sugar-independent reactions of aminoguanidine

The possibility of a sugar-independent fluorescence-generating reaction between either of the model proteins and aminoguanidine was tested by incubating each native protein for three weeks, either alone or with 25mM aminoguanidine. Aliquots were removed at 0, 1, 2 and 3 weeks incubation, and fluorescence monitored.

4.3 RESULTS

All figures referred to in the text below are presented at the end of the chapter.

4.3.1 Preliminary trial with DTPA and aminoguanidine

Development of fluorescence after one week sugar-free re-incubation is shown in Fig. 4.1. Pre-glycated proteins gave more fluorescence after one week re-incubation when DTPA had been present during glycation. The inclusion of 25mM aminoguanidine during glycation had little impact over this re-incubation period.

No protein cross-linking could be detected on SDS-PAGE after one week re-incubation. Prolonging the re-incubation period to three weeks yielded cross-linked product both in the absence of inhibitors (Fig. 4.2, lane 2) and in the presence of inhibitors. Cross-linking of pre-glycated proteins was reduced by aminoguanidine present during glycation (lane 5), but not by DTPA (lane 6). Levels of heterodimer detected when proteins were glycated in the presence of DTPA were not as strongly enhanced as was

fluorescence under the same conditions. No heterodimer band was visible in native proteins (sugar-free incubations) for the inhibitor-free (lane 1) and DTPA-containing (lane 7) treatments. Laser densitometry revealed traces of heterodimer in both native and glycated proteins pre-treated with aminoguanidine (results not shown), but this was not readily visible upon visual inspection of the gels.

Thus:

i) In pre-glycated proteins, DTPA present during glycation enhanced fluorescence during re-incubation and did not impede cross-linking. Aminoguanidine had no effect on fluorescence, but reduced cross-linking.

ii) Native proteins pre-incubated alone or with DTPA did not appear to cross-link during re-incubation. There may have been traces of cross-linked product when proteins were pre-incubated with aminoguanidine.

This indicates that the mechanism by which aminoguanidine acts differs from that of DTPA (*viz.* by blocking metal catalysed oxidation which occurs after formation of Amadori product).

4.3.2 Aminoguanidine present in either first (glycating) or second (sugar-free) incubation

The preliminary trial reported above was expanded to try to elucidate the effect of aminoguanidine.

Results are presented in Fig. 4.3 - 4.8, and are discussed below. Protein fluorescence and cross-linking measured when aminoguanidine was present in the first glycating incubation (protocol i) are shown in Fig. 4.3 (A-D). The same parameters measured with aminoguanidine present in the second sugar-free re-incubation (protocol ii) are shown in Fig. 4.4 (A-D). Representative SDS-PAGE gels after three weeks sugar-free ageing are given in Fig. 4.5 and 4.6 for protocol (i) and protocol (ii), respectively. Fig. 4.7 and 4.8 show nett glycation-associated change observed under protocol (i) and protocol (ii), respectively.

Fluorescence

Native proteins

Mean fluorescence was low and changed little during ageing of native proteins, whether these had been pre-incubated with aminoguanidine or in buffer alone (Fig. 4.3A, Native protein). However, re-incubation of native proteins with aminoguanidine continuously present for three weeks enhanced fluorescence significantly. This was most marked in the first two weeks of re-incubation. (Fig. 4.4A, Native protein). Indeed, this aminoguanidine-associated increase in fluorescence appeared to start within minutes of adding aminoguanidine.

Glycated protein

Overall, mean fluorescence increased during sugar-free ageing in all pre-glycated treatments under both protocols. However, the rate of fluorescence increase was affected differently by aminoguanidine under the two protocols. Pre-treatment with aminoguanidine (protocol i) reduced the rate of increase in fluorescence during ageing

of glycated proteins (Fig. 4.3A, Glycated protein). The reduction was significant with 25mM aminoguanidine, and became more marked with time over the re-incubation period. This is surprising since free aminoguanidine was removed, together with glucose, before re-incubation.

In contrast, once proteins were glycated, the rate of increase in fluorescence was promoted by the presence of aminoguanidine during ageing (Fig. 4.4A, Glycated protein). This finding is consistent with the observed effect of aminoguanidine on native protein. Fluorescence at the highest aminoguanidine concentration appeared to reach a maximum relatively early in the re-incubation period. This may indicate a saturation of sites for fluorescence development.

Mean fluorescence was higher in glycated proteins than in native proteins under protocol (i) (Fig. 4.3A, Glycated *vs* Native protein). Under protocol (ii), mean fluorescence of native protein was similar to that of glycated protein (Fig. 4.4B, Native *vs* Glycated protein). Aminoguanidine suppressed fluorescence in glycated protein under protocol (i). It enhanced fluorescence in both native and glycated proteins under protocol (ii).

Nett glycation-associated change

The arithmetic difference between mean changes observed in glycated proteins and those in native protein was taken to represent the change associated only with exposure to glucose, termed nett glycation-associated change.

Under protocol (i), nett glycation-associated fluorescence increased during sugar-free ageing in both the presence and absence of aminoguanidine. The rate of increase was

lowered by aminoguanidine in a concentration-dependent manner (Fig. 4.7A). Under protocol (ii), with aminoguanidine present throughout the second incubation, nett fluorescence was low and erratic, reflecting the observed similarity in fluorescence for native and pre-glycated protein (Fig. 4.8A).

Protein cross-linking

Qualitative observations from SDS-PAGE gels

(a) Native proteins

SDS-PAGE gels showed that lysozyme and β-lactoglobulin homodimers were present from the beginning of the second incubation under both protocols. In neither case was any heterodimer visible at this point (results not shown). Fig. 4.5 and 4.6 show protein dimer formation after three weeks re-incubation.

Slight traces of heterodimer formed during re-incubation of native proteins, but only if aminoguanidine had been present in the initial incubation (protocol i, Fig. 4.5, lanes 3, 5 *vs* lane 1). Thus it appears that aminoguanidine pre-treatment of native proteins may promote cross-linking during sugar-free ageing, but the effect was small and only became visible after prolonged incubation.

In native proteins re-incubated with aminoguanidine (protocol ii, Fig. 4.6), both homodimers and the heterodimer were markedly enhanced by 25mM aminoguanidine (lane 5). This was particularly noticeable for the β-lactoglobulin homodimer. Similar effects may be seen at 1mM aminoguanidine, but these are relatively faint. In addition,

the 25mM aminoguanidine treatment caused smearing and increased low molecular weight bands, indicative of protein fragmentation. Thus the presence of aminoguanidine during sugar-free ageing of native protein clearly enhance cross-linking to both the same and different proteins. This effect appeared particularly pronounced for dimerisation of lipid-associated proteins.

(b) Glycated proteins

Homodimer bands were visible in pre-glycated proteins on SDS-PAGE from the beginning of the second incubation. A clear heterodimer band appeared in aminoguanidine-free treatments under both experimental protocols at week 1 and increased in intensity with time (results not shown). As before, Fig. 4.5 and 4.6 show protein dimerisation after three weeks sugar-free re-incubation.

Under protocol (i), aminoguanidine pre-treatment initially suppressed heterodimer formation during ageing (results not shown), but after three weeks re-incubation all treatments showed a clear heterodimer band (Fig. 4.5, lanes 2, 4, 6). There was little difference in intensity of heterodimer or homodimer bands between the aminoguanidine-free (lane 2) and 1mM aminoguanidine treatment (lane 4), although 25mM aminoguanidine appeared to slightly inhibit dimerisation (lane 6). Thus aminoguanidine present during glycation initially suppressed protein dimerisation, but this effect was barely visible after three weeks sugar-free ageing.

Glycated proteins allowed to age for three weeks in the presence of aminoguanidine (protocol ii, Fig. 4.6) also showed heterodimer in all treatments. There was little difference between heter- and homodimer levels in the aminoguanidine-free (lane 2) and 1mM aminoguanidine samples (lane 4), although dimerisation was possibly slightly

suppressed by 1mM aminoguanidine. However, all protein dimers were clearly enhanced by 25mM aminoguanidine (lane 6). Enhanced cross-linking was observed in both native and glycated proteins at this concentration. Interestingly, the β-lactoglobulin homodimer was more strongly enhanced in native proteins (lane 5), while the lysozyme homodimer appeared slightly more enhanced in glycated protein (lane 6). As noted for native protein, the presence of 25mM aminoguanidine was associated with smearing and increased low molecular weight bands, again indicative of protein fragmentation. This smearing complicated comparison of intensities of bands in native (lane 5) and glycated (lane 6) protein. Thus the presence of aminoguanidine during sugar-free ageing of pre-glycated protein appeared to promote formation of homo- and heterodimers, as it did with native proteins. The presence of lipid may have altered the susceptibility of protein to aminoguanidine-enhanced cross-linking.

Quantitative results from laser densitometry

Laser densitometry was used to confirm the trends noted on visual inspection of SDS-PAGE gels by quantifying the percentage stain associated with protein bands.

(a) Native proteins

The mean percentage stain associated with heterodimer bands in native protein increased with inclusion of aminoguanidine in either the first or the second incubation, indicating that this promoted the formation of heterodimer (Fig. 4.3B and 4.4B, Native protein). Aminoguanidine in the second incubation, particularly at the higher concentration, also promoted homodimer formation. (Fig. 4.4C, D - Native protein). This was especially notable with β-lactoglobulin, which may be related to the presence

of oxidisable lipid on the protein. Promotion of dimerisation was generally significant with 25mM aminoguanidine, although it was variable at 1mM aminoguanidine.

(b) Glycated proteins

No aminoguanidine (Protocols i and ii)

Quantitation of the mean percentage stain located in each band showed that glycated lysozyme slowly cross-linked to native β-lactoglobulin when the proteins were left to age together (Fig. 4.3B, 4.4B - Glycated protein, no AG). Lysozyme homodimer also appeared to increase with time (Fig. 4.3C, 4.4C - Glycated protein). Levels of β-lactoglobulin homodimer in aminoguanidine-free treatments showed no significant change, although there appeared to be a slight decreasing trend (Fig. 4.3D, 4.4D - Glycated protein). These observations are similar to the cross-linking observed under varying pre-glycation conditions, as reported in Chap. 3.

Protein cross-linking to form homodimers is well documented in the literature. However, the formation of heterodimers as reported here and by Liggins and Furth (1996) has not been widely investigated, and has not been previously reported for proteins glycated with glucose.

Protocol (i)

Glycation of proteins in the presence of aminoguanidine reduced dimerisation during week 1 of sugar-free re-incubation, particularly for the heterodimer. The effect was small and by week 2, mean dimer levels were similar whether or not aminoguanidine had been present initially (Fig. 4.3B, C, D).

Protocol (ii)

As with native proteins, re-incubation of glycated proteins with aminoguanidine promoted formation of all three dimers (Fig. 4.4B-D, Glycated protein). The promotion was variable with 1mM aminoguanidine, but was generally significant with 25mM. Aminoguanidine-enhanced β-lactoglobulin homodimer levels were slightly lower in glycated proteins than in native proteins. Overall, homodimer and heterodimer formed to a greater extent when aminoguanidine was present in the second sugar-free incubation than when it was present in the glycating medium .

Nett glycation-associated change

If the arithmetic difference in cross-linking between glycated and native proteins does indeed pinpoint purely glycation-associated effects, then aminoguanidine present during early stages of the Maillard reaction (protocol i) clearly inhibited the subsequent development of cross-linking (Fig. 4.7B-D), as was also observed for fluorescence (Fig. 4.7A). (A possible exception was lysozyme homodimer (Fig. 4.7C), where pre-treatment with 1mM aminoguanidine appeared to promote dimerisation after one week, although it decreased thereafter.)

When aminoguanidine was present only during sugar-free ageing (protocol ii), its nett effects were small and variable. Heterodimerisation (Fig. 4.8B) was clearly inhibited, but other processes showed no clear trend (Fig. 4.8C, D).

Hence by calculating nett glycation-associated changes in this way, the original view of aminoguanidine as an inhibitor of advanced Maillard products can be broadly supported. However, absolute changes in both native and glycated proteins are clearly promoted rather than inhibited by aminoguanidine. The discrepancy between overall

effects and nett glycation-associated change shows clearly that aminoguanidine has effects which are independent of its interaction with the Maillard reaction (*i.e.* with that group of reactions dependent at least initially on the presence of sugar).

4.3.3 Investigating sugar-independent reactions of aminoguanidine

Fig. 4.9 shows results of additional trials, in which lysozyme and β-lactoglobulin were incubated individually, with or without aminoguanidine, to investigate the possibility of direct reaction between the model proteins and the inhibitor. Lysozyme fluorescence remained low, but increased slowly over three weeks both with and without aminoguanidine. The effect of aminoguanidine was variable, but by three weeks, slightly inhibitory. Fluorescence of β-lactoglobulin incubated without aminoguanidine increased to saturation after one week, with a plateau value approximately half that of lysozyme fluorescence after three weeks. However, with aminoguanidine, results were quite different. Fluorescence continued to increase throughout the incubation period, and after three weeks was almost double that of lysozyme. Hence much of the aminoguanidine-promoted fluorescence seen in the re-incubation experiments reported under 4.2.2 appears to have come from β-lactoglobulin, probably arising from protein-associated lipid.

4.4 DISCUSSION

4.4.1 Preliminary trial with DTPA and aminoguanidine

When present during glycation, DTPA enhanced formation of some early Maillard products, presumably by blocking their onward reaction to later Maillard products. After removal of DTPA, re-incubation in the absence of sugar lead to enhanced formation of fluorescent Maillard products as the accumulated early products reacted further. The same effect was less evident for cross-linked Maillard products. This discrepancy may indicate that more fluorophore precursors than cross-link precursors were formed under the anti-oxidative conditions of the DTPA-containing glycating medium.

By contrast, pre-incubation with aminoguanidine neither boosted nor inhibited fluorescent Maillard products over the first week of re-incubation. Cross-linked products were reduced, but aminoguanidine may additionally have had some effect on cross-linking independent of glycation. Thus it is evident that the inhibitory mechanism of aminoguanidine does not comprise a simple blocking of oxidative (post-Amadori) reactions, as is the case for DTPA.

Previous work using a similar experimental system (Liggins, 1996; Liggins and Furth, 1997) also indicated differential effects on different Maillard end-points. Aminoguanidine was found to inhibit fluorescence development at extremely low concentrations (50-60% inhibition at 1 mM aminoguanidine) while blocking of protein-bound carbonyl groups as measured by a dinitrophenyl hydrazine assay was considerably less effective. This runs counter to the hypothesis that aminoguanidine

inhibits advanced glycation reactions (including those giving rise to fluorescence), primarily by blocking glycation-derived carbonyl groups. It supports published findings that blocking of protein-bound carbonyls is not the overriding factor in aminoguanidine action (Requena *et al.*, 1993). It now appears likely that a significant proportion of the fluorescence which was inhibited by aminoguanidine, as reported by Liggins *et al.* (1998), was associated with reactions of protein-associated lipid, rather than directly with protein. Thus, lipid may be more vulnerable to aminoguanidine than protein.

4.4.2 Aminoguanidine present in either first (glycating) or second (sugar-free) incubation

Effect of aminoguanidine on early relative to later Maillard reactions

When aminoguanidine was present in the first incubation, levels of earlier Maillard products (indicated by PPM and reported in Chapter 5) were higher and those of later Maillard products (protein cross-links, fluorescence) were lower than when aminoguanidine was present in the second incubation. Similarly, aminoguanidine inhibited fluorescence and cross-linking when it was present during early glycation (protocol i), but not when it was present during later Maillard reactions (protocol ii). This suggests that aminoguanidine was more effective at preventing conversion of early glycation products to advanced Maillard products when present during glycation than during later sugar-independent Maillard reactions. Support for this interpretation derives from observations in the trial experiment comparing DTPA and aminoguanidine,

which indicated that results of pre-incubation with aminoguanidine were inconsistent with simple blocking of later oxidative Maillard reactions.

Booth *et al.* (1996, 1997), studying the formation of immunogenic AGEs during sugar-free re-incubation of RNase A incubated with ribose for 24 hours, reported similar observations. Aminoguanidine added only to the sugar-free (post-Amadori) incubation had little inhibitory effect. These authors also suggested that aminoguanidine appeared more successful at inhibiting formation of Maillard products when present during early stages of the Maillard reaction, than when present during later sugar-Independent stages.

These authors further reported that aminoguanidine delayed the formation of immunogenic AGEs, rather than reducing final levels thereof. The present study supports such observations at least partially, since the overall inhibitory effect of aminoguanidine pretreatment on cross-linking of glycated protein decreased over time (Fig. 4.3, B-D - Glycated protein). However, inhibition of nett glycation-associated cross-linking (difference between cross-linking in native and glycated proteins) was maintained over time in some treatments, as was overall inhibition of fluorescence. This suggests that the effect of aminoguanidine is multifactorial.

Unfortunately the studies reported by Booth *et al.* (1996, 1997) must be viewed with some caution since the reactive intermediate claimed by the authors to be formed during ribation could not be detected in their experimental system when glucose was used as the glycating sugar. Furthermore, the anti-AGE antibodies used to quantify advanced Maillard products were raised against uncharacterised products resulting from incubation of protein with glucose (not ribose) for an extended period.

Results of the present investigation indicate that the overall effect of aminoguanidine represents the balance of competing reactions, some reducing Maillard products and others promoting reactions also yielding fluorescent and cross-linked products. Inhibitory reactions of aminoguanidine appeared to dominate earlier in the Maillard reaction, while reactions enhancing levels of cross-links and fluorophores appeared to emerge during later stages of the Maillard reaction or during prolonged exposure to aminoguanidine. Therefore the effect of aminoguanidine on glycated protein may be critically dependent on the timing of its introduction. (The promotion of such products by aminoguanidine in native proteins is addressed in the next section.)

Other authors have reported competing reactions of aminoguanidine. Skamarauskas *et al.* (1996) proposed competing oxidative processes such as dicarbonyl formation *vs* fragmentation as an explanation for the observed effect of aminoguanidine on pre-glycated BSA. While fragmentation was not directly investigated in the experiments reported here, the presence of 25mM aminoguanidine in the second incubation resulted in marked smearing and increased low molecular weight bands on SDS-PAGE in both native and glycated proteins (Fig. 4.6 - lanes 5 and 6). These effects increased with time and are indicative of protein fragmentation. The same was not observed when aminoguanidine was present only in the first incubation, indirectly supporting the observations of Skamarauskas *et al.* (1996) and providing further evidence that aminoguanidine interacts differently with early and late Maillard products.

Philis-Tsimikas *et al.* (1995) reported a balance of pro-oxidant and anti-oxidant activities of aminoguanidine when incubated with lipoprotein in the absence of sugar. Pro-oxidant activity was observed at low concentrations of aminoguanidine when the

compound was incubated with LDL. Higher concentrations were associated with anti-oxidant activity. This dual activity was hypothesised to occur subsequent to nucleophilic attack of aminoguanidine on a PUFA carbon, resulting in the release of perhydroxy radical. The authors propose that at low aminoguanidine concentrations the liberated radical may promote peroxidation of protein-associated lipid, whereas at high aminoguanidine concentrations it reacts preferentially with aminoguanidine. Pro-oxidant effects were detected at aminoguanidine concentrations up to 0.1mM, though this increased as the pre-existing level of oxidation of the LDL preparations increased. Thus their results depended on aminoguanidine concentration, rather than on the timing of its introduction into a lipoprotein system.

Aminoguanidine promotion of fluorescence and cross-linking under sugar-free conditions

Aminoguanidine present during the initial incubation appeared to promote cross-linking of native proteins during re-incubation (Fig. 4.3B - Native protein). Aminoguanidine present in the second incubation promoted cross-linking and fluorophore formation in both native and glycated protein (Fig. 4.4A-D, Native and Glycated protein). In the latter case, the effect on cross-linking and fluorescence was broadly concentration-dependent. The common feature of all these treatments was the presence of aminoguanidine during ageing of glycated and native proteins in sugar-free solution at 37°C. As a consequence of aminoguanidine affecting both native and pre-glycated protein, nett effects of aminoguanidine differed from absolute effects on glycated protein in a number of instances.

This finding is supported by the results of Skamarauskas *et al.* (1996), who similarly showed protein fluorescence to be increased by aminoguanidine in a control incubation system resembling the native protein treatments of this investigation. Their re-incubation of pre-glycated protein showed decreasing fluorescence in the presence of aminoguanidine, unlike results reported here. However, their glycation was conducted in the presence of DTPA which blocks oxidative post-Amadori reactions, therefore only early glycation products would have been present. These have been shown to be susceptible to aminoguanidine, both in this investigation and by Booth *et al.* (1996).

In the present study, fluorescence results strongly point towards sugar-independent reactions occurring between aminoguanidine and lipoproteins. Firstly, fluorescence appeared to increase to saturation level in both native and glycated proteins when aminoguanidine was present during sugar-free ageing. This suggests that a sugar-independent interaction between aminoguanidine and protein provided a source of fluorophores (and potentially also of cross-linking agents). The obvious candidate for such action would be β-lactoglobulin, or possibly more precisely, the bound lipid on β-lactoglobulin. Secondly, fluorescence of β-lactoglobulin - but not of lysozyme - was promoted by aminoguanidine in sugar-free incubations (Fig. 4.9). This provides further evidence for reactions between aminoguanidine and lipoproteins which are independent of sugar and which produce fluorophores. Fluorescence results presented in Fig. 4.9 were supported by periodate assay of the same samples (reported in Chapter 5), which also indicated sugar-independent reactions between aminoguanidine and β-lactoglobulin. On the basis of present investigations, it is not possible to state conclusively whether aminoguanidine reacts initially with the lipid

component of lipoproteins, or whether the presence of lipid amplifies the reaction of aminoguanidine with protein. However, results such as those in Fig. 4.9, indicating substantially different reactions with lysozyme than with β-lactoglobulin, do suggest that lipid itself reacts directly with aminoguanidine and that the extent of this reaction may mask concurrent reactions between aminoguanidine and protein. This would be in agreement with the recognized pro-oxidant activity of aminoguanidine, and with the relatively greater susceptibility of lipid to oxidation.

Fluorescence results were largely supported by cross-linking results in this investigation. When aminoguanidine had been present in the first short incubation (protocol i), native proteins showed a low, slow formation of heterodimer during sugar-free ageing (Fig. 4.3B, no AG) which was enhanced by aminoguanidine (Fig. 4.3B, 1mM AG and 25mM AG). This time lag in heterodimer formation indicates a requirement for oxidation, probably of lipid on β-lactoglobulin (Picard *et al.*, 1992). In pre-glycated proteins, heterodimer formation during sugar-free ageing was more extensive and rapid, and aminoguanidine present during glycation had little long-term effect. When aminoguanidine was present during ageing (protocol ii), it enhanced heterodimer formation in both native and glycated proteins. Again, the observed time lag suggests a requirement for oxidation. More heterodimer was observed in glycated than in native protein re-incubated in the absence of aminoguanidine or with 1mM aminoguanidine (Fig. 4.4B). However, maximum levels at 25mM aminoguanidine were similar in both native and glycated protein, suggesting that sites available for cross-linking on β-lactoglobulin became saturated (as seen for fluorescence). This could explain the apparent inhibitory effect of aminoguanidine on nett glycation-associated cross-linking. It may be a consequence of aminoguanidine increasing heterodimer

formation to saturation levels in both native and glycated proteins. Hence aminoguanidine action at the concentrations present during sugar-free ageing in this study may be independent of, or additive to, previous exposure to sugar. Similar observations apply to the relative effects of aminoguanidine and glucose on formation of lysozyme and β-lactoglobulin homodimers. While exposure to glycated lysozyme had virtually no effect on β-lactoglobulin dimerisation, this was boosted by aminoguanidine. This indicates that aminoguanidine was a stronger promoter of cross-linking than reactive intermediates arising either from glycated lysozyme (Zyzack *et al.*, 1995; Thornalley *et al.*, 1999) or from oxidation of lipid associated with β-lactoglobulin (Esterbauer *et al.*, 1992; Requena *et al.*, 1997a). Both glucose and aminoguanidine enhanced lysozyme homodimer formation, although the effect of aminoguanidine appeared greater. Again, the two effects may be additive.

A potential explanation for the apparent promotion of fluorescent and cross-linked products by aminoguanidine was suggested by Skamarauskas *et al.* (1996). These investigators demonstrated that aminoguanidine enhances copper (II)-dependent dicarbonyl generation from glycated BSA and has copper (II)-dependent pro-oxidant activity. Aminoguanidine was used at concentrations up to 10mM, and pro-oxidative conditions were provided by the addition of copper (up to 100μM). The authors argue these properties are responsible for the increase in fluorescence produced by aminoguanidine in their sugar-free control (native BSA). The enhanced cross-linking and fluorophore formation observed under sugar-free conditions in experiments reported here may be associated with enhanced dicarbonyl generation and other oxidative processes promoted by aminoguanidine.

Philis-Tsimikas *et al.* (1995) also observed a pro-oxidant effect of aminoguanidine. This was thought to be free radical-mediated and was detected at considerably lower concentrations. They studied freshly isolated LDL with varying inherent oxidation status and found that aminoguanidine had pro-oxidant activity at concentrations near 0.1mM. The degree of glycation of the LDL was not considered.

The upper aminoguanidine concentration used in the present investigation was selected with the aim of avoiding the pro-oxidant effect reported by Philis-Tsimikas *et al.* (1995). One possible explanation of the results observed is that, despite this pre-caution, the enhancement of fluorescent and cross-linked products by aminoguanidine at both 1mM and 25mM was indeed due to aminoguanidine-promoted oxidation. Since the effect of aminoguanidine appeared to be broadly concentration-dependent in many instances (cf Fig. 4.3D, Native protein; Fig. 4.4A-D), present results suggest that the pro-oxidant properties of aminoguanidine may extend to even higher concentrations than those tested here and therefore may be significant even in experimental systems in which the inhibitory/anti-oxidant properties of aminoguanidine are expected to dominate. In this context, it is worth noting observations reported by Skamarauskas *et al.* (1996) when measuring oxidant formation by aminoguanidine in the presence of either native or pre-glycated BSA. Firstly, a pro-oxidant effect was detected at an aminoguanidine concentration of 10mM, well into the range reported to be anti-oxidant in LDL (Picard *et al.*, 1992; Philis-Tsimikas *et al.*, 1995). Secondly, oxidant production was found to be greater in native BSA than in pre-glycated BSA. These observations are similar to results in the present system, and provide further evidence that a pro-oxidant effect of aminoguanidine is not restricted simply to low, physiologically irrelevant concentrations. While this may be true of LDL, it appears that the effect of aminoguanidine may vary from protein to protein. This variation may be dependent on

protein-specific factors such as lipid content or presence of inherent anti-oxidants (such as in LDL), or on localised conditions such as the pro-oxidising environment of atherosclerotic plaques or other areas of generalised inflammation.

It may be noted that the aminoguanidine/glucose ratio used in the present study was selected to be representative of that occurring *in vivo*. Under these conditions, aminoguanidine present during ageing was a stronger promoter of fluorescent and cross-linked products than the glycation status of the protein. Studies conducted *in vitro* can be only imperfect predictors of biochemical interactions in living systems. However, if the relative contributions of pre-glycation and of aminoguanidine-promoted reactions in a sugar-free system have any parallel under low sugar conditions *in vivo*, then the pro-oxidant properties of aminoguanidine may merit greater attention than they are presently accorded.

Another interpretation of the present observations is that the reactions of aminoguanidine in the absence of free sugar are linked to the relative availability of mono- and dicarbonyl compounds. Aminoguanidine has been shown to react preferentially with dicarbonyl products of sugar autoxidation or other early glycation reactions. When such dicarbonyls have been depleted (or, in this case, removed by dialysis), aminoguanidine may react more slowly with the less reactive monocarbonyls on glycated or oxidised protein. Scavenging of reactive dicarbonyls by aminoguanidine may limit their ability to promote cross-linking and fluorescence. However, reactions of aminoguanidine itself with monocarbonyls may promote protein modification, particularly in the presence of protein-associated lipid. Formation of Schiff bases between aminoguanidine and monocarbonyls on protein or lipid (and possible maturation to more stable adducts) provides a convenient explanation for the apparent

persistence of the effect of aminoguanidine through dialysis under protocol (i). Since Schiff bases are unstable, associations of this type which do survive dialysis would presumably dissociate to release free aminoguanidine during subsequent re-incubation. However, it is possible that in the absence of reactive dicarbonyls, some may react further with either other monocarbonyls or other aminoguanidine-derived Schiff bases.

Interestingly, Ganea *et al.* (1994a, b) reported similar sugar-independent reactions between lens crystallins and the glycation inhibitor pyridoxal-5-phosphate (PLP). This compound decreased protein glycation by glucose and galactose (Ganea *et al.*, 1994a), and prevented glycation-associated protein aggregation (Ganea *et al.*, 1994b). However, PLP itself bound to protein amino groups as a Schiff base (Ganea *et al.*, 1994a), enhanced protein fluorescence (Ganea *et al.*, 1994a), and caused protein aggregation in the absence of sugar (Ganea *et al.*, 1994b). The last observation was interpreted as a possible manifestation of the molecular chaperone properties of α-crystallin binding to partially unfolded protein. These reports provide another instance in which a glycation inhibitor is itself capable of binding protein through a Schiff base, and produces the very effects it is intended to prevent if it is present in the absence of sugar.

Two possible reaction paths can be suggested for aminoguanidine-lipoprotein Schiff base adducts. Since aminoguanidine has two free amino groups, cross-linking may occur by a 'bridging' through Schiff base formation with monocarbonyl groups on different lipoprotein molecules. This resembles the way MDA has been proposed to form cross-links by di-Schiff base formation with amino groups on different proteins (Requena *et al.*, 1997a). This putative aminoguanidine-derived cross-link is depicted in Fig. 4.10.

