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**ADVANCED GLYCOSYLATION ENDPRODUCTS IN THE
PATHOGENESIS OF THE LATE COMPLICATIONS OF DIABETES
AND ATHEROSCLEROSIS.**

A thesis presented in part fulfilment of the requirements for the admittance to the
degree of Doctor of Philosophy of the University of Glasgow.

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ABSTRACT

The accumulation of advanced glycosylation endproducts (AGE) is important in the pathogenesis of the late complications of diabetes and atherosclerosis. This thesis examined the levels of AGE in proteins incubated with glucose *in vitro*, in tissues from diabetic animals, in human autopsy material and in patients with end stage renal disease.

The measurement of AGE was carried out using protein-linked fluorescence. I have attempted to determine the contribution of oxidative processes to generation of protein-linked fluorescence by studying the effect of glucose, aminoguanidine and antioxidants on the formation of the early and late products of the Maillard reaction. We have demonstrated that the Maillard reaction is affected by oxidative processes at the early stages of Amadori product formation, rather than at the stage of AGE formation.

In animal studies, the role of AGE in diabetic complications has been studied using alloxan and streptozotocin (STZ) to induce experimental diabetes in animals. We measured AGE in the tissues of spontaneously diabetic BB/E rats. We demonstrated that the levels of AGE are increased in diabetic rats when compared to non-diabetic rats and that the rate of AGE formation differs in different tissues. The BB/E rat offers a good model for the investigation of AGE.

Interactions of lipoprotein with extracellular matrix components contribute to atherogenesis. Oxidation of LDL has been shown to affect LDL-collagen interactions. We studied the binding of native and oxidised LDL to unmodified and AGE-modified type I collagen. We have demonstrated that both AGE modification and oxidation of LDL affect LDL-collagen interactions.

While the Maillard reaction is ubiquitous, changes observed in diabetic complications and atherosclerosis are tissue-specific. In this study we have demonstrated AGE-specific fluorescence in different forms of human atherosclerotic plaque. Importantly, AGE content was altered amongst the different types of atherosclerotic plaques. Also individuals with mild to moderate atheroma have lower CLF in superficial atherosclerotic plaques than patients with severe atheroma.

Plasma levels of AGE are elevated in patients with end stage renal disease. It has also been shown that after renal transplantation plasma AGE decrease. Our study

has focused on tissue levels of AGE in non-diabetic and diabetic patients with renal disease before and after transplantation. We have demonstrated that tissue levels of AGE decrease after renal transplantation.

In summary, studies described in this thesis contributed to the validation of the measurement of collagen-linked fluorescence as a method of measurement of tissue AGE accumulation. We have demonstrated tissue differences in AGE accumulation in a spontaneously diabetic animal. Focusing on human atherosclerotic plaque we observed local differences in AGE concentration in atherosclerotic aorta. We also demonstrated that AGE-modification affects the interaction of native and oxidised LDL with type I collagen. Finally our studies were first to demonstrate that renal transplantation decreases AGE level in tissues.

DEDICATION

This thesis is dedicated to both my wife and mother, both women have supported me in different ways in which this work would not of otherwise been possible. To my wife thank you for your loving support, guidance and unending patience. To my mother whose unfaltering faith will never be forgotten.

ACKNOWLEDGEMENTS

I would like to thank the staff of the Department of Pathological Biochemistry, Western Infirmary and Gartnavel General Hospital, Glasgow, especially those from the Lipid/Diabetes Laboratory section, for their help and encouragement during the work of this thesis. In particular I would also like to thank my supervisor Dr Marek H Dominiczak for his valuable advice and guidance.

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Chapter 1.

General introduction.

1.1. Summary.

This chapter describes:

- (1.) The chemistry of the early and late stages of the Maillard reaction.
- (2.) The isolation and characterisation of advanced glycosylation endproducts.
- (3.) The measurement of advanced glycosylation endproducts.
- (4.) The mechanisms of accumulation, removal and degradation of advanced glycosylation endproducts in vivo.
- (5.) Means of inhibiting the production and accumulation of advanced glycosylation endproducts.
- (6.) The pathological significance of advanced glycosylation endproducts.

1.2. The Maillard reaction: an overview.

The Maillard (browning) reaction was discovered by Louis Camille Maillard in 1912 when upon heating glycine with glucose, he observed the glycine-glucose solution turning brown [1]. Maillard repeated the experiment with different amino acids and saccharides, and found that glutamic acid and xylose were the most reactive. He also noted that the reaction proceeded regardless of whether oxygen, nitrogen and hydrogen were present. He postulated that this reaction would have relevance to a variety of areas in science and medicine, especially to chronic hyperglycaemia (diabetes).

The Maillard reaction occurs in three distinct stages; the formation of the labile Schiff base (initial protein glucose adduct: aldimine), the formation of the Amadori (ketoamine) product and finally the formation of advanced glycosylation endproducts (AGE) from reactive intermediates such as 3-deoxyglucosone. Reactions leading to the formation of the Schiff base and the Amadori products are well characterised and are known as "glycation" [2,3,4]. Schiff base and Amadori products are termed as "early glycation products", whereas AGE are called "late products".

Most research following the discovery of the Maillard reaction has focused upon the field of food science. Food containing protein stored in the presence of reducing sugars are susceptible to the Maillard reaction. This results in the formation of brown pigmented products during the late stages of the reaction, the consequence of which is a reduction in the nutritive value of food [5]. It is only in the past decade, that a number of investigators have studied the Maillard reaction in vivo and its relationship to the ageing process and diabetic complications. Maillard's suggestion of the importance of sugar induced protein modification in vivo gained substance when Mohammed et al demonstrated that

the browning of proteins could occur under physiological conditions [6]. Later, Rahbar discovered increased concentrations of haemoglobin A₁ (HbA₁), a glycosylated form of haemoglobin, in the blood of diabetic individuals [7,8]. Subsequently Bookchin and Gallop used borohydride reduction to label and quantitate the Amadori (ketoamine) product on haemoglobin [9]. Amadori products formed as a result of the rearrangement, of the initial protein glucose adduct Schiff base. Bookchin and Gallop isolated and quantitated the Amadori product by acid hydrolysis exploiting the stable nature of the ketoamine.

In addition to the products formed at the early stages of the Maillard reaction, advanced glycosylation endproducts have also been shown to exist in vivo. Lens proteins are long-lived in vivo and are therefore susceptible to postsynthetic modifications by non-enzymatic glycosylation. Monnier et al have shown that brown pigments with fluorescent properties characteristic of AGE exist in human lens protein [10,11,12]. In vitro studies have shown that the formation of brown pigments is possible under physiological conditions by incubating lens proteins with glucose. Monnier et al postulated that lens proteins could be used as a model for the investigation of non-enzymatic glycosylation and the accumulation of AGE in vivo.

It has since been proposed that the long term complications of diabetes develop at least in part as a consequence of structural and functional modifications of proteins resulting from the Maillard reaction [13,14]. The formation of protein glucose adducts via the Maillard reaction leads to the irreversible formation of crosslinks (AGE) between adjacent protein molecules. These crosslinks have been implicated in the loss of elasticity of ageing tissue [15]. The late complications of diabetes (affecting the eyes, kidneys, nerves and arteries) are thought to be due, at least in part, to the chronic hyperglycaemia that exists in diabetic patients [16,17,18].

1.3. Chemistry of the Maillard reaction.

1.3.1. Schiff base and Amadori product formation.

Aldimine (Schiff base) is formed as a result of the non-enzymatic condensation of a reducing sugar with a free amino group of protein. Biological molecules that have free amino groups such as proteins, nucleic acids and low molecular weight amines are susceptible to formation of the Schiff base [19,20,21]. Reducing sugars exist in both open and ring form in vivo (fig 1.1), but it is only the open form that reacts with amino groups of proteins to form Schiff bases. The ϵ -amino groups of lysine and hydroxylysine residues and the α -amino groups of N-terminal amino acids, act as a nucleophile and attacks the carbonyl group of sugar. Lysine is the most important amino acid involved in the formation of the Schiff base in vivo (fig 1.2).

The next stage of the Maillard reaction is the dehydration and enolisation of aldimine in a process known as Amadori rearrangement to form the Amadori product (fig 1.2). Unlike the Schiff base, which can readily dissociate back to its original constituent molecules, the Amadori product is much more stable. In vivo, lysine and valine found on the N-terminus of the α and β chains of human haemoglobin react with reducing sugars. This results in the formation of a chromatographically distinct minor haemoglobin component known as HbA_{1c} [22]. The Amadori products formed on haemoglobin are produced continuously throughout the 120 day life span of the red blood cell. There is a two to three fold increase in HbA_{1c} in the red cells isolated from subjects with diabetes mellitus [23,24]. The measurement of glycosylated haemoglobin provides an index of the mean concentration of blood glucose during the preceding two to

three months, complementing more traditional measures of glycaemic control such as glucose testing in urine and blood [25,26,27].

The rate of formation of Amadori products is dependent on the ambient concentration of glucose and the concentration of susceptible amino group glycation sites in proteins. The relatively short time (14-20 days) required for the formation of Amadori products [28] allows their accumulation on both short-lived proteins (haemoglobin, albumin and immunoglobulin G) and long-lived proteins such as collagen and elastin. Sugars other than glucose such as mannose, fructose (both aldose) and ribulose (ketose) have also been shown to be involved in non-enzymatic glycation. Even though the extracellular concentrations of these sugars are low, their high reactivity with proteins makes them strong candidates for the mediation of molecular damage to long-lived molecules via the Maillard reaction [19,29,30].

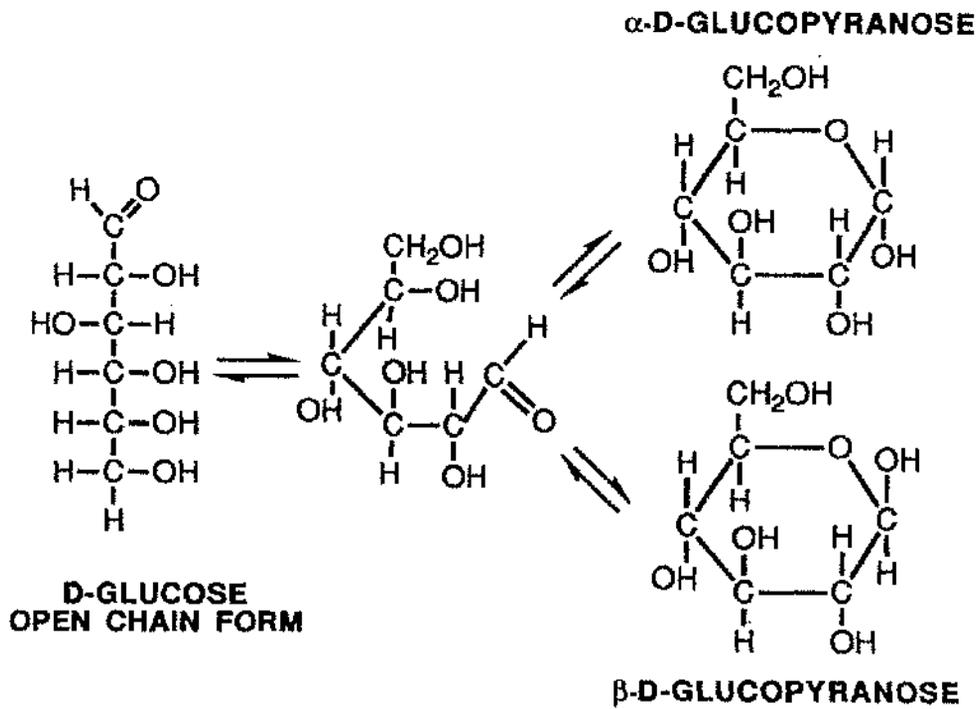


Figure 1.1

The structures of the different forms of glucose that exist *in vivo* are comprised of α and β -D-glucopyranose (36% and 64% respectively).

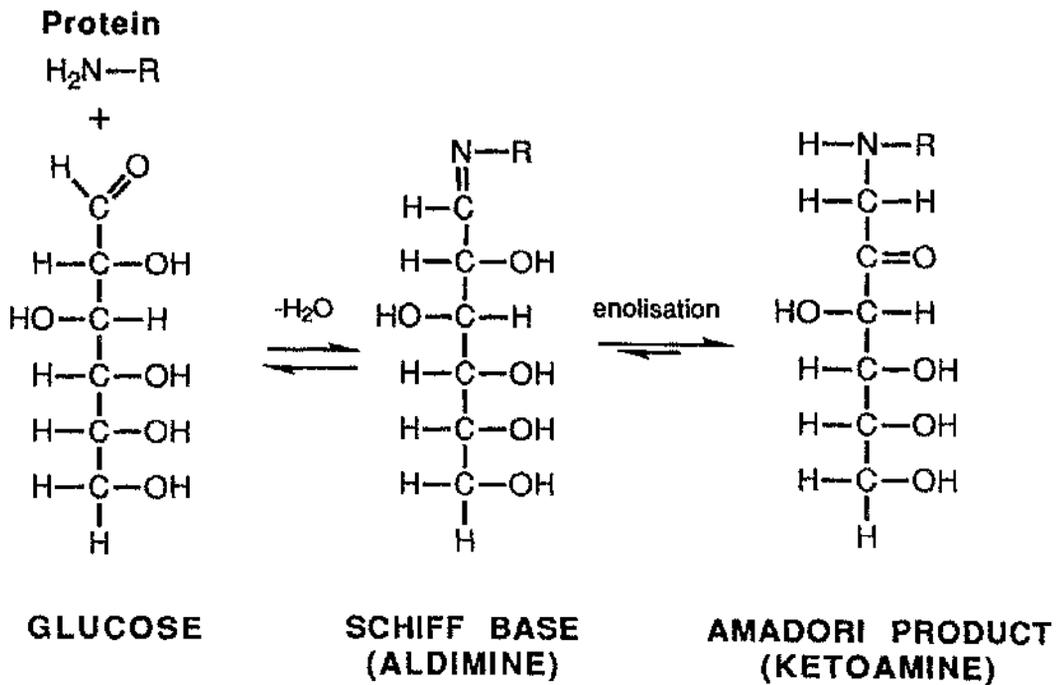


Figure 1.2

Formation of the Schiff base and Amadori product.

1.3.2. Factors affecting the rate of Amadori product formation.

The non-enzymatic glycation of proteins is affected by temperature, pH, substrate concentration and the type of reducing sugar. An important factor in the formation of Amadori product is the biological half life of the protein in vivo. Rates and sites of glycation on proteins vary depending on buffer species and concentration of reactants (amines and reducing sugars). The glycation rate of RNase, human serum albumin, lysozyme and haemoglobin is greatly enhanced when phosphate buffers rather than organic buffers such as 3-[N-morpholino] propanesulfonic acid (MOPS) are used. Similar increases were observed with bicarbonate buffers, suggesting a general effect of anionic buffers on the glycation rate of proteins [31,32,33].

The rate of non-enzymatic glycation is affected by the pKa of amino groups on protein. Lower pKa of amino groups by virtue of its greater nucleophilicity, increases the rate of formation of Schiff base adducts [34]. Also, acidic and basic amino acids present in the vicinity of the glycation site are known to modulate the reactivity of lysine residues with glucose. The presence of binding sites for fatty acids and glycosaminoglycans on proteins could either block or enhance the glycation rate of specific lysine residues [35,36].

1.3.3. The effect of Amadori products on the function of proteins.

In non-diabetic subjects the extent of Amadori product formation on proteins is relatively constant with age. This is in contrast to patients with diabetes, where levels of Amadori products can vary dramatically over a short period of time [37,38]. Amadori products affect the function of proteins *in vivo*. For instance, the presence of ketoamine on the NH₂ terminal amino group of haemoglobin (HbA₁) reduces the reactivity with 2,3-diphosphoglycerate (2,3-DPG). Although 2,3-DPG binding controls the affinity of haemoglobin to oxygen [39], the presence of glycosylated haemoglobin does not alter whole blood oxygen saturation curves [40,41]. The clinical significance of this is unclear.

In vitro studies have shown that glycation of lysine residues in or near the active site of RNase inactivates the enzyme [32]. The *in vitro* glycation of low density lipoproteins (LDL), to a degree comparable to that seen in diabetes decreases the rate of their catabolism by human fibroblasts. The glycation of LDL also affects their recognition and catabolism by macrophages [42]. LDL, isolated from diabetic patients, was shown to stimulate cholesteryl ester synthesis in macrophages, resulting in increased intracellular cholesteryl ester accumulation. This could be a mechanism by which hyperglycaemia contributes to the atherosclerosis in diabetes [43,44].

Glycation of the components of kidney basement membrane such as fibronectin and laminin affects their interaction with heparin and collagen [45,46], suggesting a possible mechanism for basement membrane alterations in diabetes. Platelet membranes isolated from the diabetic patients have increased microviscosity. This is due, at least in part, to the non-enzymatic glycation of membrane proteins rather than to an increased cholesterol to phospholipid molar

ratio which would have the same effect [47]. Increased microviscosity of the membrane could affect the functions of the membrane channels as well as receptors on the surface of the cell.

In vitro glycation of antithrombin III results in the inhibition of its heparin binding ability and consequently a reduction in its thrombin inhibiting activity [48,49].

In diabetes, the glycation of immunoglobulins is increased with a subsequent decline in their function [50,51]. To date, there is little evidence that Amadori products change the properties of collagen in diabetic patients [52]. Although the concentration of Amadori products on collagen is elevated in diabetes, it is not related to the presence or severity of diabetic complications [53,54]. Antibodies to the reduced form of Amadori product have been found in the plasma of some diabetics and could elicit an immunological reaction against proteins modified by Amadori products in vivo [55,56].

1.4. Advanced glycosylation endproducts (AGE) formation.

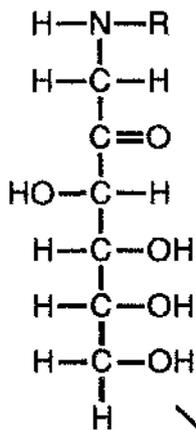
Reactions leading to the formation of AGE will be termed as either "advanced glycosylation" or "browning". Properties of AGE include the formation of brown chromophores that fluoresce at 370nm/440nm (excitation/emission wavelength) and the formation of inter and intramolecular crosslinks between proteins. The elucidation and identification of specific products formed in tissues during the latter stages of the Maillard reaction has remained difficult since AGE are a whole family of compounds.

In contrast to the formation of Schiff base and Amadori products, the reactions involved in the late stages of the Maillard reaction are less clearly defined. It is

certain that AGE are formed during reactions that involve the dehydration, fragmentation and rearrangement of the Amadori product. To date, only a few structures such as 2-furoyl-4-(5)-(2-furanyl)-1H-imidazole [57], pentosidine and pyrraline have been characterised. The latter two have been shown to exist in vivo [58,59,60].

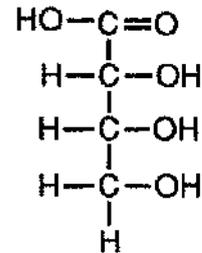
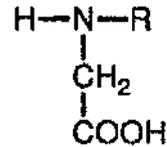
Glycooxidation and glucose autoxidation are both oxidative processes that are involved in AGE formation [61]. Glycooxidation products are formed from the oxidative cleavage of fructose-lysine (FL) [62,63,64]. The oxidative fragmentation of FL by oxygen free radicals produces pentosidine and the non-reactive products carboxymethyllysine (CML) and erythronic acid (fig 1.3). CML and pentosidine have been isolated from the tissues of diabetic patients [37,52,65]. CML could serve as a marker for the formation of glycooxidation products. Glucose in common with other α -hydroxyaldehydes, is prone to transition metal-catalysed oxidation (a process known as glucose autoxidation). Glucose autoxidation generates hydrogen peroxide, as well as reactive intermediates such as the hydroxy radical and ketoaldehydes [66,67,68]. It has been speculated that autoxidation of glucose contributes to the structural alterations observed on proteins when exposed to glucose [69].

**AMADORI PRODUCT
(KETOAMINE)**



Oxidative cleavage
in the presence
of O₂

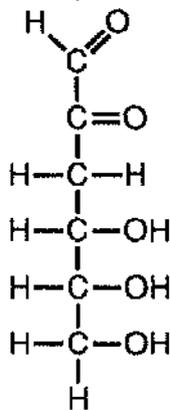
**CARBOXYMETHYLLYSINE
(CML)**



ERYTHRONIC ACID

AGE

Fluorescent inter and intra
molecular crosslinks



3-deoxyglucosone, an example of
the intermediate products of
Maillard reaction (propagators)

3-DEOXYGLUCOSONE

Figure 1.3

Structure of carboxymethyllysine and 3-deoxyglucosone produced from the degradation of Amadori product. 3-deoxyglucosone reacts with other primary amines to form brown fluorescent pigments.

The Amadori product can also spontaneously fragment to form dicarbonyl compounds, of which 3-deoxyglucosone (3DG) is thought to be an important precursor of AGE [70,71] (fig 1.3). In vitro incubation of 3DG with lysozyme and bovine serum albumin results in the polymerisation of proteins and produces a fluorescent peak characteristic of AGE to that seen in vivo [72]. The fluorescence of the products of the reaction of bovine serum albumin with 3DG is higher than that resulting from the reaction of an equivalent amount of bovine serum albumin with glucose. In addition to 3DG, 1-deoxyglucosone and 4-deoxyglucosone can be also formed from the degradation and dehydration of Amadori product. Deoxyglucosones are thought to degrade into a range of products including pyrroles, pyridines and pyrrolinone reductones [60,73,74] which are UV active. It has recently been shown that the concentration of 3DG is increased in plasma isolated from diabetic rats [75] and in the urine and plasma of human diabetic subjects [76].

1.5. The chemical structures of AGE.

Several AGE have been identified to date. Pongor et al isolated the yellow fluorescent chromophore, 2-furoyl-4-(5)-(2-furanyl)-1H-imidazole (FFI) from the acid hydrolysate of bovine serum albumin (BSA) incubated with excess glucose under physiological conditions [77]. The fluorescent properties of FFI had striking similarities to those of browned BSA and poly-L-lysine, suggesting that FFI was indeed glucose derived crosslink (fig 1.4). FFI was the first AGE characterised, but it is now unlikely that it has any biological significance. Data concerning its presence in vivo are inconsistent. Acid hydrolysis used to measure FFI is notorious for producing an array of fluorescent artefacts, Njoroge et al questioned whether FFI existed in vivo. Njoroge synthesised an FFI-like molecule by the condensation of two furosine

molecules which are themselves acid hydrolytic break-down products of glycated lysine [57].