An alternative route by which aminoguanidine bound to protein or lipid through a Schiff base can lead to cross-linked and fluorescent products has been demonstrated by Stein and co-workers (Kramer *et al.*, 1984; Schelenz *et al.*, 1984; Stein *et al.*, 1985). These authors demonstrated oxidative condensation and cyclisation of 1-amino-3-aryl- and 1-amino-3-alkyl-guanidines to the corresponding 3,6,-diaryl- or 3,6-dialkyl-tetrazines. The 1-amino-3-alkyl-guanidines could be likened to the Schiff base formed by reaction of aminoguanidine with a monocarbonyl on either protein or lipid. Essentially, the mechanism involves condensation of two molecules of aminoguanidine by reaction of the amino nitrogen of the aminoguanidino group of one aminoguanidine with the =NH of the guanidine group of the other aminoguanidine molecule. This repeats in a cyclic fashion to form a ring which is not yet aromatic. It requires an oxidant to oxidise this intermediate structure to the aromatic tetrazine. In the experimental system used by Stein and co-workers, the oxidant was cerium (IV) in a perchloric acid medium - a clearly unphysiological system. However, it is conceivable that the same reaction could occur in a buffered lipoprotein system incubated in air and at normal pH. Localised microenvironments on protein could provide the necessary acidic conditions, while oxidants could be provided by atmospheric oxygen and potentially by the products of oxidative reactions on lipoproteins. If aminoguanidine forms a Schiff base with a carbonyl from glycated protein or lipid via the guanidino nitrogen, rather than the aminoguanidino nitrogen, then the Schiff base has the requisite functionality to cross-link by this reaction. Given the aromatic nature of the resultant tetrazine cross-link, it is also likely to be fluorescent. Thus one reaction mechanism would explain the increase in both fluorescence and cross-linking observed with aminoguanidine in the absence of free sugar. The reaction is depicted in Fig. 4.11.

Irrespective of mechanism, the findings reported here further support observations that inhibition of advanced Maillard product formation by aminoguanidine is most effective during the early sugar-dependent stages of the glycation reaction sequence (Booth *et al.*, 1996). Furthermore, during sugar-free ageing aminoguanidine itself appears to promote fluorescence and cross-linking of native or pre-glycated protein. This may be a consequence of its pro-oxidant activity, which has been previously reported both in non-glycating systems (Philis-Tsimikas *et al.*, 1995) and in pre-glycated protein under oxidising conditions (Skamarauskas *et al.*, 1996). Alternatively, it may indicate oxidative reactions between aminoguanidine and monocarbonyls on protein or protein-associated lipid. The formation of fluorescent and cross-linked products by glycation-independent reactions of aminoguanidine is unlikely to have emerged from many of the presently available studies on aminoguanidine, either *in vitro* or *in vivo*, since the effect of aminoguanidine on proteins in the absence and presence of free sugar is not generally compared. Exceptions are studies such as those reported by Skamarauskas *et al.* (1996) and Booth *et al.* (1996, 1997), which included re-incubation of pre-glycated protein with aminoguanidine in the absence of the glycating sugar (respectively glucose and ribose). This highlights the earlier observation that the overall effect of aminoguanidine reflects a complex interaction of reactions. Only some of these inhibit Maillard processes, while others appear to promote the formation of fluorescent and cross-linked compounds. This study did not investigate whether the latter compounds simply resemble Maillard products in their superficial functionality (fluorescence, cross-linking), or whether their chemical composition indeed allows them to be identified as Maillard products.

4.5 SUMMARY

- The mode of action of aminoguanidine (unlike that of DTPA) was not consistent with simple blocking of oxidative post-Amadori reactions.

- Aminoguanidine inhibited fluorescence and cross-linking in protocol (i), but enhanced these parameters in protocol (ii). Thus, aminoguanidine showed differential effects on early glycation and later Maillard reactions.

- Aminoguanidine reduced cross-linking and fluorescence when present together with free sugar during early glycation (protocol i). This is consistent with its established inhibitory action, and shows that inhibition is most marked during early glycation reactions.

- The inhibitory effect of aminoguanidine present with sugar appeared to persist after removal of the free compound by dialysis. This raises the possibility that aminoguanidine may form an association with protein which allows it to survive dialysis and continue to exert some inhibitory function during re-incubation.

- Aminoguanidine present during prolonged incubation without free sugar (protocol ii) enhanced cross-linking and fluorescence of both native and glycated proteins, particularly where protein had an associated lipid component. Native proteins pre-treated with aminoguanidine also showed enhanced cross-linking. This may be attributable to pro-oxidant properties of aminoguanidine. Alternatively, it may be linked to Schiff base formation of aminoguanidine with

monocarbonyls on lipids or proteins, and subsequent oxidative reactions thereof.

- Both prior exposure to glucose and the presence of aminoguanidine without sugar were predictors of cross-linking during sugar-free ageing of proteins. They may act either independently or additively. Aminoguanidine was more strongly associated with cross-linking of lipoprotein than was glycation.

- The overall effect of aminoguanidine appears to represent a balance of competing sugar-dependent reactions and sugar-independent oxidative reactions. Some of these prevent formation of advanced Maillard products and others promote formation of functionally similar (*i.e.* fluorescent and cross-linked) products. Chemical similarity of fluorescent and cross-linked products promoted by aminoguanidine with advanced Maillard products was not investigated.

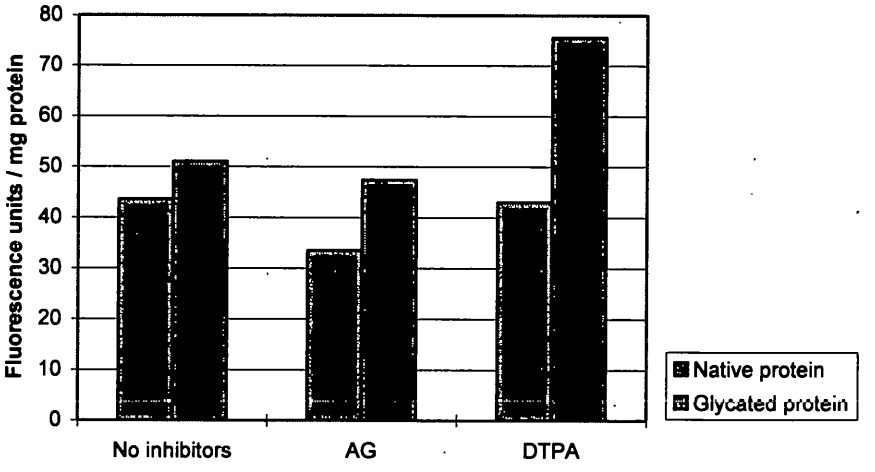


Fig. 4.1

Fluorescence development (ex/em 350/420nm) in co-incubations of lysozyme and β-lactoglobulin, pre-treated with inhibitors, after 1 week sugar-free ageing. Glycated protein treatments contained pre-glycated lysozyme (0.5M glucose, 24 hours, 37°C) and native β-lactoglobulin. Inhibitors were included in the pre-treatment as indicated: no inhibitors, 25mM aminoguanidine (AG) or 1mM DTPA. Native protein treatments resembled those for glycated proteins in all respects, but excluded glucose.

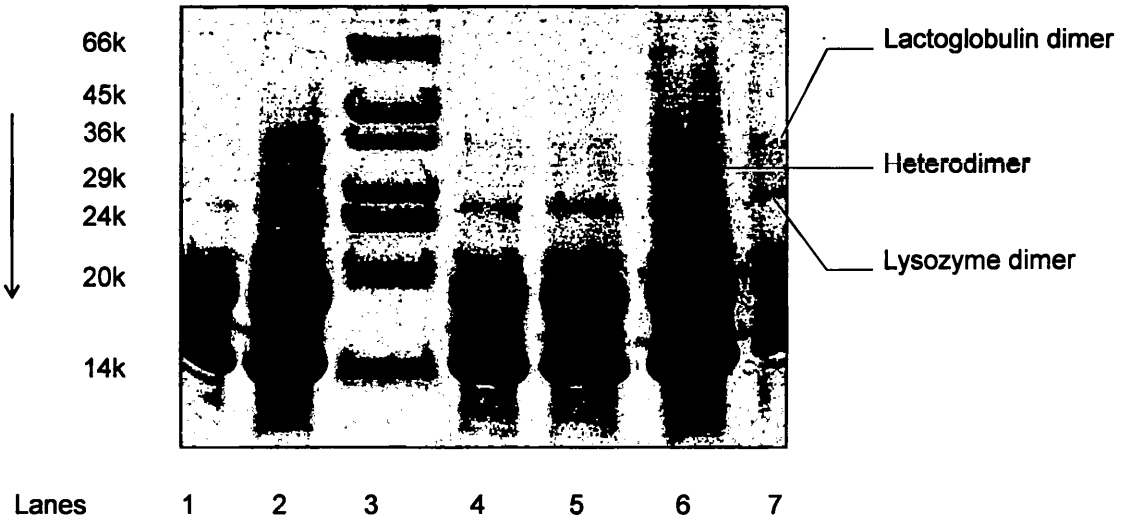


Fig. 4.2

The effect of including inhibitors in the glycating medium on protein cross-linking after subsequent sugar-free ageing for 3 weeks.

Lane 1	Native lysozyme, no inhibitors
Lane 2	Pre-glycated lysozyme, no inhibitor
Lane 3	Marker
Lane 4	Native lysozyme, pre-treated with aminoguanidine (25mM)
Lane 5	Pre-glycated lysozyme, pre-treated with aminoguanidine (25mM)
Lane 6	Pre-glycated lysozyme, pre-treated with DTPA (1mM)
Lane 7	Native lysozyme, pre-treated with DTPA (1mM)

Native β-lactoglobulin present in all treatments

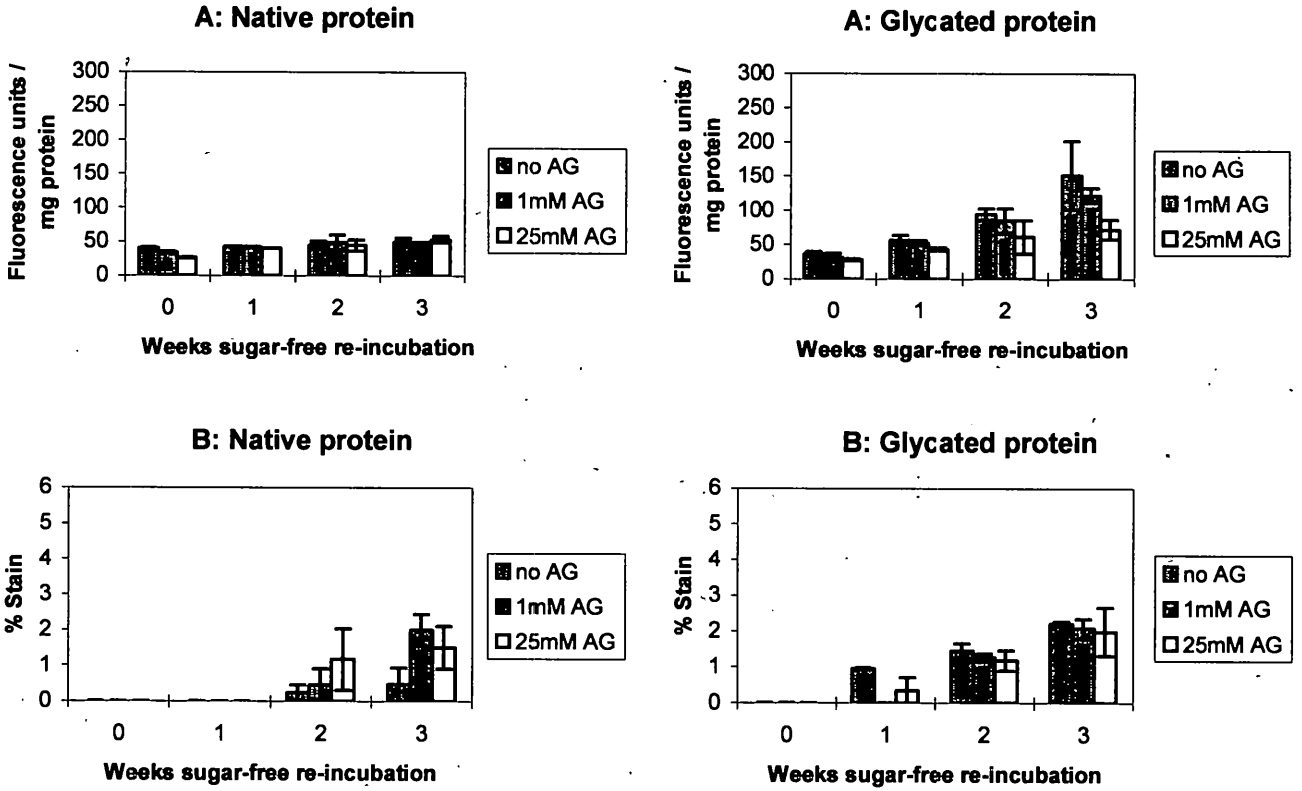


Fig. 4.3

Fluorescence development (ex/em 350/420nm) and cross-link formation during 3 weeks sugar-free ageing in co-incubations of β-lactoglobulin and lysozyme pre-treated with aminoguanidine (protocol i).

A: Fluorescence development; B: Formation of lysozyme/β-lactoglobulin heterodimer

Native protein: Lysozyme incubated initially without glucose; Glycated protein: Lysozyme incubated initially with glucose (0.5M, 24hr, 37°C)

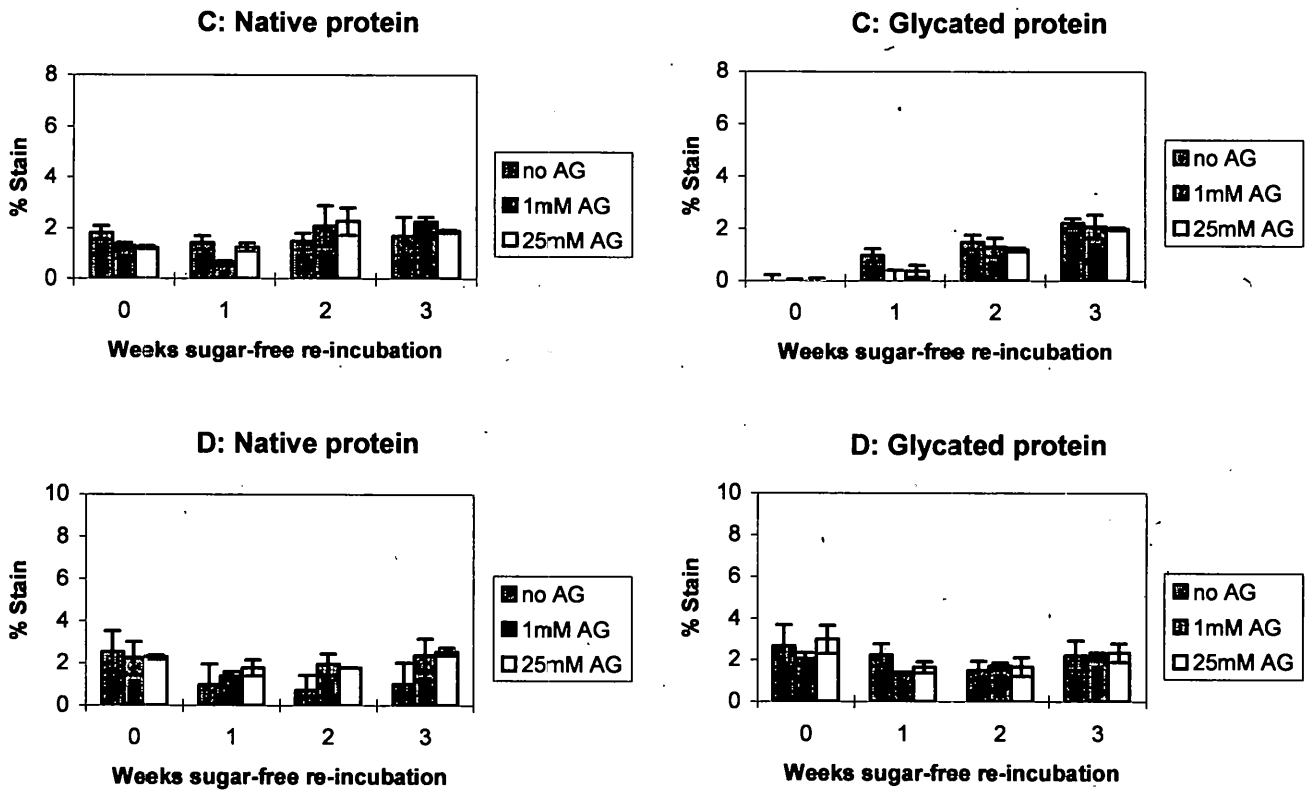


Fig. 4.3 (cont).
Fluorescence development (ex/em 350/420nm) and cross-link formation during 3 weeks sugar-free ageing in co-incubations of β-lactoglobulin and lysozyme pre-treated with aminoguanidine (protocol i).
C: Formation of lysozyme homodimer; D: Formation of β-lactoglobulin homodimer
Native protein: Lysozyme incubated initially without glucose; Glycated protein: Lysozyme incubated initially with glucose (0.5M, 24hr, 37°C)

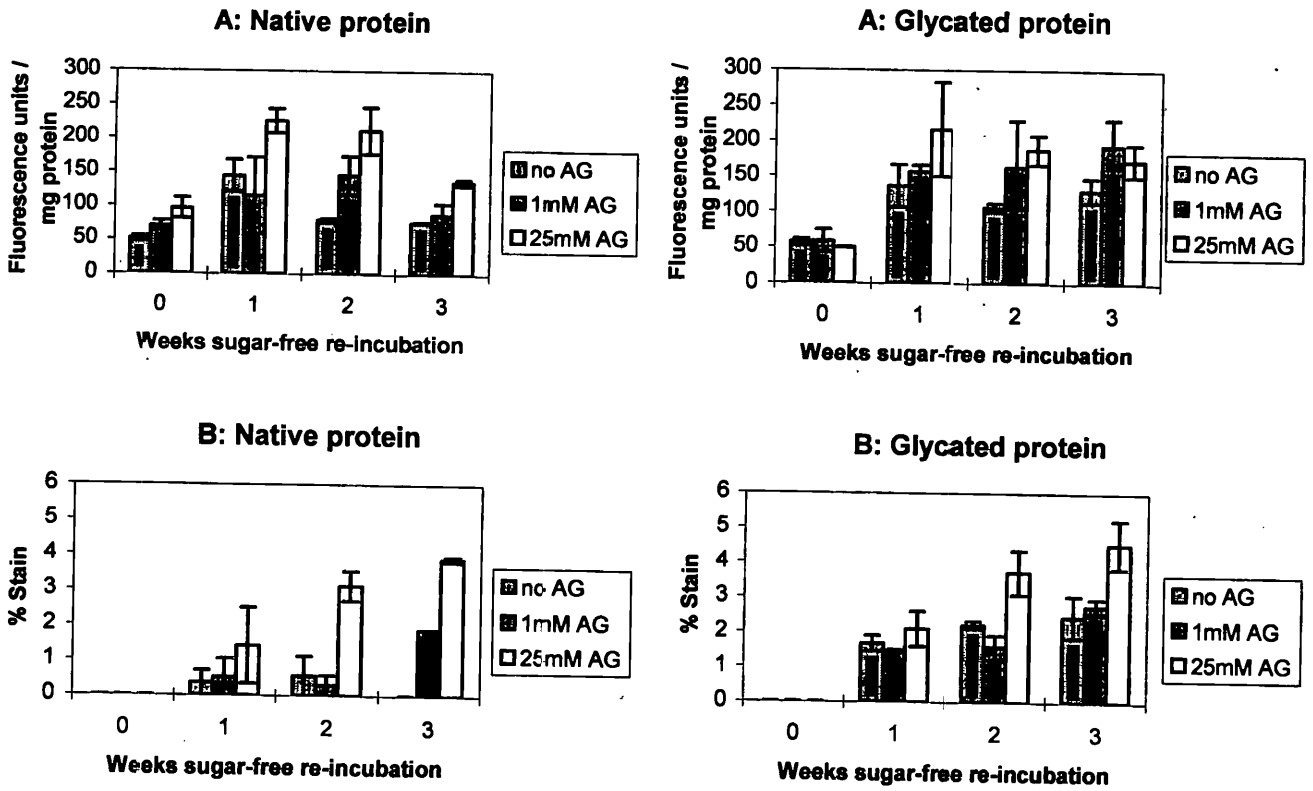


Fig. 4.4
Fluorescence development (ex/em 350/420nm) and cross-link formation during 3 weeks sugar-free ageing in the presence of aminoguanidine in co-incubations of lysozyme and β-lactoglobulin (protocol ii).
A: Fluorescence development; B: Formation of lysozyme/β-lactoglobulin heterodimer
Native protein: Lysozyme incubated initially without glucose; Glycated protein: Lysozyme incubated initially with glucose (0.5M, 24hr, 37°C)

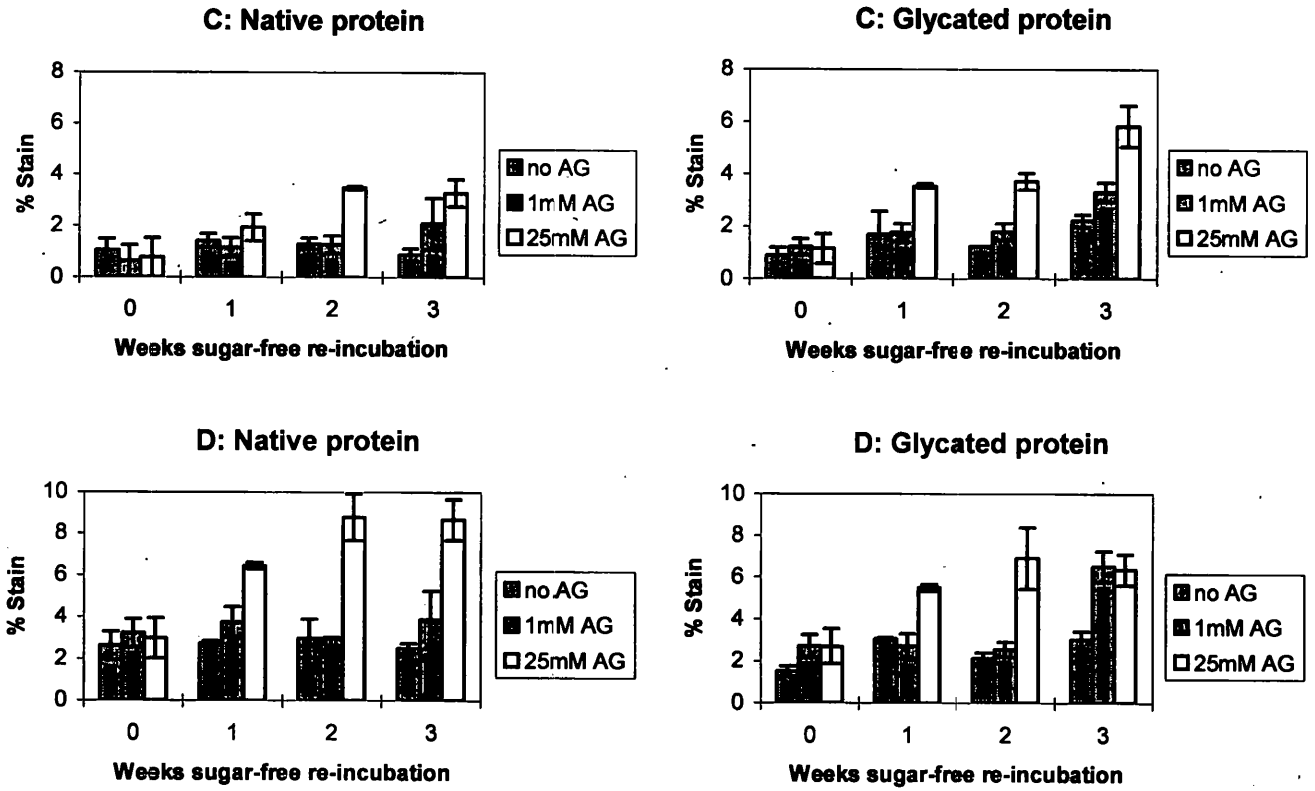


Fig. 4.4 (cont).
Fluorescence development (ex/em 350/420nm) and cross-link formation during 3 weeks sugar-free ageing in the presence of aminoguanidine in co-incubations of lysozyme and β-lactoglobulin (protocol ii).
C: Formation of lysozyme homodimer; D: Formation of β-lactoglobulin homodimer
Native protein: Lysozyme incubated initially without glucose; Glycated protein: Lysozyme incubated initially with glucose (0.5M, 24hr, 37°C)

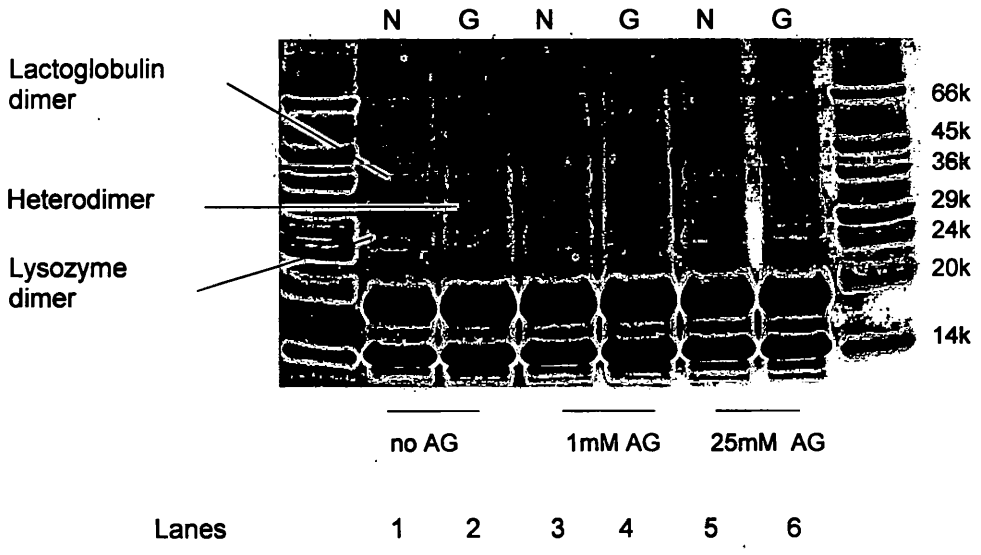


Fig. 4.5

Cross-linking, after 3 weeks sugar-free ageing, of native β-lactoglobulin and lysozyme pre-treated with aminoguandine (*i.e.* aminoguanidine present in first incubation only, protocol i).

AG Aminoguanidine

N Native protein (lysozyme initially incubated without glucose)

G Pre-glycated lysozyme (0.5M glucose, 24 hr, 37°C)

Native β-lactoglobulin present in all N and G

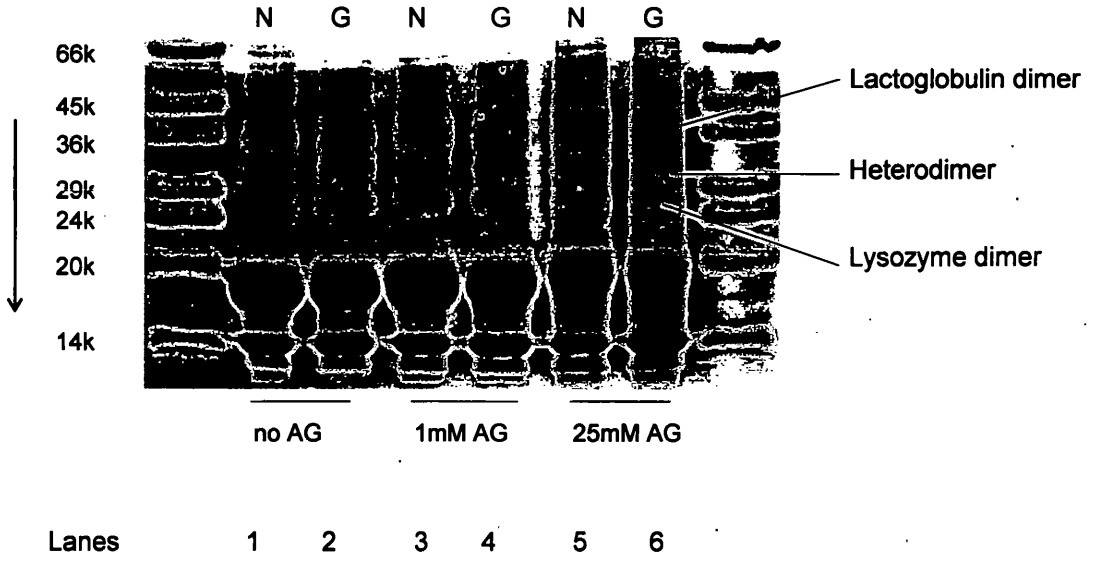


Fig. 4.6

Cross-linking of lysozyme and native β-lactoglobulin after 3 weeks sugar-free ageing in the presence of aminoguanidine (*i.e.* aminoguanidine present in second incubation only, protocol ii).

AG Aminoguanidine

N Native protein (lysozyme initially incubated without glucose)

G Pre-glycated lysozyme (0.5M glucose, 24hr, 37°C)

Native β-lactoglobulin present in all N and G

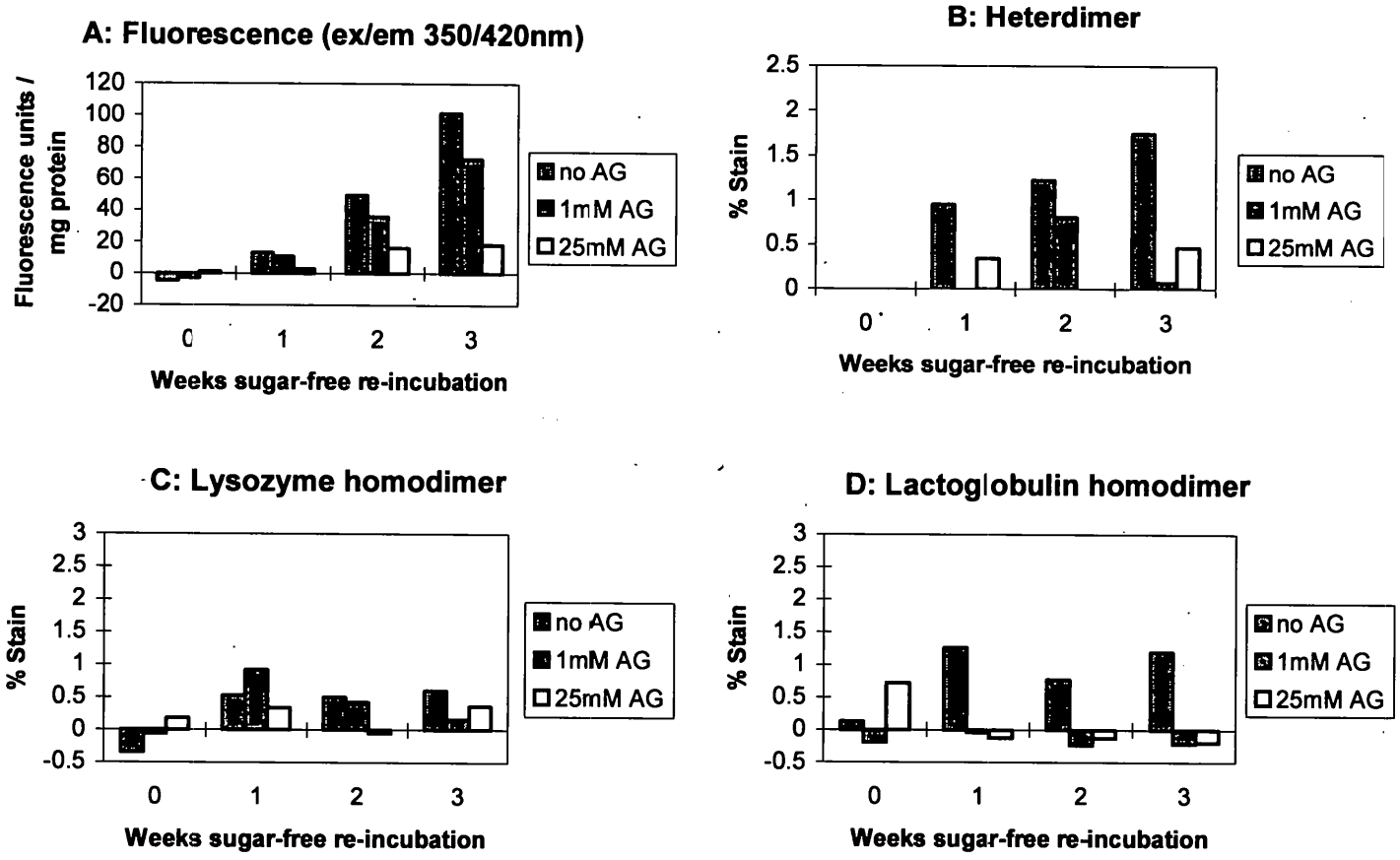


Fig. 4.7
Nett glycation-associated change (glycated protein - native protein) in fluorescence and cross-linking during 3 weeks sugar-free ageing in co-incubations of β-lactoglobulin and of lysozyme pretreated with aminoguanidine (protocol i).

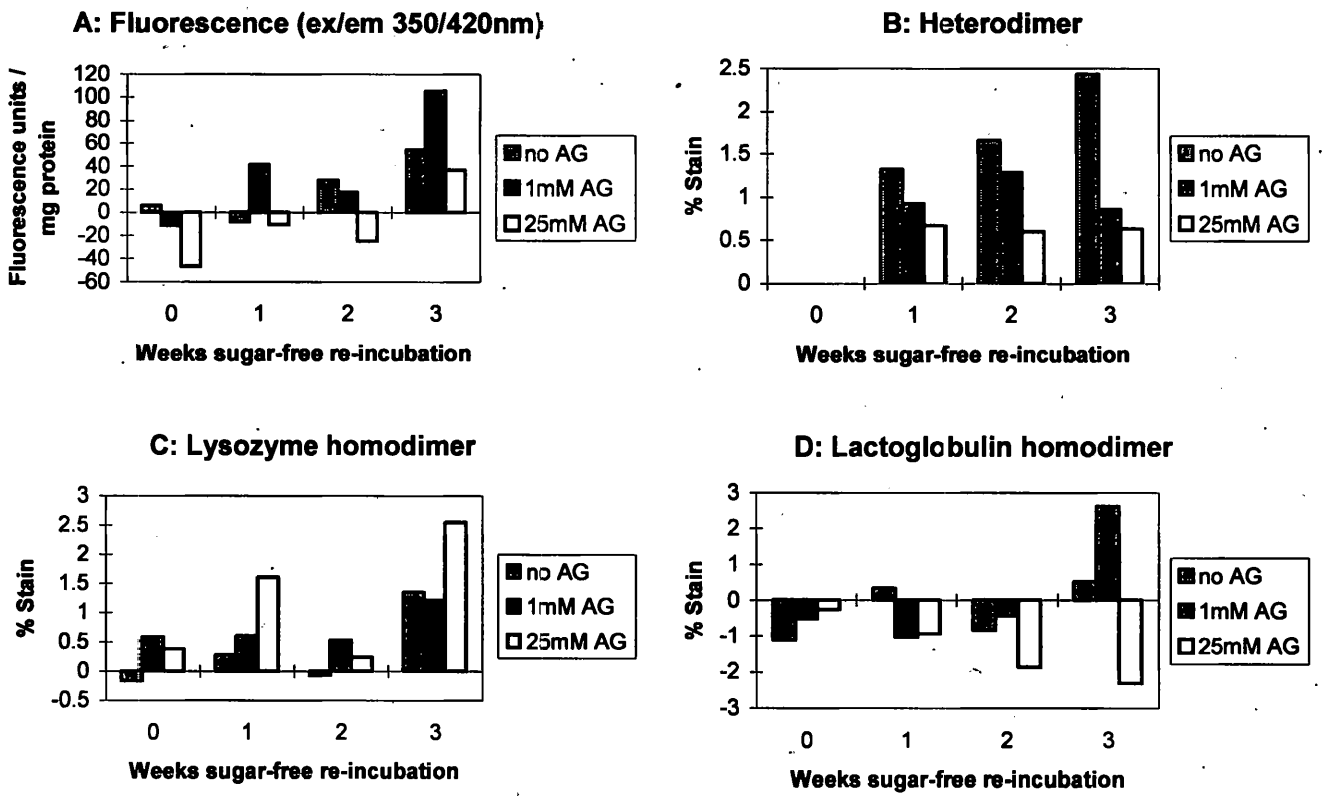


Fig. 4.8
Nett glycation-associated change (glycated protein - native protein) in fluorescence and cross-linking during 3 weeks sugar-free ageing in the presence of aminoguanidine in co-incubations of lysozyme and β-lactoglobulin (protocol ii).

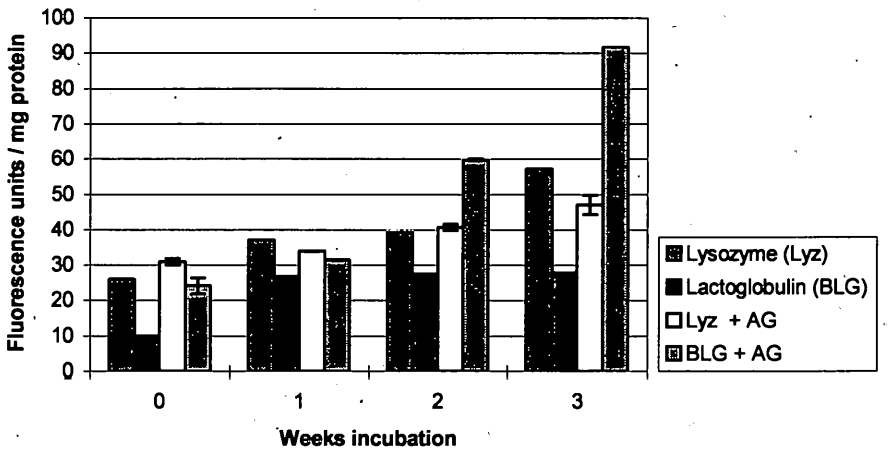


Fig. 4.9

Fluorescence development (ex/em 350/420nm) by each of the model proteins lysozyme and β-lactoglobulin, incubated either alone or together with 25mM aminoguanidine (AG).

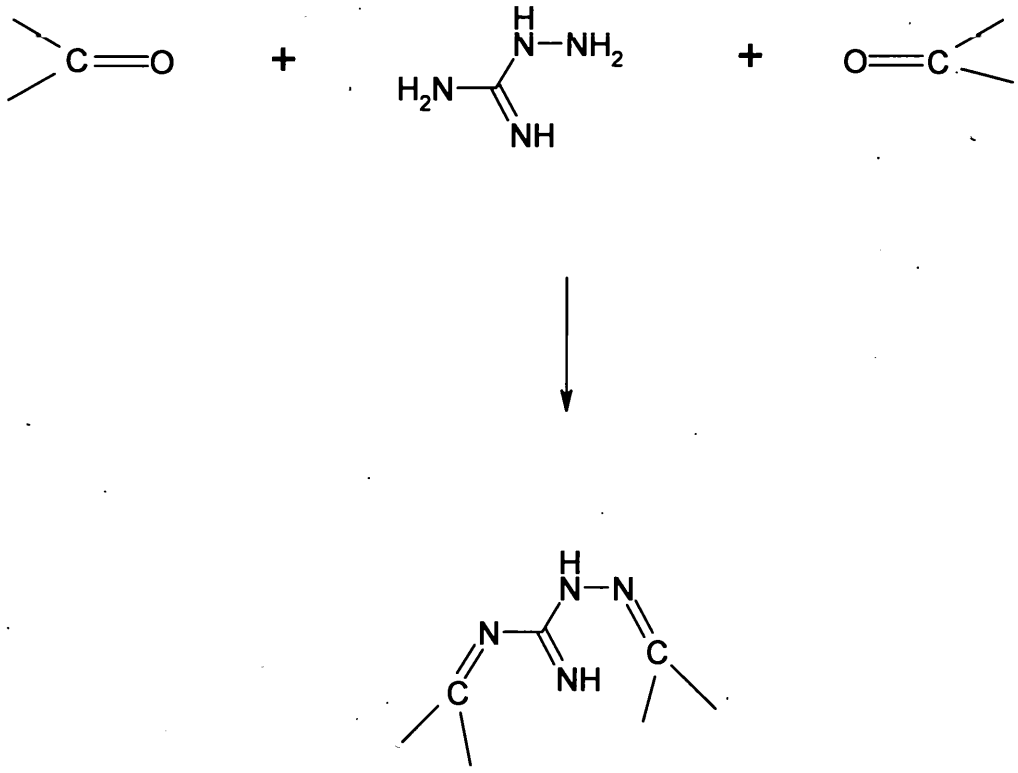


Fig. 4.10

Suggested formation of a di-Schiff base cross-link from aminoguanidine and two monocarbonyl groupings on different proteins.

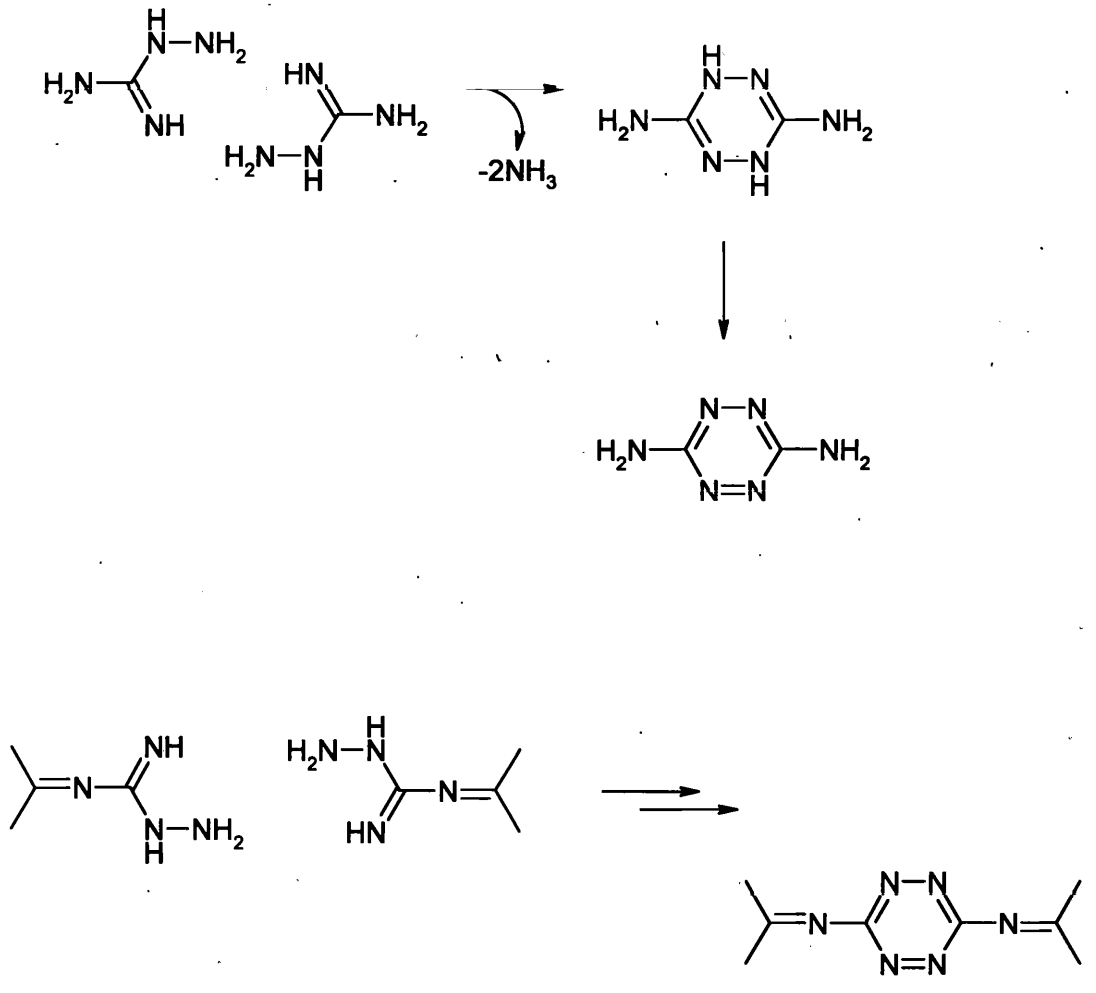


Fig. 4.11

Suggested formation of a tetrazine cross-link by dimerisation of two aminoguanidine molecules bound to different protein molecules through monocarbonyl groupings on the protein (after Kramer *et al.*, 1984, Schelenz *et al.*, 1984 and Stein *et al.*, 1985).

CHAPTER 5

EARLY GLYCATION PRODUCTS ASSESSED BY PERIODATE ASSAY

5.1 INTRODUCTION

Periodate positive material (PPM), the endpoint determined by the periodate assay, is thought to indicate the presence of Amadori product by measuring formaldehyde produced after reaction of glycated protein with sodium periodate. The periodate reaction cleaves the carbon chain of the glucose moiety between adjacent hydroxyl groups. It has been postulated that only Amadori product and Schiff base have the correct configuration to yield formaldehyde as a result of such fragmentation (Gallop *et al.*, 1981; Furth, 1988). The formaldehyde so produced is measured by a colour reaction depending on the synthesis of diacetyldihydrolutidine from acetylacetone and formaldehyde in the presence of excess ammonium salt (Nash, 1953). Dialysis after the first glycating incubation should suffice to discharge Schiff base in the assay as performed in this investigation (Ahmed and Furth, 1991). However, since Amadori product was not chemically characterised in this investigation and since a wide range of early glycation products and multiple reaction pathways are now recognised in the literature, PPM as detected here was taken as an indicator of early glycation products in general.

Proteins were glycated under various conditions, with or without inhibitors. The periodate assay was used to follow formation of PPM and thereby to assess the fate of early Maillard products during sugar-free ageing. In addition, two approaches were used to investigate the chemical characteristics of the periodate assay. Firstly, the performance of the assay in the absence of free sugar was evaluated. Secondly, the ability of the assay to detect a number of compounds which may be expected to arise during Maillard reactions was assessed.

5.2 METHODS

The incubations described under Methods in Chap. 3 (protein cross-linking under varying glycation conditions) and Chap. 4 (inhibitors of the Maillard reaction) were used to investigate the formation and fate of early glycation products, as indicated by the periodate assay (described in Chap. 2). Experimental design has been described in the relevant chapters. Aliquots taken for monitoring formation of fluorescent and cross-linked Maillard products were also subjected to periodate assay.

Furthermore, the two model proteins - lysozyme and β-lactoglobulin - were incubated (10mg/ml) in 0.1M phosphate buffer, alone or with aminoguanidine (25mM) for three weeks. Aliquots were taken weekly and assayed for PPM. (These incubations were also used to investigate the source of sugar-independent fluorescent products generated in the presence of aminoguanidine, described in Chap. 4.)

Finally, the standard periodate assay was carried out as described, but with one of a range of test compound (free of protein or sugar) introduced in place of a protein sample. Test compounds were assayed alone and in combination with aminoguanidine. The test compounds and aminoguanidine were equimolar at either 1mM or 5mM in the test solution. The compounds assayed were glycine, glycolaldehyde, glyoxal, methylglyoxal and malondialdehyde (MDA). Commercial preparations were used for all except MDA. A crude solution of MDA was prepared fresh by hydrolysing 1,1,3,3-tetraethoxypropane with 1% sulphuric acid for 15 minutes at room temperature (modified from Al-Abed and Bucala, 1997).

5.3 RESULTS

All figures referred to in the text below are presented at the end of the chapter.

5.3.1 PPM formation from two model proteins under varying glycation conditions

It was anticipated that PPM levels would decline during sugar-free re-incubation after initial glycation, as early glycation products were consumed in the formation of late Maillard products (AGEs). This was not observed. An unexpected increase in PPM was observed with time (Fig. 5.1, comparing week 0 with weeks 1-3 for each treatment). Although the increase over three weeks was significant in only one treatment (Fig. 5.1, 24hr / 0.5M glucose, week 0 *vs* week 3), the trend was consistent

throughout almost all treatments. PPM levels in the sugar-free control also increased unexpectedly over the re-incubation period.

Overall PPM levels increased as the intensity of pre-glycation increased, indicating that a significant proportion of PPM was indeed glycation-dependent (Fig. 5.1, comparing glycation treatments at each time point). In the 24 hr / 0.5M and 5 day / 0.5M glucose pre-glycation treatments, PPM differed significantly from that in the corresponding sugar-free control at all time points, including week 0 (*i.e.* immediately after glycation - Fig. 5.1). This is in accordance with the expected effect if PPM is formed predominantly as a result of early glycation reactions.

The threshold effect observed in the formation of fluorescent Maillard products (reported in Chap. 3) also appeared in the glycation-dependent fraction of PPM. Under relatively mild glycating conditions, PPM was slightly elevated, but not significantly different from that in the sugar-free control (Fig. 5.1 - minimal glycation and 24 hr / 0.05M glucose. Minimal glycation was defined in Chap. 3.). Under more intensive glycating conditions, a sharp increase in PPM was observed, both with respect to glucose concentration and glycation time (Fig. 5.1 - 24hr / 0.5M glucose and 5 days / 0.5M glucose). This appears to indicate some form of threshold condition for the formation of PPM. However, unlike the threshold observed for fluorescence development, this threshold seems to be associated more strongly with the increase in glucose concentration from 0.05M to 0.5M than with the increase in glycation duration from 24 hours to 5 days.

When PPM levels in pre-glycated treatments were adjusted for the increase observed in native proteins (nett glycation-associated change), the two mildest pre-glycation

treatments (Fig. 5.2, minimal glycation and 24h / 0.05M glucose) showed a consistent decrease in PPM during sugar-free ageing. Pre-glycation for 24 hours with 0.5M glucose yielded an initial increase in PPM, followed by a decreasing trend with time. Thus the proportion of PPM which can be associated directly with glycation did indeed exhibit the expected trend as early glycation products were converted to AGEs. Only the most intensive pre-glycation treatment (Fig. 5.2, 5 days / 0.5M glucose) showed an increase in PPM during sugar-free ageing. This last treatment is also the one most likely to represent both mixed early glycation reactions and later sugar-independent Maillard reactions in the initial incubation, which makes it difficult to isolate the fate of early glycation products.

This experiment shows that, contrary to expectation, PPM was formed during ageing of native and glycated protein, not just during early Maillard reactions in the presence of sugar.

5.3.2 Fate of PPM in the presence of glycation inhibitors - preliminary trial with DTPA and aminoguanidine

Periodate assays conducted immediately after the initial glycating incubation (before addition of β-lactoglobulin) indicated that aminoguanidine and DTPA enhanced the formation of PPM (Fig. 5.3A). After one week sugar-free ageing, there was less PPM in samples initially glycated in the presence of aminoguanidine or DTPA, than in samples which did not contain inhibitors in the initial glycation mixture (Fig. 5.4B). This indicates that early PPM, which accumulated during initial glycation in the presence of

inhibitors, was indeed consumed during subsequent re-incubation in the absence of sugar or inhibitors.

5.3.3 Fate of PPM in the presence of glycation inhibitors - aminoguanidine present in either first (glycating) or second (sugar-free) incubation

The production of PPM in the presence of aminoguanidine was further investigated by including the inhibitor either in the glycating medium or during subsequent sugar-free ageing. The specifics of the two experimental protocols and the effects modelled by the combinations of glucose and aminoguanidine have been outlined in Chap. 4.

Native protein

In the absence of aminoguanidine, a slow increase in PPM occurred in native proteins over the three week re-incubation period as the proteins underwent oxidative ageing (Fig. 5.4A and 5.5A, no AG).

Proteins briefly pre-treated with aminoguanidine (protocol i) showed slightly enhanced mean PPM levels at week 3, relative to the aminoguanidine-free pretreatment (Fig. 5.4A). In contrast, when aminoguanidine was present throughout sugar-free ageing (protocol ii), by week 3 mean PPM levels had decreased slightly relative to proteins re-incubated in buffer alone (Fig. 5.5A). It was not clear whether these changes were significant. Overall levels of PPM were similar in the two experiments.

Glycated protein

Glycated proteins also produced PPM when allowed to age in buffer alone. This can be seen particularly in the aminoguanidine-free incubations of glycated proteins under both protocols (Fig. 5.4B and 5.5B, no AG), in which PPM at week 3 had increased significantly relative to the week 0 time point.

After three weeks sugar-free ageing, mean PPM in glycated protein was lower in treatments containing 25mM free aminoguanidine in either the first or second incubation (Fig. 5.4B and 5.5B, respectively) than in treatments containing no aminoguanidine or 1mM aminoguanidine. This may represent either scavenging of PPM by aminoguanidine or suppression of PPM production.

Aminoguanidine present at 25mM during glycation was associated with increased PPM levels at the initiation of sugar-free ageing (Fig. 5.4B, week 0), followed by a sharp decrease during the first week of ageing. By contrast, an apparently immediate drop in PPM occurred when aminoguanidine was introduced at the start of the second, sugar-free, incubation (Fig. 5.5B, week 0), indicating a very rapid reaction between compounds making up PPM and aminoguanidine. Levels of PPM then increased over the remainder of sugar-free ageing, whether or not aminoguanidine was present. However, levels in aminoguanidine-containing treatments remained below those in the aminoguanidine-free treatment.

Levels of PPM in glycated protein were considerably higher overall when aminoguanidine was present in the first glycating incubation (Fig. 5.4B) than when the inhibitor was present in the second sugar-free incubation (Fig. 5.5B).

Nett glycation-associated change

The definition of nett glycation-associated change has been given in Chap. 4.

Under both experimental protocols, nett glycation-associated PPM levels were decreased by aminoguanidine over the three week re-incubation period (Fig. 5.4C and 5.5C). However, nett levels were very low when aminoguanidine was incorporated in the second incubation (Fig. 5.5C), making meaningful comparison difficult.

To summarise:

In native proteins

i) aminoguanidine pre-treatment had little effect on PPM formation during ageing, except slight enhancement after three weeks of ageing.

ii) when aminoguanidine was present at 25mM throughout ageing, there may have been a slight inhibition of PPM formation.

In glycated proteins

i) aminoguanidine present at 25mM (but not 1mM) during glycation enhanced PPM formation (Fig. 5.4B, week 0).

ii) however, protein pre-treated with 25mM aminoguanidine developed less PPM during ageing (Fig. 5.4B, week 3).

iii) the presence of 25mM aminoguanidine throughout ageing resulted in slightly reduced PPM production (Fig. 5.5B, week 3).

5.3.4 Evaluation of the periodate assay

Samples used to investigate the source of sugar-independent aminoguanidine-promoted fluorescence of the model proteins (described in Chap. 3) were also assayed for PPM. Results of periodate assay on lysozyme and β-lactoglobulin, incubated alone or with 25mM aminoguanidine, are shown in Fig. 5.6. Native β-lactoglobulin yielded more PPM than lysozyme after three weeks. Levels increased for both proteins during the incubation period. PPM from lysozyme appeared to reach a plateau, while PPM from β-lactoglobulin was still increasing in an approximately linear fashion after three weeks.

Aminoguanidine reduced PPM production from lysozyme (lipid-free) immediately upon its introduction to the incubation medium (Fig. 5.6, Lyz+AG, week 0) and even further during the first week of incubation. Thereafter there was little change in PPM over the remainder of the incubation period. Thus aminoguanidine appears to block periodate-positive groups on lysozyme. This may explain the drop in PPM observed under protocol (ii) above, when aminoguanidine was introduced at the beginning of the second incubation (Fig. 5.5B, week 0).

In contrast, aminoguanidine strongly boosted PPM production from β-lactoglobulin (lipid-associated) immediately upon its addition to the incubation medium (Fig. 5.6, BLG+AG, week 0). Thereafter, aminoguanidine decreased PPM over three weeks incubation. This initial boosting of PPM by aminoguanidine in lipid-associated protein may explain the boosting of PPM observed at initiation of the second incubation under protocol (i) (Fig. 5.4B, week 0). The effect may have been mediated by the interaction of aminoguanidine surviving dialysis with β-lactoglobulin introduced at the beginning of sugar-free ageing. Although theoretically aminoguanidine had been removed by dialysis prior to addition of β-lactoglobulin, the possibility of some aminoguanidine surviving dialysis in a reversible association with protein has been mentioned in Chap. 4. The alternative explanation for the boosting of PPM by aminoguanidine present during glycation - *viz.* that PPM production from lysozyme was boosted - appears unlikely in view of the results observed during co-incubation of lysozyme and aminoguanidine.

Periodate assays were also carried out with a number of test compounds (1mM or 5mM). Structures of the compounds tested are shown in Fig. 5.7. Assay results are shown in Table 5.1. When assayed alone at a concentration of 1mM, only glycolaldehyde yielded a positive periodate response, as would be expected from its chemical structure (adjacent carbonyl and hydroxyl groups). At 5mM, both glyoxal and methylglyoxal also gave a positive reaction. This was an unexpected result and the chemical nature of the reaction is not clear. Malondialdehyde, which - as a product of lipid oxidation capable of contributing to the Maillard reaction - might be suspected of interfering in the assay, gave a very weak response. Glycine also yielded very little

PPM, in accordance with expectations based on the chemical basis of the periodate reaction.

When test compounds were assayed in combination with aminoguanidine (equimolar with the test compound), some correction of PPM detected was necessary to account for the response of aminoguanidine itself in the assay. The corrections applied are indicated in the column headings of Table 5.1. Aminoguanidine assayed together with individual test compounds (Table 5.1, column 1) gave lower readings than when each was assayed separately and the readings added (column 2). More than 50% of PPM associated with each test compound appeared to be blocked by reaction with aminoguanidine, except for glycolaldehyde. This is in accordance with the reported scavenging of α-dicarbonyls (Chen and Cerami, 1993; Glomb *et al.*, 1994; Hirsch and Feather, 1994) and of aldehyde products of lipid oxidation (Al-Abed and Bucala, 1997) by aminoguanidine, and with its relatively low reactivity towards monocarbonyls. MDA showed the greatest sensitivity to aminoguanidine, although readings were very low and may not be significant.

5.4 DISCUSSION

5.4.1 PPM formation from two model proteins under varying glycation conditions

PPM is thought to indicate the presence of Amadori product, by measuring formaldehyde produced after reaction of glycated protein with periodate. It was

therefore expected that negligible PPM would form in native proteins, and that PPM in pre-glycated proteins would remain approximately constant at week 0 levels during ageing, or would decline with conversion of Amadori product to later Maillard products. These expectations were not borne out. Instead, PPM increased during sugar-free ageing of both native and glycated protein. The validity of PPM as an indicator of solely glycation-associated reactions is therefore called into question.