FFI was detected in vitro in browned albumin and polylysine by Pelli et al using collision spectroscopy [78]. In contrast Lapolla et al, found no FFI present in collagen isolated from diabetic rats [79]. Recently, FFI was detected using a radioimmunoassay in globin and serum albumin isolated from normal individuals [80]. When FFI was coupled to proteins, it was specifically taken up by macrophages suggesting the presence of a specific receptor [81]. These findings imply that even if FFI does not exist in vivo, its structure could be, in itself, representative of fluorescent chromophores which do form during the incubation of proteins with glucose.

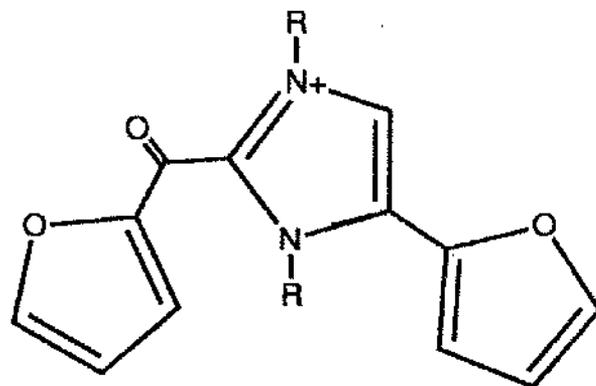
Another AGE, pentosidine, has been isolated from human extracellular matrix (fig 1.4). Pentosidine is fluorescent at excitation wavelength 335nm and emission wavelength 385nm [82,83]. The Amadori products of ribose and glucose, as well as lysine and arginine residues contribute to the formation of pentosidine. There is also a requirement for oxygen. Comparison of the ability of ribose, glucose, and their Amadori products to form pentosidine showed that ribosylated lysine had the highest reactivity, followed by ribose and then glucose. The high reactivity of ribose suggest that it may be a main precursor of pentosidine. Since plasma levels of ribose are very low, the significance of this is uncertain [84,85].

Fructose can also be a precursor of pentosidine. The non-enzymatic fructosylation and crosslinking of proteins by fructose in vitro are possible. Fructose can react with an amino group via a Heyns rearrangement to form pentosidine [86]. This could be of relevance in diabetes since fructose levels in diabetic patients are increased as a consequence of activation of the aldose

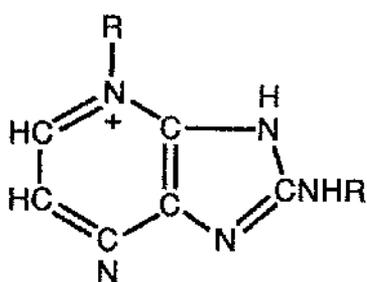
reductase pathway [87]. The aldose reductase pathway is a metabolic shunt involving the conversion of glucose to fructose with the formation of sorbitol as an intermediate. It has been shown that fructose levels in the tissue of diabetic animals are increased 2-3 fold when the aldose reductase pathway is active [88,89]. Ascorbate and dehydroascorbate are also precursors of pentosidine [81]. The breakdown products of ribonucleotide during cell death could also be a source of pentose [59,83]. However, pentosidine has been shown to account for less than 1% of the total crosslinks formed during the browning reactions with glucose in vitro [58,83]. It is necessary to search for other carbohydrates as potential precursors of pentosidine.

Finally, pyrraline is a recently discovered AGE which forms under physiological conditions [90]. Pyrraline was synthesised in vitro by reacting glucose with neopentylamine, a simple amine, in phosphate buffer at 37°C. The proposed mechanism of formation of pyrraline is thought to be the condensation of 3-deoxyglucosone and protein (fig 1.4). Quantitation of pyrraline on intact proteins was carried out by radioimmunoassay. Hayase et al showed an increased pyrraline immunoreactivity in the albumin-rich fraction of serum isolated from diabetic patients [74]. Recent studies however have cast doubt as to whether pyrraline plays a role in the pathology of diabetic complications. It has been suggested that immunoassay of pyrraline may have given positive results because of non-specific antibodies raised to impure hapten [91]. Horiuchi et al have suggested that pyrraline is an early product of the Maillard reaction, formed in the initial few days of in vitro incubation, rather than an advanced glycosylation endproduct which would form after 3-4 weeks [92].

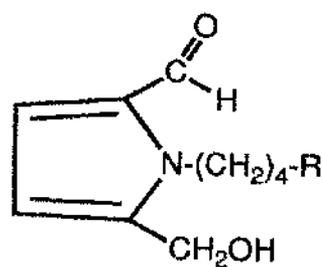
Further research is necessary to elucidate the structures of AGE present in vivo.



FFI



Pentosidine



Pyrraline

Figure 1.4

Structures of FFI, pentosidine and pyrraline.

1.6. AGE-induced protein crosslinking.

On incubating RNase with glucose, Eble et al have observed the polymerisation of RNase into dimer and trimer forms. This was accompanied by a decrease in the number of primary amino groups of the protein, as shown by a decrease in lysine recoverable by amino acid analysis [93]. Polymerisation of RNase was inhibited by the addition of lysine, suggesting that the dimerisation of RNase occurs by the condensation of an Amadori product and an amino group. Importantly, even after the removal of the reducing sugar from the medium, the protein continues to polymerise. This suggests that once glycation of the protein is initiated, the presence of a reducing sugar is not essential for AGE formation. Eble also observed that crosslinking can occur between native and glycated proteins. The formation of AGE in vivo is slow ($t_{1/2}$ = weeks to months [19]) when compared to the formation of Amadori product ($t_{1/2}$ = days to weeks [28]).

1.7. Target Proteins for AGE formation in vivo.

Because of the relatively long time required for the formation of advanced Maillard products, these reactions occur predominantly on long-lived proteins [28]. The in vivo accumulation of AGE is dependent on the biological half life of target proteins. Collagen, lens crystallin and nerve myelin protein are main target proteins for AGE formation [94,95,96].

1.7.1. Collagen.

Collagen, one of the most abundant proteins found in mammals, is found in connective tissues such as tendon, cartilage, the organic matrix of bones and the cornea of the eye. Collagen provides an extracellular framework for all

multicellular animals. It is abundant in all tissues and particularly in fibrous connective tissue [97,98]. Collagen is rich in glycine (35%), alanine (11%) and proline (21%). Two amino acids characteristic of collagen are hydroxyproline and hydroxylysine, neither of which are genetically coded and are formed from their precursor residues, proline and lysine respectively by the action of distinct oxygenases. So far five types of collagen have been characterised [98,99]. Their distribution in the body is summarised in table 1.5.

Type	Native polymer	Tissue distribution
I	Fibril	Skin, tendon, bone, dentin
II	Fibril	Cartilage
III	Fibril	Skin, uterus, blood vessels
IV	Basement membrane	Kidney glomeruli, lens capsule, basement lamina of all epithelial and endothelial cells
V	Unknown	Basement lamina of smooth and striated muscle cells.

Table 1.5

Tissue distribution of vertebrate collagens.

Collagen is composed of three left handed helical α -chains wound around each other to form a super-helical cable known as tropocollagen [97]. In addition to the triple helical structures, collagen fibres are further stabilised by covalent crosslinks, formed by lysyl oxidase-mediated oxidative deamination of lysine and hydroxylysine residues [100]. The extent and type of crosslinking varies with the physiological function and age of the tissue. Formation of such crosslinks constitutes a process of *in vivo* maturation [101]. During maturation, collagen becomes both less soluble and less susceptible to proteolytic enzymes.

The in vitro incubation of collagen with a reducing sugar leads to the formation of Amadori products. This occurs via the condensation of glucose with the α -amino group of a terminal amino acid or the ϵ -amino group of a peptide bound lysine. The end result is the formation of AGE and an associated increase in fluorescence [71,77]. Tanaka et al have shown that triple helical regions of α chains isolated from rat tail collagen incubated with ribose are both highly fluorescent and crosslinked [102].

Collagen isolated from diabetic patients, when compared to collagen isolated from age-matched controls has reduced solubility [103,104,105] and increased resistance to collagenase digestion [106,107]. Increases in AGE-related fluorescence indicative of increased crosslinking of collagen may explain the increased stiffness of arterial, skin and lung tissue from diabetic subjects [108,109,110]. The stiffening of arteries as a result of increased AGE accumulation on diabetic collagen could lead to increased blood pressure [109,111].

The increased crosslinking of collagen can occur via two pathways. One pathway begins with the lysyl oxidase-mediated oxidative deamination of lysine and hydroxylysine residues. The aldehyde moieties react further with each other to form a series of di- or trifunctional crosslinks [100,112]. AGE formation is a second pathway by which increased crosslinking of proteins occurs. Late products of the Maillard reaction contribute to increased crosslinking as seen during the in vitro incubation of RNase with glucose [113]. Studies by Andreassen et al with tail tendon isolated from streptozotocin-diabetic rats showed decreased thermal rupturing under tension [114], which was not related to the enzymic ageing process [115,116].

1.7.2. Myelin.

The basic unit of the nervous system is the individual nerve cell or neuron which is covered by myelin, a fatty material formed from the plasma membranes of specialised cells. Myelin insulates the membrane, making it more difficult for electric currents to flow between the intracellular and extracellular compartments of nerve cells.

The products of the Maillard reaction accumulate in both peripheral and central nerve myelin [117,118,119]. Vlassara et al showed that peripheral nerve myelin protein isolated from rats with long term diabetes is preferentially taken up by macrophages, when compared to either the non-diabetic rats or diabetic rats with short term diabetes. The in vitro incubation of nerve myelin protein with glucose has the same effect [96]. A long term complication of diabetes mellitus, peripheral neuropathy, is characterised by segmental demyelination. The extent and degree of this pathological change correlates with the severity and duration of hyperglycaemia [120]. These observations provide a pathophysiological link between excessive non-enzymatic glycosylation of myelin protein as induced by hyperglycaemia and segmental demyelination in diabetes. It has been suggested that the increased concentrations of pentosidine found on both cytoskeletal and myelin fractions of human nerves could affect the turnover rate and may alter the phosphorylation/dephosphorylation of axonal cytoskeletal proteins, thereby affecting their assembly and transport, and ultimately leading to axonal atrophy and degeneration [121]. This most likely acts in synergy with other pathogenetic mechanisms known to occur in diabetic peripheral nerve.

1.7.3. Lens crystallin.

The lens crystallin exists in three distinct forms (α , β and γ crystallin) and comprises more than 90% of the dry weight of lens fibre cells. The relationship between hyperglycaemia and the crosslinking of proteins in vitro and in vivo was first demonstrated using lens crystallin [122,123]. Slow turnover rate of crystallin makes the post-translational modification by the products of the Maillard reaction possible. Cataractogenesis in diabetic and senile cataracts is the result of protein aggregates in the lens [124,125]. Lens opacity, observed in lenses incubated with glucose is accompanied by the formation of brown fluorescent pigments with spectroscopic properties identical to AGE [10,126]. The concentration of Amadori products in cataractous lenses is increased in young rats with experimental diabetes, and in senile lenses. However, cataractogenesis in diabetes is a multifactorial process and apart from AGE formation, abnormalities in the aldose reductase pathway, disulfide crosslink formation, increased oxidative potential and non-enzymatic glycosylation, all play a role in the formation of protein aggregates in the lens [127].

1.7.4. Deoxyribonucleic acid.

The long life of deoxyribonucleic acid (DNA) in the cell makes it a potential target for AGE formation [128,129]. Incubation of DNA with reducing sugars produces brown pigments that have the characteristic fluorescence of AGE. Lorenzi et al have shown that the incubation of endothelial cells with high glucose concentrations (30mM) induces an accelerated unwinding rate of double stranded DNA in the presence of alkali. This is indicative of increased single strand breaks. The increase in single strand breaks was observed in endothelial cells but not in fibroblasts [130]. Studies by Lee et al have shown that reactive compounds of unknown structure formed from glucose and lysine are twenty

times more damaging to DNA than glucose alone and may contribute to the increased protein-DNA crosslinking seen in cells from aged rats [21,131].

Since endothelial cells are independent of insulin for glucose transport, the intracellular concentration of glucose in these cells is related to the extracellular concentration of glucose [132,133]. The browning of regulatory DNA binding proteins has been shown to occur in vitro and may constitute one of the pathways by which gene expression could be altered [134]. Glucose-induced crosslinking between amino acids and nucleic acids may be the mechanism responsible for increasing amounts of proteins becoming covalently attached to DNA as a person ages [135].

1.8. The degradation and elimination of AGE-modified proteins.

1.8.1. Receptors for AGE.

A possible biological pathway for the removal and degradation of AGE-modified proteins may occur via the AGE-specific receptors found on macrophages. A receptor that binds AGE (AGE-R) has been isolated and characterised and is distinct from other macrophage scavenger receptors that recognise chemically modified proteins such as acetylated LDL and maleylated albumin [81]. The in vitro modification of LDL and albumin by acetic anhydride or maleic anhydride results in macrophage recognition and uptake by specific distinct receptors [136,137]. Vlassara, using a FFI affinity column, isolated a 90 kDa polypeptide from a mouse cell derived cell line, RAW 264.7. The isolated receptor had a binding affinity of the $3.1 \times 10^7 M^{-1}$, which was close to that found on intact murine macrophages [138].

Vlassara et al have observed an increased uptake and accumulation of myelin, isolated from diabetic and non-diabetic rats, by mouse peritoneal macrophages. In vitro glycosylation of peripheral nerve myelin also resulted in increased uptake [96]. In a similar study using human autopsy material, the uptake of myelin by mouse macrophages, was 2-3 times higher in diabetic myelin than in myelin isolated from non-diabetic subjects [139]. The specificity of the AGE-myelin interaction with macrophages was assessed by incubating labelled AGE-BSA in the presence of increasing concentrations of unlabelled AGE-myelin and AGE-BSA. In similar studies Gilcrease and Hoover have observed that human monocytes preferentially degrade non-enzymatically glycosylated collagen [140].

Recently, a new receptor for AGE designated as RAGE (receptor for advanced glycosylation endproducts) has been isolated from bovine lung endothelial cells [141,142]. Schmidt et al have identified two endothelial cell surface-associated proteins, an apparently unique 35kDa polypeptide (RAGE) and a lactoferrin-like polypeptide (LF-L). Molecular cloning studies have shown RAGE to be a member of the immunoglobulin superfamily [143]. Binding of the AGE to cultured endothelial cells results in endocytosis of AGE, alterations in endothelial cell growth, coagulant and barrier functions [144]. The identification of RAGE as a member of the immunoglobulin superfamily suggested that in addition to interacting with AGE, it might serve other functions, including mediation of cell-cell recognition or binding of growth factors and cytokines.

1.8.2. Enzymatic degradation of Maillard products.

As described above 3-deoxyglucosone (3DG) is an intermediate in the Maillard reaction [70,145]. Therefore, enzymes degrading 3DG could possibly prevent the accumulation of the products of the Maillard reaction. Oimomi et al have

extracted the enzyme, α -ketoaldehyde dehydrogenase, from human liver and have shown that 3DG was a substrate for this enzyme [146]. Another enzyme, 2-oxoaldehyde reductase, isolated by Liang et al, reduces 3DG to 3-deoxyfructose and has been found in the liver and kidneys of pigs [147,148]. The activity of this enzyme was highest in the liver and kidney (200-350U per 100mg tissue). This kidney enzyme might prevent the propagation of the Maillard reaction, in particular crosslink formation.

1.9. Measurement of the endproducts of the Maillard reaction.

Studies on the relationship between the formation of AGE in tissues and diabetic complications have been impeded by the lack of detailed information on the chemistry of the late stages of the Maillard reaction and difficulties in the measurement of AGE. The early evidence for the occurrence of AGE in vivo has been based primarily on criteria such as impaired digestibility of proteins by proteolytic enzymes, reduced solubility and increased resistance to heat denaturation [149]. Increased fluorescence at excitation 370nm, emission 440nm has been found in tissues isolated from older subjects, as well as diabetic subjects [13]. Fluorescence measurement has been the most commonly used method of AGE determination in vivo. Other methods that include a radioreceptor assay, radioimmunoassay and high performance liquid chromatography (HPLC) have now been developed to measure AGE in vivo. Each of these methods will be discussed in detail below.

1.9.1. Protein-linked fluorescence.

Since many AGE exhibit natural fluorescence, they have traditionally been measured by fluorimetry at distinct excitation and emission wavelengths. AGE are brown, fluorescent chromophores [150,151,152]. One of the first

fluorescent yellow-brown product to be identified from the acid hydrolysates of bovine serum albumin and polylysine that have undergone nonenzymatic browning was 2-furoyl-4-(5)-(2-furanyl)-1H-imidazole (FFI), characterised by spectroscopic (fluorescence, absorbance and mass spectrometry) methods [77]. Dyer et al, also using fluorescence at specific excitation and emission wavelengths detected pentosidine in in vitro browned lysozyme as well as human lenses and skin [58]. They observed that pentosidine accounted for only a small fraction (<1%) of glucose derived crosslinks.

Studies on the effect of free radicals and products of lipid peroxidation on collagen-linked fluorescence (CLF) have been carried out [153,154]. There was no significant decrease in CLF in streptozotocin (STZ) diabetic rats supplemented with vitamin E, a free radical scavenger [155], even though lipid peroxidation measured as thiobarbituric acid (TBA) reactivity was considerably lower than in control animals [156]. This would suggest that the generation of CLF in vivo is not affected by oxidative stress, at least in the case of lipid mediated peroxidative reactions. In contrast, a study by Bucala et al have shown that phospholipids do react directly with glucose to form AGE that in turn initiate lipid peroxidation in vitro [157]. In the same study LDL isolated from diabetic patients had increased levels of both apoprotein-linked and lipid-linked AGE, when compared to LDL from non-diabetic patients. Circulating levels of oxidised LDL were elevated in diabetic patients and correlated with AGE-lipid levels. This study would suggest that AGE could in fact play a role in initiating LDL oxidation in vivo.

The measurement of AGE-modified proteins using fluorescence at excitation wavelength 370nm, emission wavelength 440nm has proved useful in assessing the level of AGE in tissues [11,158]. The fluorescence of collagen samples obtained from dura matter of normal subjects has been observed to

linearly increase with age [109,159]. Fluorescence of AGE in diabetic skin has been related to the severity of retinopathy, arterial stiffness and joint stiffness [160]. Unlike radioimmunoassay, HPLC and radioreceptor assay, AGE in plasma cannot be measured using fluorescence, since there are too many interfering substances in plasma [80,109,137]. This problem does not occur when purified tissue collagen is used.

1.9.2. Radioreceptor assay (RRA).

Radioreceptor assay (RRA) utilises the presence of a membrane receptor specific for AGE-modified proteins on mouse macrophages [161,162]. The binding of labelled AGE-BSA by macrophages is inhibited by adding increasing amounts of unlabelled AGE-BSA. BSA chemically linked to FFI also inhibits [¹²⁵I]-AGE-BSA binding suggesting that the macrophage receptor recognises an AGE structure homologous to FFI [163]. RRA has also been used to quantitate AGE on proteins other than BSA such as collagen, LDL and RNase modified by glucose *in vitro*. Amadori products do not inhibit the binding of AGE-modified proteins to macrophage receptor.

RRA has been used for the measurement of AGE content on tissue proteins in a number of studies. Makita et al measured AGE on arterial collagen isolated from diabetic patients and showed that tissue AGE were higher in the diabetic group when compared to the non-diabetic individuals. Using RRA, AGE levels in the femoral and coronary arteries isolated from the same patient were similar. In contrast, fluorescence measurement of the same arterial samples showed no such similarity. In the same study, serum of diabetic patients with and without nephropathy, were separated into a high and low molecular weight peptide fraction (< 10kDa) containing AGE. Separation of the serum into low and high molecular weight components showed that AGE levels in the high molecular

weight component did not differ much between the groups [164], whereas a significant difference was observed when measuring AGE levels in the low molecular weight component. This probably reflects the poor removal of low molecular weight AGE-peptides in patients with diabetic nephropathy. The AGE-specific receptor is a useful tool for the measurement of AGE in tissue and plasma proteins.

1.9.3. Enzyme linked immunoabsorbent assay (ELISA).

An immunochemical assay for AGE has been developed by Makita et al. AGE-specific antisera were produced by the immunisation of rabbits with AGE-modified RNase. These antibodies reacted with AGE-modified BSA. However, none of the known AGE such as FFI, carboxymethyllysine and pentosidine were found to compete for the binding of anti-AGE antibody. Amadori products were also not recognised by this antiserum. In vitro time course studies have shown that anti-AGE serum is specific for the "late" AGE which occur after fluorophore formation [165]. Anti-AGE antibody did not react with proteins incubated with glucose in the presence of aminoguanidine, an inhibitor of AGE formation [166,167]. Anti-AGE serum bound more arterial collagen isolated from diabetic rats than from non-diabetic rats. Using the immunoassay and RRA, comparable results were obtained when measuring circulating serum AGE in diabetic patients. Serum AGE concentration was highest in patients with diabetic nephropathy [164,168]. Monoclonal antibodies have also been used to detect AGE in atherosclerotic lesions of human and rabbit aorta [169,170]. It is now known that the predominant AGE detected by immunoassay using antibodies raised to glucose-derived AGE proteins is N_ε-carboxymethyl lysine [349]

1.9.4. High performance liquid chromatography (HPLC).

HPLC has been used to isolate and characterise AGE-fluorophores from the insoluble collagen rich fraction of human dura matter, lens crystallin and plasma proteins [82,124,171]. Sell and Monnier were first to isolate, purify and characterise AGE-fluorophores by studying the tryptic digests of collagen peptides from human dura matter. Collagen was digested with trypsin and separated into high and low molecular weight fractions. The high molecular weight fraction containing fluorescent material was further digested with collagenase and separated using gel filtration chromatography. Fractions obtained from gel filtration were injected into HPLC column linked to a fluorimeter. Two fluorescent peaks were obtained, designated as P and M with excitation/emission wavelengths 335/385 and 360/460nm respectively. To avoid fluorescent artefacts, proteolytic digestion of collagen was used rather than acid hydrolysis. It is now known that fluorescent peak P is pentosidine [59]. Peak M probably represented other fluorescent AGE detected in increasing amounts in the skin and dura matter of diabetic patients [109]. Pentosidine levels were found to increase with the biological age of individuals regardless of whether they were non-diabetic or diabetic. Highest pentosidine concentrations were found in individuals with end stage renal failure [143]. Pentosidine was also detected in red blood cells and plasma proteins [83].