Despite this consideration, a significant association between glycation and PPM was found. Mean PPM increased strongly with increasing intensity of the pre-glycation treatment (Fig. 5.1) and, as predicted, nett glycation-associated PPM levels decreased during re-incubation in all but the most intensively pre-glycated treatment (Fig. 5.2). This latter treatment probably represents a mixture of early sugar-dependent reactions and later sugar-independent changes, whereas the shorter pre-incubation times of the less intensive pre-glycation treatments are likely to eliminate most later Maillard reactions during the first incubation.

It has been noted that there was a threshold in the development of glycation-associated PPM. Little PPM formed under mild glycating conditions, followed by a sharp increase in PPM as some threshold condition appeared to be exceeded under the more intensive glycation conditions tested (Fig. 5.1). This corresponds with results reported in Chap. 3 for fluorescence and contrasts with the formation of cross-linked products, both under the same glycating conditions. As noted in that chapter, since the observed threshold has a time component, it may reflect a requirement for oxidation. However, unlike the threshold observed for fluorescence, PPM formation appeared to increase more strongly with increasing glucose concentration (from 0.05M to 0.5M glucose) than

with increasing glycation duration (from 24 hours to 5 days). Thus this threshold might be a stronger indicator of a requirement for precursor accumulation than for oxidation.

Wells-Knecht *et al.* (1995b) reported that, at high phosphate concentrations (0.2M) *in vitro*, glycoxidation products formed primarily via autoxidation of glucose or of glycation intermediates preceding Amadori product (*eg.* Schiff base). The Amadori product route became more significant at low phosphate concentrations, similar to those found *in vivo*. It is possible that, in the present system, early glycation products derived from autoxidation of glucose and Schiff base were the predominant species formed during the glycating incubation at low glucose concentrations, and that Amadori product (and hence PPM) formation only became significant at a glucose concentration of 0.5M.

In view of the unexpected increase observed in PPM during ageing, it appears likely that the periodate assay also detects a component which is not dependent on the presence of free sugar for its formation. This complicates the interpretation of results from the periodate assay.

5.4.2 Fate of PPM in the presence of glycation inhibitors - preliminary trial with DTPA and aminoguanidine

DTPA is thought to block post-Amadori Maillard reactions by scavenging free metals (Fu *et al.*, 1992, 1994), thereby preventing metal catalysed oxidation of early glycation products. Aminoguanidine is thought to scavenge reactive dicarbonyl compounds arising from the Amadori product or by other reaction routes occurring predominantly

during early glycation (Chen and Cerami, 1993; Requena *et al.*, 1993; Glomb *et al*, 1994; Booth *et al.*, 1996, 1997). It was therefore postulated that the presence of either inhibitor during early glycation reactions would block conversion of early products to AGEs, boosting levels of early glycation products. This was indeed reflected in the observed elevation of PPM in lysozyme glycated in the presence of DTPA or aminoguanidine (Fig. 5.3A).

It was further postulated that removal of free inhibitor would allow the accumulated early products to react further to form late Maillard products (AGEs), resulting in a drop in PPM and higher final levels of Maillard products than in inhibitor-free controls. This expected drop in PPM during re-incubation was indeed observed, especially with aminoguanidine (Fig. 5.3B). However, only DTPA produced the expected increase in late Maillard products (reported in Chap. 4). Glycated lysozyme formed more fluorescent Maillard products on re-incubation with native β-lactoglobulin, if it had been pre-treated with DTPA. However, aminoguanidine had a different effect. Virtually no additional fluorescent Maillard products were formed during one week re-incubation, and cross-linked Maillard products after three weeks were lower than in the corresponding inhibitor-free control (Chap. 4, Fig. 4.1 and 4.2). Thus aminoguanidine failed to boost formation of late Maillard products. This was puzzling, since it had more effect on PPM formed during glycation than did DTPA.

These results emphasise that the inhibition of late Maillard reactions by DTPA and by aminoguanidine is achieved by significantly different chemical routes.

5.4.3 Fate of PPM in the presence of glycation inhibitors - aminoguanidine present in either first (glycating) or second (sugar-free) incubation

Interpretation of PPM results from investigations with aminoguanidine requires the consideration of two questions: firstly, the possible identity of compounds detected as PPM; and secondly, the nature of the reaction of aminoguanidine with PPM.

Identity of PPM formed during sugar-free ageing

It was observed, both in inhibitor studies (5.2.3, Fig. 5.4 and 5.5) and in studies of varying glycation treatments (5.2.1, Fig. 5.1), that PPM increased during three weeks sugar-free ageing in both pre-glycated and native proteins. PPM in native proteins at initiation of the sugar-free re-incubation may have originated from *in vivo* glycation or oxidation of the commercial protein preparations. However, this fails to explain the rise in PPM observed during sugar-free ageing under both experimental protocols in the inhibitor study. Both native and glycated proteins produced PPM during ageing, but the effect was more marked in glycated proteins. A number of possible explanations may be suggested from the literature:

- Zyzak *et al.* (1995) described the release of glucose, mannose, tetroses and pentoses from model Amadori products. These sugars would yield formaldehyde in the periodate assay. However, since presumably one mole of sugar would be produced per mole of Amadori product, it is difficult to see how this could result in an increase in PPM during re-incubation.

- Various studies have demonstrated the generation of low molecular weight dicarbonyl compounds from early glycation products on protein. Among those identified to date are 3-deoxyglucosone, glycolaldehyde, glyoxal and methylglyoxal (Glomb and Monnier, 1995; Zyzak *et al.*, 1995; Ahmed *et al.*, 1997; Thornalley *et al*, 1999). They are thought to arise from acid-base catalysed enolisation of dicarbonyl derivatives of the Amadori product; from reverse aldol reaction; or from hydrolysis, oxidative cleavage and rearrangement reactions of Amadori product or Schiff base or Amadori product derivatives. It is possible that one of the rearrangement and cleavage reactions may produce the monocarbonyl formaldehyde in addition to small dicarbonyls. This formaldehyde would be detected as PPM in the periodate assay here, provided it were produced from protein-bound glycation products only after dialysis.

- The dicarbonyl derivatives described above may react in a similar way to formaldehyde in the periodate assay, or may themselves yield formaldehyde under the assay conditions.

- A protein-bound derivative of the Amadori product, such as carboxymethyllysine, may unexpectedly either generate formaldehyde or react in the same way as formaldehyde and thus be detected as PPM in the assay.

- Some aspect of the assay conditions (such as pH changes) may cause fragmentation or rearrangement of any of the products described above in such a way that formaldehyde is produced.

Periodate assays were conducted with glycine (as an analogue of carboxymethylated protein- cf Fig. 5.7), glycolaldehyde, glyoxal, methylglyoxal and malondialdehyde in order to test some of the possible candidates for PPM formed during sugar-free ageing of protein. With the exception of glycine, these products have all been shown to form from glycated protein (Glomb and Monnier, 1995; Zyzack *et al.*, 1995; Ahmed *et al.*, 1997; Thornalley *et al*, 1999) or from oxidation of protein-associated lipid (Esterbauer *et al*, 1992). Results presented in Table 5.1 indicate that, in addition to formaldehyde, the periodate reaction may detect glycolaldehyde, glyoxal or methylglyoxal, and presumably other compounds with similar functionality. However, the assay response for the α-dicarbonyls, glyoxal and methylglyoxal, was weaker than for compounds having adjacent carbonyl and hydroxyl groups, such as glycolaldehyde. MDA and glycine showed very little response, indicating that carboxymethylated protein or MDA from lipid oxidation are unlikely to interfere significantly with the assay.

Whatever the origin of PPM, differences between protocols (i) and (ii) remain to be explained. All glycated samples in protocol (ii) (Fig. 5.5B) had markedly lower PPM levels than those in protocol (i) (Fig. 5.4B). This was especially surprising for the aminoguanidine-free glycated controls under both protocols (Fig. 5.4B and 5.5B, week 0, no AG), since these received almost identical treatment. The only difference was that all protocol (ii) samples received a second dialysis, after the ageing step. This was to remove any aminoguanidine added immediately before ageing, and was carried out on aminoguanidine-free controls as well as aminoguanidine treatments. Treatments are compared in Fig. 5.8.

This observation allows certain conclusions to be drawn regarding the nature of PPM generated during ageing as compared with that generated during glycation.

- The drop in PPM in glycated proteins during sugar-free ageing under protocol (ii) can only be attributed to removal of PPM during the second dialysis. Thus PPM generated during sugar-free ageing of glycated proteins must have comprised small non-protein-bound compounds which were removed by dialysis. This contrasts with PPM formed during glycation. Glycated proteins under protocol (i) displayed much higher PPM at initiation of the second incubation than did native proteins (Fig. 5.4B vs A, week 0). Thus, a significant fraction of PPM formed during glycation must have been protein-bound sufficiently strongly to survive dialysis after the first incubation.

- PPM in the aminoguanidine-free glycated control at the initiation of the second incubation under protocol (ii) (Fig. 5.5B, week 0, no AG) was approximately half of that in the corresponding sample under protocol (i) (Fig. 5.4B, week 0, no AG). This discrepancy in PPM between the two parallel treatments at week 0 cannot be attributed to formation of new dialysable PPM during ageing since neither sample was re-incubated at 37°C. Hence some PPM which was protein-bound shortly after glycation appears to be released even during the short period of sugar-free ageing represented by the second dialysis (4 days at 4°C). This also suggests that there is likely to have been some loss of PPM during the first dialysis, although enough remains to mediate late Mallard product formation during ageing.

- Although low molecular weight compounds from early glycation reactions were removed by the first dialysis, both fluorescent and cross-linked Maillard products were still formed during sugar-free ageing of pre-glycated proteins under both protocols. Thus significant late Maillard reaction routes must exist which proceed initially via protein-bound intermediates, possibly Amadori product. This contrasts with hypotheses that small free dicarbonyl compounds are the major contributors to the formation of Maillard products (Glomb and Monnier, 1995; Wells-Knecht *et al.*, 1995a). Brinkman Frye *et al.* (1998) and Thornalley *et al.* (1999) point out that glyoxal and methylglyoxal (forming GOLD and MOLD imadozolium salt cross-links, respectively) may arise from either protein-bound or free dicarbonyl precursors formed during early glycation.

- PPM levels were similar under protocols (i) and (ii) for native protein (Fig. 5.4A and 5.5A), but differed between protocols (i) and (ii) for glycated proteins (Fig. 5.4B and 5.5B). Hence in native protein - in contrast with glycated protein - it appears unlikely that much PPM was lost during the second dialysis after sugar-free ageing. This suggests that dialysable low molecular weight PPM arising during ageing originates predominantly from glucose exposure.

The chemical identity of PPM which accumulated during sugar-free re-incubation in the present system therefore remains unclear. The periodate assay, as employed in this investigation, appears to detect early glycation products, possibly including Schiff base, Amadori product and their derivatives. However, products with the same assay response continue to form during ageing of glycated protein (*i.e.* over longer time periods than are required for formation of Schiff base or Amadori product) and may form independently of free sugar (presumably by oxidation). They are at least initially

protein-bound, but appear to include a significant low molecular weight component after prolonged incubation under oxidative conditions. They are susceptible to reaction with aminoguanidine and may have a dicarbonyl functionality.

In addition, PPM includes a component generated during ageing of unglycated protein. In order to clarify whether this component originated from protein or from protein-associated lipid, native lysozyme and β-lactoglobulin were incubated in the absence of sugar for three weeks. Results indicate that the sugar-independent component of PPM appears to be generated at higher levels from lipid-associated protein than from lipid-free protein, but originates at least in part from the protein component itself.

Taken together, the evidence presented here indicates that most of the PPM comprises early glycation products and, as such, is dependent on glucose for its formation. A smaller proportion is independent of glucose exposure and forms during ageing, probably through oxidative processes. This ageing-associated PPM is enhanced in pre-glycated protein, presumably because glycation increases the susceptibility of protein to oxidation (Traverso *et al.*, 1997). The presence of lipid further enhances oxidation and hence, presumably, PPM formation. The synergistic effects of glycation and oxidation have been shown to be enhanced in lipoprotein, where glycation occurs on both protein and lipid fractions and enhances lipid oxidation (Bucala *et al.*, 1993; Fu *et al.*, 1996; Menzel *et al.*, 1997).

Nature of reaction between aminoguanidine and PPM

Aminoguanidine suppressed PPM under both protocols, *i.e.* when present either during glycation only or throughout sugar-free ageing.

One possible explanation for the suppression of PPM levels by aminoguanidine pre-treatment during ageing is that some aminoguanidine survived dialysis by forming a reversible association with glycated protein, as previously suggested in Chap. 4. The same association does not appear to occur with native protein since here aminoguanidine pre-treatment slightly increased PPM during the second incubation (Fig. 5.4A, week 3).

Aminoguanidine at 25mM reduced PPM formation in glycated protein during ageing under both protocols, indicating that the chemical species reacting with the inhibitor was probably still present after the first dialysis. Free low molecular weight compounds arising from autoxidation of sugar or of early glycation intermediates, which have been suggested as the likely targets of aminoguanidine, would presumably have been removed during dialysis at the end of the first incubation. This suggests again that the product(s) which appear(s) to be scavenged by aminoguanidine in this experimental system must initially be protein-bound with sufficient stability for at least a proportion thereof to survive exhaustive dialysis. It may be a low molecular weight derivative of the Schiff base or Amadori product which is released slowly from early glycation products on protein under oxidative conditions. Candidate compounds described in the literature include pentose and tetrose sugars, glyoxal, methylglyoxal and 3-deoxyglucosone (Glomb and Monnier 1995; Zyzack *et al*, 1995 ; Thornalley *et al*,

1999). Some of these have been considered as candidates for PPM in the preceding section.

The probable loss of PPM during dialysis after sugar-free ageing (protocol ii) has been discussed (cf Fig. 5.8). It is not possible to say how aminoguanidine may have reacted with this dialysable component. However, it was noted that levels of the remaining protein-bound PPM were reduced by aminoguanidine. Even if aminoguanidine had no effect on the low molecular weight component of PPM, the total inhibitory effect would still remain valid for all time points except week 3 (Fig. 5.5B). If aminoguanidine scavenged low molecular weight PPM at least as efficiently as it did the protein-bound component of PPM, then the total effect of aminoguanidine would appear more pronounced. Indeed, given what is known about the interaction of aminoguanidine with low molecular weight products of the Maillard reaction, it appears likely that aminoguanidine would remove the dialysable fraction of PPM more efficiently than it would the protein-bound fraction. Given the uncertainty regarding absolute levels of PPM detected after re-incubation under protocol (ii), absolute values of nett glycation-associated change in PPM (Fig. 5.5C) are not useful. However, In the light of the foregoing discussion, the indicated trend - *viz.* that levels of glycation-associated PPM are reduced by aminoguanidine during sugar-free ageing - is likely to remain valid.

Introduction of aminoguanidine at the beginning of sugar-free ageing resulted in a virtually immediate drop in mean levels of protein-bound PPM, indicating that the reaction of aminoguanidine with PPM was extremely rapid (Fig. 5.5B, week 0). This is in accordance with the known reaction of aminoguanidine with small dicarbonyl compounds. By contrast, reaction of aminoguanidine with larger monocarbonyl compounds such as Amadori product has been shown to occur far more slowly

(Feather, 1994). However, Skamarauskas *et al.* (1996) demonstrated that aminoguanidine increases the release of sugar-derived carbon from pre-glycated protein, also suggesting that small compounds - either sugars or sugar-derived - are generated during ageing in the presence of aminoguanidine. The same study further showed increased dicarbonyl production from pre-glycated protein in the presence of aminoguanidine. This latter report suggests that, if small dicarbonyl compounds are detected as PPM, this parameter may increase rather than decrease in the presence of aminoguanidine.

To investigate the possibility that small dicarbonyl compounds contribute to PPM during sugar-free ageing, several candidate compounds were tested in the present investigation and were found to give a positive reaction (already discussed). When aminoguanidine was assayed in combination with these test compounds, PPM levels fell by more than 50% for all except glycolaldehyde (Table 5.1). The greatest drop (70%) occurred with MDA, although this may not be significant because the periodate reaction of MDA itself was slight. The apparent loss of PPM in the model system resulting from co-incubation with aminoguanidine may therefore reflect reaction of the inhibitor with dicarbonyl groups on glycation or oxidation products on protein, or generated from such protein-bound products.

In addition to uncertainty regarding the specific glycation products which are detected by the periodate assay and which react with aminoguanidine, glycation-independent interactions were observed between PPM and aminoguanidine (Fig. 5.7). When the model proteins were incubated individually in the absence of sugar, PPM associated with lysozyme was suppressed immediately upon addition of aminoguanidine. This suppression was maintained for at least one week. PPM from β-lactoglobulin was

boosted by addition of aminoguanidine, but thereafter PPM was suppressed throughout the incubation period. Since all other variables were comparable between the incubations, the differences in the effect of aminoguanidine can best be attributed to the presence of protein-associated lipid in β-lactoglobulin, but not in lysozyme. This reflects observations for fluorescence and cross-linking (Chap. 4), in which aminoguanidine was linked with glycation-independent lipid-associated increases in both end-points.

Thus, the chemical characteristics of the periodate assay and the nature of the reaction observed between PPM and aminoguanidine can be seen to be somewhat conflicting. Dicarbonyl compounds (arising from glycation products, native protein or protein-associated lipid) would be expected to react rapidly and to form stable triazine end-products with aminoguanidine (Hirsch *et al.*, 1991; Hirsch *et al.*, 1995a; Araki *et al.*, 1998), thereby removing them from further reaction and detection as PPM. While this is in accordance with observed results, the periodate reaction as originally described would not be expected to proceed on such compounds (Nordin, 1983). On the other hand, the reaction product of a monocarbonyl compound with aminoguanidine would be a labile Schiff base. This would be expected to dissociate readily under the assay conditions and regenerate PPM. Thus a monocarbonyl which yielded PPM would not be expected to be scavenged by aminoguanidine as clearly and as rapidly as was observed. What is clear is that the chemistry of the periodate assay, as used here to indicate early products of glycation, is considerably more complex than originally anticipated.

Conclusions from the periodate assay

In view of the obviously diverse pool of compounds detected here using the periodate assay, it has shown itself to be a dubious indicator of early glycation products. However, the assay has yielded a number of interesting insights into products formed from native and glycated proteins during sugar-free ageing. These have been discussed in some detail above and are summarised in Fig. 5.9. Briefly, a group of compounds giving similar reactions in the periodate assay were seen to arise during glycation. Compounds giving the same reaction were also formed during ageing of native and glycated protein, but arose to a greater extent from glycated protein. If arising from glycated protein, they were initially protein-bound, but were released from protein during sugar-free ageing. If arising from native protein, such compounds appeared to remain mostly protein-bound. A proportion of this pool of compounds appeared to be dicarbonyls.

5.5 SUMMARY

- When ageing in phosphate buffer at 37°C, both native and glycated proteins produce PPM.

- Both DTPA and aminoguanidine block later Maillard reactions to allow accumulation of early (periodate positive) products. These are consumed during inhibitor-free and sugar-free re-incubation. In protein pre-treated with DTPA, PPM consumption is accompanied by formation of fluorescent and

cross-linked late Maillard products. By contrast, in protein pre-treated with aminoguanidine, formation of late Maillard products is more complex.

- The periodate assay, as used in this study, is not restricted to detecting only Amadori product. It also detected other glycation-dependent compounds, and compounds arising by routes other than glycation alone. This diverse group is formed during sugar-free ageing of both native and pre-glycated protein, probably by oxidative processes. Pre-glycation enhanced PPM formation both as a result of glycation and during sugar-free ageing.

- The compounds which make up PPM (both those arising by glycation and those formed independently of glycation) are initially protein-bound with sufficient stability to survive prolonged dialysis.

- Glycation-dependent PPM appears to become susceptible to removal by dialysis during sugar-free ageing.

- A proportion of PPM may have a dicarbonyl functionality.

- PPM is partially blocked by aminoguanidine.

- As noted in Chap. 4, aminoguanidine present during glycation continues to exert an effect during sugar-free ageing following removal of the free compound by dialysis. This may represent a reversible association between aminoguanidine and protein.

- Aminoguanidine has different effects on sugar-independent PPM arising from lipid-free proteins and from lipid-associated proteins.

- Results with aminoguanidine incorporated either during glycation or during sugar-free ageing support multiple routes to the formation of PPM.

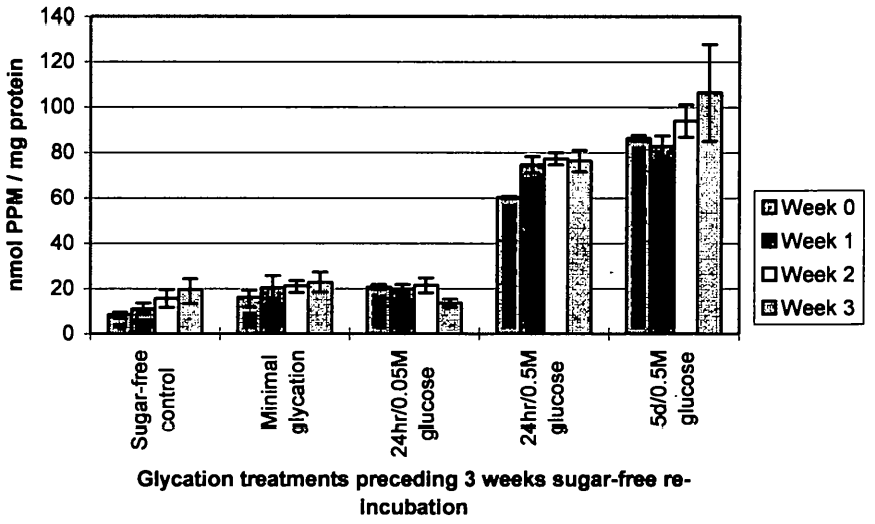


Fig. 5.1

Formation of early glycation products, indicated by periodate positive material (PPM), produced after a range of glycating conditions and during 3 weeks subsequent sugar-free ageing. Glycating incubations contained only lysozyme. Re-incubations contained a 1:1 mixture of preglycated lysozyme and native β-lactoglobulin.

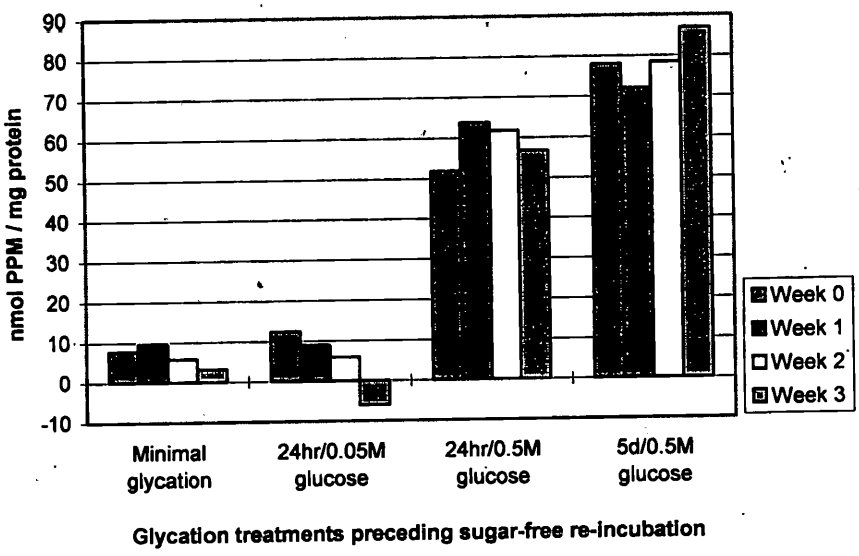


Fig. 5.2

Nett glycation-associated change (glycated protein - native protein) in PPM produced after a range of glycating conditions and during 3 weeks subsequent sugar-free ageing. Glycating incubations contained only lysozyme. Re-incubations contained a 1:1 mixture of preglycated lysozyme and native β-lactoglobulin.

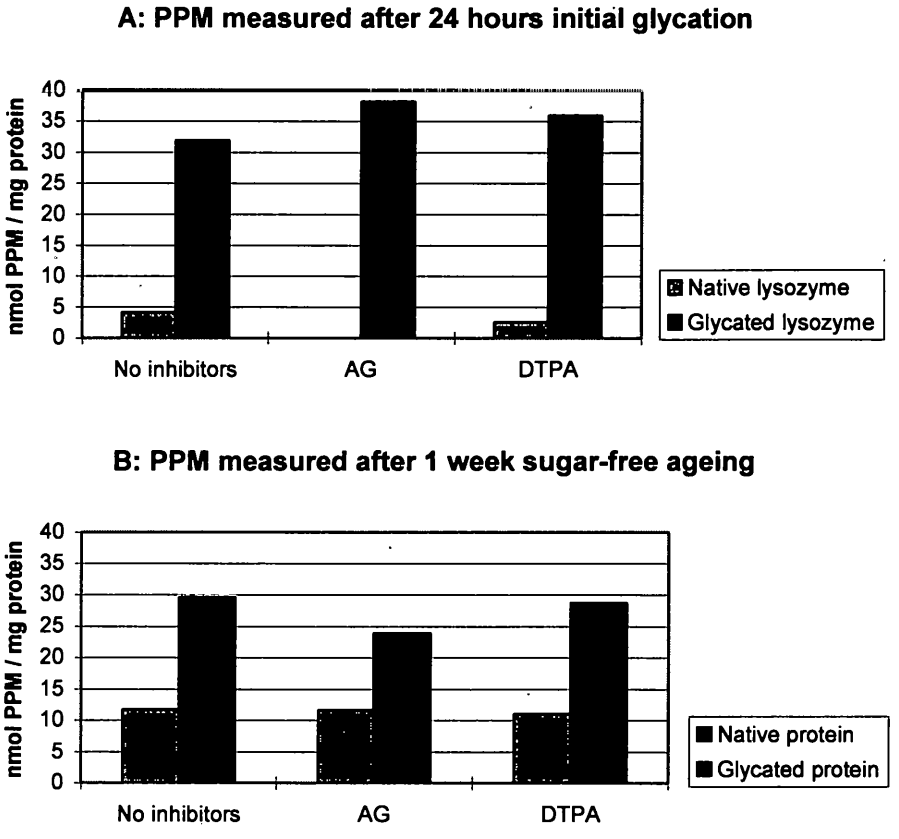


Fig. 5.3

A Formation of PPM during glycation of lysozyme (10mg/ml) for 24 hours at 37°C in 0.5M glucose. The glycation mixture contained no inhibitors, 25mM aminoguanidine (AG), or 1mM DTPA. A sugar-free control (native lysozyme) was similarly treated.

B Formation of PPM after 1 week sugar-free ageing of the pre-glycated lysozyme (5mg/ml), shown in Fig. 5.3A, with native β-lactoglobulin (5mg/ml). The sugar-free control shown in Fig. 5.3A was similarly re-incubated with native β-lactoglobulin (native protein).

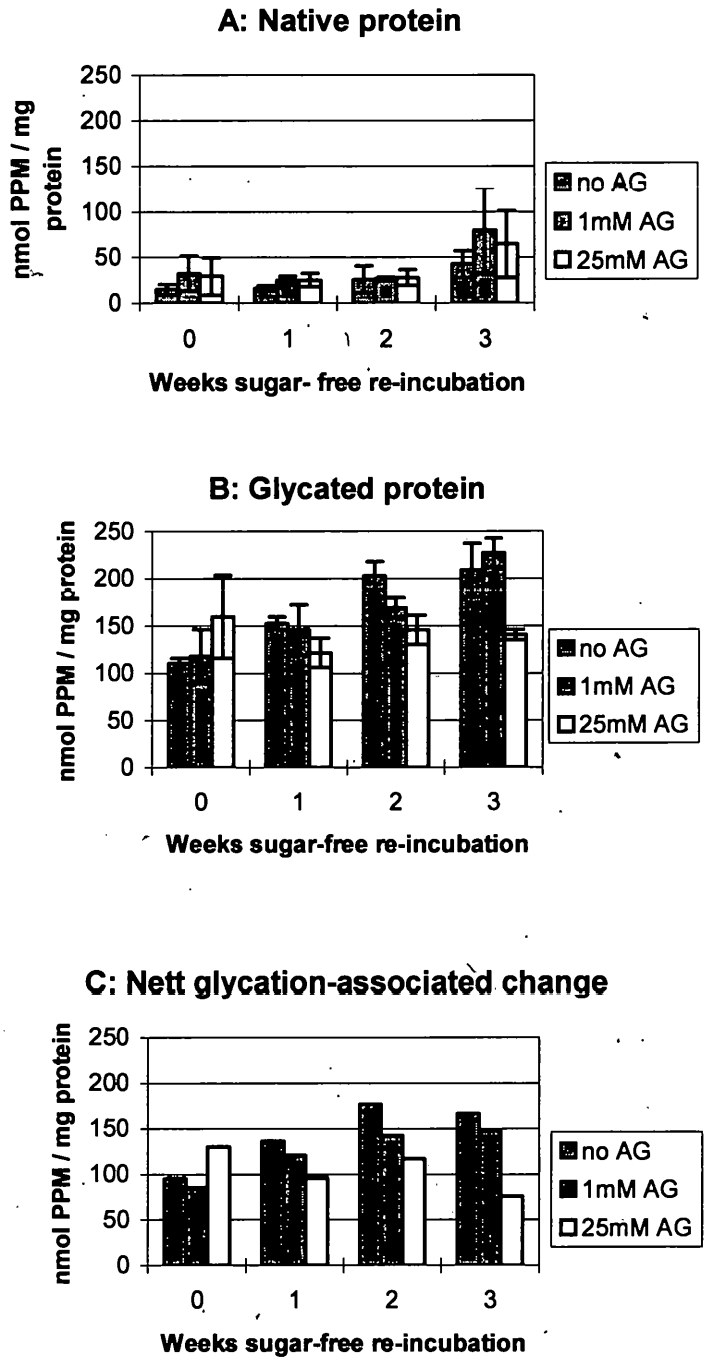
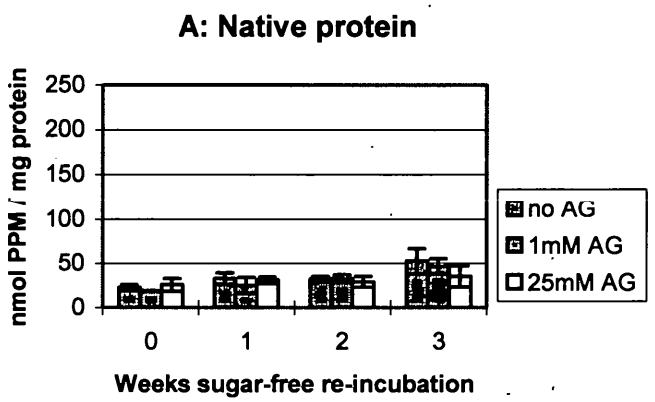
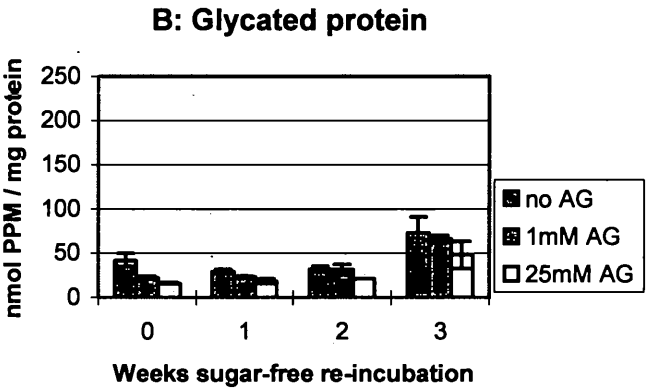


Fig. 5.4

Changes in PPM in native protein (A) and glycated protein (B), and nett glycation-associated change in PPM (glycated protein - native protein, C), during 3 weeks sugar-free ageing in co-incubations of β-lactoglobulin and lysozyme pretreated with aminoguanidine (protocol i).





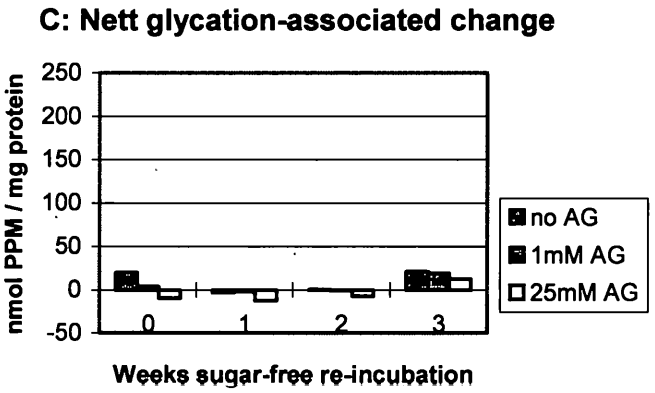


Fig. 5.5

Changes in PPM in native protein (A) and glycated protein (B), and nett glycation-associated change in PPM (glycated protein - native protein, C) during 3 weeks sugar-free ageing in the presence of aminoguanidine in co-incubations of lysozyme and β-lactoglobulin (protocol ii).

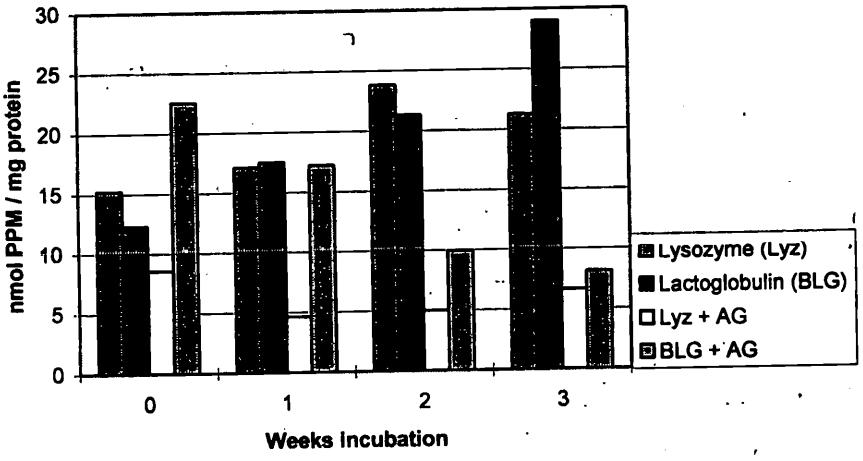
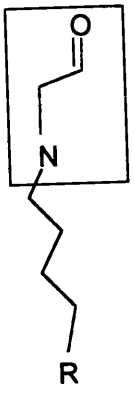


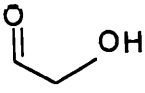
Fig. 5.6

Formation of PPM by each of the model proteins lysozyme and β-lactoglobulin, incubated either alone or together with 25mM aminoguanidine (AG).

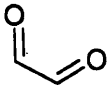
Glycine



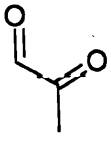
Carboxymethyllysine



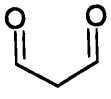
Glycolaldehyde



Glyoxal



Methylglyoxal



Malondialdehyde

Fig. 5.7

Structures of compounds tested for reactivity in the periodate assay.

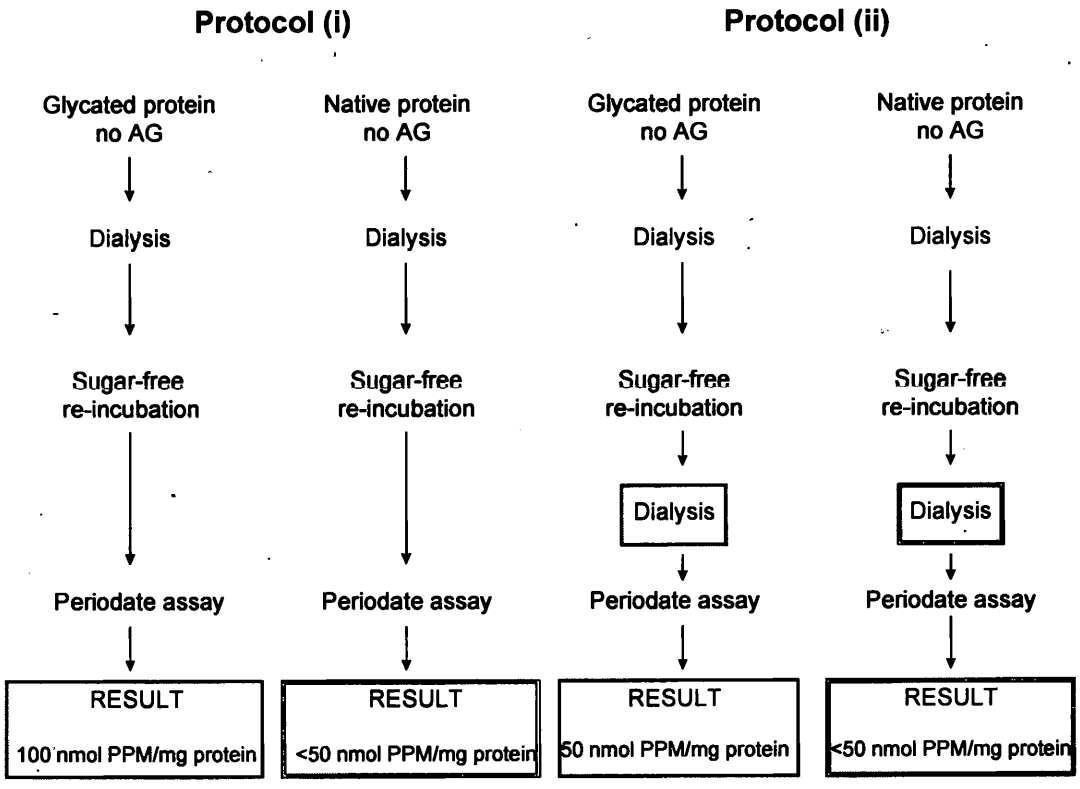


Fig. 5.8

Comparison of aminoguanidine (AG)-free incubations under protocol (i) and protocol (ii). Boxes with the same outline indicate equivalent treatments, with the exception of the second dialysis under protocol (ii).

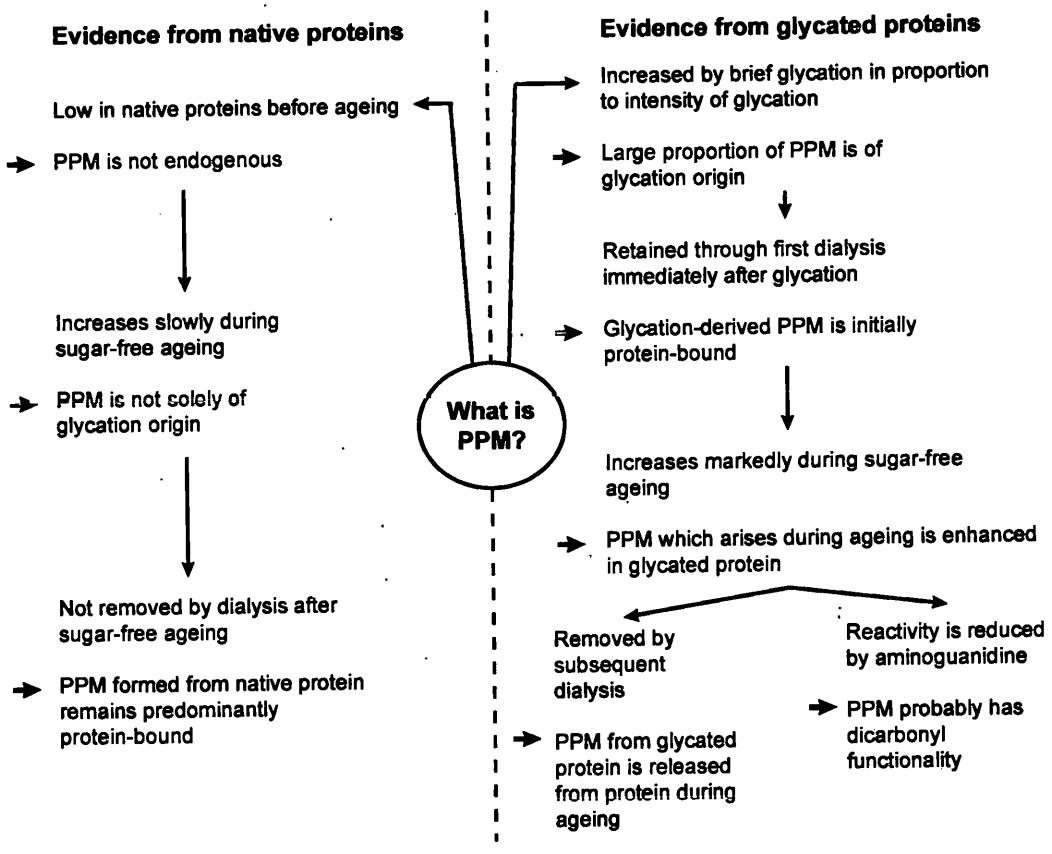


Fig. 5.9

Summary of lines of evidence emerging from the periodate assay.

CHAPTER 6

MAILLARD CROSS-LINKING BETWEEN SERUM AND STRUCTURAL PROTEINS INVESTIGATED BY X-RAY DIFFRACTION

6.1 INTRODUCTION

HSA has been shown to form glycation-dependent cross-links with another protein (Chap. 3). This chapter reports attempts to use X-ray diffraction to demonstrate glycation-associated cross-linking between HSA and collagen in intact tendon. Relevant aspects of collagen structure and the use of X-ray diffraction to investigate Maillard-derived cross-linking of collagen are briefly reviewed below.

6.1.1 Collagen structure

Collagen is the most abundant protein in mammals. It is a fibrous protein found in bone, tendons, skin, blood vessels and the cornea of the eye. In its most common form (type 1 collagen, as found in tendon), it exists as a triple helical structure. It is best studied as the collagen precursor, tropocollagen. This is rod-shaped, has a length of about 300nm, a diameter of 1.5nm, and a mass of about 285kD. It comprises three polypeptide chains of approximately equal size. Two are identical (termed α1(I)), while the third differs (termed α2). Each of these polypeptide strands consists of approximately 1000 amino acid residues. The strands are coiled

around each other to form a triple helical structure of great tensile strength. The strands are held together by hydrogen bonding. Each third amino acid residue in the sequence is glycine (which has no side chain) to accommodate the close proximity of the three strands at the centre of the structure. Other amino acids which occur with high frequency are proline and hydroxyproline, with the sequence glycine-proline-hydroxyproline recurring frequently (Stryer, 1981: Marks *et al.*, 1996).

Collagen molecules spontaneously associate to form collagen fibrils. X-ray diffraction and electron microscopy studies have shown that these fibrils exhibit striations which result from staggering of adjacent rows of tropocollagen by approximately one-quarter of the length of the basic unit. Thus the fundamental structure of a collagen fibril is a quarter-staggered array of collagen molecules. Tropocollagens are not linked end-to-end in this array. There is a gap of approximately 40nm between the end of one tropocollagen and the start of another (Stryer, 1981). The quarter-staggered staggered arrangement gives rise to an axial periodicity, referred to as the D-period, comprising a gap region and an overlap region. One D-period is about 67nm long and corresponds to 234 amino acid residues (Meek *et al.*, 1979). The structure of the collagen fibril, indicating the D-period, is shown diagrammatically in Fig. 6.1 (presented at the end of the chapter).

The large size and extensive cross-linking of mature collagen makes it difficult to study biochemically. It is accessible only to methods which break collagen into smaller fragments by enzyme action (*e.g.* pepsin digestion) or by chemical means (*e.g.* cyanogen bromide cleavage). The regular axial arrangement and periodicity of molecules in collagen makes intact tissue (particularly highly ordered tissues such as tendon) particularly suitable for study by electron microscopy or X-ray diffraction. The electron density distribution along the collagen fibre lends itself to staining

methods which allow the periodic structure to be studied at the electron microscope. However, this requires extensive sample processing, particularly dehydration. X-ray diffraction offers the possibility of studying unmodified tissue samples in the hydrated state.

6.1.2 Collagen cross-linking and the Maillard reaction

Enzymatic post-translational modifications add to the strength of collagen. They also reduce its solubility, contributing to the difficulties of studying mature collagen biochemically. Products of non-enzymatic Maillard reactions result in the accumulation of coloured, fluorescent and cross-linked products on collagen. These are associated with decreases in solubility and susceptibility to protease digestion, and increases in stiffness, mechanical strength and thermal stability. In the context of this thesis, it is important to distinguish between this *enzymatic* glycosylation and cross-linking, and cross-linking associated with *non-enzymatic* Maillard reactions. Such Maillard cross-linking - and the use of biophysical methods to characterise products thereof on collagen - have been reviewed in Chap. 1.

6.1.3 X-ray diffraction

X-ray diffraction has been used to study native collagen structure for several decades. Tendon collagen was the original material of choice since the fibres are well oriented and align almost parallel to the tendon axis. This gives rise to well-defined X-ray diffraction patterns, and underlies the choice of rat tail tendon (RTT)

for use in the present study. Subsequently, various investigators have extended studies to tissues with less organised arrangement of collagen.

X-ray diffraction patterns reflect the scattering of X-rays by collagen. Perturbations in collagen structure, either along the axis of collagen fibres or perpendicular to the axis, result in changes in the diffraction pattern. Two types of diffraction pattern are distinguished. Diffraction in the region perpendicular to the fibre axis is derived from the lateral packing of collagen. Diffraction parallel to the fibre axis is termed meridional diffraction and is derived from the axial structure of the tissue specimen. Changes in meridional diffraction patterns reflect changes in electron density distribution along the collagen molecule or fibre. Cross-linking of collagen to other collagen fibres or to other proteins would cause changes in this parameter and could therefore be expected to produce changes in the meridional diffraction pattern (Brodsky *et al.*, 1998; Tanaka *et al.*, 1988b). This study concerns itself with meridional diffraction patterns of glycated tendon collagen incubated with native HSA, or with native tendon collagen incubated with glycated HSA.

High angle and low angle X-ray diffraction patterns can be used to obtain information about structure at different resolutions. Structures of large dimensions (5-100nm) can be examined by low angle diffraction. The diffraction patterns provide information about the interfibrillar spacing of collagen and contain the low order meridional reflections of collagen arising from the D-periodicity of collagen fibrils. Only meridional reflections were considered in this study. High angle X-ray diffraction patterns allow structural details of the collagen helix to be elucidated. The diffraction patterns provide information about the intermolecular spacing of collagen within fibres and contain the higher order meridional reflections (Brodsky *et al.*, 1998; Tanaka *et al.*, 1988b). Once again, only the meridional reflections were considered in this study.

Meridional reflections appear in X-ray diffraction patters as arcs in the vertical plane, corresponding to the orders of D-periodicity of RTT collagen. The reflections appear as arcs rather than spots because the molecules in tendon are not perfectly aligned. Structural modifications, such as cross-links, along the collagen axis result in changes in electron density distribution and hence in the intensity of the meridional reflections. Changes in the electron density within the D-period are calculated by computing the Patterson functions. These give the Fourier syntheses of the intensity data without requiring phase information (Hadley, 1999).

6.2 MATERIALS AND METHODS

6.2.1 Pre-glycation and sugar-free ageing

Healthy normoglycaemic young rats were obtained from the University of Oxford (Department of Physiology). The age of the rats when sacrificed was 30 days. Rat tail tendons from three rats were excised, washed in distilled water, cut into sections of 2-3cm, blotted dry and weighed. RTT segments were thoroughly mixed to ensure randomisation of RTT assigned to each experimental treatment. Approximately 0.15g RTT (2-3 sections) was assigned to each experimental treatment.

RTT to be pre-glycated was incubated for 24 hours at 37°C in 0.1M phosphate buffer containing 0.5M glucose, with gentle shaking. The buffer was supplemented with sodium azide (3mM), benzamide hydrochloride (2.5mM) and trypsin inhibitor (0.01%) to inhibit proteases and microbial growth. After the glycating incubation,

RTT sections were washed three times in distilled water and three times in clean phosphate buffer to remove sugar. Each wash comprised immersing RTT sections in clean water or clean buffer and shaking at medium speed for 5 minutes.

HSA (purity >99%) was glycated by incubating the protein (10mg/ml) in 0.1M phosphate buffer containing 0.5M glucose at 37°C for 24 hours. The buffer was supplemented with 3mM sodium azide to prevent microbial growth. After glycation, free glucose was removed by dialysing against phosphate buffer at 4°C for 4 days. The final HSA concentration was 6.1mg/ml.

The experimental treatments are listed below. The concentration of pre-glycated or native HSA in the incubation medium (where used) was 6.1 - 6.5mg/ml. As before, incubations were performed in 0.1M phosphate buffer, pH 7.4, supplemented as above with inhibitors of proteases and microbial growth.

A Native (unglycated) RTT washed in distilled water and frozen immediately

B Native RTT incubated in phosphate buffer at 37°C for 3 weeks

C Glycated RTT washed in distilled water only and frozen immediately

D Glycated RTT incubated in phosphate buffer at 37°C for 3 weeks

E Native RTT and native HSA incubated in phosphate buffer at 37°C for 3 weeks

F Glycated RTT and native HSA incubated in phosphate buffer at 37°C for 3 weeks

G Native RTT and glycated HSA incubated in phosphate buffer at 37°C for 3 weeks

After incubation, all samples were stored at -20°C until analysis by X-ray diffraction.

6.2.2 X-ray diffraction

X-ray diffraction patterns of the RTT samples were obtained on Stations 2.1 and 7.2 at the Daresbury Laboratory Synchrotron Radiation Source (SRS).

Station 2.1

Station 2.1 is one of the highest intensity low angle X-ray diffraction stations at the Daresbury SRS. X-ray patterns were collected on a multiwire, area detector, with a working area of 20cm x 20cm, 512 x 512 pixels. The focussing optics consisted of a triangularly bent germanium monochromator, asymmetrically cut at 10.5°. This focussed the beam in the horizontal plane, with a selected wavelength of 0.154nm. A fused quartz mirror (75cm long) focussed the beam vertically by total reflection of X-rays. Further definition of the beam was performed by 4 sets of collimating slits to yield a beam with dimension 5mm x 1mm (Hadley, 1999). A camera length of 6m was used. Exposure time was 3 minutes.

Station 7.2

Beamline 7.2 (high angle) uses a monochromatic beam of wavelength 0.1488nm. The beam was focussed in the vertical plane by a fused quartz mirror, and in the horizontal plane by reflection from the plane of a germanium (Ge-111) crystal monochromator. Focal spot size was 0.5mm x 0.3mm. The interior of the camera was filled with helium gas to minimise gas scatter. X-ray diffraction patterns were

collected by the online phosphor image plate (MAR Research), which provided a circular detector with a plate diameter of 18cm. The images were recorded at 1200 x 1200 pixel size (Hadley, 1999). A 0.6mm circular collimator was used and the camera length was 55.7cm. The camera was kindly loaned by T Wess (University of Stirling). Exposure times were 5 - 20 minutes (usually 5 minutes).

6.2.3 Data analysis

Data from Station 2.1

The 2-dimensional images obtained from Station 2.1 were corrected for X-ray intensity, background scatter and detector response. Images were normalised using the ion chamber count in order to eliminate variations in the X-ray intensity between exposures. An empty cell X-ray pattern was subtracted from sample X-ray patterns to remove any X-ray scatter due to the cell. A detector response pattern was obtained using a radioactive source. Each sample pattern was divided by this to reduce errors or artefacts arising from the detector. These corrections of the X-ray images were carried out using the BSL software (CCP13 suite, Daresbury SRS). A 1-dimensional (1-D) scan was made vertically across each pattern to allow further analysis of the intensities of meridional reflections.

Further manipulations of the 1-D traces were performed in XVGR (Shareware), Statistica (StatSoft Inc, Release 5.1A) or Microsoft Excel (Windows 95, Version 7.0a).

Data from Station 7.2

The software IPDISP (Daresbury SRS) was used to view the X-ray diffraction patterns obtained from Station 7.2. The images were transformed from 1200 x 1200 2-byte integer format to 512 x 512 4-byte floating point format (MAR2BSL, Oxford Research Unit) and vertical 1-D scans were performed (BSL, Daresbury SRS).

Again, further data manipulations were performed using the software XVGR, Statistica or Microsoft Excel.

Background removal

Background scatter in X-ray diffraction patterns arises mostly from non-fibrillar proteins (*e.g.* type VI collagen, proteoglycan cores) present in the interfibrillar matrix. Removal of background is both important and difficult, since X-rays scattered from background components mask the first and second meridional reflections. This complicates resolution of the first few meridional intensities.

Background was removed by fitting a curve to each of the 1-D traces obtained from the corrected X-ray diffraction patterns, and subtracting this from the trace. For data obtained on Station 2.1, this was performed in Statistica, using a power law expression (Equation 6.1).

Equation 6.1 $y=bx^a$

For data obtained on Station 7.2, background fitting and subtraction was performed in Statistica, using either a third order or a fourth order polynomial function (Equation 6.2, third order polynomial).

Equation 6.2 $y=a+bx+cx^2+dx^3$

Comparing peak height and peak area as measures of peak intensity

To determine whether peak heights could be used as measures of peak intensity in place of peak areas, corrected and background-adjusted 1-D X-ray traces for native RTT (treatment A) from both stations were printed out. Peak heights were recorded, and the peaks were cut out and weighed. The peak masses were used as measures of peak area. Peak height was plotted against peak mass (in Excel) for each trace and the linear correlation coefficient was calculated.

Combining meridional peak intensities from low angle and high angle X-ray diffraction patterns

Peak positions were identified from the traces derived for native RTT from Stations 2.1 and 7.2. The corresponding peak intensities were extracted from all corrected and background-adjusted data sets. All peak intensities were scaled to the 9th order (set to an arbitrary value of 100). Mean peak intensities and min/max deviations were calculated for duplicate treatments.

Mean peak intensities were combined for corresponding experimental treatments, using data from both stations. The number of orders which could be reliably distinguished in all scans was 22. Orders 1-9 were extracted from Station 2.1 data; orders 9-22 were extracted from Station 7.2 data (the 9th order having been set to 100 in both as an earlier step).

Patterson functions

Scaled and combined mean peak intensities of meridional reflections were subjected to Fourier analysis. The Patterson function was used for this purpose since it expresses the relative frequencies of intra-period electron density-connecting vectors without requiring knowledge of the phases of the component vectors. The mathematical expression of the function is given in Equation 6.3. Its derivation from experimental data is shown in Fig. 6.2 (presented at the end of the chapter).

Equation 6.3 $$P(x) = \sum I(h)(\cos 2\pi hx)(\exp(-h^2B))$$

where h = order

I = intensity

x = relative shift of data (from 0 to 234 residues)

$\exp(-h^2B)$ is a smoothing factor which reduces termination errors.

The value of B was chosen by a process of trial and error. A starting value of B=0.0027 (Hulmes *et al.*, 1977) was used, and adjusted iteratively until curves

plotted from the output of the Patterson functions represented an acceptable balance between under-smoothing and over-smoothing. A value of 0.1 was identified as suitable.

Patterson functions for all RTT samples were calculated using a computer programme written by J Hadley and provided by K Meek (Hadley, 1999 - see Appendix).

6.3 RESULTS

All figures referred to in the text are presented at the end of the chapter.

The meridional reflections with which this study concerned itself appear as arcs in the vertical plane, corresponding to the orders of D-periodicity of the RTT collagen. Traces (1-D) through the corrected patterns produced a scan with peaks corresponding to the meridional orders superimposed on a background curve. Subtraction of the background left a series of peaks with their bases on the baseline. Examples of such background-corrected scans for native RTT in presented in Fig. 6.3A (Station 2.1) and 6.3B (Station 7.2).

6.3.1 Comparing peak height and peak area as measures of peak intensity

Plots of peak height (read from 1-D traces as arbitrary units) against peak mass (as indicator of peak area) are shown in Fig. 6.4A (native RTT, Station 2.1) and Fig. 6.4B (native RTT, Station 7.2). The linear correlation coefficient was greater than

0.99 in both instances. This was taken as sufficient grounds for using peak heights in place of peak areas as measures of peak intensity.

6.3.2 Combining meridional peak intensities from low angle and high angle X-ray diffraction patterns

The combined mean peak intensities from Station 2.1 and Station 7.2 for each experimental treatment are presented in Fig. 6.5A-G. Error bars show the min/max deviation around the mean. In three instances, X-ray data were available for only one replicate (Treatment D, Station 2.1; Treatment E, Station 7.2; Treatment F, Station 7.2).

Comparing Fig. 6.5A through Fig. 6.5G, it is apparent that differences among mean peak intensities for different treatments are small, and variability within each treatment is quite large. Tanaka *et al.* (1988b) reported changes in the intensities of meridional orders 14 through 19 in RTT exposed to ribose for several days or glucose for several months. Comparing order 10 and upwards for the experimental treatments represented here, a number of observations can be made. (Note that differences were only considered if the min/max error bars did not overlap.)

Effect of phosphate buffer

Hadley (1999) reported that phosphate itself binds to collagen and that this effect complicates the interpretation of changes in electron density distribution arising from glycation. For this reason, the experimental treatments in the present study were

designed to allow the effects of phosphate and those of glycation to be separated as far as possible.

To assess the effect of phosphate, native RTT was compared without or with incubation in phosphate buffer (Fig. 6.5A, B), and similarly glycated RTT without or with incubation in phosphate buffer (Fig. 6.5C, D). Phosphate was associated with decreases in orders 15-19, irrespective of whether collagen had been glycated prior to incubation. In addition, there were some increases in lower orders (10-13), but these were less consistent between native and glycated RTT. This indicates that phosphate buffer binds to collagen in the native state, and probably also in the glycated state. However, it should be noted that the difference between Fig. 6.5C and 6.5D are due to the effect of *both* the presence of phosphate and sugar-independent Maillard reactions which continue on glycated collagen after removal of free sugar.

Effect of glycation and co-incubation with HSA

Comparison of the effect of native RTT ageing in phosphate alone with that of RTT ageing in phosphate together with native HSA (Fig. 6.5B, E) shows that HSA made little difference in the mean peak intensities. Some changes occurred in the lower orders. There were some small decreases orders 13-17, but it is not clear where any decrases were significant.

By contrast, comparison of native RTT not incubated in phosphate with co-incubation of native RTT and native HSA (Fig 6.5A, E) shows changes in a number of the orders from 11 to 18. Thus the effect of phosphate appears greater than that

of native HSA, indicating more widespread binding of phosphate to collagen than of HSA to collagen.

Comparing native and glycated collagen without the influence of phosphate (Fig. 6.5A, C), differences in peak intensities attributable to 24-hour glycation of collagen were slight. There were small decreases in the intensities of orders 10 and 12, and a small increase in order 19. However, the patterns of mean peak intensities were similar between the two treatments.

Comparison of the effect on collagen of ageing in phosphate alone with the combination of pre-glycation and ageing in phosphate (Fig. 6.5B, D) showed numerous small differences in the pattern of mean peak intensities, but few of these appeared significant. Ageing of pre-glycated collagen in phosphate was associated with increases in orders 16 and 17, and with decreases in orders 13 and 19, relative to the effect ageing in phosphate alone.