1.9.5. Disadvantages of RRA, ELISA and HPLC in the measurement of AGE.

A common disadvantage of RRA, ELISA and HPLC methods used for the measurement of AGE is the assumption that the use of standards, prepared by either the in vitro incubation of a reducing sugar with protein or the chemical synthesis of one particular AGE moiety are the same as AGE found in vivo.

All these methodologies are useful tools in the measurement of AGE in tissue and serum samples, providing us with an understanding of the relevance of AGE in the pathology of disease, especially the late complications of diabetes.

1.10. Elevated AGE and its relationship to disease processes.

Over recent years, our understanding of the involvement of AGE in the late complications of diabetes and atherosclerosis has improved. Diabetic patients have a 2-3 times higher risk of cardiovascular disease than non-diabetic patients. The effect of diabetes is independent of all other known cardiovascular risk factors: plasma lipids and lipoproteins, family history of coronary heart disease, obesity, age, cigarette smoking, hypertension, thrombogenic risk factors and sex [172,173,174,175]. Chronic hyperglycaemia is believed to play a major role in the pathophysiology of diabetic complications such as retinopathy, neuropathy, nephropathy. Its role in cardiovascular disease is less clear. Increased AGE levels in tissue and plasma proteins have been linked with each of these complications at the structural (crosslinking of proteins) [63,65,176,177] and cellular (release of growth and chemotactic factors) levels [178,179,180].

Many studies have examined the relationships between elevated AGE levels and the presence and severity of diabetic complications. Monnier et al observed that collagen-linked fluorescence (CLF), was twice as high in diabetic subjects as in control patients. CLF also correlated with the severity of retinopathy and arterial and joint stiffness [54,160,181]. Limited joint mobility demonstrated in patients with diabetes mellitus is thought to be the result of the stiffening of connective tissue. Such stiffening has been linked to the crosslinking properties of AGE. Dominiczak et al observed that CLF was increased in the skin of young patients with IDDM and was related to the duration of diabetes and severity of diabetic

retinopathy [182]. Beisswenger et al studied the relationship between levels of pentosidine on collagen and the severity of retinopathy and nephropathy in diabetic patients. They observed elevated tissue pentosidine levels in patients with diabetes. Diabetic patients with nephropathy or retinopathy were found to have significantly higher pentosidine levels than diabetic patients without either of these complications. Also, elevated pentosidine levels correlated with age and duration of diabetes [158].

1.11. Potential mechanisms by which elevated AGE could contribute to atherosclerosis.

Previously mentioned is the crosslinking property of AGE and how this may contribute to structural and functional changes in collagen. The interaction of AGE with specific receptors on macrophages, mesangial and endothelial cells leads to the release of several growth factors which in turn may contribute to the formation of the atherosclerotic plaque through the remodelling of vascular tissue [144,178,183].

Atherosclerosis is defined as "arterial lesions characterised by thickening of the intima, the thickenings comprised of fat and collagen-like fibres, both being present in widely varying proportions" [184,185]. Atherosclerosis is a multifactorial phenomenon involving complex interactions among environmental and genetic factors. The formation of an atherosclerotic plaque is a localised phenomenon and occurs with consistent topography in relation to flow dividers, branching sites and areas of arterial curvature. The focal distribution of atherosclerotic lesions suggests that there is a role for hemodynamic forces in either the initiation or augmentation of the disease [186]. There is still controversy as to what constitutes the earliest lesion of human atherosclerosis, though major morphological hallmarks of

atherosclerosis are lipid deposition, intimal penetration of blood-borne monocytes, smooth muscle cell proliferation and fibrosis [187,188]. Normal arterial intima is constituted of proteoglycans, collagens, elastin, fibronectin and laminin. These components contribute to the normal function and integrity of the arterial wall [99,189,190,191]. The extracellular matrix is the medium through which essential nutrients are transported across the intima, the site for accumulation of products released by intimal cells, the site of the accumulation of cell debris, and the avenue for the migration of cells entering and transversing the intima. Endothelial and smooth muscle cells are the principal cellular components of human arterial intima although isolated macrophages are also always present [192,193]. The presence of macrophages in the arterial intima have substantial implications, not only for lipid clearance (LDL receptor pathway) and accumulation, but in relation to connective tissue proliferation which is one of the hallmarks of a mature atherosclerotic lesion.

The arterial wall is composed of four components: the endothelium, intima, media and adventitia. The endothelium serves a number of important functions which include acting as a permeability barrier, the mediation of vascular tone [194,195], the synthesis and secretion of plasminogen activator [196] and the rapid metabolism of platelet aggregating agents [184,197]. The arterial intima is defined as the region of the arterial wall containing at most, endothelium, basal lamina, subendothelial connective tissue, and internal elastic lamina (which may be absent in many vessels). Surrounding the intima is the media, the largest component of the arterial wall and is composed of collagen, elastin and smooth muscle fibres. The elasticity of the media sustains blood pressure between heart beats. Finally, the adventitia surrounds the media and is composed of elastic collagen fibres and small blood vessels called vasa vasorum which enter the outer layers of the media and supply the vascular wall with nutrients.

Components of the arterial wall such as proteoglycans (PG) have been implicated in the development of atherosclerosis through their ionic binding and retention of low density lipoproteins and lipoprotein Lp(a) [198,199]. Lp(a) is a lipid molecule similar in lipid composition to LDL, except that Lp(a) has an additional apolipoprotein (apo a) attached via one disulfide bridge [199]. It is thought that Lp(a) may interfere with fibrinolysis *in vivo* by interfering with the binding of plasminogen to fibrin clots thereby inhibiting the conversion of plasminogen to active plasmin (proteolytic enzyme). The synthesis of PG by smooth muscle cells can be modulated by IL-1, prostaglandins and platelet derived growth factor [200,201]. In atherosclerotic lesions, the concentration of arterial PG such as sulphated glycosaminoglycans increases [189,202].

Accumulation of AGE on collagen increases its mechanical stiffness and its resistance to proteolytic degradation [114,151]. AGE content increases with age of the human subject and hyperglycaemic states. It has been suggested that chronic tissue damage associated with diabetes mellitus may arise in part from the continuous accumulation of serum proteins and subsequent *in situ* immune complex formation. Immunoglobulins have been found to bind to long-lived structural proteins that have undergone excessive non-enzymatic glycosylation [203,204]. Increased accumulation of AGE in tissue could lead to the increased deposition of plasma proteins within the arterial wall. In addition to the effects that AGE have on the mechanical properties of collagen, AGE-modified proteins can be recognised and degraded by macrophages. Receptors for AGE have now been isolated and characterised in macrophages, monocytes and endothelial cells [81,205]. AGE interaction with macrophages initiates a sequence of cytokine-mediated processes known to promote tissue remodelling [178] by initiating cellular proliferation, new matrix protein synthesis and release of extracellular proteases [206]. These processes may affect the development of atherosclerotic plaques.

Specific receptors for AGE have been found on mouse macrophages/monocytes, rat mesangial cells and bovine endothelial cells. The binding of AGE to receptors on the surface of mesangial cells have been found to increase extracellular matrix production [207,208], as well as increasing the endothelial monolayer permeability and to modulate the surface anticoagulant properties of endothelial cells [144,209]. AGE/macrophage reactions are also known to trigger the synthesis and release of cytokines; in particular interleukin-1 β (IL-1 β), tumour necrosis factor- α (TNF- α) which promote growth responses in smooth muscle cells [210,211,212] and fibroblasts [213] and contribute to the removal and replacement of extracellular tissue components, thereby promoting atherosclerotic plaque formation [178,214]. The release of such factors can in turn prompt the release of additional mediators such as platelet derived growth factor [215,216], as well as proteolytic enzymes such as collagenase [217], which collectively provide the additional signals required for tissue remodelling. The increase in connective tissue content of the plaque is brought about by the proliferation of smooth muscle cells within the arterial intima [218] and by an increase in the synthesis of extracellular connective tissue elements such as collagen and elastin [185] by cells of mesenchymal origin. This increase in connective tissue content contributes to the occlusion of vessels and affects flow of blood.

The binding of AGE to specific receptors found on monocytes induces the release of insulin growth factor (IGF-1) [219]. IGF-1 is known to be a growth promoting peptide [220,221].

AGE-modified proteins are chemotactic for monocytes, therefore the accumulation of AGE in the vessel wall, either on the structural components or in the subendothelial space would induce monocyte migration, AGE uptake,

intracellular degradation and cytokine secretion which could eventually lead to tissue remodelling [178,214]. Data so far accumulated support the notion that AGE proteins might contribute to the progressive tissue disorganisation by interacting with AGE receptors found on T-lymphocytes, fibroblasts and smooth muscle cell thereby promoting abnormal cellular proliferation and protein synthesis observed during atherosclerotic plaque formation [222].

Esposito et al observed that monolayer permeability and the coagulant properties of endothelial cells were altered by binding of AGE-BSA [144]. Endothelial binding of AGE-BSA was inhibited by AGE-modified ribonuclease and haemoglobin. Increased monolayer permeability could result in the increased passage of atherogenic molecules such as low density lipoproteins [223,224]. The entrapment of LDL in the intima of the arterial wall makes it more susceptible to lipid peroxidation. Modified LDL are avidly taken up by macrophages to form the foam cells [187,225]. Foam cells are found in the early stages of atherosclerotic plaque formation. AGE binding also led to the down regulation of the anticoagulant endothelial cofactor thrombomodulin and induced the synthesis of procoagulant cofactor and could promote clot formation [226,227]. The consequences of the accumulation of AGE in tissue are summarised in table 1.6.

Schmidt et al have shown that AGE can modulate mononuclear phagocyte migration, indicating a possible mechanism by which AGE may contribute to the pathogenesis of vascular lesions [228]. Soluble AGE attract mononuclear phagocytes into the vessel wall, whereas matrix-associated AGE cause retention and activation of mononuclear phagocytes in the subendothelium [214]. The ability of AGE to modulate phagocyte migration could contribute to the pathogenesis of vascular lesions.

The exposure of mouse mesangial cells to AGE-modified proteins increases extracellular matrix production [187]. This would be of importance in renal disease as it could lead to a progressive obliteration of vascular spaces and cause renal failure [229,230].

There is enhanced binding of erythrocytes from diabetic patients to endothelial cells which can be inhibited by anti-AGE IgG or antibodies to the receptor for AGE [231]. Binding of diabetic erythrocytes to endothelium generates oxidant stress and induces cytokine secretion. The over production of factors such as IL-1, TNF, PDGF may contribute to abnormal cellular proliferation and synthesis of extracellular matrix, which are hallmarks of atherosclerotic plaque formation.

Table 1.6

The effects of AGE accumulation in vascular tissue.

Tissue component involved	Mechanism	Consequences
Endothelium	Monokine response to AGE (INF, IL-1).	Increase in vascular permeability
Extracellular matrix	Decrease in binding of proteoglycans to matrix proteins with AGE.	Increase in vascular permeability
Extracellular matrix	Degradation of proteoglycans in the matrix, in response to AGE/macrophage binding [232].	Increase in vascular permeability
Extracellular matrix	Resistance of AGE crosslinked proteins to enzymatic degradation	Thickened, inelastic vascular wall
Extracellular matrix	Plasma protein deposits through AGE entrapment of proteins such as albumin and immunoglobulins	Structural changes in the vascular wall
Vessel wall	Proliferation of matrix and cells in response to AGE-induced secretion of growth promoting monokines from macrophages and endothelial cells.	Cell proliferation

The formation of AGE in tissue proteins is relevant to the development of the late complications of diabetes and atherosclerosis but it has been stressed that these diseases are multifactorial. The accumulation of AGE causes structural and functional modifications of tissue proteins. This induces changes in biosynthetic/secretory patterns of macrophages, endothelial and mesangial cells. Data so far accumulated has led to the formulation of a hypothesis which proposes a central role for the Maillard products in atherosclerosis and in the development of late complications of diabetes.

1.12. Mechanisms affecting AGE formation and accumulation.

1.12.1. Glycaemic control

A recently completed Diabetes Control and Complications Trial (DCCT) has shown that intensive treatment of patients with insulin-dependent diabetes mellitus, with the goal of maintaining blood glucose concentrations close to the normal range, delays the onset and slows the progression of diabetic complications such as retinopathy, nephropathy and neuropathy [233]. AGE have been implicated in the late complications of diabetes. The relationship between hyperglycaemia and macrovascular disease is complicated by other factors such as abnormal changes in lipoprotein metabolism, platelet aggregation and the secretion of factors with growth promoting potential which influence atherogenesis [16,19,234]. Hyperglycaemia has been linked to the increase in non-enzymatic glycosylation of proteins leading to the formation of AGE [61].

Beisswenger et al found that tissue levels of AGE increase significantly with worsening glycaemic control in patients with type I diabetes [235]. The most obvious way to modify the levels of AGE would be by improved glycaemic

control. Lyons et al in a 4 month study using diabetic patients investigated the effect of improved glycaemic control on skin glycation and AGE levels [63]. Skin glycation decreased in patients after improvement of glycaemic control but AGE levels measured as pentosidine, carboxymethyllysine and fluorescence levels, remained unchanged. This confirms that AGE formation is irreversible. However improved glycaemic control decreases Amadori product formation thereby inhibiting the further formation and accumulation of AGE. The normalisation of glucose levels in patients with diabetes mellitus by pancreatic transplantation should decrease the rate of accumulation of AGE on long-lived proteins.

1.12.2. Renal transplantation.

Patients with chronic renal failure (CRF) have a cardiovascular mortality 20 times higher than the general population [236,237]. Cardiovascular mortality is particularly high in recipients of renal transplants. In addition to an increased prevalence of hyperlipidaemia, other, as yet unknown causative factors presumably exist in these patients. Several studies have shown that elevated AGE levels correlate with the severity of nephropathy [160,182,238]. The metabolism of AGE-modified proteins, which are both chemically and biologically active, has not as yet been fully elucidated [239]. If the breakdown products of AGE-modified proteins are eliminated through kidneys, loss of renal function could lead to the accumulation of AGE in blood and tissue.

Renal dysfunction involves two major processes. Firstly there is the obliteration of glomerular capillaries due to the expansion of mesangial tissue [229,230]. This expansion involves the accumulation of mesangial matrix which is a complex extracellular material that includes type IV and type V collagen, fibronectin, laminin and proteoglycans [240,241,242]. In most experimental

models, progressive renal disease is not limited to the matrix deposition but includes mesangial cell proliferation and accumulation of macrophages in the kidney [243]. Mesangial cells are primarily responsible for the maintenance of glomerular mesangium by providing a structural supportive role, and are contractile and phagocytic [244]. When interacting with AGE-modified proteins, macrophages are known to release IL-1 β , TNF, PDGF and insulin like growth factor [219]. In response to these, mesangial cell proliferate [214,245] and extracellular matrix is synthesised and deposited. Mesangial cells have receptors for AGE. Mesangial cells cultured on dishes coated with AGE-BSA increase both matrix production and secretion. Increased matrix synthesis leading to the progressive obliteration of the vascular spaces is central to the disease process of end stage renal disease (ERSD) [246].

Three types of therapy are used to treat renal failure: haemodialysis, continuous ambulatory peritoneal dialysis (CAPD) and renal transplantation. CAPD employs the peritoneum as a dialysis membrane. Presently used dialysis fluids contain high concentrations of glucose. Little attention has been given to the potential detrimental effects of continued exposure of the peritoneal membrane to high glucose concentrations in the dialysis fluid. Recently, the study of peritoneal biopsies in CAPD patients suggested that non-enzymatic glycosylation of proteins is responsible for changes in the stromal texture and the reduplication of basement membrane leading to reduced fluid transfer across the peritoneal membrane [247,248, 249].

Makita et al have shown elevated levels of serum AGE in patients with diabetic nephropathy. Serum AGE decreased in both patients treated with haemodialysis and renal transplantation [164,250]. Hricik et al hypothesised that combined pancreatic-kidney transplantation would have a greater effect on lowering pentosidine levels than kidney transplantation alone, as the effect of renal

transplantation would be reinforced by improved glycaemic control [171]. However, improved glycaemic control and restoration of renal function had no obvious benefit when compared to the kidney transplants alone. Hricik et al concluded that renal transplantation was the key factor in reducing plasma pentosidine levels.

1.12.3. Pharmacological intervention.

Therapeutic agents which inhibit AGE accumulation on proteins could act by competing with glucose for the glycation site on the protein molecule or react with the Schiff base or Amadori product thereby inhibiting the generation of AGE [238]. Some potential agents are listed below:

(1.) Ascorbic acid (vitamin C) and acetylsalicylic acid have been shown to inhibit glycation of serum proteins and collagen by competing with glucose as a reducing agent for amino groups [251,252]. Both compounds also inhibit the formation of high molecular weight aggregates in lens crystallin [253,254,255].

(2.) Aminoguanidine, a nucleophilic hydrazine compound inhibits AGE formation by reacting with α -oxoaldehydes, 3-deoxyglucosones and 2-glucosulose, and α,β -dicarbonyl compounds formed during the degradation of fructosamines [350] and methylglyoxal [351]. Brownlee et al showed that aminoguanidine prevented the glucose induced crosslinking of collagen *in vitro*. Cyanogen bromide digestion of collagen incubated with glucose showed that amounts of high molecular weight crosslinked peptides increased with incubation time. Arterial collagen, isolated from diabetic rats treated with aminoguanidine, also showed decreased crosslinking compared to untreated diabetic rats [167,256,257,258]. The accumulation of fluorescent AGE was

greater in the aortic tissue of the untreated diabetic rats than in the diabetic rats treated with aminoguanidine. When compared to the collagen from untreated animals, the collagen isolated from diabetic rats treated with aminoguanidine, was more susceptible to solubilisation by acetic acid, chemical cleavage by CNBr and proteolytic degradation by pepsin [167,255,257]. Aminoguanidine has been shown to prevent diabetes induced collagen crosslinking and, glucose induced vascular dysfunction in retina, peripheral nerve, aorta, and kidney [259,260,261,262,263].

Recent studies have shown that aminoguanidine also prevents increases in CLF in glomeruli and renal tubules isolated from the kidneys of diabetic rats [261].

(3.) D-lysine reacts directly with free glucose and thus prevents protein glycation [149,264]. The administration of L-arginine to diabetic mice led to increased solubility and therefore decreased crosslinking of collagen. This is thought to occur by a mechanism similar to that of aminoguanidine [265].

(4.) D-penicillamine, which is used in the treatment of rheumatic disease, inhibits hexose-induced crosslinking of RNase *in vitro* [29] and has been shown to reduce crosslinking of collagen from rats with experimental diabetes [266].

These agents are useful in determining the role that AGE play in the development of diabetic complications and atherosclerosis. The use of the agents mentioned above either alone or in combination to inhibit the development of diabetic complications and atherosclerosis remains a clinical possibility, at least in respect to AGE formation and accumulation. Clinical trials of aminoguanidine in the treatment of diabetic complications are now in progress.

Chapter 2.

Protein-linked fluorescence as a marker of AGE formation.

2.1 Summary.

This Chapter describes:

- (1.) In vitro formation of AGE-modified bovine serum albumin.
- (2.) The measurement of Amadori product.
- (3.) The effect of oxidative processes on the formation of advanced glycosylation endproducts.

2.2. Introduction.

Bovine serum albumin (BSA) has a molecular mass of 66,267 Da. BSA is a nonglycoprotein and is one of the few secreted proteins that lacks carbohydrate. BSA has a low tryptophan and methionine content and a high content of charged amino acids such as lysine and arginine [267]. The high content of lysine residues (10.1%) makes this protein susceptible to glycation. BSA has been used as a model protein in numerous advanced glycosylation studies. AGE-BSA has been used as a standard in both radioimmunoassay and radioreceptor assays for AGE.

The aim of this study was to determine if fluorescence is a reliable marker of AGE accumulation on proteins and what effect oxidative processes have on fluorescence generation. We incubated BSA with glucose to produce AGE with a characteristic fluorescence at excitation wavelength 370nm and emission 440nm). The effect of glycooxidation was investigated by incubating the protein with butylated hydroxytoluene (BHT); a known antioxidant [268,269]. BHT is used in protecting foodstuffs, animal feed and petrol from oxidative damage. The effect of glucose autoxidation was studied using sorbitol, a potent scavenger of the hydroxyl radical [270,271].

Aminoguanidine, an inhibitor of AGE formation, was added to confirm that the generation of fluorescence was due to the accumulation of AGE on albumin.

2.3. Materials and methods.

2.3.1. In vitro glycosylation of bovine serum albumin using D-glucose.

BSA (essentially fatty acid free, fraction V, lot No 126F-9350, Sigma Chemical Co Ltd, Dorset Poole, UK), (100mg/ml) was dissolved in 0.5M phosphate buffered saline (PBS) pH 7.4 and D-glucose was added to a final concentration of 1.67M. The BSA-glucose solution was filtered through a Millex-GV (0.22µm) filter unit (Millipore S.A. 67 Molesheim, France). Toluene (10µl) was added to prevent bacterial growth. The sample was incubated at 37°C over a period of 3-28 days. BSA incubated without D-glucose was used as a control. To determine the effect of an antioxidant on AGE formation, BHT (0.005%) or sorbitol was (200mM) was added to BSA and glucose, at the start of the incubation. To inhibit AGE formation, aminoguanidine (200mM) was added to the mixture of BSA and D-glucose.