However, the most noticeable effect of pre-glycation was not differences among the peak intensity patterns of different combinations of pre-glycated proteins. Rather, it was the close correspondence of results for all treatments which incorporated at least one glycated protein. Patterns of mean peak intensity were almost identical for glycated RTT incubated in phosphate (Fig. 6.5B), glycated RTT and native HSA incubated in phosphate (Fig. 6.5F), and native RTT and glycated HSA incubated in phosphate (Fig. 6.5G). Thus glycation appeared to cause similar changes along collagen fibres, irrespective of whether collagen was itself glycated or whether it was left in contact with a different glycated protein. This would be conveniently explained by cross-linking between the glycated and non-glycated proteins, and suggests a role for collagen in glycation-mediated cross-linking to serum proteins.

Data variability

There was considerable variability in results obtained with two or three replicate RTT segments for the same experimental treatment. The extent of the variability differed among treatments, with some showing noticeably greater variability than others. This was particularly marked when intensities were plotted on a linear scale (not shown); the log scale used in Fig. 6.5 serves to minimise this effect. There are numerous factors contributing to this. Some are associated with variability inherent in living systems. Tissues show variation among individuals, even when matched for strain and age. Thus RTT from different rats may display variations in the extent of inherent collagen cross-linking (both enzymatic and non-enzymatic), in collagen-associated matrix components, and in susceptibility to glycation and oxidative modification.

Added to this is variability arising from the experimental system. Different thicknesses and degrees of abrasion of the excised tendon segments would result in variation in the surface area exposed to glucose and to phosphate in the incubating medium. This would be associated with variations in the sites exposed for sugar binding, phosphate binding and cross-link formation. Furthermore, since RTT collagen is not a soluble protein, there may have been variations in contact between the incubation buffer and RTT segments.

Additional sources of variability are introduced by the nature of X-ray diffraction studies. Variations in the alignment of the sample within the cell for exposure to X-rays mean that the beam may not always pass through the centre of the tissue sample, or that the axis of the collagen may not be exactly perpendicular to the beam. Differences in the thickness of the tendon sample result in variations in X-

ray scattering. Variation in the extent of stretching of the tendon segment, and of hydration of the tendon can also serve to introduce variability in the scattering of X-rays which are not related to sample treatment.

These considerations serve to highlight the problems with data variability which are inherent in X-ray diffraction studies of animal tissues subjected to experimental modification.

6.3.3 Patterson functions

Both Patterson functions and difference Patterson functions were calculated. More information was revealed in the Patterson functions, therefore the following discussion concentrates on these. Graphical representations of the Patterson functions are shown in Fig. 6.6A - G.

The Patterson function of native RTT collagen (Fig. 6.4A), as calculated here, shows an underlying steeply sloping function with main vectors at residue positions ~63 and ~171. These are complementary vectors, with that at residue ~171 being effectively the same as that at ~63 residues, thus only the first appearance of this vector is considered. It is highly likely that other vectors are superimposed on the Patterson function, but that these have been suppressed by the choice of smoothing factor. For this reason, the vector which is clearly visible is referred to here as the main vector.

Effect of phosphate

The effect of phosphate can be seen by comparing the Patterson function for native RTT (Fig 6.6A) with that for native RTT incubated in phosphate (Fig. 6.6B). It is clear that phosphate alters electron density, indicating that phosphate binds to collagen. Most notably, ageing of native RTT in phosphate strongly suppressed the vector at ~63 residues. It also shifted it to ~77 residues, a shift of 14 residues. The resolution of the data is given by dividing the D-period (234 residues) by the number of orders represented (22). This is 11 (less than the observed shift), so the shift may be significant.

Comparisons of the Patterson function for briefly glycated RTT (Fig. 6.6C) with that for pre-glycated RTT incubated in phosphate (Fig. 6.6D) are difficult to interpret. These Patterson functions are clearly different, and also differ from the effect of phosphate on native RTT. However, changes in electron density distribution due to phosphate occur simultaneously with changes arising from sugar-independent Maillard reactions of pre-glycated collagen during sugar-free ageing. It is unclear how much each process contributed to the observed changes. In glycated RTT, three weeks ageing in phosphate strongly *enhanced* the vector which was *suppressed* by these conditions in native RTT. The shift in peak position was not significant in this instance.

Effect of glycation of RTT

The effect of glycation alone on RTT can be seen by comparing the Patterson functions for native RTT (Fig. 6.6A) with glycated RTT (Fig. 6.6C). The combined

effect of pre-glycation and subsequent sugar-free ageing in phosphate can be seen by comparing native RTT (Fig. 6.6A) with glycated RTT incubated in phosphate (Fig. 6.6D). Glycation alone slightly suppressed the vector at ~63 residues and slightly flattened the underlying function. There was a slight shift in peak position, but this was not significant. This indicates that early glycation products in collagen did not markedly alter the electron density distribution along collagen

The combination of pre-glycation and ageing in phosphate buffer resulted in marked differences in the Patterson function. The main vector was enhanced and the peak shift (to ~77 residues) became significant. This indicates that sugar-independent Maillard reactions in the presence of phosphate resulted in considerable modifications along the collagen fibre. It parallels findings from biochemical studies that cross-linked and fluorescent protein products are formed during sugar-free ageing of pre-glycated protein in phosphate buffer (reported in Chap. 3).

Effect of co-incubation of RTT with HSA

Comparing native RTT incubated in phosphate (Fig. 6.6B) with native RTT and native HSA similarly incubated in phosphate (Fig. 6.6E), it can be seen that the presence of HSA partly mitigated the suppression of the main vector by phosphate. Fig. 6.6E is intermediate in shape between Fig 6.6A (RTT without phosphate) and Fig 6.6B (RTT incubated with phosphate). Peak position was not significantly different between Fig 6.6B (~77 residues) and Fig. 6.6E (~85 residues), but did differ significantly between Fig. 6.6A (~63 residues) and Fig. 6.6E.

Comparing native RTT (Fig. 6.6A), native RTT incubated in phosphate (Fig. 6.6B), and native RTT and glycated HSA incubated in phosphate (Fig. 6.6G) shows that, unexpectedly, glycated HSA almost completely cancelled the effect of phosphate on native RTT. The intensity and position of the vector and the shape of the underlying function in Fig. 6.6G closely approach those in Fig. 6.6A. A similar effect was observed upon co-incubation of native HSA and native RTT in phosphate (Fig. 6.6E), but comparison of Fig. 6.6G, E and A reveals that glycated HSA was noticeably more effective than native HSA at counteracting the effect of phosphate on native RTT collagen,

Comparing glycated RTT (Fig. 6.6C), pre-glycated RTT incubated in phosphate (Fig. 6.6D), and pre-glycated RTT and native HSA incubated in phosphate (Fig. 6.6F), the effect of glycated HSA appeared slight. The increase in the main vector associated with incubation of pre-glycated collagen in phosphate was slightly counteracted, producing a Patterson function which was intermediate between Fig. 6.6C and Fig. 6.6D, but with a greater resemblance to Fig. 6.6D. Peak position did not differ significantly among the three treatments, and was identical in Fig 6.6C and 6.6F.

6.4 DISCUSSION

Observations arising from Patterson functions for RTT collagen with respect to phosphate buffer, brief glycation and co-incubation with serum protein can be summarised as follows:

- Incubation of native RTT in phosphate buffer alters the electron density along the collagen, probably as a result of phosphate binding to collagen

- Glycation of RTT does not greatly impact electron density along collagen.

- Ageing of glycated RTT in phosphate buffer results in profound changes in electron density along the collagen fibre. These changes differ markedly from those observed in native RTT similarly aged in phosphate buffer. They probably indicate formation of cross-linked products during sugar-independent Maillard reactions involving early glycation products on RTT.

- Both native and glycated HSA interact with native RTT collagen in a manner which reduces the effects on electron density distribution of ageing in phosphate buffer. Glycated HSA almost completely cancels the effect of phosphate on native collagen.

- Native HSA interacts with glycated collagen, but the effect is relatively small.

The following discussion considers possible explanations for the observed effects on electron density distribution along collagen.

The Patterson function for native RTT not incubated in phosphate shows a main vector at ~63 residues. The suppression of this vector as a consequence of incubation in phosphate buffer suggests that phosphate binds approximately uniformly along the collagen axis, except in the region that contributes to the vector at ~63 residues. This minimises the apparent changes in electron density seen in native RTT without phosphate.

There is a precedent for the interaction of phosphate with native RTT collagen, shown here by X-ray diffraction. Biochemical studies by a number of authors have shown that phosphate buffer enhances the Maillard reaction (discussed in Chap. 3) and can affect the balance of the chemical pathways contributing the overall reaction (Wells-Knecht, 1995b). Baynes *et al.* (1989) have discussed this effect in terms of phosphate binding to proteins. They suggest that phosphate (originating from either the buffer or endogenous organic phosphates) can bind to protein in cationic pockets formed by an array of basic amino acid residues. The bound phosphate can then exert a localised catalytic effect, resulting in preferential glycation of lysine residues in basic sequences or regions. The authors propose this mechanism as a possible explanation for promotion of glycation by phosphate in RNase, haemoglobin and HSA. They suggest that binding of the buffering species may determine promotion of glycation in phosphate buffer, rather than direct catalysis by functional groups on basic amino acid residues in the vicinity of lysine (and possibly arginine) residues.

Catalysis of the Maillard reaction by phosphate buffer occurs by at least two routes. The phosphate anion itself catalyses the Amadori rearrangement, which has been identified as the rate-limiting step in the classical glycation pathway (glucose + protein → Schiff base → Amadori product → → late Maillard products, including protein cross-links) (Watkins *et al.*, 1987). In addition, trace metal ions, present as contaminants in phosphate buffer, catalyse oxidative cross-linking of collagen by metal-catalysed oxidation. Fu *et al.* (1992) found that inclusion of metal chelators inhibited cross-linking of collagen by glucose under oxidative conditions. Therefore, both phosphate and trace metal ions in phosphate buffer may be binding to collagen and catalysing either the formation of Amadori product as a pre-requisite to cross-

linking, or cross-linking itself. Thornalley *et al.* (1999) demonstrated that the model Amadori product, fructoselysine, generates the reactive intermediates glyoxal, methylglyoxal and 3-deoxyglucosone during glucose-free incubation, and that the rate at which this occurs increases with increasing phosphate concentration. They suggest that the phosphate dianion (HPO_4^{2-}) is involved in activating deprotonation reactions and trace metal ions promote oxidation, including the oxidative formation of glyoxal. Thus there are a number of routes by which components of phosphate buffer, bound to collagen near glycation sites, can catalyse protein cross-linking, either directly or indirectly.

The similarity between native RTT frozen without incubation in phosphate and glycated RTT frozen immediately after glycation is not unexpected. The glycated RTT at this stage carries early glycation products which do not represent major structural modifications of the protein. Flattening of the underlying function and slight suppression of the main vector may represent glucose binding to lysine (or arginine) residues involved in glycation.

The changes observed in pre-glycated RTT after three weeks ageing in phosphate buffer probably represents a combination of the maturation of early glycation products to late Maillard products (including cross-links) and of the effects of the buffer. Since Patterson functions reflect changes in electron density within the D-period, it is necessary to consider how formation of Maillard products could achieve this. Suppression both of the main vector and of the underlying shape of the Patterson function, as observed with native RTT incubated in phosphate, has been assumed to reflect binding of phosphate along the collagen fibre. Hence, the enhancement of this vector by Maillard products presumably signifies the development of new sites of increased electron density. This probably represents post-Amadori reactions of pre-glycated collagen to form collagen/collagen cross-

links. The vector at ~77 residues corresponds closely to the length of the overlap region of the collagen fibril, so it is possible that these new Maillard products are formed near the gap/overlap junctions. This is depicted schematically in Fig. 6.7. The contribution of phosphate buffer to the observed changes in the Patterson function of glycated collagen during incubation in phosphate buffer probably involves catalytic effects of bound phosphate or trace metals on sugar-independent oxidative Maillard reactions of glycation products.

The mechanism by which HSA (and particularly glycated HSA) appears to mitigate the effect of phosphate on native collagen also remains to be explained. Two possibilities present themselves: (1) that phosphate binds preferentially to HSA and particularly to glycated HSA, or (2) that HSA binds to collagen, thereby re-introducing electron density changes masked by phosphate binding.

The first possibility appears unlikely. Phosphate is present in the buffer in vast excess over both collagen and HSA. Thus even if all possible phosphate-binding sites on HSA were saturated, it is unlikely that phosphate would be sufficiently depleted to reduce its binding to collagen significantly.

The second possibility is supported by results reported in Chap. 3, which clearly demonstrated enhanced cross-linking of HSA to a different protein, provided one of the proteins had been briefly pre-glycated. The Patterson functions appear to indicate that HSA cancels the changes in electron density distribution on collagen which are introduced by phosphate buffer, or introduces additional localised changes in electron density. This could occur if HSA binds to collagen near the gap/overlap region where phosphate binding appears to be the least, and where early glycation products appear to bind.

Native HSA contains some glycated protein as a consequence of *in vivo* glycation of the commercial protein preparation. This could explain why mitigation of the electron density perturbations associated with phosphate was observed even with native HSA and native RTT. Alternatively, cross-linking between native HSA and native RTT may be mediated by lipid oxidation products generated from lipid associated with HSA during ageing in phosphate buffer. Without additional information, it is not possible to determine which mechanism is the more likely. However, the observed residue shift of the vector in Patterson functions for co-incubation of RTT with native HSA and with glycated HSA does suggest that the different forms of HSA bind at slightly different positions and therefore possibly by different mechanisms. Whatever the mechanism, cross-linking of native HSA and a second native protein is supported by results reported in Chap. 3. Some cross-linking was observed even between native HSA and native lysozyme, but this was greatly enhanced if either protein had been pre-glycated.

The Patterson functions for incubation of glycated RTT alone, and for glycated RTT incubated with native HSA, are similar in shape. This indicates that the collagen/ collagen cross-links formed in glycated RTT, also form in the presence of HSA. This appears to be the over-riding process on pre-glycated collagen, even in the presence of another protein. The Patterson function for co-incubation of glycated RTT and HSA shows some reduction in the intensity of the main vector and some flattening of the underlying function. This may indicate that some cross-linking with native HSA occurs at or near sites involved in collagen/collagen cross-linking, thereby spreading the changes in electron density over a slightly broader region.

Within the context of the postulated mechanisms, it is not immediately clear why a smaller mixed protein interaction was observed when RTT rather than HSA was the

glycated protein. It seems probable that some form of cross-link formed between glycated collagen and native HSA. Biochemical studies showed that co-incubations of HSA and lysozyme produced similar mixed oligomers, irrespective of which protein had been pre-glycated (Chap. 3). Parallel with the formation of HSA/collagen cross-links, early glycation products on the glycated RTT also continue to form late Maillard products on collagen during incubation. This cross-linking between early glycation products on glycated RTT and other collagen molecules may leave fewer sites available for cross-linking to HSA. Thus concurrent cross-linking processes occur in co-incubations of glycated RTT and native HSA. This contrasts with co-incubations of native RTT and HSA (native or glycated), in which no significant collagen/collagen cross-linking would expected to occur. Preferential formation of collagen/collagen cross-links on glycated RTT incubated with native HSA may be related to the greater proximity of early glycation products on collagen to other collagen fibres than to HSA in solution. It may also reflect the greater penetration of glucose than of HSA into collagen, resulting in the formation of early glycation products at sites inaccessible to HSA.

Like Patterson functions, mean peak intensities of meridional orders also indicated binding of phosphate buffer to RTT collagen. Unlike Patterson functions, they indicated differences between glycated and unglycated treatments, but no significant differences among treatments in which glycated protein was allowed to age in phosphate buffer (glycated RTT incubated in phosphate; glycated RTT incubated with native HSA; native RTT incubated with glycated HSA). Since Patterson functions were derived from mean peak intensities, this observation is a little unexpected. It may be attributable to the choice of the smoothing factor in the Patterson function. It may also be that Patterson functions have revealed information additional to that visible by comparing peak intensities alone. Taken together, this may indicate that cross-links form whenever significant levels of

glycated protein are present in co-incubations of intact collagen and another protein, but that the nature of these cross-link structures differs depending on which protein has been pre-glycated.

The formation of Maillard-derived collagen cross-links during sugar-free ageing of mixed protein systems has been previously demonstrated biochemically. Sajithlal *et al.* (1998) incubated heavily AGE-modified BSA with native RTT collagen in the absence of free sugar, and observed enhanced cross-linking of collagen. Their experimental design differs from that of the systems examined in this investigation (both biochemically and by X-ray diffraction) in terms of the types of Maillard products likely to be found on the glucose-modified protein, and in that no attempt was made to determine whether mixed protein cross-linking occurred. However, their results support collagen cross-linking in mixed protein systems, as reported here.

Previous investigations of the effect of glycation on intensities of meridional reflections of RTT collagen indicated changes in orders 14-19 (Tanaka *et al.*, 1988b). Results in the present study were similar to some extent, since glycation treatments were associated with changes in intensities of the 16th, 17th and 19th orders, as well as some changes in lower orders (10-13). However, these changes were very small. This probably reflects differences in experimental procedures. Tanaka *et al.* (1988b) allowed RTT to age for several weeks with ribose, or several months with glucose. They also followed changes in RTT aged with the sugar present in the glycating medium. In the present study, proteins were pre-glycated for just 24 hours, and changes during subsequent sugar-free ageing were followed by X-ray diffraction. As has been discussed in earlier chapters, these differences hold considerable implications for the nature of the Maillard reactions monitored.

Unlike results reported here, Tanaka *et al.* (1988b) detected changes in glycated RTT, but found no changes in native RTT incubated in phosphate buffer without sugar for several months. In the present study, phosphate itself was found to have an effect during incubation. However, glycation conditions employed by Tanaka *et al.* (1988b) (elevated glucose for several months) were unphysiological and likely to result in high levels of modification of collagen at sites which would probably not be glycated *in vivo*. Under such conditions, the effect of phosphate is likely to have been masked by the far greater effect of extended sugar exposure. These authors also reported cross-linking of glycated collagen to occur predominantly in the overlap zone of the D-period. Present results indicate that collagen/collagen and collagen/HSA cross-linking was concentrated at the gap/overlap junctions. This difference may again be due to the intensive glycation conditions used by Tanaka *et al.* (1988b), with collagen modification possibly occurring at sites unlikely to be involved under far milder physiological glycating conditions.

To the author's knowledge, this study represents the first attempt at using X-ray diffraction to study sugar-independent Maillard reactions of pre-glycated proteins, or to investigate Maillard cross-linking between collagen and another protein. The use of X-ray diffraction to investigate changes occurring on collagen as a consequence of glucose glycation under physiological conditions *in vitro* has also not been previously reported. Present results highlight a number of questions which have yet to be addressed adequately in investigations using X-ray diffraction to investigate effects of Maillard reactions on collagen.

- The nature of the interaction between collagen and phosphate buffer (and possibly other anionic buffering species) needs to be clarified if such buffers are to be used in X-ray diffraction studies. A further interesting question is

the extent to which endogenous organic phosphates and phosphorylated metabolic intermediates (Baynes *et al.*, 1989) are able to exert effects on collagen such as those observed here with inorganic phosphate.

- Results reported here suggest that only subtle changes are seen when collagen is glycated *in vitro* under physiological conditions. Physiological concentrations of the glycating species and physiological exposure durations have yet to investigated systematically by biophysical methods *in vitro,* as does sugar-free ageing of glycated protein. Similar concerns have been raised for *in vivo* systems Wess *et al.* (1993, 1996).

- The changes in structural properties (as reflected by changes in X-ray diffraction) associated with specific collagen modifications have not been studied. In particular, specific Maillard products formed by reactive dicarbonyl intermediates such as glyoxal, methylglyoxal and 3-deoxyglucosone have not been investigated by biophysical methods. The importance of imidazolone adducts on collagen has also been highlighted recently (Paul *et al.*, 1998, 1999). The X-ray diffraction characteristics of such adducts are not presently known, therefore it is possible that changes in elctron density distribution which have been interpreted here as indicating cross-link formation, may also indicate the formation of non-cross-linking imidazolones.

Given proposals for 'carbonyl stress' as a major contributor to the complications of both diabetes (Baynes and Thorpe, 1999) and uraemia (Miyata *et al.*, 1999), and given that both conditions occur together in a significant proportion of diabetic patients, the research needs identified in the above paragraphs represent questions

of direct biological relevance. Similar questions have been raised in biochemical studies of Maillard-associated cross-linking, discussed in Chap. 3-5.

6.5 SUMMARY

- Phosphate (and possibly trace metal ions) binds in numerous regions along collagen fibres during prolonged incubation of RTT in phosphate buffer.

- Collagen shows little structural modification immediately following 24 hour glycation, but proceeds to cross-link during subsequent sugar-free ageing in phosphate buffer.

- Glycated collagen forms collagen/collagen cross-links in preference to cross-linking with a different native protein during sugar-free ageing in phosphate buffer.

- Native collagen forms cross-links with glycated and native HSA during sugar-free ageing in phosphate buffer, but cross-links preferentially with glcyated HSA.

- Glycation-associated changes in electron density distribution (indicating cross-link formation) occur predominantly near the gap/overlap junction in the D-period of collagen.

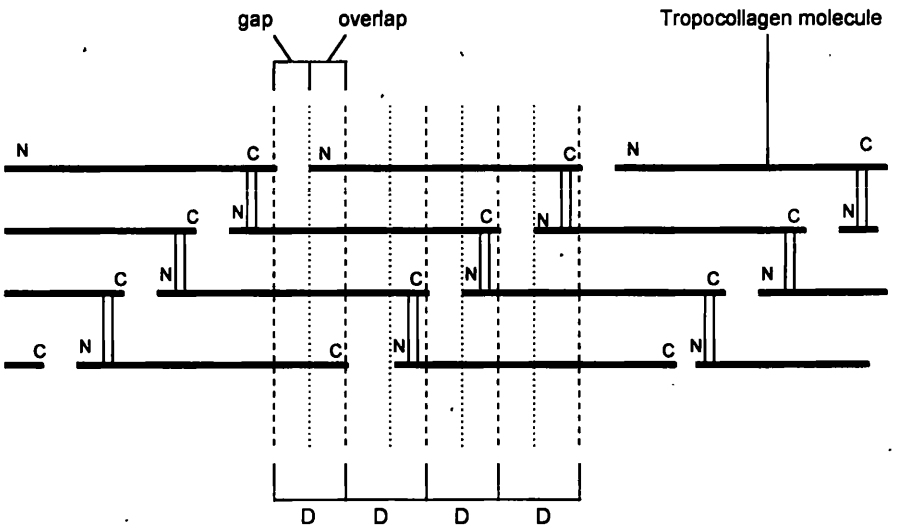


Fig. 6.1

Schematic representation of the arrangement of tropocollagen molecules in Type I collagen fibrils.

Gap	40nm
Tropocollagen	300nm
D-period	67nm / 234 amino acid residues
‖	enzymatic cross-links

It should be noted that the representation of enzymatic cross-links as presented here is limited by the two-dimensional nature of the diagram. While such cross-links are shown above as linking only adjacent molecules with a 1-D stagger, in three-dimensional space such cross-links may extend to molecules two or three D-periods displaced (*i.e.* the molecule next-but-one down or the one below that, in the two-dimensional representation above).

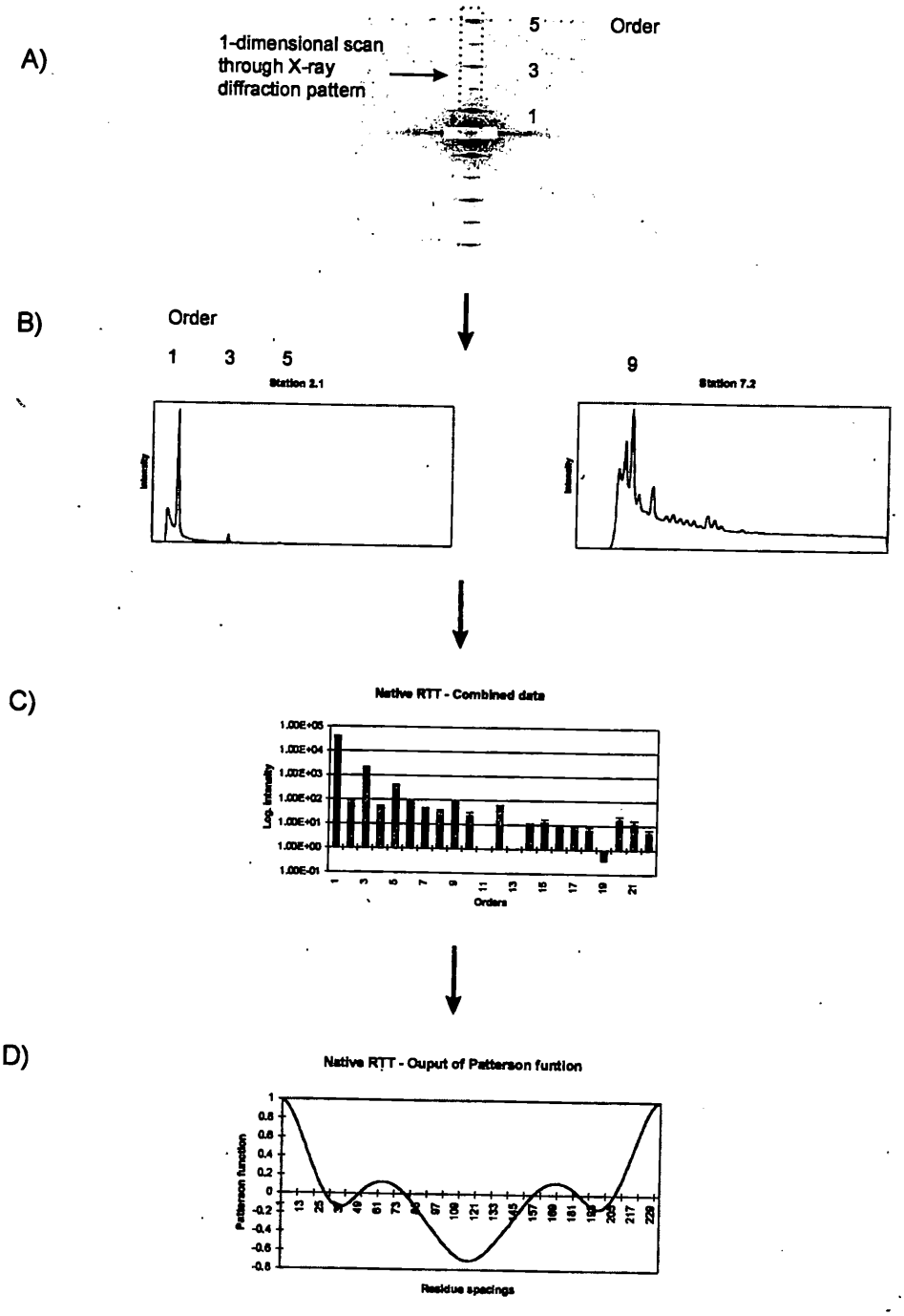


Fig. 6.2

Derivation of the Patterson function from experimental data.

A) X-ray diffraction pattern of native RTT collagen, showing arcs corresponding to meridional reflections. The numbers indicate the orders arising from the 67nm D-periodicity of Type I collagen. The relative intensities of the

reflections are related to the distribution of electron density along one D-period of the collagen fibril axis, and may be used to produce the autocorrelation function of this electron density (the Patterson function), as depicted in B-E.

B) One-dimensional (1-D) scans through diffraction patterns arising from Type I collagen, as recorded on Stations 2.1 and 7.2 at Daresbury SRS, corresponding to the rectangular box marked in A.

C) Peak meridional reflection intensities, extracted from normalised, background corrected, scaled and averaged 1-D scans. These provide the component intensities for the Fourier transform represented by the Patterson function, thereby avoiding the need for phase information on electron density vectors.

D) Output of the Patterson function (Equation 6.3 in text). This is an autocorrelation function which steps the electron density function past itself, one residue position at a time. The autocorrelation function is the sum obtained by taking the electron density at each residue position and multiplying it by a copy of itself. This sum gives the value of the autocorrelation function at position zero on the x-axis. The electron density is then translated by one residue position with respect to its copy (with wrap-around) and the sum is recalculated to give the value of the autocorrelation function at x=1 residue. The process is repeated with a shift of x=2, 3, ..., 234 residues. Overall maxima occur where the function and its copy coincide perfectly, *i.e.* at x=0 and x=234. Because of the wrap-around, the function is symmetrical about (d=234)/2 residues. A local maximum will occur where a peak in the electron density corresponds with a different peak

in the translated copy of electron density. The positions of such maxima therefore indicate the separation of the peaks in the electron density distribution, although they do not show where the peaks actually occur within the D-periodicity.