Both control and glycosylated BSA were sampled at pre-determined periods (3,6,9,12,15,28 days). After each of the time periods, incubated BSA was extensively dialysed against deionised water over 2 days with changes of deionised water every 3hours. A molecular porous dialysis membrane with a molecular cut off weight of 12-14 kDa was used (Pierce and Warriner (UK)).

2.3.2. Determination of protein concentration.

The protein concentration of the dialysed sample was determined using a colorimetric (Lowry) method [272].

A BSA stock standard (1000 $\mu\text{g/ml}$) was diluted to give protein standards ranging from 0-1000 $\mu\text{g/ml}$. Lowry reagent (1ml) was added to the samples and BSA and allowed to stand at room temperature for 20min. Stock Lowry reagent 100ml (2% sodium tartrate), 1ml (1% Na_2CO_3 in 0.1M NaOH), 1ml (2% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$).

Folin and Ciocalteu's phenol reagent (2M phenol reagent diluted 1:1 with distilled water) was added (0.1ml), with rapid mixing, and the colour was allowed to develop over 30 minutes. Absorbance was read within 30 minutes on a Sp8-100 Ultraviolet spectrophotometer (Pye Unicam Ltd, Cambridge, England, UK) at 750nm. Absorbance values were plotted against the protein standard concentrations to obtain a calibration curve. A typical standard curve is shown in fig 2.1. The protein concentrations of both control and glycosylated BSA were calculated using the standard curve.

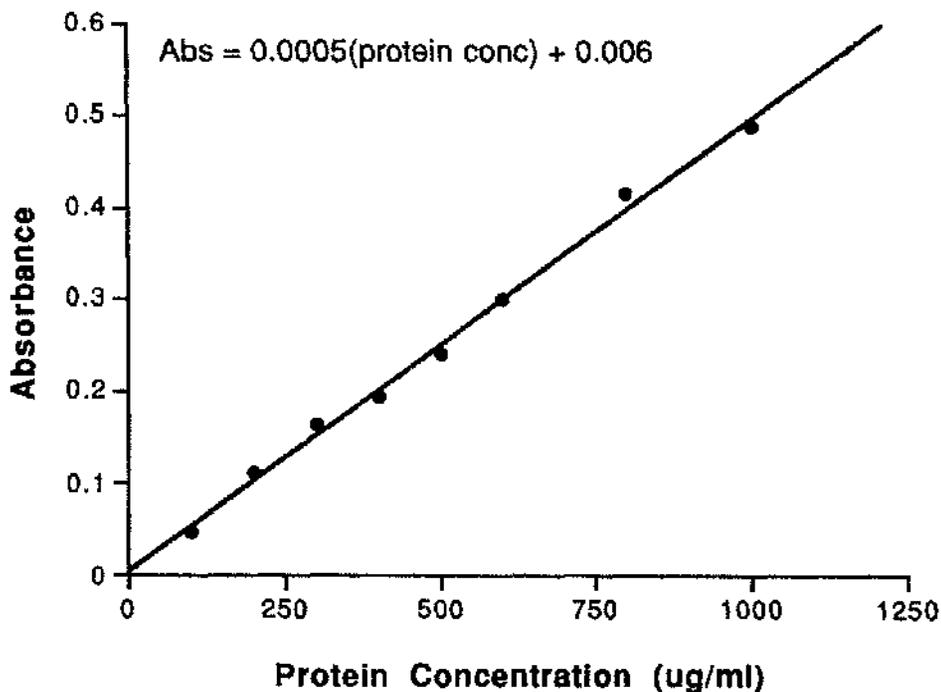


Figure 2.1

A typical standard curve from a Lowry protein assay. Each point is an average of two readings.

2.3.3. Measurement of Amadori products.

The amount of glycosylated BSA was determined using two methods: affinity chromatography, using glycogel columns, and the measurement of fructosamine content.

2.3.4. Affinity chromatography.

Affinity chromatography was carried out using glycogel columns (Pierce Warriner, UK). Glycogel is a chromatographic support (6% beaded agarose) which incorporates an immobilised ligand, *m*-aminophenyl boronic acid. The Amadori product on proteins reacts with the immobilised

boronic acid and forms a covalent five member ring complex which can be dissociated by sorbitol. Glycogel is insensitive to moderate temperature and pH fluctuations.

The columns were stored at 4°C and were equilibrated using 5.0ml equilibration/wash buffer (EWB: 250mmol/L ammonium acetate, 50mmol/L magnesium chloride, 500mmol/L sodium chloride, 3mmol/L sodium azide, 0.1% Tween 20 detergent, pH 8.5). The buffer was allowed to flow through the columns and the effluent was discarded. The columns were suspended over an appropriate reservoir.

The protein concentration of control and glycosylated samples was adjusted to 10mg/ml and 200µl of each sample was loaded onto the affinity column. The samples were allowed to pass into the columns and 0.5ml of EWB was added to ensure complete incorporation of the samples into the chromatographic support. More buffer (EWB; 19.5ml) was added and the total effluent was collected (total volume 20.2ml). Native BSA (non-glycosylated) was present in the this fraction (non-bound fraction; NBF).

To elute the bound fraction (BF) which contained glycosylated BSA, 3.0ml of albumin elution buffer (Alb-EB: 200mmol/L sorbitol, 500mmol/L sodium chloride, 50mmol/L Na₂ EDTA, 100mmol/L Tris, 0.1% Tween, pH 8.5) was added and the entire fraction was collected. The measurement of the amount of glycosylated protein was performed by measuring the protein concentration of the non-bound and bound fraction by the method of Lowry. Blank corrections were made using distilled water that had been run through a separate column. Protein yield from the columns was >98%.

The degree of glycosylation (%) was calculated as follows:-

$$\frac{\text{BF } (\mu\text{g})}{\text{BF } (\mu\text{g}) + \text{NBF } (\mu\text{g})} \times 100 = \% \text{Glycosylated BSA.}$$

2.3.5. Fructosamine measurement.

Fructosamine (1-amino-1-deoxyfructose) is a ketoamine; a derivative of the non-enzymatic reaction product of a reducing sugar and a protein. Colorimetric measurement of fructosamine was carried out using a Cobas Bio centrifugal analyser (Roche Diagnostics, Welwyn, Garden City, UK). Fructosamine was measured using a Roche fructosamine calibrator which was standardised by the manufacturer against glycated polylysine and human serum albumin glycated with ¹⁴C-glucose. Unlike the use of affinity columns there is no need for the separation of glycosylated and non-glycosylated protein. The method was devised by Johnson et al [273] and is based upon the reducing ability of fructosamines in alkaline solution.

Samples were diluted appropriately, added to a carbonate buffer (pH 10.35) at 37°C and their absorbance was measured at 550nm, after 10 and 15 minutes. The 10 minute incubation period is necessary to allow fast-reacting, interfering reducing substances to react. Fructosamine concentrations were expressed as umol of fructosamine per gram of BSA (umol/g).

2.3.6. Measurement of AGE content using protein-linked fluorescence.

The fluorescence at excitation wavelength 370nm and emission wavelength 440nm is characteristic of advanced glycosylation endproducts.

After extensive dialysis of the glycosylated BSA, the sample was diluted with distilled water and placed into clear four sided cuvettes (Sarstedt, Beaumont Leys, Leicester, England, UK.) and read using the LS-3B fluorescence spectrophotometer (Perkin-Elmer, Beaconsfield, Bucks, England, UK.).

Fluorescence of samples was expressed as arbitrary fluorescence units per milligram of protein (U/mg). The concentration of protein was measured by the method of Lowry. The sensitivity of the fluorescence measurement was reduced with readings over 80 fluorescence units (U), therefore glycosylated BSA with fluorescence over 80U was diluted with distilled water. Distilled water was used as a blank. The within assay coefficient of variation (CV) for protein-linked fluorescence was 3.2% and the between assay CV was 6.2%.

2.3.7. Statistics.

Results are expressed as mean \pm standard error of the mean (SEM). Statistical analysis was performed using the Student's unpaired t-test. Simple regression was calculated by assuming a linear model ($y=ax+B$) where appropriate and minimising the sum of the squares of the residuals for the fitted line. All analyses were carried out using Statgraphics software (Statistical Graphics System. (1986) Statistical Graphics Corporation Inc., Rockville, Maryland, USA).

2.4. Results.

2.4.1. The fluorescence of BSA incubated with D-glucose.

Emission spectra of BSA incubated over the 28 day period were obtained using excitation wavelength 370nm (fig 2.2). The fluorescence of BSA incubated with 1.67M D-glucose over a period of 28 days increased from 6.76 ± 0.43 U/mg at day 3 to 169.65 ± 8.31 U/mg at day 28. In the control sample, there was only a minimal increase in BSA fluorescence between day 3 (2.27 ± 0.26 U/mg) and day 6 (3.01 ± 0.23 U/mg). Fluorescence of the control sample remained unchanged thereafter (Table 2.3). Results are means of 3 independent experiments using the same batch of BSA. All protein measurements were carried out in duplicate and fluorescence measurements in triplicate.

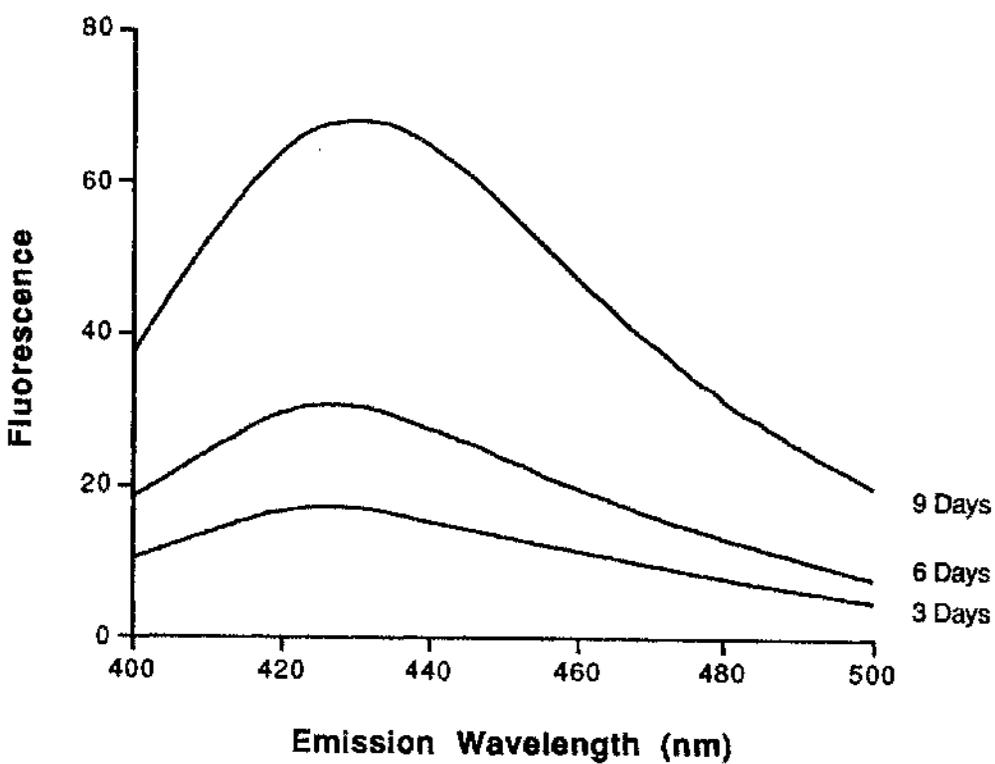


Figure 2.2

Emission spectra of BSA incubated with 1.67M D-glucose, excitation wavelength 370nm.

A. BSA incubated in the absence of D-glucose.

Incubation period (days)	Fluorescence (U/mg) mean	SEM	Compared to 3 day point (Students t-Test)
3	2.26	0.105	
6	3.01	0.227	p=0.016
9	3.44	0.437	p=0.034
12	3.98	0.497	p=0.007
15	3.20	0.097	p=0.006
28	3.38	0.389	p=0.010

B. BSA incubated in the presence of D-glucose.

Incubation period (days)	Fluorescence (U/mg) mean	SEM	Compared to 3 day point (Students t-Test)
3	6.75	0.43	p<0.001
6	18.86	1.65	p<0.001
9	39.14	1.66	p<0.001
12	54.25	3.12	p<0.001
15	77.98	4.54	p<0.001
28	169.65	8.31	p<0.001

Table 2.3

Incubation of BSA in the absence (A) or presence (B) of 1.67M D-glucose (0.5M PBS, pH7.4 at 37°C) over a period of 28 days.

2.4.2. The effect of aminoguanidine on AGE-related fluorescence.

Aminoguanidine, an inhibitor of AGE formation, was added to the mixture of BSA and glucose at the start of the incubation period and fluorescence was measured at excitation wavelength 370nm and emission wavelength 440nm over 28 days as described above. Aminoguanidine incubated with BSA in the absence of D-glucose was used as a control.

Aminoguanidine inhibited glucose-induced increase in fluorescence by 81% at day 28. There was only a slight increase in the fluorescence of BSA incubated with D-glucose in the presence of aminoguanidine between day 3 (2.7 ± 0.15 U/mg) and day 28 (32.2 ± 4.86 U/mg), fig 2.4.

The incubation of BSA with aminoguanidine in glucose-free media resulted in an increase in AGE-related fluorescence between days 15 and 28. No increase in AGE-related fluorescence was observed between day 3 (1.9 ± 0.18 U/mg,) and day 15 (3.7 ± 1.13 U/mg). Only at day 28 (15.0 ± 2.32 U/mg, $p=0.006$) was there a significant increase in fluorescence when compared to day 3.

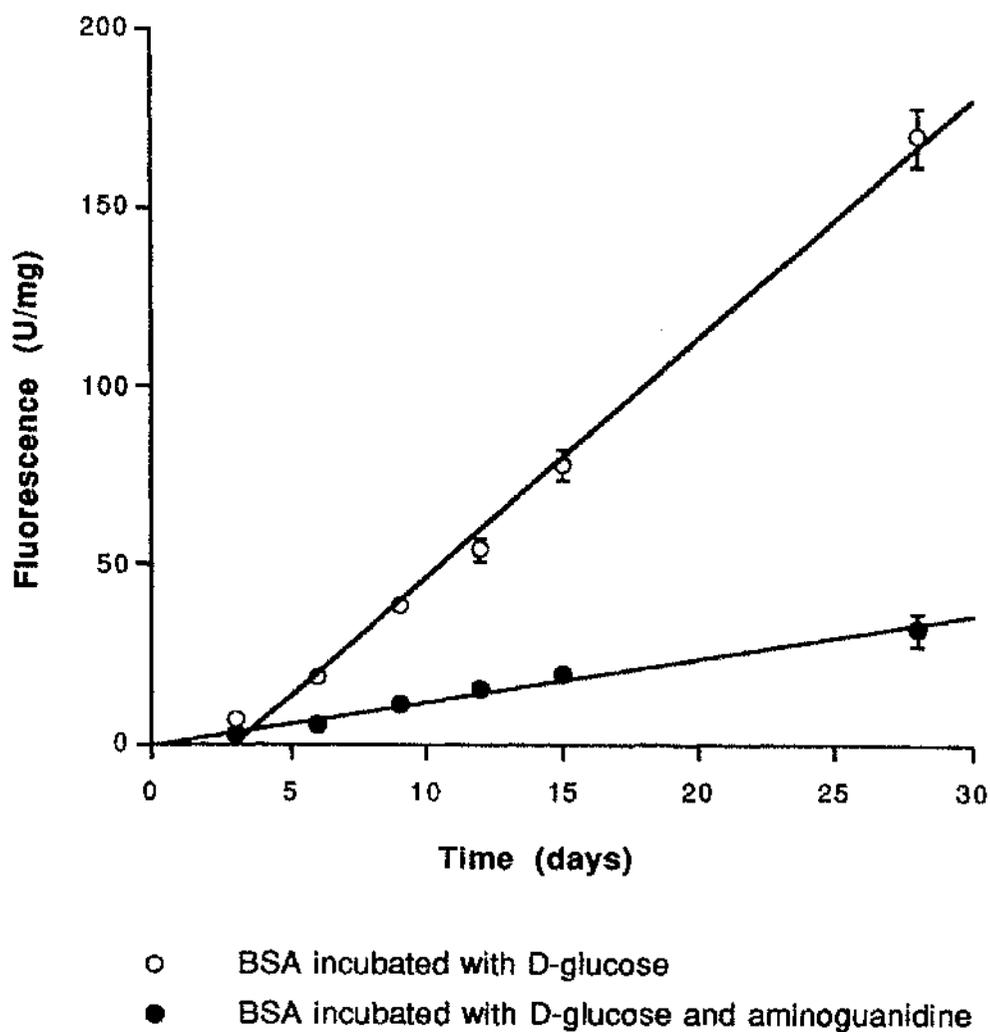


Figure 2.4

The effect of D-glucose (1.67M) and aminoguanidine (200mM) on AGE-related fluorescence over a 28 day period. Each point represents three individual experiments. Error bars denote SEM.

2.4.3. Effect of the antioxidant, butylated hydroxytoluene (BHT), on AGE fluorescence.

The addition of BHT to BSA and D-glucose also results in the increase of AGE-related fluorescence over the incubation period. BHT had no effect on the glucose-induced increase in fluorescence between day 3 (6.5 ± 0.47 U/mg) and day 9 (44.9 ± 2.63 U/mg) when compared to the fluorescence of BSA incubated with D-glucose alone (fig 2.5). In contrast, the addition of BHT resulted in an increase in fluorescence between day 12 (66.9 ± 3.64 U/mg, $p=0.020$) and day 28 (236.0 ± 11.88 U/mg, $p=0.023$), fig 2.5.

The addition of BHT to BSA in glucose free media resulted in a minimal increase of AGE-related fluorescence between day 3 (2.4 ± 0.38 U/mg) and day 28 (5.9 ± 1.45 U/mg, $p=0.001$). This increase was no different from fluorescence observed in control sample without glucose. Therefore BHT alone had no effect on AGE-related fluorescence in the absence of glucose.

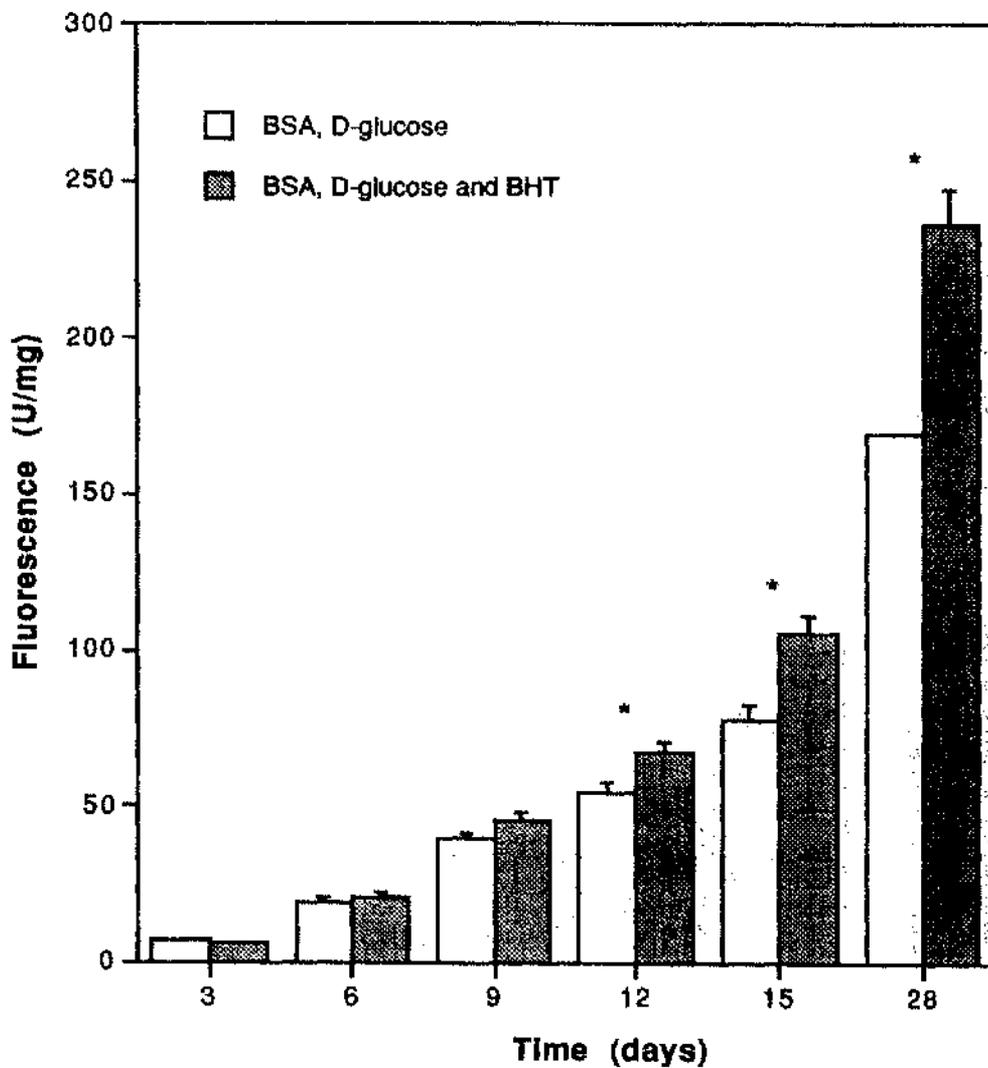


Figure 2.5

Effect of 0.005% BHT on the fluorescence of BSA incubated with 1.67M D-glucose. Excitation wavelength 370nm, emission wavelength 440nm. Error bars denote SEM. Results are the means of 3 individual experiments. * $p < 0.02$ from unpaired Students t-test.

2.4.4. Levels of Amadori products measured by affinity chromatography.

The incubation of BSA with D-glucose resulted in virtually complete Amadori product formation as assessed by affinity chromatography. No significant differences in Amadori products assessed by affinity chromatography were found between BSA incubated with D-glucose and BSA with D-glucose and BHT or aminoguanidine (table 2.6). In contrast to the increases in fluorescence, levels of Amadori product in glycosylated BSA remained unchanged after three days (fig 2.7).

	3 days	6 days	9 days	12 days	15 days	28 days
BSA	5%	4%	3%	3%	3.5%	4%
BSA + GLUCOSE	95%	99%	99%	100%	100%	100%
BSA + BHT	4%	4%	5%	4%	5%	4%
BSA+GLUCOSE+BHT	96%	97%	96%	99%	100%	100%
BSA + AG	4%	3%	4%	4%	4%	5%
BSA+GLUCOSE+AG	95%	96%	98%	98%	99%	100%

Table 2.6

Amadori product formation on BSA expressed as percent glycation after incubating BSA with and without glucose, BHT and aminoguanidine (AG) over 28 days. Results are the means of 3 individual experiments.

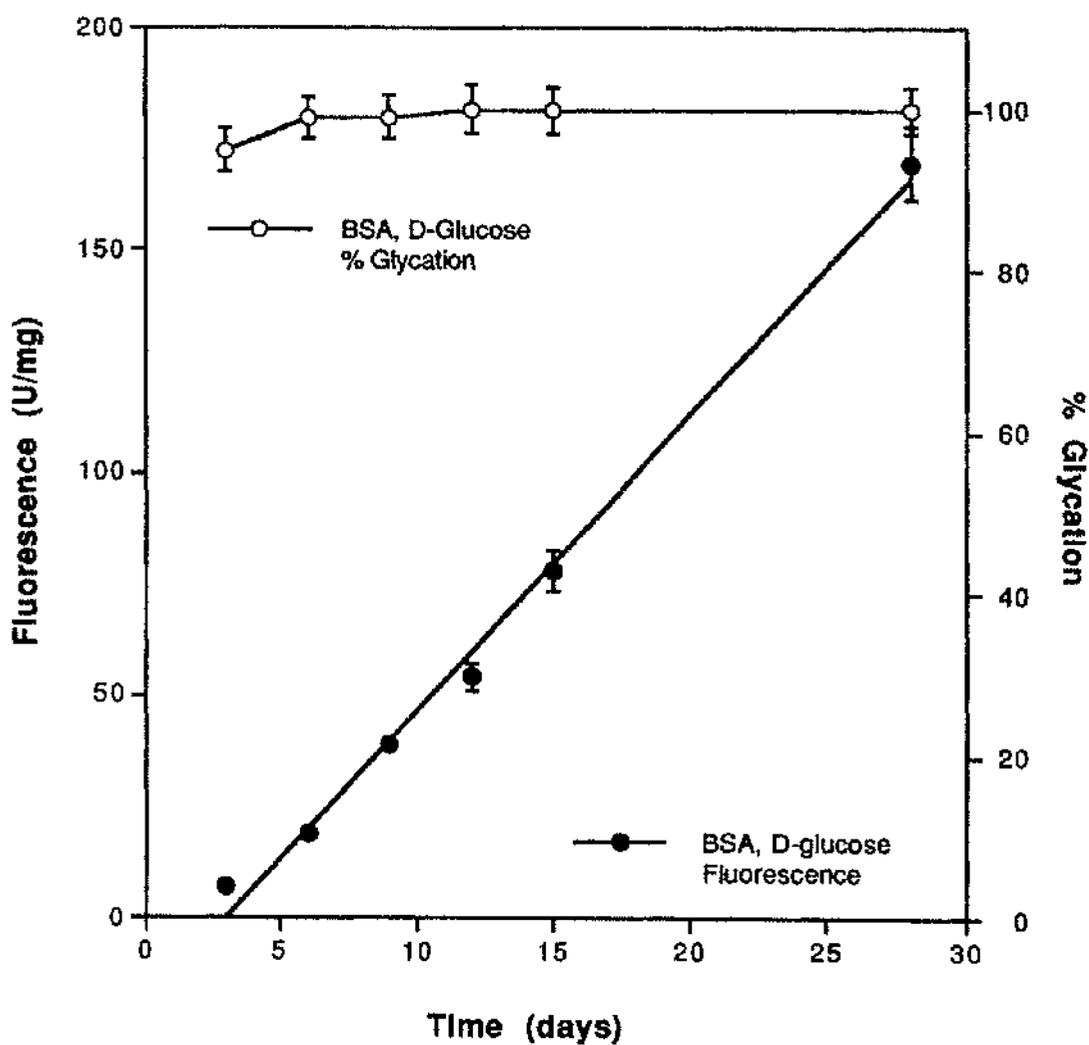


Figure 2.7

The effect of D-glucose on BSA glycation and fluorescence as assessed by affinity chromatography and fluorescence measurement. Error bars denote SEM.

2.4.5. The effect of sorbitol on the fluorescence of BSA incubated with D-glucose.

In this experiment, a different batch of BSA (Sigma (Lot No 11H-9312)) was incubated in the presence of 1.67M D-glucose, 0.005% BHT and 200mM sorbitol over a period of 9 days.

Fluorescence of BSA incubated in glucose free media remained constant over the 9 day period. The fluorescence of the BSA incubated with D-glucose increased from 11.8 ± 1.5 U/mg at day 3 to 54.2 ± 4.6 U/mg at day 9. BHT added to BSA in the presence of D-glucose caused a further increase in AGE-related fluorescence (12.4 ± 0.33 U/mg and 79.1 ± 2.36 U/mg) at day 3 and 9 respectively (fig 2.8).

In contrast, the addition of sorbitol resulted in a decrease of AGE-related fluorescence between day 3 (10.3 ± 0.8 U/mg) and day 9 (47.5 ± 4.1 U/mg) compared to BSA incubated with D-glucose alone at day 6 ($p=0.010$) and day 9 ($p=0.025$) (fig 2.8).

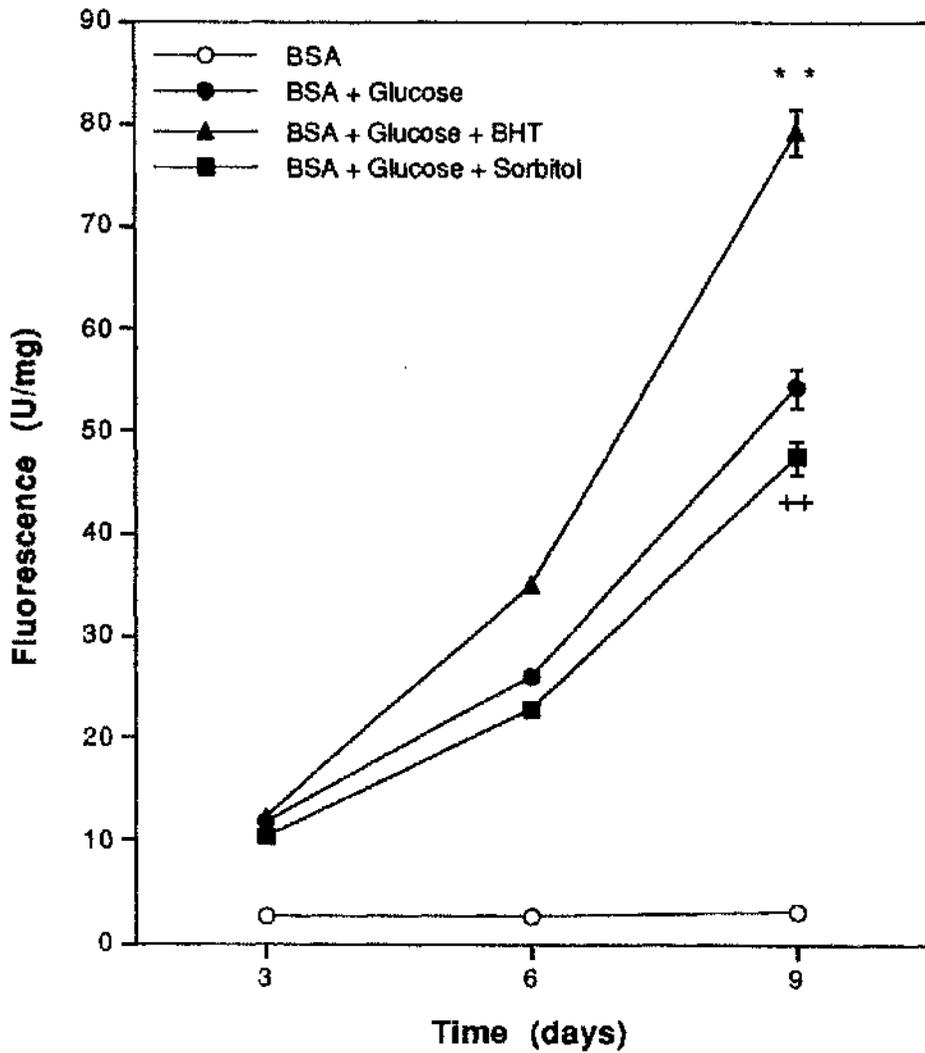


Figure 2.8

The effect of BHT and sorbitol on AGE-related fluorescence. Results are the means of 3 individual experiments.

** BSA + Glucose + BHT vs BSA + Glucose, $p < 0.001$

** BSA + Glucose + BHT vs BSA + Glucose + Sorbitol, $p < 0.001$

++ BSA + Glucose + Sorbitol vs BSA + Glucose, $p = 0.025$

2.4.6. The effect of BHT and sorbitol on the fructosamine content of BSA incubated with D-glucose.

In the absence of D-glucose, fructosamine levels of BSA decreased significantly over the 9 day period. D-glucose resulted in an increase in BSA fructosamine content from 277.2 ± 9.4 $\mu\text{mol/g}$ at day 3 to 475.0 ± 27.2 $\mu\text{mol/g}$ at day 9 (fig 2.9).

In the presence of glucose, further increases in fructosamine levels of BSA were observed on addition of BHT. At day 9, levels of fructosamine were higher in BSA incubated with D-glucose and BHT compared to BSA incubated with D-glucose alone ($p=0.02$). In contrast when sorbitol was added, the levels of fructosamine were lower compared to BSA incubated with D-glucose alone (fig 2.9).

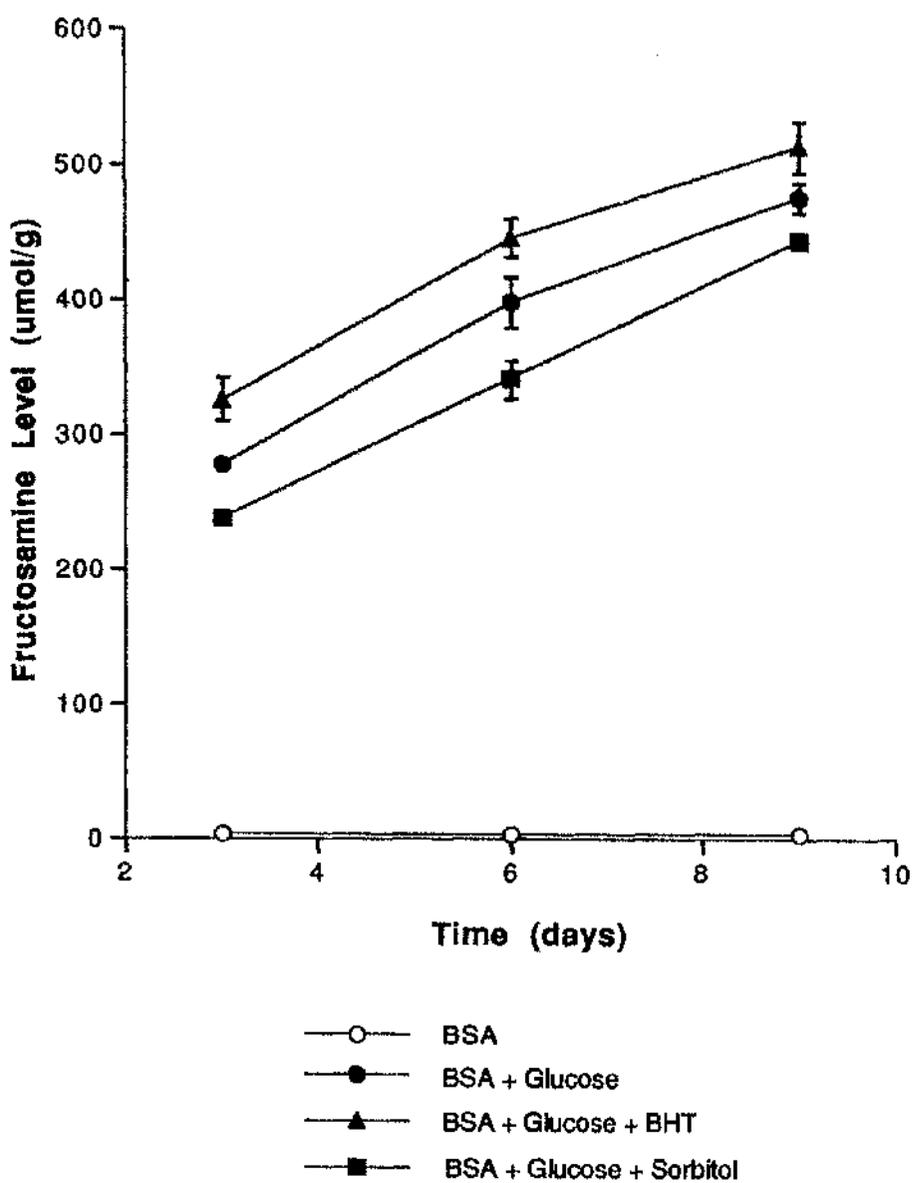


Figure 2.9

The effect of D-glucose, BHT and sorbitol on BSA fructosamine level.

Experiments were carried out in triplicate and error bars denote SEM.

2.4.7. Number of glucose adducts on each BSA molecule.

The amount of protein-glucose adducts on each BSA molecule can be calculated from fructosamine content. This was carried out by using Avogadro's constant (6.022×10^{23}) and assuming the molecular mass of BSA is 66,267 and that all BSA molecules are glycosylated to the same degree. Thus as an example BSA incubated with D-glucose at day 3 (277.2 $\mu\text{mol/g}$) :-

- (1.) $277.2 \mu\text{mol} = 1.67 \times 10^{20}$ protein glucose adducts per gram of BSA.
- (2.) $1.67 \times 10^{20} / 9.09 \times 10^{18} = 18.4$ protein glucose adducts found per BSA molecule.

This gives an insight to the number of glucose adducts found on each protein molecule, table 2.10. This calculation is based on the assumption that all BSA molecules are glycosylated to the same degree.

Days	BSA alone	BSA + glucose	BSA + glucose + BHT	BSA + glucose + sorbitol
3	0.3	18.4	21.5	15.7
6	0.25	26.3	29.4	22.4
9	0.2	31.4	33.8	29.3

Table 2.10

Fructosamine levels expressed as the number of protein glucose adducts per BSA molecule.

2.5. Discussion.

2.5.1. Fluorescence of BSA incubated with D-glucose.

Fluorescence at excitation wavelength 370nm and emission wavelength 440nm is a marker for AGE formation on proteins and correlates with increased protein crosslinking [151,242,274]. However the inherent lack of specificity of fluorescence measurement remains a problem. The use of fluorescence spectroscopy in the measurement in plasma is not possible since there are too many interfering substances. This problem of interfering substances does not occur when the fluorescence of an isolated, purified protein is measured. It has been suggested that fluorescence at similar wavelength may also be generated as a result of oxidative processes. Recent research has shown that the autoxidation of glucose is slow under physiological conditions and only becomes significant in protein glycation processes at high phosphate and glucose concentrations (>200mM). Moreover the major products of glucose autoxidation are arabinose and glyoxal [352,353]. Wolff and Dean showed that autoxidative glycosylation, in which α -ketoaldehydes are formed from the metal-catalysed oxidation of glucose react with proteins and initiate the advanced stages of the Maillard reaction [66,67,68]. This can contribute to total protein-glucose adduct formation and changes to fluorophoric properties [270].

In the present study the fluorescence of BSA incubated in the absence of glucose increased slightly between day 3 and day 6 of the incubation period but remained unchanged thereafter. This presumably reflects the conversion of endogenous Amadori products to AGE. Fluorescence of BSA incubated with glucose increased in a linear fashion. In the presence of glucose, protein-linked fluorescence was higher than the control as early as day 3. However, no plateau of fluorescence was observed during the 28 day incubation period.

2.5.2. Inhibitory effect of aminoguanidine on AGE formation.

Aminoguanidine inhibited fluorescence generated during the incubation of BSA with D-glucose. Aminoguanidine is a nucleophilic hydrazine compound. Edelstein and Brownlee, using ^{14}C labelled glucose, showed that in contrast to the inhibition of AGE formation, Amadori product formation was not inhibited by aminoguanidine and suggested that aminoguanidine reacts with the Amadori-derived fragmentation products such as 3-deoxyglucosone [275]. Aminoguanidine has been shown to prevent AGE formation on arterial collagen and inhibit protein crosslinking [167]. Collagen isolated from aminoguanidine-treated streptozotocin-diabetic rats shows a decreased level of fluorescence compared to diabetic rats on no treatment [276]. Soulis-Liparota studied the effect of aminoguanidine on experimental diabetic nephropathy and observed that aminoguanidine attenuated the rise in albuminuria, glomerular basement thickening and mesangial expansion [229,261,277]. Malik and Meek have suggested that aminoguanidine causes structural alterations of protein even in the absence of sugar. They observed that the intermolecular spacing of collagen decreased, indicative of increased cross-linking, when collagen isolated from corneal tissue was incubated with aminoguanidine [255]. The small rise in fluorescence between day 15 and day 28 in BSA incubated with aminoguanidine only, observed in the present study could reflect a change in the fluorescence properties of the protein itself rather than the formation of AGE.

In our study, aminoguanidine inhibited the increase in AGE-related fluorescence by 81% at day 28 of the incubation. On the other hand, the levels of Amadori products measured by affinity chromatography did not decrease. Percentage glycation of BSA incubated in the presence of glucose was comparable to BSA

incubated with glucose and aminoguanidine. This would suggest that aminoguanidine does not inhibit the early stages of Maillard reaction.

Affinity columns when using such a high glucose concentration (1.67M) to glycate the BSA, may have limited value, since it can be postulated that all BSA molecules with their abundance in glycation sites (lysine) will be glycosylated. This method does not measure the number of Amadori products formed on each BSA molecule but only indicates the percentage of glycosylated proteins found.

2.5.3. The effect of BHT and sorbitol on the levels of Amadori products formed on BSA.

As assessed by affinity chromatography, BHT and sorbitol had no effect on Amadori product formation of BSA incubated with D-glucose.

In addition to affinity chromatography, the number of glucose-protein adducts on the glycosylated BSA can be determined using the fructosamine assay. Fructosamine was detected on the control BSA suggesting that albumin isolated at source has residual glucose protein adducts. Levels of fructosamine in the BSA incubated in the absence of glucose decreased over the nine day incubation. This suggests that the Amadori product either dissociates to form sugar and protein or undergoes reactions which produce AGE. It would seem more likely that the dissociation of the adduct back to its constituent forms is more likely, since the absence of glucose substrate would favour the production of Schiff base from Amadori product (fig 1.2). However Eble et al on removing glucose from the media of browning proteins observed that AGE formation still continued [92]. Fructosamine content of control BSA indicates that not every BSA molecule is glycosylated, as the number of protein glucose adducts found on each BSA molecule is less than 1.

Fructosamine content of BSA incubated in the presence of glucose increased with time. At day 3 there were approximately 18 glucose protein adducts per BSA molecule and this increased to 31 at day 9. The addition of BHT to BSA-glucose mixture resulted in an increase in the Amadori product formation. There was also an increase in AGE-related fluorescence in BSA incubated with glucose and BHT. In contrast, sorbitol decreased the level of Amadori products and AGE-related fluorescence. This contrasts the studies of Hunt et al who showed that exposure of sorbitol to hydroxy radicals produced by the radiolysis of water produces ketoaldehydes which can contribute to glycofluorophore development [270]. The varying incubation conditions (protein concentration, buffer concentration and incubation time) could explain these differences [278]. Our results suggest that BHT and sorbitol affect the Maillard reaction at the stage of Amadori product formation

2.5.4. The effect of BHT and sorbitol on the generation of AGE-related fluorescence.

In the presence of trace amounts of transition metals, glycation is complicated by the ability of glucose to oxidise and generate reactive intermediates such as hydroxyl radicals, hydrogen peroxide and ketoaldehydes [69,279]. These species, produced by glucose autoxidation, are thought to contribute to the development of fluorescence and may cause structural changes such as fragmentation. Protein fragmentation can be inhibited by benzoic acid, deoxyribose and sorbitol [280]. Glucose autoxidation is inhibited by DETAPAC (diethylenetriaminepentaacetic acid) which sequesters the transition metals necessary for glucose autoxidation.

In our studies, BHT increased protein-linked fluorescence of BSA incubated with glucose by 39%. If oxidative species were to be produced by glucose autoxidation, antioxidant should have inhibited fluorescence generation. We used a high concentration of D-glucose (1.67M), compared to 25 mM in other studies. It seems that at this glucose concentration, BHT does not inhibit the generation of AGE. Fructosamine measurements suggests that the addition of BHT to browning BSA aids the production of AGE by increasing the number of Amadori products. Although free radicals generated from the autoxidation of glucose have been reported to increase the fluorescence of proteins (Wolff and Dean), the wavelengths (ex 350nm, em415nm) and incubation conditions used in fluorescence measurement were different to that used in the present study [69,153].

Sorbitol, a polyhydric alcohol and hydroxyl radical scavenger, decreased AGE-related fluorescence. This is in contrast to that reported by Woolf and Dean who observed an increase in fluorophore generation. Again it should be noted that measurement of fluorescence was carried out at different excitation and emission wavelengths. Fluorophore generation from BSA incubated with sorbitol and glucose has been reported to decrease in the presence of DETAPAC suggesting that transition metals are necessary for this process. DETAPAC has less influence on fluorescence at higher protein and glucose concentrations [271].

In the present study, sorbitol decreased the level of AGE-related fluorescence by 13% at day 9. This suggests that sorbitol acts as an inhibitor of AGE formation. Fructosamine measurements performed in this study suggest that sorbitol decreases AGE formation through a decrease in Amadori product formation and thus the generation of AGE fluorescence.