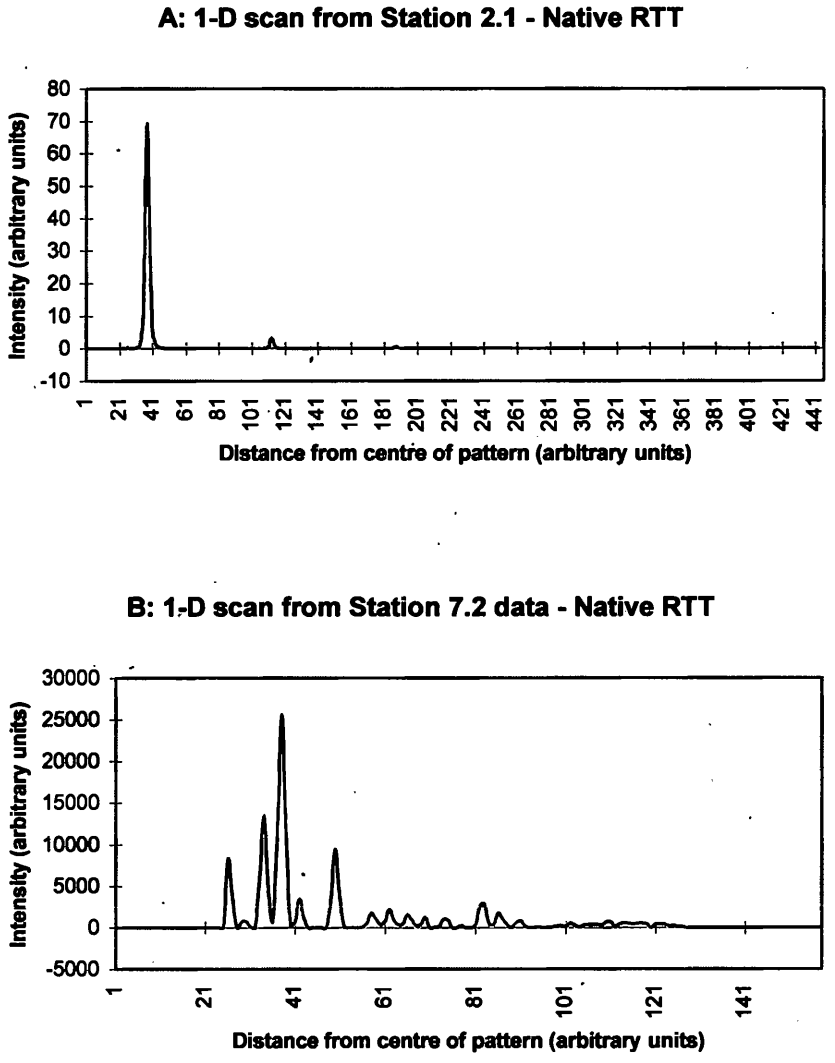


Fig. 6.3

Examples of background-corrected one-dimensional scans through X-ray diffraction patterns of native rat tail tendon (RTT) collagen.

A: Daresbury SRS Station 2.1

B: Daresbury SRS Station 7.2

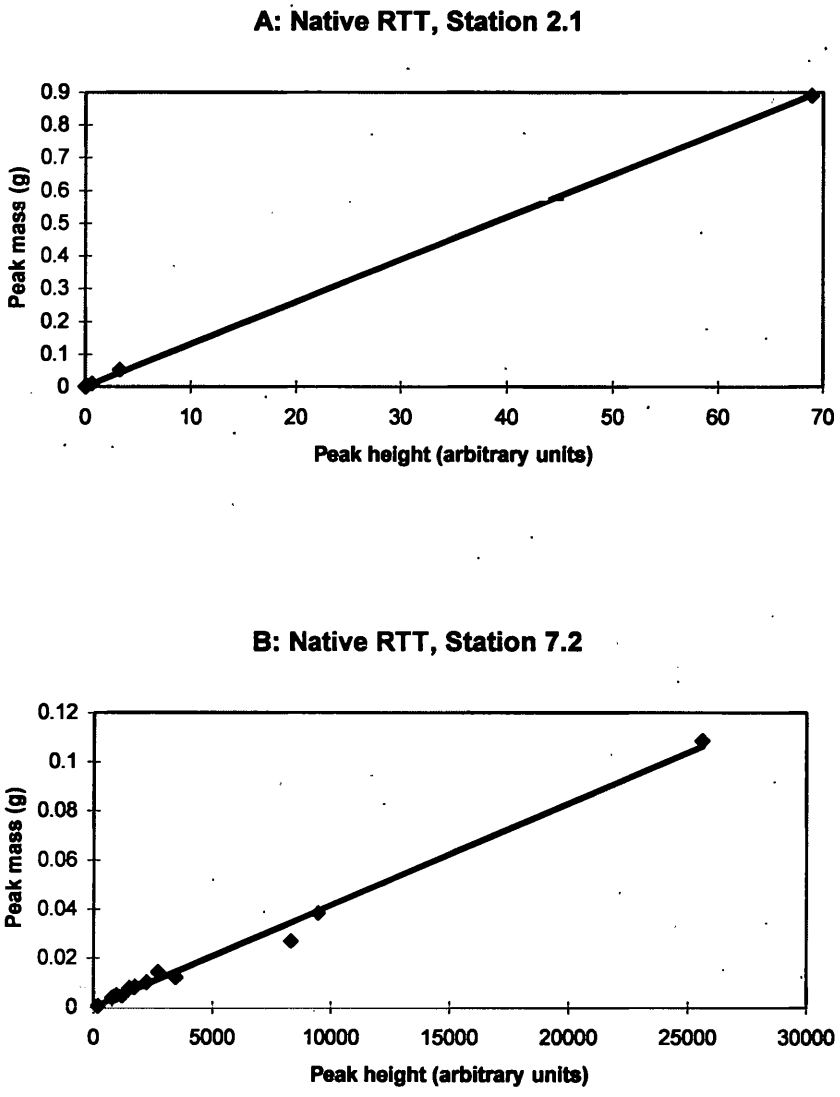
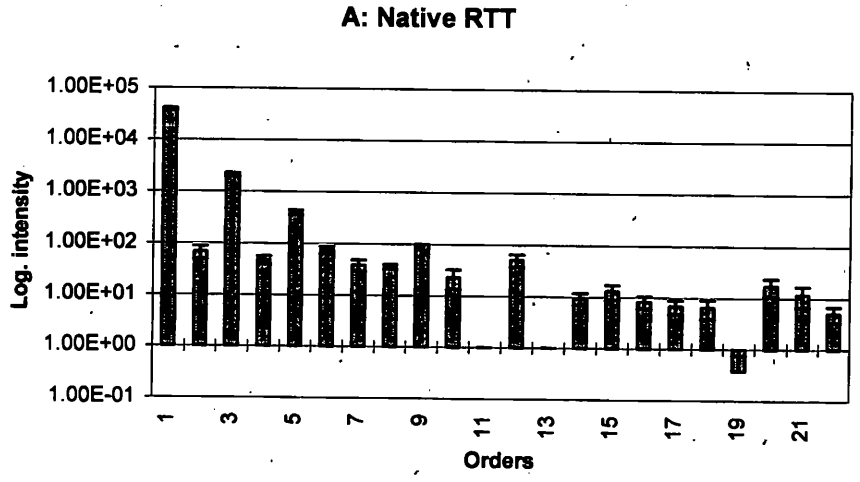


Fig. 6.4

Linear correlations of peak mass (as estimate of peak area) with peak height (measured in arbitrary units from 1-D scans), to determine whether peak area could be estimated by peak height. Both were based on scans obtained for native RTT.

A: Daresbury SRS Station 2.1

B: Daresbury SRS Station 7.2



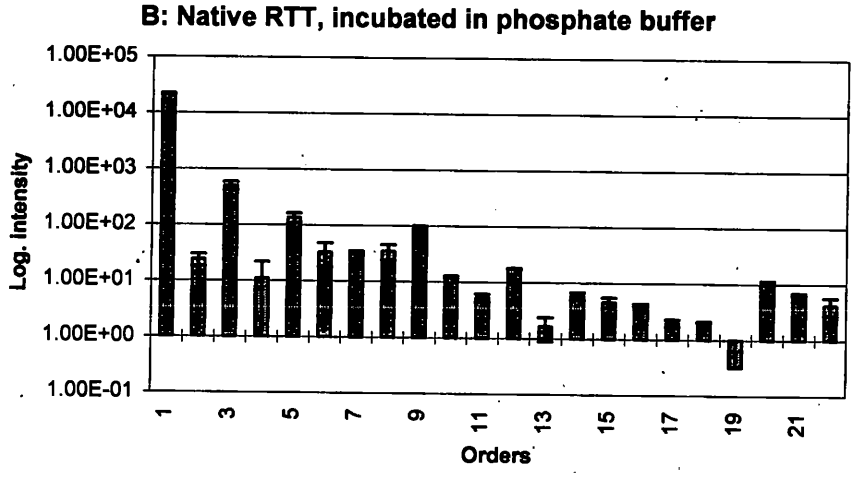
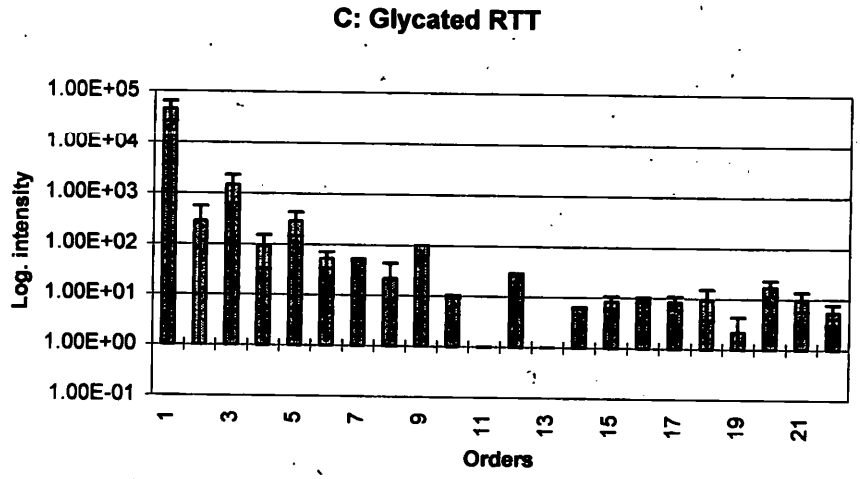


Fig. 6.5

Mean peak intensities of 22 orders of meridional reflections of RTT, variously pre-glycated or co-incubated with pre-glycated HSA, as measured after 3 weeks sugar-free ageing in phosphate buffer. Relevant native RTT controls are also shown.

A: Native RTT, frozen immediately after excision and washing in distilled water

B: Native RTT, aged for 3 weeks in phosphate buffer



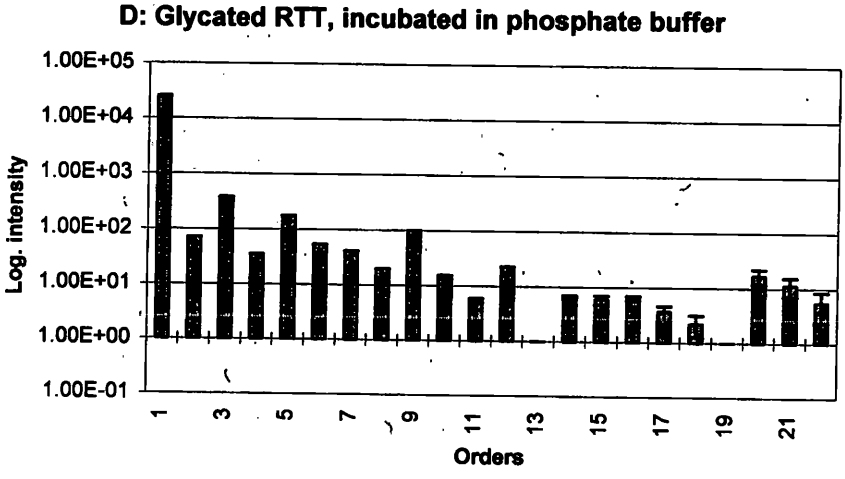
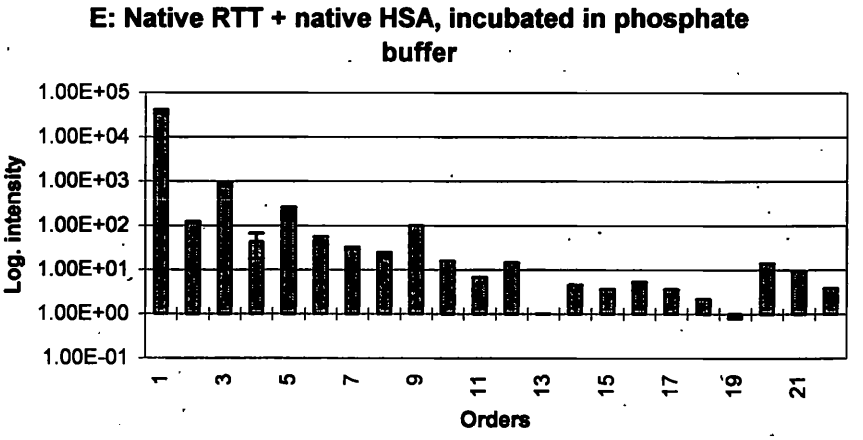


Fig. 6.5 (cont.)

Mean peak intensities of 22 orders of meridional reflections of RTT, variously pre-glycated or co-incubated with pre-glycated HSA, as measured after 3 weeks sugar-free ageing in phosphate buffer. Relevant native RTT controls are also shown.

C: Glycated RTT (0.5M glucose, 24 hours, 37°C), frozen immediately after washing in distilled water

D: Pre-glycated RTT (as above), aged for 3 weeks in phosphate buffer



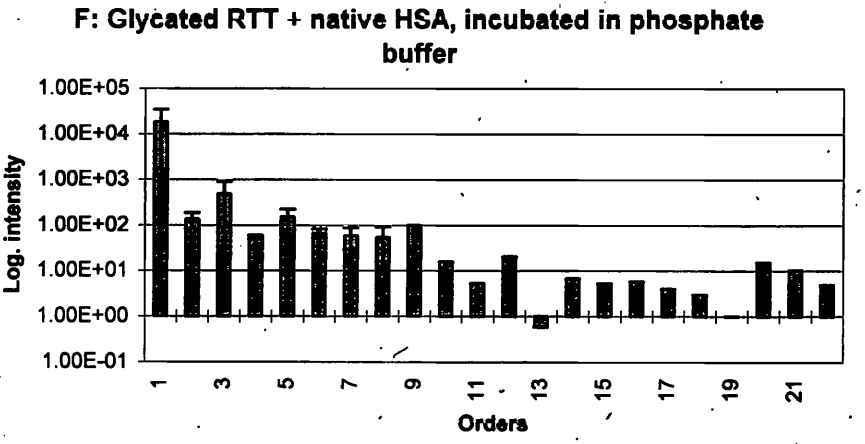


Fig. 6.5 (cont.)

Mean peak intensities of 22 orders of meridional reflections of RTT, variously pre-glycated or co-incubated with pre-glycated HSA, as measured after 3 weeks sugar-free ageing in phosphate buffer. Relevant native RTT controls are also shown.

E: Native RTT and native HSA, aged together for 3 weeks in phosphate buffer

F: Pre-glycated RTT and native HSA, aged together for 3 weeks in phosphate buffer

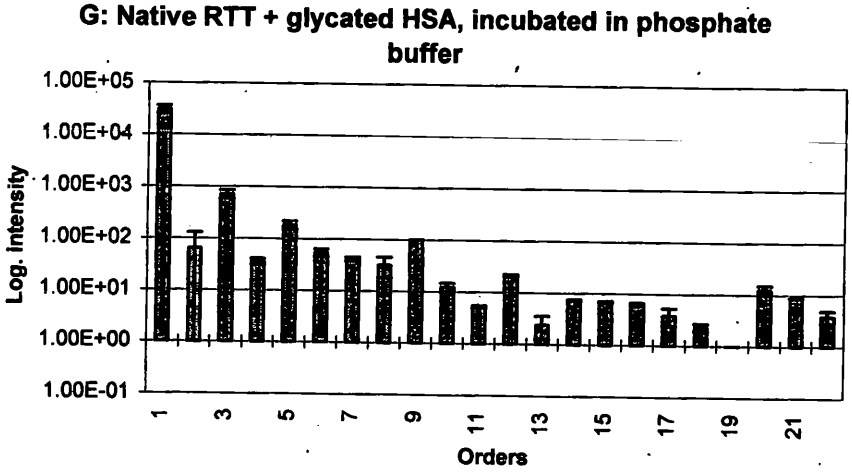
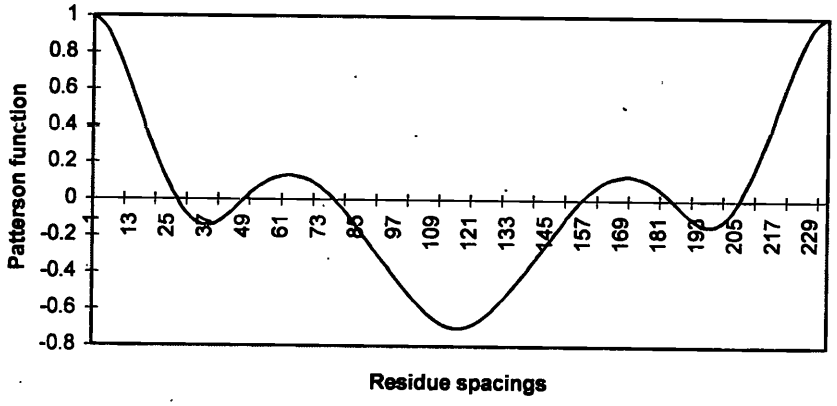


Fig. 6.5 (cont.)

Mean peak intensities of 22 orders of meridional reflections of RTT, variously pre-glycated or co-incubated with pre-glycated HSA, as measured after 3 weeks sugar-free ageing in phosphate buffer. Relevant native RTT controls are also shown.

G: Native RTT and pre-glycated HSA (0.5M glucose, 24 hours, 37°C), aged together for 3 weeks in phosphate buffer

A: Native RTT



B: Native RTT, incubated in phosphate buffer

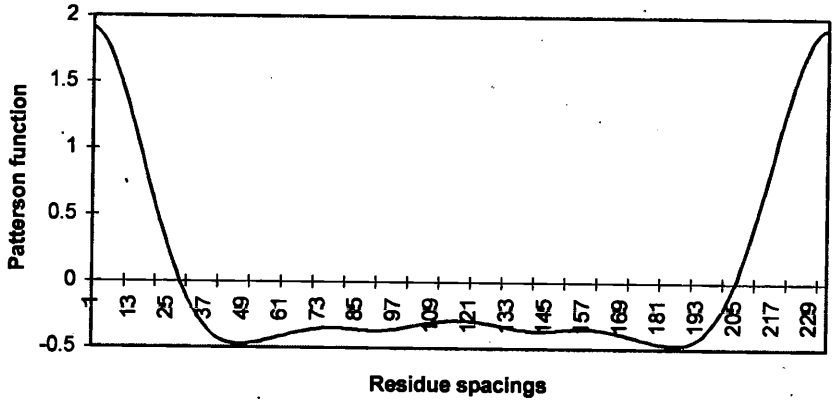
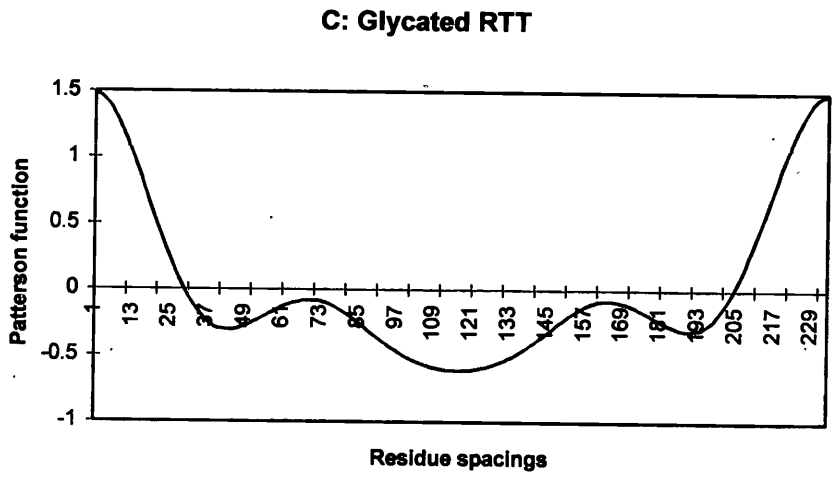


Fig. 6.6

Patterson functions for RTT, variously pre-glycated or co-incubated with pre-glycated HSA, as measured after 3 weeks sugar-free ageing in phosphate buffer. Patterson functions for relevant native RTT controls are also shown.

A: Native RTT, frozen immediately after excision and washing in distilled water

B: Native RTT, aged for 3 weeks in phosphate buffer



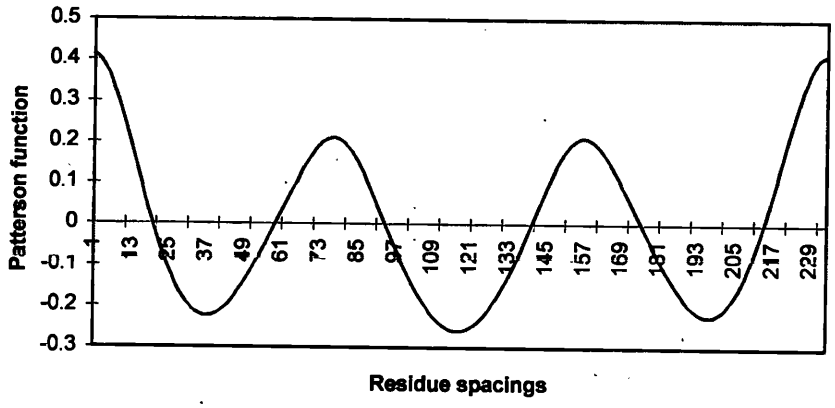
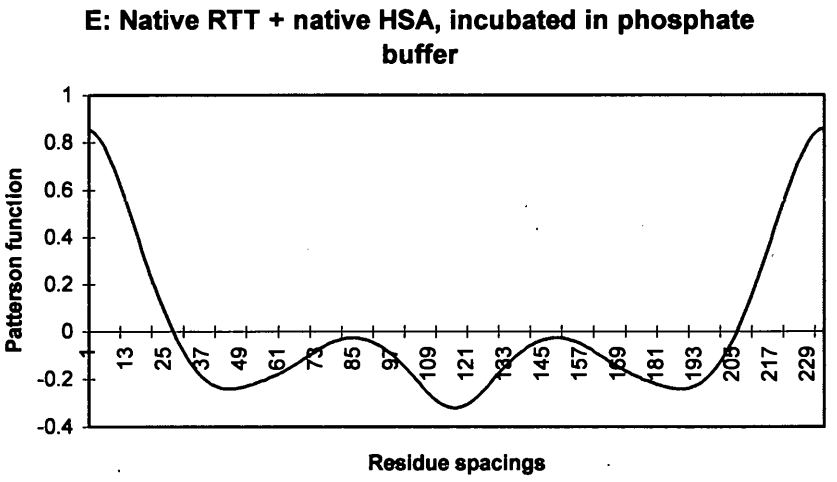


Fig. 6.6 (cont.)

Patterson functions for RTT, variously pre-glycated or co-incubated with pre-glycated HSA, as measured after 3 weeks sugar-free ageing in phosphate buffer. Patterson functions for relevant native RTT controls are also shown.

C: Glycated RTT (0.5M glucose, 24 hours, 37°C), frozen immediately after washing in distilled water

D: Pre-glycated RTT (as above), aged for 3 weeks in phosphate buffer



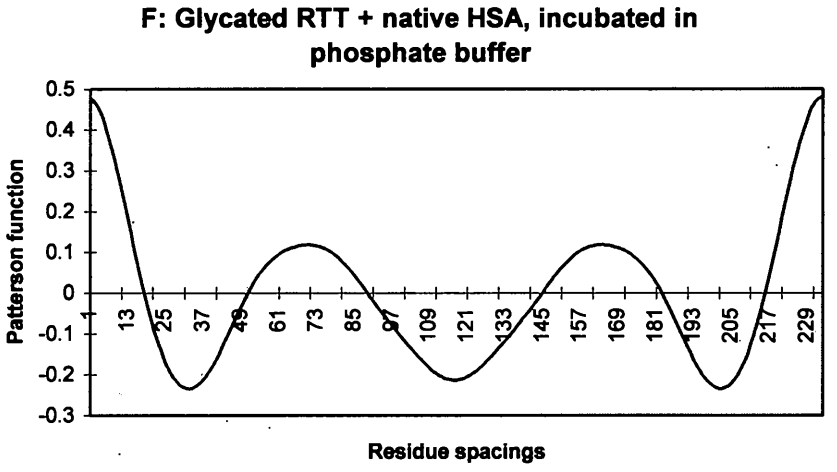


Fig. 6.6 (cont.)

Patterson functions for RTT, variously pre-glycated or co-incubated with pre-glycated HSA, as measured after 3 weeks sugar-free ageing in phosphate buffer. Patterson functions for relevant native RTT controls are also shown.

E: Native RTT and native HSA, aged together for 3 weeks in phosphate buffer

F: Pre-glycated RTT and native HSA, aged together for 3 weeks in phosphate buffer

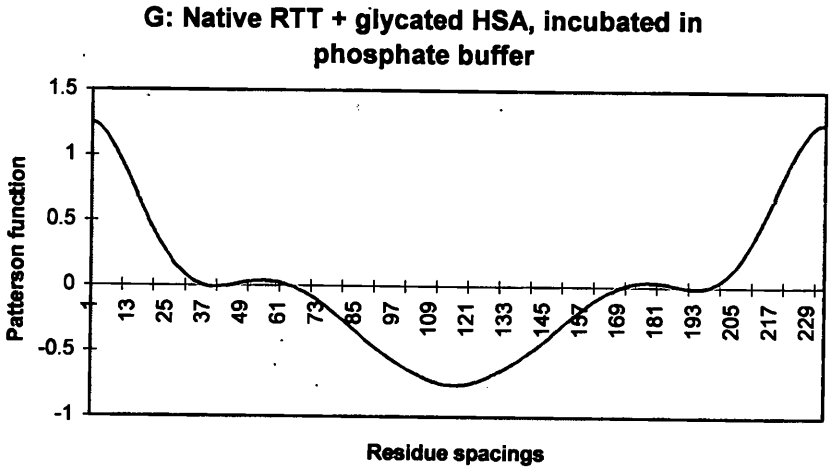


Fig. 6.6 (cont.)

Patterson functions for RTT, variously pre-glycated or co-incubated with pre-glycated HSA, as measured after 3 weeks sugar-free ageing in phosphate buffer. Patterson functions for relevant native RTT controls are also shown.

G: Native RTT and pre-glycated HSA (0.5M glucose, 24 hours, 37°C), aged together for 3 weeks in phosphate buffer

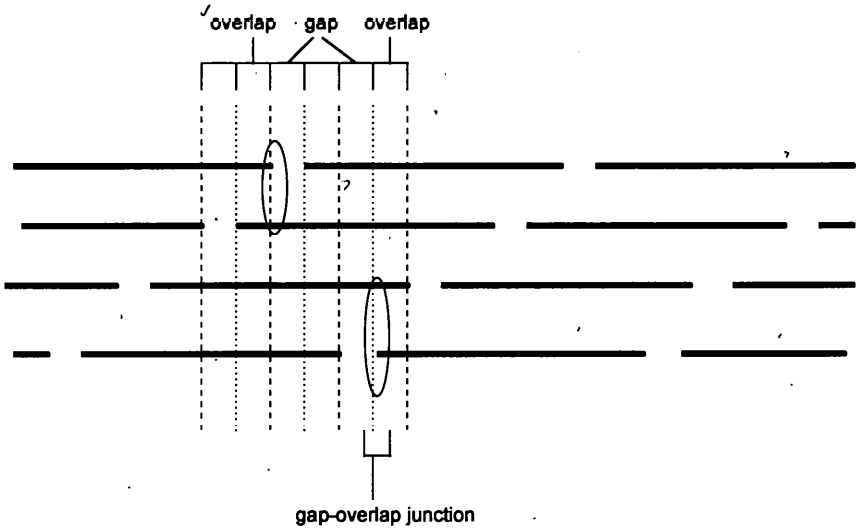


Fig. 6.7

Schematic representation of the location of sites of altered electron density distribution (probably representing new cross-link formation), as detected by X-ray diffraction.

Note that, as described for enzymatic cross-links in Fig. 6.1, the representation of new non-enzymatic cross-links is limited by the two-dimensional nature of the diagram.

CHAPTER 7

DISCUSSION

7.1 INTRODUCTION

This chapter recaps the aims of the present study, and the experimental approaches used to address them. The major findings from all the results chapters are summarised, and are discussed together. Disadvantages and shortcomings of the study are considered. Finally, implications of the results are addressed within the broader context of therapeutic approaches to diabetic complications.

7.2 SUMMARY OF AIMS AND EXPERIMENTAL APPROACH

The study aimed to investigate the fate of proteins briefly pre-treated with glucose during subsequent sugar-free ageing, and to compare this with the fate of native proteins similarly aged in air in the absence of free sugar. The experimental system aimed to model *in vitro* the sugar-independent reactions which might proceed *in vivo* subsequent to brief hyperglycaemic episodes. As such, interactions between glycated protein and a different native protein were considered. The protein exposed to sugar was lipid-free in most instances, in order to ensure that initial glycating events occurred only on protein. The native protein introduced during sugar-free ageing carried an associated lipid fraction, in most instances. The purpose of this was to allow both protein- and lipid-associated reactions to influence the formation of advanced Maillard products, as would be expected to occur *in vivo*.

The effect of aminoguanidine on early sugar-dependent and later sugar-independent reactions was assessed, and was compared to its effect on ageing native protein in the absence of sugar.

7.3 SUMMARY OF MAIN FINDINGS

- The periodate assay, used here to indicate early glycation products (putative Amadori product) detects products of glycation and ageing in addition to Amadori product.