2.5.5. Fluorescence as a marker of AGE accumulation in tissues.

Protein-linked fluorescence reflects the majority, but not all of AGE present. At one time many structurally different AGE form on a given protein molecule. There is to date no ideal method of AGE measurement. The presently used methods include fluorescence, macrophage based radioreceptor, ELISA and HPLC. Each of these methods has disadvantages. The radioreceptor assay, although theoretically more specific than CLF, employs a non-physiological labelled ligand such as AGE-BSA, that has been produced in vitro. An immunochemical assay developed using AGE-RNase to produce anti-AGE antisera, has shown that the formation of AGE fluorophores precedes the formation of antibody-reactive material during the incubation of BSA with glucose. In the same study, using arterial tissues obtained from experimentally induced diabetic rats fluorescence and AGE content measured by ELISA increased two fold compared to age-matched non-diabetic rats [165]. Measurements of pentosidine, pyrraline or N-ε-(carboxymethyl)lysine using HPLC have the advantage of measuring chemically defined compounds, but again do not measure all AGE present. It has been reported that pentosidine accounts for less than 1% of the total AGE in vivo.

Using in vitro browned BSA, we validated fluorescence measurement as a marker of AGE accumulation in tissues. We demonstrated that aminoguanidine inhibited AGE formation. It has also recently been demonstrated by Hepburn et al that tissue CLF correlates well with pentosidine concentrations as measured by HPLC (unpublished data).

2.6. Conclusions.

- 1. Incubation of BSA with a high concentration of glucose generates fluorescence, at an excitation/emission spectra characteristic of AGE. Aminoguanidine, an inhibitor of AGE formation inhibits fluorescence generation. Thus protein-linked fluorescence reflects AGE formation.**
- 2. Oxidative processes affect the Maillard reaction. BHT, a chain breaking antioxidant, increases both fructosamine content and fluorescence of BSA incubated with glucose. This suggests that BHT aids the production of the Amadori product and consequently increases rate of AGE formation. Sorbitol, a hydroxyl radical scavenger, inhibits fluorescence of BSA incubated with a high concentration of glucose. This decrease is a consequence of the inhibition of the Amadori product formation as suggested by the decreased levels of fructosamine. The above experiments suggest that AGE-associated fluorescence was produced by processes that did not involve free radicals that could be scavenged by BHT or sorbitol in the rate-limiting step.**

Chapter 3

The binding of native and oxidised low density lipoprotein to AGE-modified type I collagen.

3.1. Summary.

This chapter describes:

- (1.) The in vitro preparation of AGE-modified type I calf skin collagen.
- (2.) The isolation of low density lipoprotein (LDL) from the plasma of normolipaemic subjects.
- (3.) The binding of native and oxidised LDL to native and AGE-modified type I collagen.

3.2. Introduction.

Collagen is one of the main targets for AGE accumulation. Recently, MacDonald et al measured AGE fluorescence in the mesenteric artery and observed a relationship between the age of the patient and AGE fluorescence [152]. Increased AGE formation on collagen may be one of the biochemical links between excessive LDL accumulation in the arterial wall and persistent hyperglycaemia. Binding of LDL to AGE-modified collagen could prevent the diffusion of LDL out of the intima and may make the LDL more susceptible to oxidation by a variety of cells present within the arterial wall: macrophages, smooth muscle cells and endothelial cells [281,282,283]. The oxidation of LDL results in it being taken up more readily by macrophages to form the foam cell; an important stage in atherogenesis [284]. In atherosclerotic plaques, LDL appears to be attached covalently to the protein components of the arterial wall [285,286]. LDL found in normal intima can be readily removed from the tissue samples by electrophoresis whereas LDL found in a fibrous plaque can only be released from the lesion by the use of proteolytic enzymes. Most of the LDL found in these lesions is extracellular. The reported ability of AGE-modified collagen to bind covalently to LDL could promote excessive fibrous plaque lipid formation. It is known that elastin prepared from atherosclerotic aortic intima binds more LDL [287,288]. The binding of LDL to AGE-modified collagen could indirectly facilitate its own glycation by prolonging its half life in vivo.

The aim of this study was to determine the extent of LDL binding to native and AGE-modified type I collagen in vitro and to investigate the effect of LDL oxidation on its ability to bind native and AGE-modified collagen.

3.3. Materials and Methods.

3.3.1. The preparation of AGE-modified type I collagen.

Type I calf skin collagen (Sigma, Poole, Dorset, UK) was AGE-modified by incubating collagen with D-glucose-6-phosphate (G6P) monosodium salt (Sigma, Poole, Dorset, UK) for 2 weeks. G6P (500mg) was dissolved in 7 ml of 0.5M PBS pH 7.4 to give a final concentration of 250mmol/L. Media (6ml) containing 250-1000mmol/L was added to type I calf skin collagen (100mg) and incubated for 2 weeks at 37°C. As a control, 100mg of calf skin collagen was incubated with 6ml of G6P-free 0.5M PBS pH7.4. Streptomycin (100µg) and penicillin (100 IU) were added to each incubation to prevent bacterial growth.

At the end of the incubation period, 10ml of 0.1M PBS pH 7.4 were added to both control and AGE-modified samples. The samples were centrifuged (Sorvall RT-6000 refrigerated centrifuge, Dupont (UK) Ltd, Stevenage, Herts, England, UK.) at 2000g for 15 minutes at room temperature, a pellet was obtained and the supernatant was discarded. This procedure was repeated 8 times to wash off any excess glucose-6-phosphate. The pellets were then washed three times with distilled water. These collagen preparations were used to study the binding of native and oxidised LDL.

3.3.2. Treatment of collagen for use in LDL binding studies.

Control and AGE-modified collagen samples were partially dried with filter paper (Whatman, Qualitative No 1) and lyophilised. Following lyophilisation the collagen samples were aliquotted into 5mg portions and stored at -20°C until analysis.

3.3.3. Measurement of collagen-linked fluorescence (CLF).

Aliquots of native and AGE-modified collagen were thawed and digested with 250U type VII bacterial collagenase (EC 3.4.24.3, Sigma, Poole, UK) in 1ml of buffer H for 24h at 37°C in a shaking water bath. After incubation the samples were centrifuged at 1000g on a bench top centrifuge at room temperature. Fluorescence was measured using the LS3B fluorescence spectrometer at excitation wavelength 370nm and emission wavelength 440nm and corrected against a collagenase blank. CLF was expressed as arbitrary fluorescence units per milligram of collagen (U/mg) as described above.

3.3.4. Measurement of hydroxyproline.

Procedure.

Solubilised collagen (50µl) was hydrolysed with 0.5ml of 6M HCl at 115°C on a Techne Dri Block DB4 (Fisons Scientific Equipment, Leicestershire, UK) in a stoppered glass hydrolysis test tube for 24 hours [289]. Glass beads were added to prevent bumping. Cis-4-hydroxy-1-proline (50µl of 300 µg/ml) solution was processed in parallel with the experimental samples and used to calculate the recovery of hydroxyproline after hydrolysis. After completion of hydrolysis, the samples were evaporated to dryness and distilled water (2.5ml) was added. A sample of the hydrolysate (1.0ml) was transferred into a glass reaction tube and chloramine T solution (1.0ml) was added, mixed vigorously and left standing at room temperature for 20min. A further 1ml of benzaldehyde/perchloric acid solution was added, stoppered and heated in a water bath at 60°C for 15min. After incubation, the test tubes were cooled

under water and the absorbance was read at 550nm on a Sp8-100 ultraviolet spectrophotometer (PYE) within 15 minutes.

The concentration of hydroxyproline in the solubilised collagen was calculated from the standard curve constructed using cis-4-hydroxy-1-proline (300 μ g/ml). Standard concentrations ranged from 0.3 μ g/ml to 6.0 μ g/ml (fig 3.1). The amount of collagen was calculated assuming 14% hydroxyproline content. The measurement of hydroxyproline was carried out in duplicate.

Reagents.

Citrate/acetic acid buffer (0.16M/1.07mM), sodium acetate trihydrate (0.59M) and sodium hydroxide (0.57M) were dissolved in 75ml of n-propanol and made up to a total volume of 375ml with distilled water.

Chloramine T solution (0.1M) was dissolved in 10ml of n-propanol and 10ml of distilled water then made up to a total volume of 100ml with citrate/acetic acid buffer.

Benzaldehyde/perchloric acid solution was prepared with 1M p-dimethylaminobenzaldehyde (Ehrlich's reagent) using 13ml of perchloric acid and made up to a volume of 50ml with n-propanol using a volumetric flask.

Quality control and assay precision.

To assess the degree of hydroxyproline degradation during hydrolysis an internal standard was run in parallel with the digested collagen samples. A 300 μ g/ml (50 μ l) of cis-4-hydroxy-1-proline solution was used to assess the

amount of hydroxyproline degradation. If the recovery of the internal standard was lower than 95%, the collagen sample was re-hydrolysed.

The within assay coefficient of variation (CV) at a CLF value of 12.4 U/mg was 4.5%. The between assay CV at a CLF value of 10.2 U/mg was 9%.

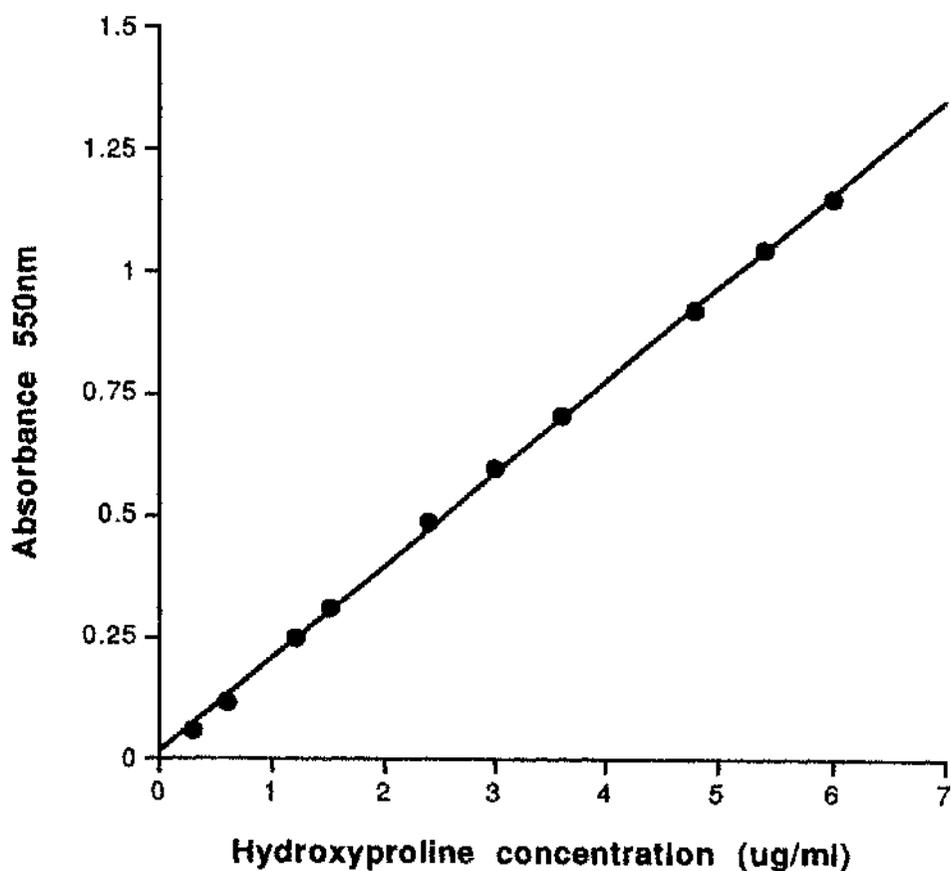


Figure 3.1

A hydroxyproline standard curve. Each point represents the mean of duplicate measurements.

3.3.5. Preparation of LDL.

Low density lipoprotein (LDL) was isolated from plasma obtained from normolipaemic subjects using flotation ultracentrifugation. Whole blood (70ml) was collected in EDTA vacutainers (Becton Dickinson, New Jersey, USA) and centrifuged at 400g at room temperature for 15min on a RT-6000 Sorvall centrifuge (Sorvall, Hertfordshire, UK). After centrifugation, 4ml plasma were transferred to a 13x64mm Beckman Ultraclear centrifuge tubes (Beckman Instruments Inc Spinco Division, Palo Alto, CA 94304, USA) containing 0.32ml of 0.195M NaCl and 0.5mM EDTA Na₂ (density, d=1.182g/ml). The tube was mixed by inversion then layered with 1.68ml NaCl solution of d=1.019g/ml and centrifuged at 100000g for 24h at 4°C on a L8-70M Beckman ultracentrifuge using a Ti 50 rotor (Beckman Instruments UK Ltd, High Wycombe, Bucks, England, UK.).

After ultracentrifugation, the top 2ml containing chylomicrons and VLDL were removed and discarded. To the remaining 4ml, 1.47ml of 2.44M NaBr solution (d=1.182g/ml) was added, mixed by inversion and then layered with 0.53ml of 1.063 g/ml NaCl solution. This was centrifuged at 100000g for 24h at 4°C. The top 2ml of the tube contents, containing LDL, were removed and the remainder was discarded.

3.3.6. Iodination of LDL.

Procedure.

The concentration of LDL was determined by measuring the UV absorbance at 280nm (1 absorbance unit = 1mg/ml) as a rough approximation, and then the protein concentration was measured by the method of Lowry. The concentration

converted into nanomoles (assuming 1mg of LDL is equal to 15.6nM of LDL, molecular mass of LDL = 64kDa). The Lowry method described in chapter (2.3.2) was modified by the addition of 1% sodium dodecyl sulphate to the Lowry reagent containing copper sulphate. LDL was radiolabelled with ^{125}I iodine by the method of McFarlane using iodine monochloride (ICl, 25mM) and 1M glycine, pH10. A ICl:LDL protein ratio of 2.5:1 was used., ICl was added to 1.5ml of LDL prediluted with 375 μl of glycine. Finally 0.01mCi ^{125}I (West of Scotland Radionuclide Dispensary, Western Infirmary, Glasgow, Scotland, UK) was added [290]. To separate the free ^{125}I from the labelled LDL, the ^{125}I -LDL was loaded onto PD-10 Sephadex columns. The columns were equilibrated with 25ml binding buffer before use. The sample was loaded on and allowed to run in to the column. Subsequently, LDL binding buffer was added to elute the ^{125}I -LDL. Fractions (0.5ml) were collected and the radioactivity measured with a Geiger counter (Series 900 mini monitor, Mini Instruments, Essex, UK). Fractions with the highest radioactivity were pooled and used in collagen binding experiments. A small aliquot (10 μl) of ^{125}I -LDL was measured on a NE 1612 Gamma counter (Nuclear Enterprises Ltd, Beenham, Reading, England, UK.) for 1 minute and LDL concentration was calculated. Protein yields from the Sephadex columns were greater than 90%. The specific activity of LDL was calculated as follows:-

$$\text{Specific activity} = \frac{\text{counts per minute}}{\text{total LDL concentration } (\mu\text{g})} = \text{cpm}/\mu\text{g LDL}$$

Reagents.

PD-10 Sephadex columns Pharmacia (Pharmacia Biosystems Ltd, Knowlhill, Central Milton Keynes, England, UK) containing Sephadex G-25M swollen in distilled water and 0.15% Kathon as a preservative.

LDL binding buffer contained 13.5mM KH₂PO₄, 46.6 mM Na₂PO₄ at pH 7.35.

3.3.7. Binding studies.

The ¹²⁵I-LDL was diluted to a final concentration of 200ug/ml and 300μl was added to the control and AGE-modified collagen in 5ml polypropylene tubes in the presence and absence of 0.5% albumin. The samples were incubated for 16h at 37°C. Control samples containing labelled LDL and no collagen were set-up alongside LDL/collagen incubations and were treated in exactly the same manner. On completion of the incubation period the samples were washed with LDL binding buffer until radioactivity measured in the control tubes decreased to 5% of the level in the tubes containing LDL and collagen. Radioactivity was measured on a NE 1612 Gamma counter for 1 minute.

The quantity of LDL bound to collagen was calculated as follows:-

$$(1.) \quad \frac{\text{LDL bound (cpm)}}{\text{Specific activity (cpm/}\mu\text{g LDL)}} = \text{LDL bound } (\mu\text{g})$$

$$(2.) \quad \frac{\text{LDL bound } (\mu\text{g})}{\text{Collagen weight (mg)}} = \text{LDL bound } (\mu\text{g)/collagen (mg)}$$

3.3.8. Oxidation of LDL using copper chloride.

LDL isolated from plasma was dialysed to remove excess EDTA using Spectrapor dialysis membrane with a molecular weight cut-off of 12-14kDa. Dialysis was performed against 0.01M PBS pH 7.4 for 24h at 4°C in a shaking

water bath. After dialysis, the LDL concentration was adjusted to 250 μ g/ml with 0.01M PBS pH 7.4. Copper chloride was added to the LDL to a final concentration of 5 μ M, mixed and incubated for 24h at 37°C. Oxidation was assessed by measuring fluorescence before and after the addition of CuCl₂ at excitation wavelength 320nm/emission 420nm and excitation 360nm/emission 420nm. The oxidised LDL was labelled with ¹²⁵I, purified on a Sephadex column and used in binding studies as described for native LDL.

3.3.9. Statistics.

Data were analysed on Minitab Statistics package (version 8, State college: Minitab Inc, 1991) using unpaired Students t-test and reported as mean \pm SEM.

3.4. Results.

3.4.1. The effect of glucose-6-phosphate on collagen-linked fluorescence.

Incubation of collagen with G6P over a 2 week period resulted in a gradual increase in CLF with increasing G6P concentrations. CLF of control collagen incubated in the absence of G6P, was 4.4 ± 0.35 U/mg. This was significantly lower than the G6P-incubated collagen at each of the G6P concentrations used. CLF increased from 12.4 ± 0.07 U/mg after incubation with 250mM G6P to 13.6 ± 0.04 U/mg after incubation with 1M G6P ($p < 0.001$; fig 3.2).

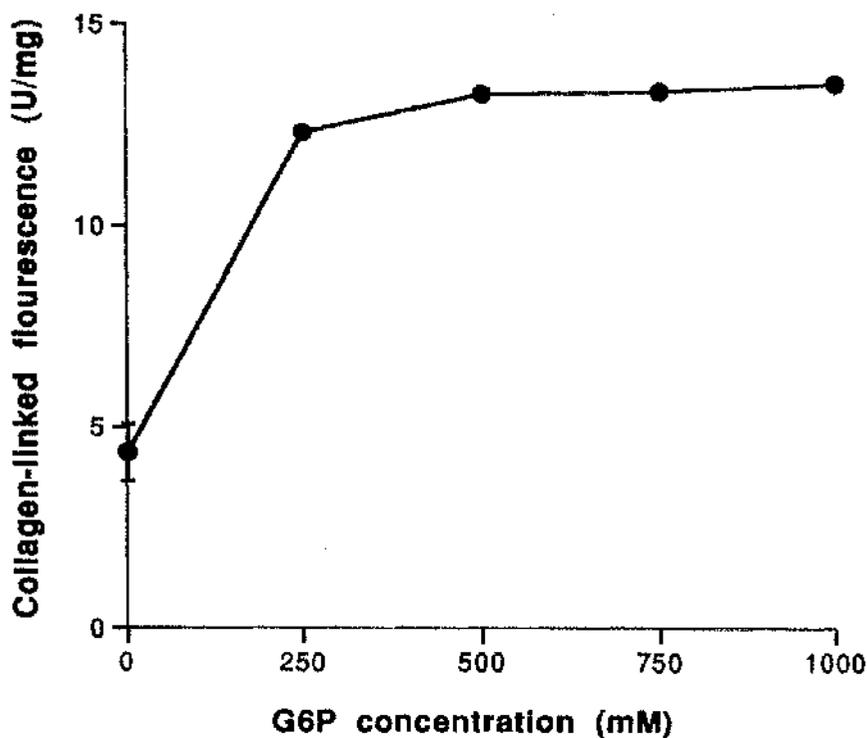


Figure 3.2

The effect of G6P on the generation of collagen-linked fluorescence. Results are the mean of 5 separate experiments and error bars denote SEM.

3.4.2. The effect of LDL concentration on its binding to collagen.

The binding of non-modified LDL to native collagen and to collagen modified by AGE was concentration dependent at LDL concentrations 200-1000 $\mu\text{g/ml}$. In this range of LDL concentrations, the binding was non saturable (fig 3.3). A LDL concentration of 200 $\mu\text{g/ml}$ (60 μg radiolabelled LDL) and AGE-modified collagen browned with 250mM G6P were used in subsequent experiments.

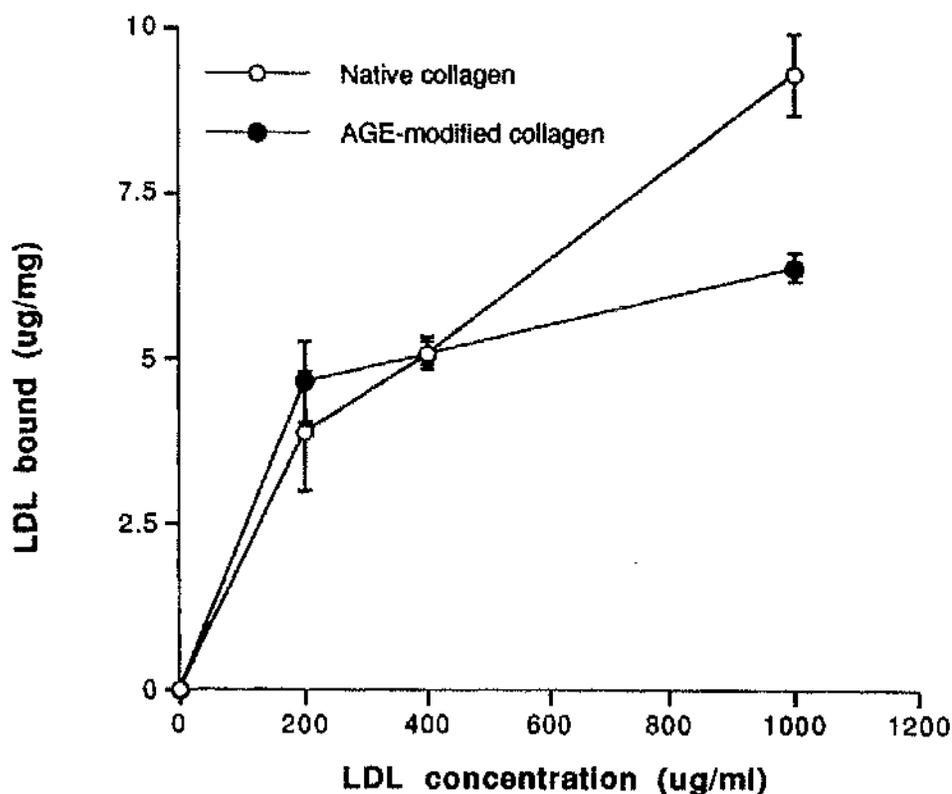


Figure 3.3

The effect of LDL concentration on its binding to native and AGE-modified collagen (browned with 250mM G6P). Results are the mean of 5 separate experiments.

3.4.3. The oxidation of LDL.

After the incubation of LDL isolated from normolipaemic subjects with CuCl_2 , an increase in fluorescence of LDL at excitation/emission wavelengths 320nm/420nm and 360nm/420nm was observed. The increase in fluorescence of LDL at the excitation wavelength 360nm is much greater than that seen at 320nm indicating that LDL is oxidised (fig 3.4). Measurements of LDL fluorescence before and after the addition of copper chloride were carried out in triplicate. LDL fluorescence after the addition of $5\mu\text{M}$ CuCl_2 varied between different batches of LDL prepared from normolipaemic subjects; due largely to different oxidative potentials of each of the subjects. (concentrations of antioxidants such as vitamin E may vary in different subjects).

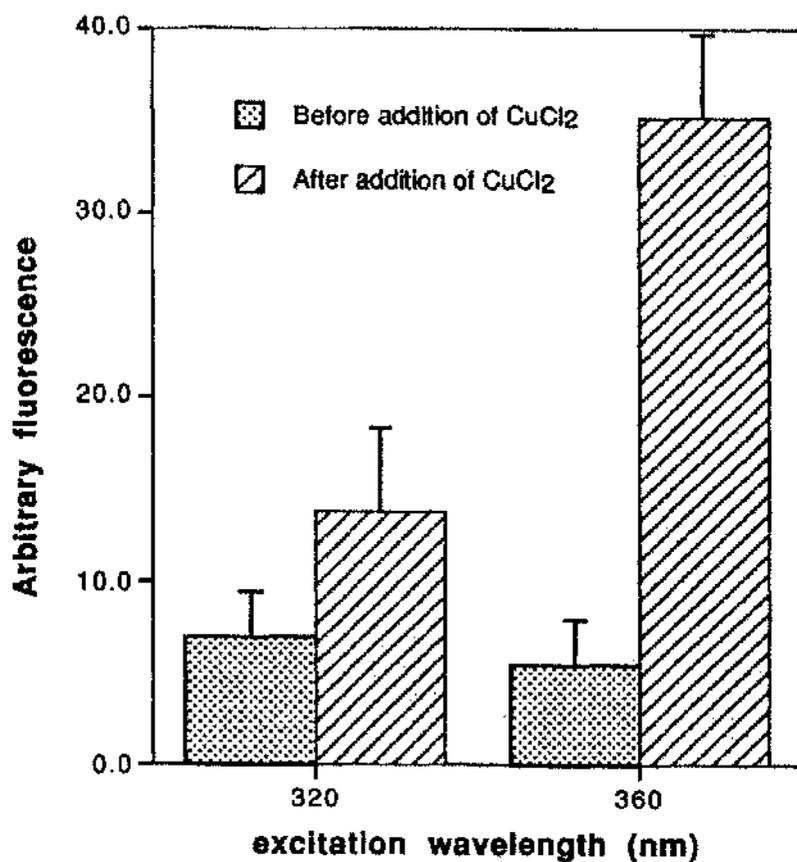


Figure 3.4

Fluorescence of LDL before and after the addition of 5 μ M copper chloride. Each measurement was carried out in triplicate). Emission wavelength was 420nm for both excitation wavelengths used. Error bars denote SEM.

3.4.4. Non-specific binding of LDL to collagen.

Increasing concentrations of BSA (0 - 1.0%) were added at the start of the LDL/collagen incubation. The binding of LDL to both native and browned collagen decreased with increasing concentrations of BSA (0.75% - 1.0%; fig 3.5). Binding of LDL to native collagen decreased from $7.2 \pm 0.26 \mu\text{g}/\text{mg}$ in the absence of BSA to $5.3 \pm 0.24 \mu\text{g}/\text{mg}$ in the presence of 1% BSA. The binding of LDL to AGE-modified collagen decreased from $4.9 \pm 0.12 \mu\text{g}/\text{mg}$ in the absence of BSA to $2.0 \pm 0.13 \mu\text{g}/\text{mg}$ in the presence of 1% BSA.

The binding of oxidised LDL to native collagen decreased from $6.5 \pm 0.34 \mu\text{g}/\text{mg}$ in the absence of BSA to $5.0 \pm 0.24 \mu\text{g}/\text{mg}$ in the presence of 1% BSA. The binding of oxidised LDL to AGE-modified collagen also decreased from $3.5 \pm 0.21 \mu\text{g}/\text{mg}$ in the absence of BSA to $1.9 \pm 0.14 \mu\text{g}/\text{mg}$ in the presence of 1% BSA. Decreases in binding of native and oxidised LDL to native and AGE-modified collagen are comparable (fig 3.5).

Binding of native and oxidised LDL to AGE-modified collagen was not significantly different in the presence of 0.5% compared to 1% BSA. Thus 0.5% BSA was used in subsequent experiments. This BSA concentration used is approximately equal to the albumin concentration in normal human aortic intima [287].

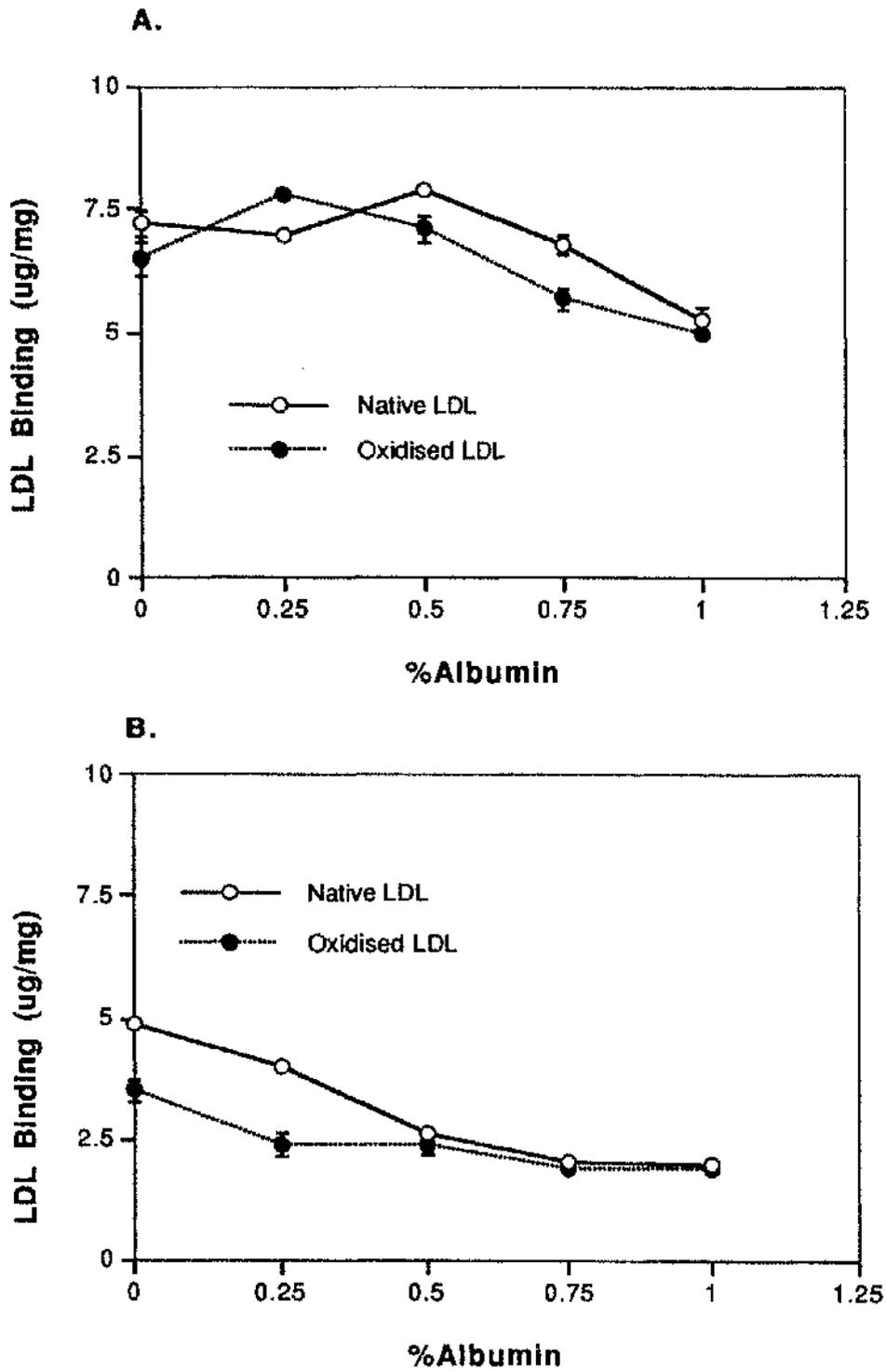


Figure 3.5

The binding of native and oxidised LDL to native (A) and AGE-modified (B) collagen in the presence of increasing BSA concentrations. Error bars denote SEM (n=6).

3.4.5. The binding of native and oxidised LDL to AGE-modified collagen.

Native collagen bound more native LDL ($4.3 \pm 0.28 \mu\text{g}/\text{mg}$) than oxidised LDL ($2.7 \pm 0.53 \mu\text{g}/\text{mg}$, $p=0.015$). This trend was also evident for the binding of native LDL ($4.0 \pm 0.32 \mu\text{g}/\text{mg}$) and oxidised LDL ($2.26 \pm 0.25 \mu\text{g}/\text{mg}$, $p=0.0001$) to AGE-modified collagen browned with 250mM G6P.

Binding of either native or oxidised LDL decreased as AGE-modified collagen was browned to different degrees (using 250mM-1000mM G6P) (fig 3.6). A linear relationship was observed for native and oxidised LDL binding to collagen browned to different degrees ($r=0.98$, $p=0.004$ and $r=0.98$, $p=0.003$ respectively).

At each of the G6P concentrations AGE-modified collagen bound more native LDL than oxidised LDL ($p<0.001$ for all points). Compared to native LDL, oxidised LDL showed a 37% decrease in binding in the absence of G6P and a 56% decrease at 1000mM G6P. These experiments were based on 28-43 individual incubations at each concentration.

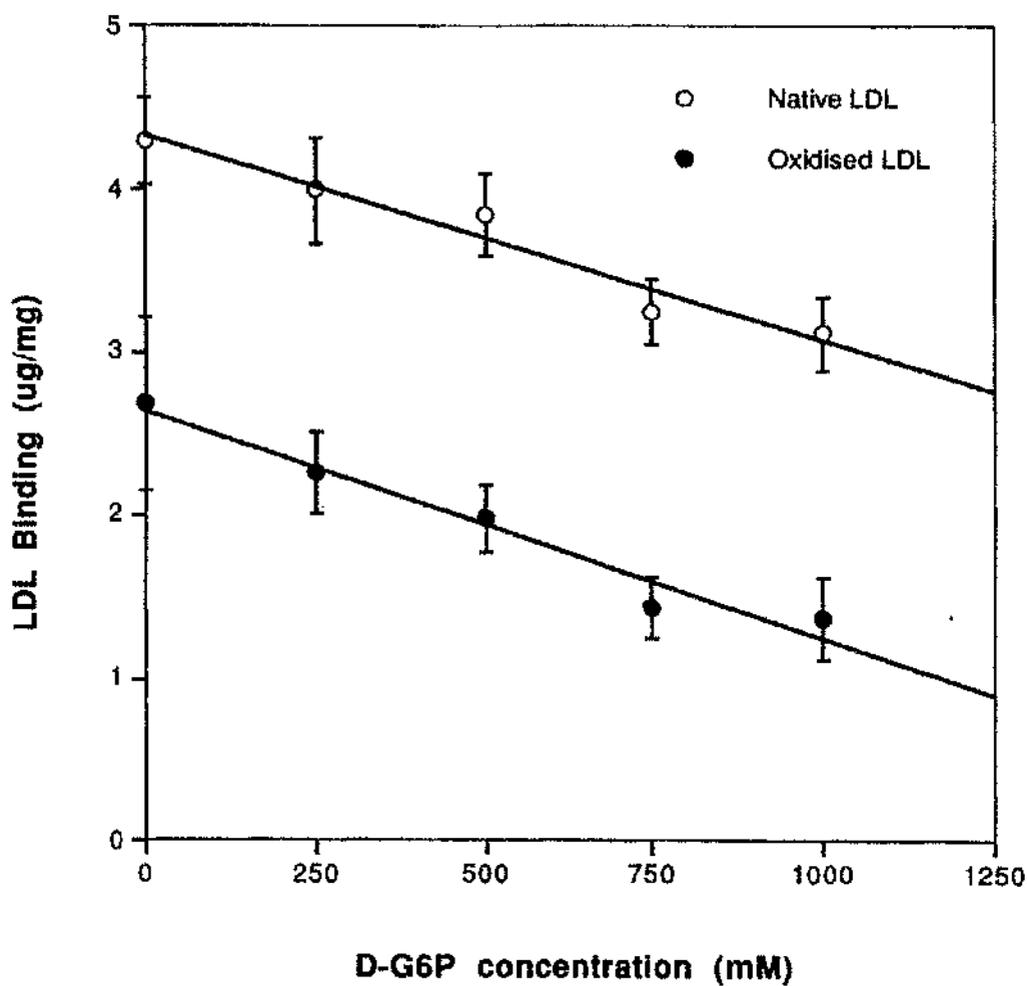


Figure 3.6

The effect of collagen browning on the binding of native and oxidised LDL.

Error bars denote SEM.

3.5 Discussion.

3.5.1. In vitro glycosylation of collagen.

Many studies on diabetic tissues have indicated that various proteins become non-enzymatically glycosylated. Advanced glycation of collagen increases covalent cross-linking between and results in decreased solubility of collagen [266,274]. Kent et al demonstrated, using SDS polyacrylamide electrophoresis, that there was increased covalent crosslinking of rabbit tendons incubated with glucose [291].

We observed a small increase in collagen-linked fluorescence when increasing concentrations of G6P (250mM-1000mM) were used to brown collagen. In most proteins, lysine is a primary target for non-enzymatic glycation. The number of sites on collagen available for lysine-derived AGE is limited compared to BSA, since less than 4% of amino acids is lysine as compared to 10% found in BSA [292]. The majority of amino acids found in collagen are glycine (35%), hydroxyproline (14%) and alanine (11%). Though lysine residues are present in collagen, lysine residue is either converted into hydroxylysine by oxygenases or is involved in the maturation (covalent crosslinking) of collagen [101]. Yamauchi et al have suggested a role for glycosylated hydroxylysine in collagen crosslinking [293]. The small increase in CLF when using higher concentrations of G6P (above 250mM) would suggest that the non-enzymatic glycation of collagen is saturable at low concentrations of G6P.

3.5.2. The effect of LDL on the binding of native and AGE-modified collagen.

Apart from changes in the mechanical properties of collagen such as decreased solubility and increased resistance to proteolytic degradation, it has also been reported that the collagen binding of proteins such as albumin, immunoglobulin and LDL is also increased when collagen is modified by AGE [204,209,294].

Accumulation of foam cells of monocyte origin in the aortic intima is an early event in atherosclerosis [185,295]. The clearance of LDL from plasma is dependent on a receptor-mediated endocytosis involving the recognition of apolipoprotein B (apo B), the major protein of the LDL particle, by a high affinity receptor present on cell membranes. There are few LDL receptors on macrophages, but macrophages can internalise LDL by the LDL pathway. This leads to lysosomal degradation of apo B and LDL cholesterol ester hydrolysis. Acetylated LDL, and other chemically modified forms, are taken up preferentially by the scavenger (acetyl LDL) receptor. The lack of regulation of the scavenger receptor leads to lipid deposition within the cells [296,297]. To date it has not been possible to identify a biologically generated form of LDL that behaves like acetyl LDL. Henriksen et al incubated native LDL with cultured endothelial cells, and observed a modification that converts LDL to a form recognised by the scavenger receptor [225,298]. Atherosclerotic lesions contain higher levels of esterified cholesterol than normal intima. This esterified cholesterol is thought to derive from the esterification of cholesterol within foam cells [184,188,299,300]. Early lipid deposits in human artery are often associated with small extracellular droplets of cholesteryl ester. It could be speculated that LDL bound to the extracellular matrix would be more prone to modifications by smooth muscle cells and macrophages since the diffusion of lipoproteins out of the vascular wall would be impeded. Binding of LDL to the extracellular matrix could act in synergy with other mechanisms to produce accelerated large vessel disease [199,301].

There is a well documented association between elevated plasma cholesterol concentrations and the prevalence of atherosclerosis-related disease [223,302]. Diabetes mellitus increases the risk of myocardial infarction by a factor of 2-3 [172]. It has been postulated that the increased formation of AGE on diabetic collagen may contribute to the increased cardiovascular risk.

We observed increased binding of LDL to collagen with increasing concentrations of LDL. This is in agreement with previous studies by Brownlee et al and Kalant et al [303,304]. No plateau was observed even at concentrations of 1000 μ g/ml. This would support the hypothesis that higher levels of LDL (found in patients with hyperlipidaemia) could result in an increased LDL binding to collagen in the aortic intima. This LDL would then be "trapped" and be more susceptible to modifications such as oxidation by macrophages.

However, in contrast to other studies by Brownlee et al, we found that AGE-modified collagen bound less LDL than unmodified collagen when higher concentrations of LDL (1000 μ g/ml) were used. Experimental methods used in this study differed to those by other groups, since in our study we used intact native and AGE-modified collagen and LDL isolated by ultracentrifugation from individual patients, rather than a pooled normolipidaemic source. Brownlee et al used denatured type I collagen from calf skin which was immobilised on an agarose matrix and non-enzymatically glycosylated at 44 $^{\circ}$ C for 2 weeks. It is not clear whether intermolecular crosslinks that exist between collagen fibrils in vivo would be preserved in denatured collagen bound to agarose. Since thermal denaturing of type I collagen occurs over the range of 35-40 $^{\circ}$ C, the process of denaturation and advanced glycation of collagen may have altered LDL binding sites and could account for the difference results observed in our study [52].

Clearly using either soluble or insoluble collagen in these binding studies has a significant effect on the binding of LDL and needs further study.

3.5.3. Binding of native and oxidised LDL to AGE-modified collagen.

Collagen modified by AGE to different degrees, as indicated by higher CLF levels, bound less native and oxidised LDL. Thus AGE modification of collagen inhibits LDL binding. The mechanism of inhibition of LDL binding by AGE-modified collagen is unknown, but it could be that the formation of AGE blocks LDL binding sites normally associated with native collagen. It has been reported that proteoglycans (glycosaminoglycans (GAG)) isolated from human intima have a high binding affinity for LDL and lipoprotein Lp(a). It is now accepted that interaction of apo B containing lipoproteins with GAG is one of the key features in the sequence of events leading to lipid deposition and plaque formation [202,305].

In the present study, the binding of oxidised LDL to both unmodified and AGE-modified collagen was significantly lower than that of native LDL. Thus both LDL oxidation and modification of collagen by AGE affect LDL binding to collagen. This is in agreement with a study carried out by Kalant et al who found that the non-enzymatic glycation of collagen gels resulted in the decreased binding of oxidised LDL [304]. The oxidation of LDL and the consequent damage to apo B may inhibit interactions of LDL with collagen binding sites. AGE formation on collagen could sterically block the binding of LDL to collagen. These results do not agree with the proposal that AGE-modified collagen is involved in the increased "trapping" of LDL.

A hypothesis, consistent with our results is that inhibition of binding of LDL to collagen by AGE modification of collagen and by oxidation of LDL makes more LDL more available for the scavenger receptor and thus may stimulate cellular cholesterol which leads to the transformation of monocyte-derived macrophages into foam cells [281].

3.6. Conclusions.

- (1) Collagen binds native and oxidised LDL in a non saturable concentration dependent manner. AGE formation on collagen is saturable at low concentrations of G6P.
- (2) Modification of collagen by AGE decreases LDL binding to collagen.
- (3) The decrease in LDL binding to AGE-modified collagen is enhanced when LDL is oxidised.

Chapter 4

AGE accumulation in tissues of spontaneously diabetic rats.

4.1. Summary.

This chapter describes:

- (1.) Measurement of collagen-linked fluorescence (CLF) in tissues from non-diabetic and spontaneously diabetic BB/E rats.
- (2.) Relationship between glycosylated haemoglobin concentration (HbA_{1c}), plasma glucose and CLF in spontaneously diabetic BB/E rats.

4.2. Introduction.

In man, AGE form slowly and increase with age in both non-diabetic and diabetic subjects. Numerous studies have shown that AGE accumulation is increased the tissues of diabetic patients. In young insulin-dependent diabetic patients who had diabetes for less than ten years, AGE measured as CLF were greater than their age matched non-diabetic counterparts [182]. The relationship between hyperglycaemia and macrovascular disease is complicated by other factors such as changes in lipoprotein metabolism, the aggregation of platelets and the secretion of growth factors which can influence atherogenesis.