- Both native and glycated proteins formed early glycation products (indicated by PPM) when ageing in phosphate buffer alone at 37°C. This was in addition to PPM formed by glycated proteins as a consequence of their exposure to sugar (Chap. 5). There may also have been slight dimerisation of native proteins during ageing. These changes were greater with glycated proteins, and were linked to the extent of glycation (Chap. 3). Glycated, but not native, proteins also produced fluorescence. This relationship (unlike cross-linking) showed a threshold condition, linked mainly to glycation time (Chap. 3). PPM production also showed a threshold, more strongly linked to glucose concentration (Chap. 5). Cross-linking involving a physiologically relevant protein (HSA) was also demonstrated (Chap. 3).

- When aminoguanidine was present together with sugar (Chap. 4, protocol i, glycated protein), it had an immediate effect in reducing fluorescence (week 0). This is consistent with its published role as an inhibitor of advanced Maillard product formation.

The inhibitory effect on fluorescence was maintained throughout 3 weeks of ageing, with formation of PPM and heterodimer also being reduced. Thus glycating in the presence of aminoguanidine reduced Maillard product formation during subsequent ageing, even though free aminoguanidine (and sugar) had been removed by dialysis.

- Native proteins that had been briefly pre-treated with aminoguanidine (Chap. 4, protocol i, native protein), also retained an effect of aminoguanidine during subsequent ageing. However, this differed markedly from the effect of aminoguanidine on glycated protein. Native proteins formed *more* PPM and *more* lipid-associated dimers than proteins ageing in the absence of aminoguanidine, even through free aminoguanidine had been removed by dialysis before ageing. Aminoguanidine pre-treatment appeared to have no effect on fluorescence, nor on cross-linking of lipid-free protein.

- When aminoguanidine was added to the ageing buffer (Chap. 4, protocol ii) there were distinct changes. These were similar for both native and glycated proteins, but again contrasted with the effect of aminoguanidine present together with sugar. There was little apparent effect on PPM (although this may be misleading, as discussed in Chap. 5). More significantly, aminoguanidine *enhanced* both fluorescence and cross-linking. The enhanced cross-linking was more marked where lipid-associated proteins were involved.

- Cross-linking of glycated collagen during sugar-free ageing was demonstrated with X-ray diffraction. Glycation-associated changes in electron density distribution (indicating cross-link formation) occurred

predominantly near the gap/overlap junction in the D-period of collagen (Chap. 6).

- Glycated collagen formed collagen/collagen cross-links in preference to cross-linking with native HSA during sugar-free ageing, although some mixed cross-linking did occur. Native collagen formed cross-links with glycated HSA during sugar-free ageing (Chap. 6).

- Interpretation of X-ray diffraction results was complicated by phosphate buffer binding to collagen (Chap. 6).

7.4 EVALUATION OF THE PERIODATE ASSAY AS A TOOL FOR STUDYING MAILLARD REACTIONS

The periodate assay, originally thought to detect only Amadori product (Gallop *et al.*, 1981; Furth, 1988; Ahmed and Furth, 1991), was shown to detect a broader pool of early Maillard products, possibly including dicarbonyl compounds. Aldehyde products of lipid oxidation such as MDA did not appear to interfere in the assay. Compounds yielding the same reaction with periodate as did early glycation products were also generated from both glycated and native protein during sugar-free ageing. Glycated protein yielded more of these compounds during ageing than did native proteins.

Formation of glycation-derived PPM appeared to be associated with a threshold, as was observed for fluorescent Maillard products, which may indicate oxidative formation of PPM. This argues against the detected end-point comprising

predominantly Amadori product, since this has been shown to be formed without a requirement for oxidation (Fu *et al.*, 1992, 1994). However, unlike fluorescent products, the threshold for PPM formation was more strongly linked with glucose concentration than with length of the glycating exposure. It could therefore represent a need for accumulation of precursors (possibly Schiff base), rather than a requirement for oxidation, in the formation of PPM.

PPM on glycated protein was initially protein-bound, but became susceptible to removal by dialysis during sugar-free ageing. It was also blocked by reaction with aminoguanidine.

The cumulative evidence from the periodate assay (summarised above) is consistent with three components contributing to PPM:

(i) protein-bound products (including Amadori product) formed during early glycation and persisting through dialysis immediately after glycation,

(ii) subsequent degradation products released from early glycation products as free dialysable intermediates (susceptible to removal during second dialysis),

(iii) further intermediates produced from glycated proteins, and from native proteins and lipoproteins, during sugar-free ageing.

The last two components may include smaller sugars (pentoses, tetroses) and dicarbonyl compounds such as glyoxal, methylglyoxal or 3-deoxyglucosone (Zyzack *et al.*, 1995; Thornalley *et al.*, 1999).

The apparently anomalous reaction of dicarbonyl compounds in the periodate assay may be explained by direct complexing of small dicarbonyls with the detection reagent (diacetyldihydrolutidine). This reaction would be sterically hindered by comparison with the reaction between diacetyldihydrolutidine and formaldehyde

(originating from periodate cleavage of Amadori product), which would explain the lesser sensitivity of the assay to dicarbonyl compounds.

Some investigators may be tempted to argue that simplistic indicators such as the periodate assay have lost their relevance in the light of sophisticated analytical techniques which allow identification of specific Maillard intermediates and products. However, not all laboratories have access to the necessary equipment. Furthermore, it is important to bear in mind that concentrating on specific well-recognised compounds may result in other, as yet unrecognised, intermediates and pathways being overlooked. Thus, assays providing a broad overview of probable Maillard intermediates (whether the periodate assay as used here, or others) still have a place.

7.5 EVALUATION OF X-RAY DIFFRACTION AS A TOOL FOR STUDYING MAILLARD REACTIONS

X-ray diffraction successfully demonstrated changes in electron density distribution along the axis of the collagen fibre (detected as changes in meridional reflections) associated with late Maillard product formation during sugar-free ageing of relatively mildly glycated protein. Furthermore, X-ray diffraction proved suitable for detecting enhanced cross-linking of native collagen to glycated HSA. It also allowed comparison of cross-linking during co-incubation of glycated collagen and native HSA with that during co-incubation of native collagen and glycated HSA. Previous X-ray diffraction studies on *in vitro* glycated collagen have concentrated on heavily AGE-modified material and have not considered the post-Amadori fate of glycated collagen in isolation (Tanaka *et al.*, 1998b). Cross-linking reactions between

collagen and a serum protein have not been previously investigated by this technique. However, reports of biochemical analyses of collagen co-incubated with heavily AGE-modified BSA have indicated enhanced cross-linking of collagen in mixed protein systems during sugar-free ageing (Sajithlal *et al.*, 1998).

Interpretation of changes in X-ray diffraction were complicated by the binding of components of phosphate buffer to collagen. This effect proved a significant interference in the present study because structural modifications occurring during sugar-free ageing of relatively mildly glycated protein were subtle. It has not been previously reported, except by Hadley (1999), because protein modifications (and hence changes in X-ray diffraction) associated with physiological glycating conditions are likely to be very slight compared to the changes in heavily glycated protein, as examined in previous investigations (Tanaka *et al.*, 1988b). Thus buffer effects are likely to have been overlooked as insignificant in previous reports.

7.6 INSIGHTS INTO THE MAILLARD REACTION AND ITS INHIBITION

7.6.1 Formation of mixed dimers

Proteins briefly glycated with glucose formed both cross-linked and fluorescent Maillard products during subsequent sugar-free ageing. Cross-linking was shown to occur between glycated protein and a different native protein. Cross-linking during sugar-free ageing of a single pre-glycated protein has been previously demonstrated by Eble *et al.* (1983) and McPherson *et al.* (1988). Heterodimerisation, as reported here, has been previously demonstrated with fructose (Liggins and Furth, 1996), but not with glucose as the glycating sugar. The

formation of mixed cross-linked products was shown here both with model proteins (lysozyme, β-lactoglobulin) and with a lipoprotein of physiological relevance (human serum albumin). Glycation-mediated cross-linking of HSA has been previously reported (Sakurai *et al.*, 1984), but cross-linking of HSA to another protein as a consequence of glycation of either protein has not. Sajithlal *et al.* (1998) demonstrated that heavily AGE-modified BSA promotes cross-linking of native collagen, but did not investigate whether BSA formed mixed cross-linked products with collagen. Present results showing glycation-mediated HSA/lysozyme cross-linking indicates that the processes demonstrated here with model proteins have the potential to play a role *in vivo*.

The probable physiological relevance of mixed protein cross-linking is further supported by X-ray diffraction results in the present investigation. These indicated that pre-glycated HSA undergoes enhanced cross-linking with native collagen, while pre-glycated collagen preferentially forms collagen/collagen cross-links. Such findings suggest that glycation of circulating serum protein and subsequent sugar-independent cross-linking to native collagen may indeed provide a mechanism for entrapment of serum proteins by structural collagens (Miller and Michael, 1976; Michael and Brown, 1981; Brownlee *et al.*, 1986a; Cohen, 1996).

Cross-linking between glycated protein and native lipoprotein occurred even after extremely mild glycating exposure. Under the more intensive of the glycating conditions investigated, cross-linking could be detected within one week of glycation. This runs counter to scepticism of some workers that Maillard end-points can be effectively demonstrated using glucose as the glycating sugar (Khalifah *et al.*, 1996).

7.6.2 Thresholds for formation of late Maillard products

Formation of cross-linked Maillard products from sugar-free co-incubations of pre-glycated lipid-free protein with native lipoprotein showed no evidence of a threshold for cross-linking. Formation of fluorescent Maillard products under the same conditions did show a threshold, both in terms of glucose concentration and duration of glycation. Of these factors, glycating exposure duration was the more influential. This suggests that a threshold for fluorophore formation may involve oxidation, probably of the lipid fraction of the native lipoprotein. PPM formation also showed a threshold effect, but this appeared to be linked more strongly with glucose concentration than with the duration of glycation.

7.6.3 Identity of protein-bound late Maillard products

No chemical characterisation of cross-linked or fluorescent Maillard products was conducted in this study. However, numerous recent reports on the importance of dicarbonyl glycation intermediates, added to older literature, have lead to identification of a number of structures of cross-linked and fluorescent Maillard products. Dicarbonyl and aldehyde products of lipid oxidation can contribute to formation of similar products. Intermediates, mechanisms and Maillard structures have been reviewed in Chap. 1. This allows some speculation on the identity of the products observed here, and on intermediates in their formation.

Cross-linked and fluorescent Maillard products detected in the present investigation were formed during sugar-free ageing of pre-glycated proteins. Precursors to these products survived prolonged dialysis, indicating that they were at least initially

protein-bound. This was supported by PPM results. It appears likely that free low molecular weight protein- and lipid-derived intermediates arose from early protein-bound glycation products over time. This, too, was supported by PPM results and by reports in the literature (Zyzack *et al*, 1995; Brinkman Frye *et al*, 1998; Thornalley *et al.*, 1999). These considerations exclude a role for autoxidation products of free sugar in the formation of the products observed here, but allow for a variety of mechanisms occurring via free or protein-bound dicarbonyls (Glomb and Monnier, 1995; Degenhardt *et al.*, 1998a, b; Sajithlal *et al.*, 1998), and via products arising from glycation-promoted lipid oxidation (Menzel *et al.*, 1997)

Degenhardt *et al.* (1998a) argue that CML, CEL, GOLD and MOLD (the last two representing protein cross-links) probably represent the majority of AGEs in tissue proteins, with MOLD and GOLD being present at levels 10-50 times higher than pentosidine. Thus the cross-links formed as a consequence of glucose exposure in the present study are likely to comprise predominantly GOLD and MOLD, with possible contributions from DOLD, pentosidine and MDA-mediated structures. Lederer *et al.* (1998) have demonstrated the potential for methylglyoxal-derived cross-linking between arginine and lysine residues, which may also be represented in the cross-linked products observed here. Intermediates implicated in their formation are glyoxal, methylglyoxal, 3-deoxyglucosone and MDA generated from glycated protein and lipoprotein. Fluorescence is likely to have originated from pentosidine, argpyrimidine and some imidazolones (Wells-Knecht *et al.*, 1996; Glomb and Nagaraj, 1998). Pentose for the formation of pentosidine can originate by decomposition of Amadori product on glycated protein (Zyzack *et al.*, 1995). Methylglyoxal and 3-deoxyglucosone for formation of imidazolones and argpyrimidine may be derived from either glycated protein or lipid.

7.6.4 Effects of aminoguanidine

When aminoguanidine was present together with glucose, it demonstrated its established function of scavenging early glycation intermediates and inhibiting the formation of later Maillard products. Evidence both from early glycation products (PPM) and from advanced Maillard end-points (fluorescence, cross-linking) suggests that its inhibitory properties are preserved in some form for some time after removal of the free compound (and of free sugar). Inhibition of late Maillard reactions by brief aminoguanidine pre-treatment has not been previously reported. The possibility of a reversible association between aminoguanidine and lipid oxidation products was suggested by Scaccini *et al.* (1994), when discussing interference of aminoguanidine in assays of lipid oxidation. More significantly, aminoguanidine pre-treatment has been reported to have an anti-oxidant effect during subsequent aminoguanidine-free incubation. Giardino *et al.* (1998) pre-treated rat retinal Mueller cells in culture with aminoguanidine for 48 hours and observed subsequent reductions in hydrogen peroxide-induced apoptosis, lipid peroxidation and generation of reactive oxygen species. The effect was dose-dependent, and did not increase further following pre-treatment durations longer than 48 hours. While the experimental system and end-points differ markedly from those employed here, the persistence of an aminoguanidine-mediated effect is sufficiently similar for it to be likely that a similar mechanism facilitated persisting inhibition of Maillard cross-linking and fluorescence after removal of aminoguanidine, as observed in the present study.

The persistence of an aminoguanidine-mediated effect after dialysis held even for native proteins briefly exposed to it. In this case, however, aminoguanidine boosted

PPM slightly (but not significantly), and promoted cross-linking of lipid-associated protein. Unexpected effects were also observed when aminoguanidine (up to 25mM) was present during ageing of either native or glycated proteins in the absence of free sugar. In this instance, it reduced PPM, but promoted fluorescent and cross-linked products - particularly with lipid-associated protein. These effects appear at odds with the inhibitory effects observed when aminoguanidine was present together with sugar.

A search of the literature for reasons underlying these apparently conflicting results showed that at least four activities have been consistently suggested to explain the effects observed with aminoguanidine:

(i) binding to monocarbonyl groups on glycated proteins, specifically the monocarbonyl of Amadori product (Brownlee *et al.*, 1986b), although this mechanism has recently been discounted as a minor pathway relative to those listed below

(ii) scavenging of dicarbonyl intermediates of the Maillard reaction, or of lipid oxidation (Hirsch *et al.*, 1991; Picard *et al.*, 1992; Chen and Cerami, 1993; Hirsch *et al.*, 1995a)

(iii) classical anti-oxidant activity by preventing further reaction of free radicals originating from lipid oxidation (Picard *et al.*, 1992; Philis-Tsimikas *et al.*, 1995; Giardino *et al.*, 1998)

(iv) promotion of oxidation through generation of free radicals from lipoproteins (Philis-Tsimikas *et al.*, 1995) and through increased dicarbonyl release from glycated protein (Skamarauskas *et al.*, 1996).

The following paragraphs consider which of these best explain the results reported here, and hence which processes may have predominated in the experimental system used in the present study.

Fluorescence and cross-linking associated with reactions of glycated protein were inhibited by aminoguanidine present together with glucose. This inhibition can reasonably be attributed to scavenging of free dicarbonyls arising from early glycation products (ii above). Anti-oxidant activity (iii above) may also have contributed to inhibition by aminoguanidine which survived dialysis and was thereby able to interact with lipoprotein introduced at the start of sugar-free ageing. However, dicarbonyl scavenging is likely to have been dominant inhibitory mechanism.

By contrast, fluorescence and cross-linking were enhanced if aminoguanidine was present without free sugar. It remains to suggest a mechanism whereby aminoguanidine could *promote* the end-points it is widely considered to inhibit. Neither (ii) nor (iii) above provide plausible explanations. A number of possible alternative routes are listed below. These are based either on reactions of aminoguanidine with monocarbonyls on protein or lipid (i above), or on the pro-oxidant properties of aminoguanidine (iv above).

It is postulated that aminoguanidine may have promoted cross-linking and fluorescence in the present experimental system by:

(i) the action of aminoguanidine-promoted dicarbonyl intermediates or free radicals via the recognised pro-oxidant activity of aminoguanidine (Philis-Tsimikas *et al.*, 1995; Skamarauskas *et al.*, 1996),

(ii) formation of di-Schiff bases, formed by reaction with monocarbonyls on different protein/lipoprotein molecules through both ends of one aminoguanidine molecule (cf Fig. 4.10), or

(iii) formation of aminoguanidine-derived tetrazine cross-links by dimerisation of two aminoguanidine moieties bound through monocarbonyls on different

protein/lipoprotein molecules (Kramer *et al.*, 1984; Schelenz *et al.*, 1984; Stein *et al.*, 1985) (cf Fig. 4.11).

Since the tetrazine structure formed by dimerisation of aminoguanidine is likely to be fluorescent, the last possibility would explain the increase in both fluorescence and cross-linking observed with aminoguanidine. Formation of di-Schiff bases or tetrazine intermediates would also explain how aminoguanidine appears to survive dialysis in protein-bound form. Aminoguanidine trapped in a cross-link structure would be unavailable for further reaction, either through carbonyl scavenging or through free radical reactions.

It is worth noting that in either of the last two cases mentioned above, the products formed by aminoguanidine would be independent of Maillard processes except insofar as the monocarbonyls through which aminoguanidine binds protein (or protein-associated lipid) may arise through glycation or through glycation-promoted lipid oxidation.

7.7 DISADVANTAGES OF THE EXPERIMENTAL SYSTEM AND QUESTIONS RAISED

A number of questions arising from the present study could be clarified by additional investigation:

- Both the model proteins used (lysozyme and β-lactoglobulin) may have a tendency to self-aggregate (Aymard *et al.*, 1996; Belotti *et al.*, 1999). Lysozyme has also been demonstrated to have a high affinity for AGEs (Li

et al., 1995; Mitsuhashi *et al.*, 1997). Hence, this experimental system may be over-sensitive to the cross-linking potential of glycated protein and aminoguanidine. Despite its drawbacks, lysozyme has been utilised as a model protein in a number of published Maillard studies (Dyer *et al.*, 1991a; Prabhakaram and Ortwerth, 1994; Niwa *et al.*, 1995; Hayase *et al.*, 1996). Nonetheless, it would be desirable to establish the reproducibility of the effects noted with a number of different proteins.

- The mixture of lipid-free and lipid-associated proteins utilised in the model system was chosen as being more physiologically representative than one incorporating only a single lipid-free protein. However, it was difficult to determine to what extent the end-points observed were attributable to reactions on protein, to those on lipid, or to an interaction of the two. It would be beneficial to have results from a two-protein lipid-free system for comparison of the relative contributions of protein and lipid.

- Physiologically, glucose is the most prevalent glycating sugar and was therefore an obvious choice for glycation studies. Furthermore, by using glucose in preference to one of the reported intermediates in the Maillard reaction (*e.g.* glyoxal, methylglyoxal or 3-deoxyglucosone), more of the pathways leading to total fluorescent and cross-linked Maillard products were likely to be represented in the experimental system. However, using glucose as the glycating species carried the disadvantage of low reactivity relative to other sugars or intermediates, consequently of longer incubation times and lower yields of Maillard products. These factors complicated interpretation of the relatively non-specific end-points measured. It would be useful to conduct the same studies with a faster reacting sugar, such as fructose, or with a number of proposed glycation intermediates, in addition to

glucose.

- Uncertainty surrounding the chemical identity and origin of early glycation products detected by the periodate assay proved a limitation in data interpretation. Previous thinking regarding the nature of glycation products detected by the assay was clearly insufficient to explain the results obtained. Elucidation of the chemistry of this assay would therefore be a priority if it were to be used in a similar capacity in future. Among the questions which need to be addressed are:

  (i) Which glycation products besides Amadori product form PPM, and how do these compare with Amadori product in their reactivity in the periodate assay? (This was addressed to some extent, but needs to be investigated more comprehensively.)

  (ii) Is PPM a valid marker for early glycation products, or do late Maillard products also form PPM?

  (iii) What are the products of ageing/oxidation which form PPM, and how do these relate to Maillard products or intermediates detected by the assay?

  (iv) Can the periodate assay help identify permanent changes to protein structure that remain after dialysis and which are of physiological relevance?

- Similarly, elucidation of the chemical nature of the cross-linked or fluorescent products promoted by aminoguanidine would be a priority for future work. Clarity regarding the identity of these products and the mechanism of their formation is required before their physiological relevance can be evaluated.

- The use of X-ray diffraction to investigate subtle effects on collagen resulting from physiological glycating conditions was hampered by binding of phosphate buffer to collagen. This effect needs to be characterised.

- Better replication of the experimental data would have reduced variability and allowed for greater confidence in judging the significance of differences between treatments. This was precluded by time constraints in the present investigation, but should be a priority of any similar work.

A definite outcome of results presented here is the need for a better understanding of the complex interactions which aminoguanidine undergoes in systems incorporating glycated and ageing proteins under fluctuating sugar concentrations and in the presence of lipid. Studies conducted in the presence of persistently elevated sugar concentrations - either *in vitro* or *in vivo* - cannot be considered as adequate for judging the suitability of aminoguanidine as an inhibitor of Maillard processes if there is significant risk of protein damage when concentrations of free sugar are low.

7.8 IMPLICATIONS FOR THE THERAPEUTIC USE OF AMINOGUANIDINE IN DIABETES

Assuming the results with the present *in vitro* model system are of relevance in living systems, they hold a number of implications for the prophylactic use of aminoguanidine to prevent or ameliorate diabetic complications.

The inhibitory action of aminoguanidine was observed only when the inhibitor was present together with free sugar. This suggests that the use of aminoguanidine to prevent the development of diabetic complications would require careful timing of drug administration. It would be necessary to give aminoguanidine during the early stages of glycating reactions, *i.e.* during hyperglycaemic events, and to discontinue therapy as soon as blood glucose levels drop to near normal. Widespread use as a prophylactic agent in diabetics with good and possibly even moderate blood glucose control appears to be undesirable since aminoguanidine may cause unwanted reactions with existing glycated or oxidised proteins. Even in poorly controlled diabetes, continuous use of aminoguanidine may be questionable, since diurnal blood glucose fluctuations would result in low glucose concentration at least some of the time. This could enhance existing protein damage. In brief, present results point to the desirability of ensuring that maximum glucose concentrations coincide with maximum aminoguanidine concentrations, in order to minimise the possibility of undesirable reactions promoted by aminoguanidine.

Aminoguanidine appeared to show some residual effect after removal of the free compound in the *in vitro* system used in this investigation. This casts some doubt on whether long-term continuous therapy would be necessary to ameliorate the effect of hyperglycaemic events. Support for such suggestions arises from preliminary results from clinical trials of aminoguanidine, which indicated that beneficial effects were maintained when continuous aminoguanidine therapy was discontinued after two years (Brownlee, 1999).

Undesirable reactions of aminoguanidine were observed even at glucose/ aminoguanidine ratios representative of those which may be expected *in vivo*. These reactions were observed in the present model system when glucose and aminoguanidine were not present simultaneously. It could be argued that it is

unlikely for aminoguanidine to be present *in vivo* without the simultaneous presence of sugar, and therefore that the observed effects are not of physiological relevance. It is difficult to determine from *in vitro* evidence to what extent the reported results apply to physiological combinations of aminoguanidine and low glucose concentration (as occur intermittently in insulin-controlled diabetes). It is, however, clear that there is a complex web of glycation-associated and glycation-independent changes associated with aminoguanidine in a mixed protein/lipoprotein system *in vitro*. Considering the far greater number of variables in living systems, there may be no easy way of predicting when the balance of aminoguanidine-associated reactions *in vivo* would fall in favour of glycation-associated inhibition of cross-linking, and when in favour of glycation-independent promotion of cross-linking. This is likely to vary among individuals, as does susceptibility to diabetic complications. Thus the long-term effects of aminoguanidine may depend on factors such as susceptibility to oxidative stress (or carbonyl stress), or efficiency of protein turnover. The time scale for the development of potentially adverse effects associated with aminoguanidine may be on a scale comparable to that for the development of diabetic complications, *i.e.* beyond that considered in studies reported to date. Only long-term follow-up of aminoguanidine therapy in living models showing the blood glucose fluctuations typical of diabetics under insulin (or other) therapy can provide answers to these questions.

It may be worth noting that the situation may differ for uraemic patients, with or without diabetes. It has been postulated that the complications of both diabetes and uraemia are the consequence of an underlying state of 'carbonyl stress' (Baynes and Thorpe, 1999; Miyata *et al.*, 1999). In uraemia, this is thought to arise from increased oxidation of carbohydrates and lipids, and from impairment of the detoxification systems for the dicarbonyl products thereof (Miyata *et al.*, 1999). Evidence for this derives from elevated levels of MOLD and GOLD (Odani *et al.*,

1998), and of imidazolone adducts (Niwa *et al.*, 1997c) in uraemic patients. Degenhardt *et al.* (1998b) identified CML, CEL, GOLD and MOLD as major tissue biomarkers of the Maillard reaction, and reported that all four are elevated in plasma proteins from uraemic patients. In diabetes, carbonyl stress arises from an overloading of detoxification systems for dicarbonyl products of sugar metabolism and the Maillard reaction, both of which are increased as a consequence of hyperglycaemia. Baynes and Thorpe (1999) argue that the latter circumstances result in raised baseline levels of dicarbonyl compounds in diabetes. It remains to be established whether consistently elevated levels of dicarbonyl intermediates occur in both conditions, or whether dicarbonyl levels in diabetes show fluctuations linked to fluctuations in blood glucose. The difference is significant in terms of the results and postulated mechanisms presented in this study. Consistent elevations of dicarbonyl compounds would ensure an uninterrupted supply of reactive compounds for reaction with aminoguanidine. Under these conditions, pro-oxidative reactions of aminoguanidine or reactions with less reactive monocarbonyl compounds would be unlikely to occur. If dicarbonyl levels were to fluctuate, then a greater possibility would exist that undesirable reactions of aminoguanidine may occur at times when dicarbonyl levels were low. This could potentially result in the accumulation of aminoguanidine-based modifications on proteins and lipids over long periods of time (similar to the accumulation of Maillard products themselves).

Viewed in this context, diabetic patients with renal impairment may be a poor model in which to test aminoguanidine. Such patients combine the carbonyl stress of hyperglycaemia (overloaded detoxification systems) with that of uraemia (impaired detoxification systems). Thus not only are dicarbonyl levels chronically elevated, they are also further boosted in response to hyperglycaemic excursions. Under these conditions it is highly unlikely that aminoguanidine would display the sort of reactions which the present study indicates might, in the long-term, lead to

accumulation of undesirable protein- and lipid-bound products. This suggestion is supported by a recent study in which aminoguanidine was found to ameliorate albuminuria in diabetic rats without inhibiting increases in pentosidine and CML in skin collagen (Degenhardt *et al.*, 1999). The authors concluded that therapeutic effects of aminoguanidine on albuminuria are not related to inhibition of AGE formation. Thus, if diabetic complications are indeed causally linked to formation of advanced Maillard products, then the validity of extrapolating mechanisms by which aminoguanidine improves uraemia to diabetes must be questioned. Aminoguanidine has recently completed Phase III clinical trials for diabetic patients with nephropathy (Friedman, 1999; Brownlee, 1999). Considering the additional impact of uraemia on the availability of substrate for inhibitory aminoguanidine reactions, it is not clear whether these trials can satisfactorily establish the safety of aminoguanidine for use in diabetics in general.

APPENDIX
PATTERSON FUNCTION PROGRAM LISTING

```
/* Patterson Function ******************************************** */
#include "stdio.h"          /*                                      */
#include "stdlib.h"         /*                                      */
#include "math.h"           /*                                      */
#define SIZE 22             /* No. of orders visible in all traces  */
#define PI 3.141592653      /*                                      */
#define NELEM 234           /*                                      */
/*************************************************************** */
void main(void)
{
      int o_array[SIZE];  /*                                       */
      float i_array[SIZE];/*                                       */
      float P[NELEM];     /*                                       */
      int order;          /* Order of D-periodicity /reflection   */
      int x;              /*                                       */
      FILE *fpdat;        /*                                       */
      FILE *fpout;        /*                                       */
      char name[99];      /* Source data file name                 */
      char outfile[99];   /* Output data file name                 */

      printf("SOURCE(incl.full path): ");
      scanf("%s", name);

      if ((fpdat=fopen(name,"r"))==NULL)
      {
            fprintf(stderr,"Not found, try again!\n");
            exit(1);
      }

      for (order=1; order<=SIZE; order++)
      {
            fscanf(fpdat, "%d %f\n",
                        &o_array[order],
                        &i_array[order]);
      }

      fclose(fpdat);

      printf( "Data read.  Patterson begins \n\n");

      for (x=1; x<=NELEM; x++)
      {
            P[x]=float(0.0);
            for (order=1; order<SIZE; order++)
            {
                  P[x]+=i_array[order]
                        *cos(2.0*PI*(float)order*(float)x/234.0)
                        *exp(-(float)order*(float)order*0.1)));
            }
      }

      printf("Results to (include path name): ");
      scanf("%s", outfile);

      if ((fpout=fopen(outfile,"w"))==NULL)
      {
            puts("Unable to open output - something is wrong...\n");
      }
      else
      {
      for (x=1; x<=NELEM; x++) fprintf(fpout, "%d %f\n", x, P[x]);
      }
      fclose(fpout);
      exit(1);
}
```

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