The diversity of AGE in vivo has made their measurement difficult and has also limited clinical progress. To date, when assessing the effectiveness of aminoguanidine on tissue fluorescence in animal studies, either streptozotocin (STZ) or alloxan have been used to induce experimental diabetes. The unknown extrapancreatic toxicity of these compounds and the fact that generation of free radicals is at least partially involved in their diabetogenic action (which could affect protein glycation [69,306]), questions the suitability of these models for the study of AGE metabolism. The aim of this study was to measure AGE formation as CLF in tissues from diabetic and non-diabetic animals from the Edinburgh (BB/E) colony of spontaneously diabetic, insulin-dependent BB/rats, and to determine whether the rate of AGE formation differs between levels of AGE in skin, aorta, diaphragm and tail tendon.

4.3. Materials and methods.

4.3.1. Animals.

The BB/E colony consists of two sub-lines of animals created by selective breeding, the nucleus of which came from the colony maintained at the Animal Resources Division of Canada, Ottawa (Dr P. Thibert). The incidence of insulin-dependent diabetes in the animals of the diabetes-prone line, predominantly maintained by crossing diabetic male and non-diabetic female siblings is 55-70%. The mean age (\pm SD) at the onset of diabetes is 96 ± 18 days. The diabetes resistant BB/E subline has an incidence of diabetes less than 1%. The rats belonging to both sublimes were bred in isolators and then removed after 120 days and subsequently maintained in clean rooms with filtered air. Samples of tissues from these animals were kindly provided by Dr R.M. Lindsay, Metabolic Unit, Western General Hospital, Edinburgh.

4.3.2. Treatment of animals.

Diabetic animals were treated daily with a single subcutaneous injection of insulin (Ultratard Bovine Insulin, Novo, Denmark). The dose of insulin was adjusted individually on the basis of daily measurements of body weight and glycosuria. The rats were maintained at 20°C on 12 hour light/dark cycles and were fed SDS rat and mouse No 1. expanded feed (Special Diet Services, Witham, U.K.) and water ad libitum. On the day of sacrifice, the animals were weighed.

4.3.3. Tissue collection.

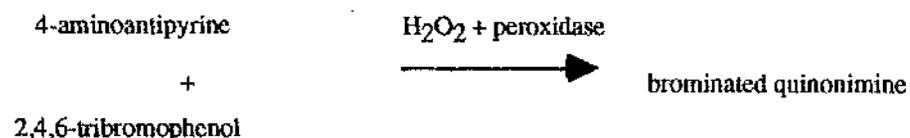
Blood samples were collected from anaesthetised (sodium pentobarbitone), age-matched (mean \pm SE, 259 \pm 4 days), female non-diabetic (n=10) and diabetic BB/E rats (n=10, mean \pm SE, duration of diabetes 161 \pm 13 days) and dispensed into lithium/heparin anticoagulant containers. Samples of aorta, diaphragm, skin and tail tendon were removed, rinsed thoroughly in isotonic saline to remove residual blood, and stored at -20°C until analysis.

4.3.4. Plasma glucose analysis.

Plasma glucose was measured by the glucose oxidase method on a Beckman Synchron AS4 automated analyser (Beckman-RIIC, High Wycombe, U.K.). Glucose oxidase is specific for β D-glucose and catalyses the conversion of D-glucose into gluconic acid and hydrogen peroxide:



The hydrogen peroxide produced in the presence of horseradish peroxidase causes a condensation reaction between 4-aminoantipyrine and 2,4,6-tribromophenol:



The reagent mixtures were incubated at 37°C for 5 minutes. Calibration of the described procedure was accomplished by the use of two standards at levels of 50 and 300mg/dl glucose. The absorbance of brominated quinonimine was read at 500nm.

4.3.5. The measurement of glycosylated haemoglobin.

Glycosylated haemoglobin (HbA₁) in rat blood samples was determined by electroendosmosis. Electroendosmosis is the movement of cations and their associated water molecules towards the cathode rather than the anode. This is caused by the presence of large anionic residues such as sulphate and pyruvate on agarose that are fixed in the matrix and cannot move in an electric field [307]. Three forms of glycosylated haemoglobin are present in the blood: HbA_{1a}, HbA_{1b} and HbA_{1c}. Aldimine (labile haemoglobin A₁) is sixty more times more likely to dissociate to free glucose than undergo the Amadori rearrangement. Separation of HbA₁ exploits the greater negative charge exhibited by the glycosylated haemoglobin (ketoamine form). Glycosylated haemoglobin travels faster through the agarose gel than the native haemoglobin (HbA₀). The measurement of glycosylated haemoglobin represents the time-averaged glycaemia. It is unaffected by short term fluctuations in blood glucose concentration.

In the present study glycosylated haemoglobin (HbA₁) was measured by using ready made agarose plates and buffers (Glytrac Haemoglobin set, Ciba Corning Diagnostics Ltd, Halstead, UK). The blood sample was haemolysed within 7 days from sampling using a haemolysing reagent (Ciba Corning Diagnostics Ltd, Halstead, UK) which contained 0.1% saponin and 0.05% EDTA in 0.5M potassium biphthalate buffer pH 5.0. The haemolysate was prepared by adding 3 parts of haemolysing reagent (30-300µl) to 1 part of the whole blood (10-100µl), mixing vigorously and incubating at 37°C for 15min. Electrophoretic buffer (95ml, 27.91g of trisodium citric acid dihydrate and 0.98g of citric acid made up to 1 litre) was poured into the chamber of an the electrophoretic cell base. The haemolysed blood sample (1µl) was loaded into preformed wells on

the agarose plate using a microlitre sample dispenser with disposable tip. A normal and abnormal quality control (haemolysates) supplied by Ciba Corning (Glyco HB I, II) were run with each batch and contained 8.2% and 13.5% HbA₁ respectively. The cell base was connected to a power supply (Shandon), switched on at 60 volts. After 40min, the plate was removed from the cassette holder and scanned using a Corning 720 densitometer at 420nm. The glycosylated haemoglobin is reported as a percentage of total haemoglobin.

4.3.6. The measurement of collagen-linked fluorescence in rat tissues.

Skin, aorta, diaphragm and tail tendon were dissected. Hair on the skin samples was scraped off using a scalpel blade. Adipose and connective tissue surrounding the skin, aorta, diaphragm and tail tendon were also stripped off. All the tissues were washed with saline. Each specimen weighing approximately 40mg was homogenised and delipidated. Three samples for CLF measurement were taken from each dissected tissue. Tissue samples were stored at -20°C until analysis.

Samples were finely minced using dissecting scissors, suspended in 10ml of 0.15M phosphate buffer pH 7.4 and homogenised for 30sec using an IKA homogeniser (IKA Labortechnik, Germany) in a polypropylene tube. The samples were then centrifuged at 1300g for 15min at 4°C on a Sorvall RT-6000 centrifuge. After centrifugation, the supernatant was discarded and the pellets were washed extensively with distilled water and centrifuged. The pellets were delipidated using 5ml of chloroform/methanol (2:1) in a shaking water bath at 4°C for 24h. Subsequently 1.0ml of methanol and 0.5ml of distilled water were added to each of the samples and the samples were again centrifuged for 15min at 1300g. The pellets were washed twice with ice cool methanol, three times

with distilled filtered water and once with Hepes Buffer (0.02M Hepes, 0.01M CaCl₂, pH7.5) then stored overnight at 4°C. Next day, 125 Units of type VII bacterial collagenase (EC 3.4.24.3, Sigma, Poole, UK) were added to each of the samples and incubated at 37°C for 24h in a shaking water bath (total volume 0.5ml). Toluene (2µl) and chloroform (2µl) were added to each of the incubations to prevent any bacterial growth. After the overnight incubation the samples were centrifuged at 10000g on a bench top microfuge (Sanyo Gallenkamp PLC, Leicester, England) for 6min at room temperature.

The supernatant containing the solubilised collagen was removed for measurement of CLF and hydroxyproline concentration. Hydroxyproline content was measured by the method of Stegemann and Stalder (see 3.3.4.) and fluorescence was measured at excitation 370nm and emission 440nm on a LS-3B fluorimeter. CLF was expressed as fluorescence units per milligram of collagen (U/mg). When measuring fluorescence, the digested collagen was placed in a clear 4 sided quartz microcuvette (total volume 300µl). A sample containing bacterial collagenase only was used as a blank.

4.3.7. Quality control.

A sample of AGE-modified collagen was prepared by the method described above (Chapter 3.3.1). Collagen was incubated with G6P, washed with 0.1M PBS pH 7.4, distilled water, and Hepes buffer (0.02M N-[2-Hydroxyethyl]piperazine-N-[2-ethanesulfonic acid], pH 7.5) and centrifuged at 3000rpm for 15min at 4°C. This process was repeated three times. To equilibrate collagen samples with buffer H (buffer H, 0.02M Hepes, 0.01M CaCl₂, pH 7.5) used in the collagenase digestion stage, the pellet was washed once with Hepes buffer containing calcium chloride, dried with filter paper, aliquotted into 10mg samples and stored at -20°C. A sample of AGE-modified

collagen was digested with bacterial collagenase and treated in exactly the same manner as tissue samples under study, except that there was no need for homogenisation. AGE-modified collagen was produced with 250mM G6P as described previously in section 3.3.1.

4.3.8. Statistics.

Unpaired Students t-test was used to compare results from the non-diabetic and diabetic sub-lines. Correlation between variables was carried out assuming a linear model. Data were analysed on Minitab Statistics package (version 8) and all results are reported as mean \pm 1SEM unless otherwise stated.

4.4. Results.

4.4.1. Animals.

Table 4.2. shows the characteristics of the diabetes resistant and diabetes prone sub-lines of BB/E rats. No differences in the age of the animals, body weight and plasma glucose concentration were observed between the two groups ($p>0.1$). As expected, the glycosylated haemoglobin (HbA₁) was higher in the diabetic rats ($P<0.001$).

4.4.2. Digestion of tissues.

The degree of digestion of tissue collagen samples by collagenase was greater than 95%. This was assessed by hydroxyproline assay on the hydrolysate of the residual pellet remaining after collagenase digestion. Recovery of hydroxyproline after hydrolysis was also greater than 95%. Inter-assay variability of CLF measurement calculated using AGE-modified collagen was 7%. Intra-assay variability was calculated using the triplicate measurements of skin, aortic, diaphragmatic and tendon CLF was 9.7%.

Table 4.2.

Characteristics of diabetic and non-diabetic sublines BB/E Wistar rats, (* = $p < 0.001$). Error bars denote SEM.

	Non-diabetic	Diabetic
Age (days)	256 ± 4	261 ± 7
Duration of diabetes (days)	-	161 ± 13
Body weight (g)	286 ± 19	268 ± 9
Daily insulin dose (U)	-	2.7 ± 0.2
Plasma glucose concentration (mmol/L)	7.2 ± 0.69	8.5 ± 4.4
HbA _{1c} (%)	4.7 ± 0.6	6.9 ± 0.8 *
n	10	10

4.4.3. Tissue collagen-linked fluorescence in BB/E rats.

CLF was significantly increased in the tissues of the diabetic animals (fig 4.3). Skin CLF in the diabetic rats was 1.5 ± 0.3 U/mg (mean ± 1SEM) compared to 0.8 ± 0.03 U/mg ($p=0.0095$) in the non-diabetic animals. Aortic CLF in the diabetic rats was 2.8 ± 0.32 U/mg compared to 1.3 ± 0.2 U/mg ($p=0.0010$) in the non-diabetic rats. Diaphragmatic CLF in the diabetic rats was 3.6 ± 0.5 U/mg and 1.5 ± 0.4 U/mg ($p=0.0018$) in the non-diabetic rats. Tendon CLF in the diabetic rat was 1.45 ± 0.2 U/mg and 0.44 ± 0.06 U/mg in the non-diabetic rats ($p=0.0012$). The diabetic to non-diabetic tissue CLF ratio was 2.0 for skin, 2.2 for aorta, 2.4 for diaphragm and 3.3 for tendon.

Correlation between CLF in different tissues was only evident in diabetic rats where the skin CLF correlated with diaphragmatic CLF ($r=0.65$, $p=0.038$), (fig 4.4). Correlation between skin and tendon CLF was of borderline significance ($r=0.62$, $p=0.054$). No correlation was observed between the CLF of the different tissues of the non-diabetic rats.

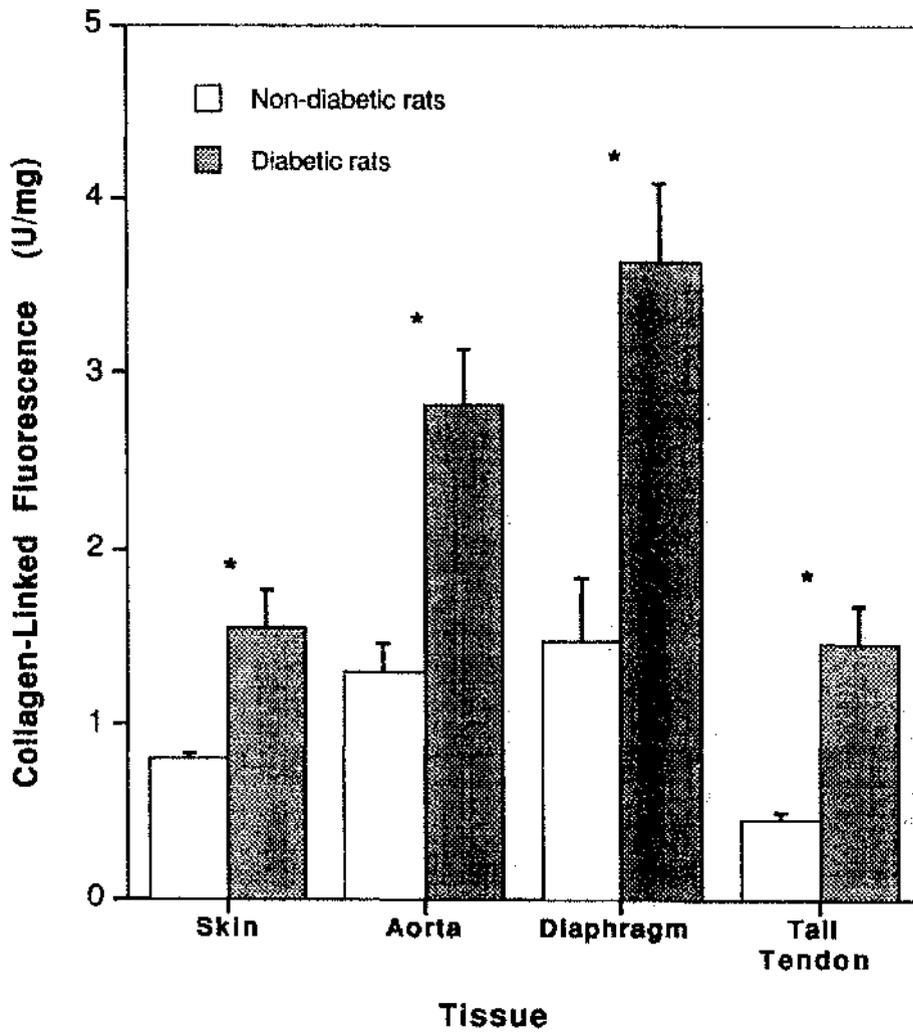


Figure 4.3

Tissue CLF values in diabetic and non-diabetic BB/E rats. (* = $P < 0.01$). Error bars denote SEM.

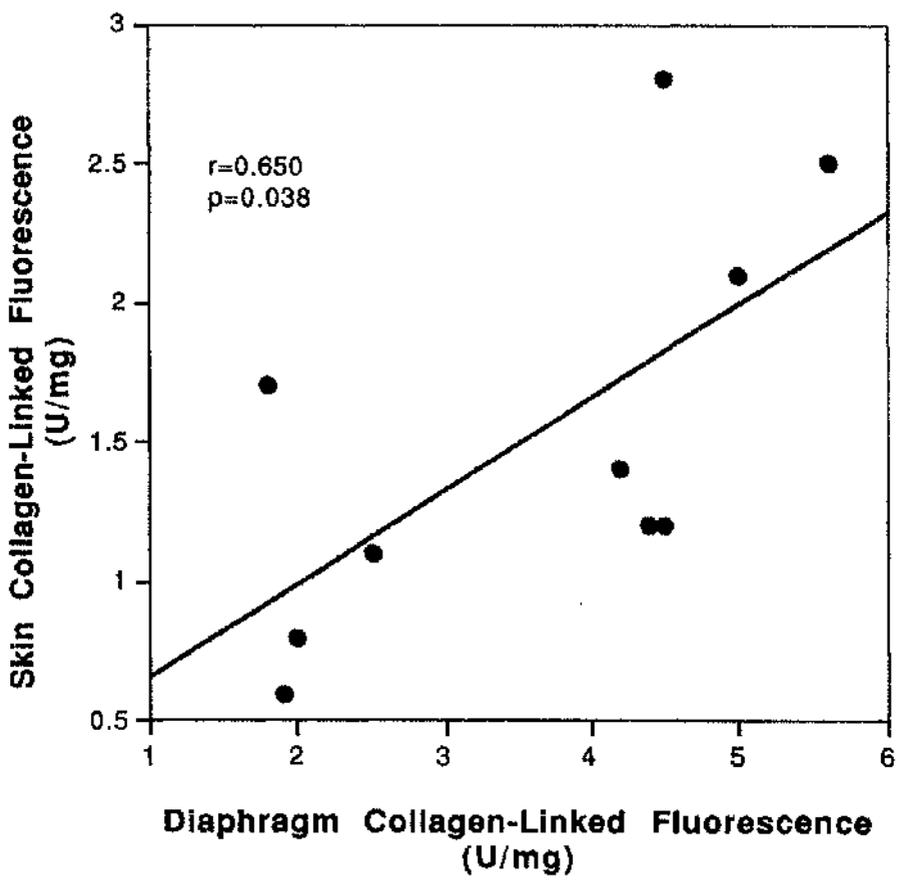


Figure 4.4

The relationship between skin and diaphragmatic CLF in diabetic BB/E rats.

4.4.4. Relationship between tissue CLF and glycosylated haemoglobin (HbA₁).

No correlation was observed between plasma glucose and glycosylated haemoglobin concentration in either non-diabetic or diabetic BB/E rats.

CLF did not correlate with plasma glucose concentration in tissues from non-diabetic and diabetic rats. Tendon CLF in non-diabetic rats was inversely related to plasma glucose concentration ($r=-0.81$, $p=0.007$; fig 4.5).

In non-diabetic rats, skin CLF correlated with glycosylated haemoglobin concentration ($r=0.72$, $p=0.017$; fig 4.6). In diabetic rats the aortic CLF correlated inversely with HbA₁ ($r=-0.74$, $p=0.012$; fig 4.7). CLF in other tissues in both sublimes was not related to HbA₁.

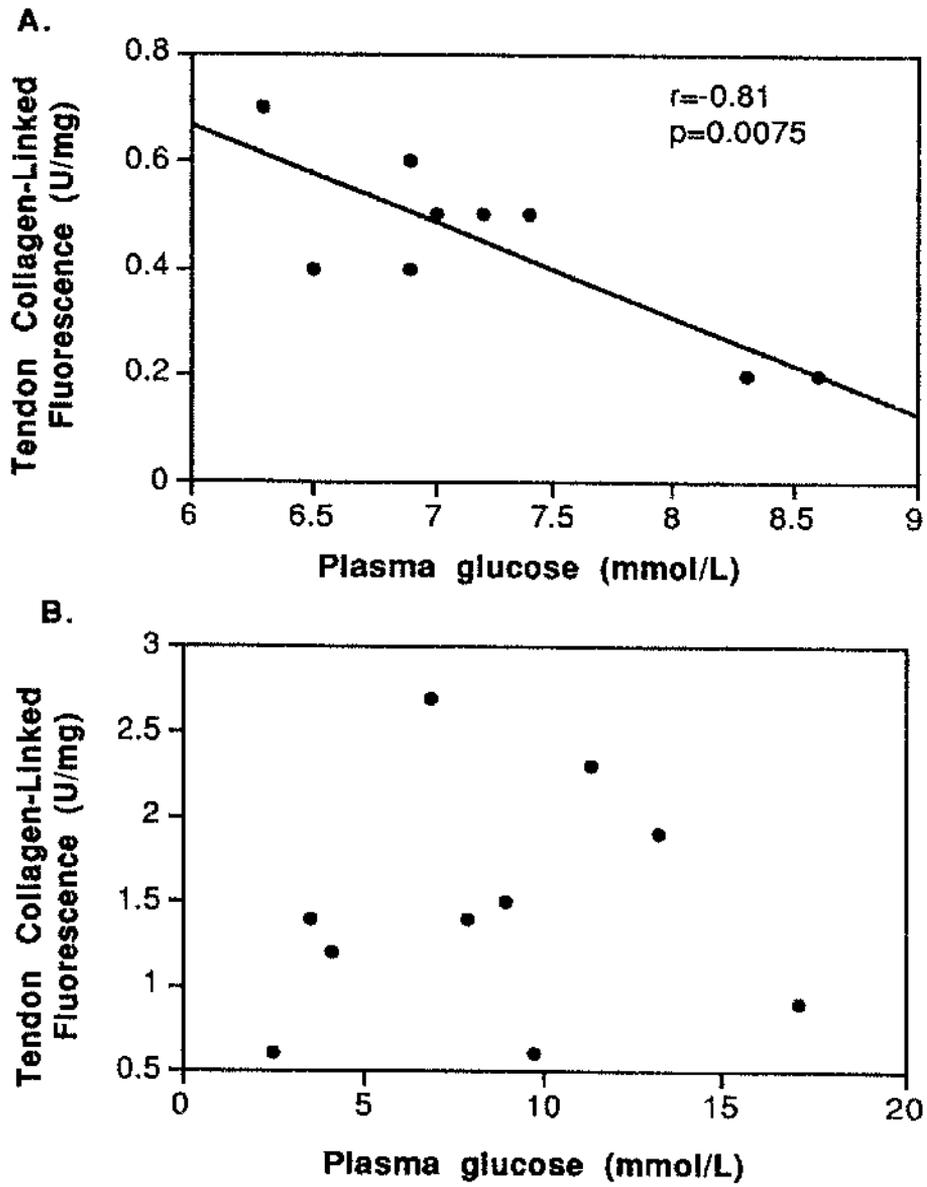


Figure 4.5

The relationship between tendon CLF and plasma glucose concentration in non-diabetic (A.) and diabetic rats (B).

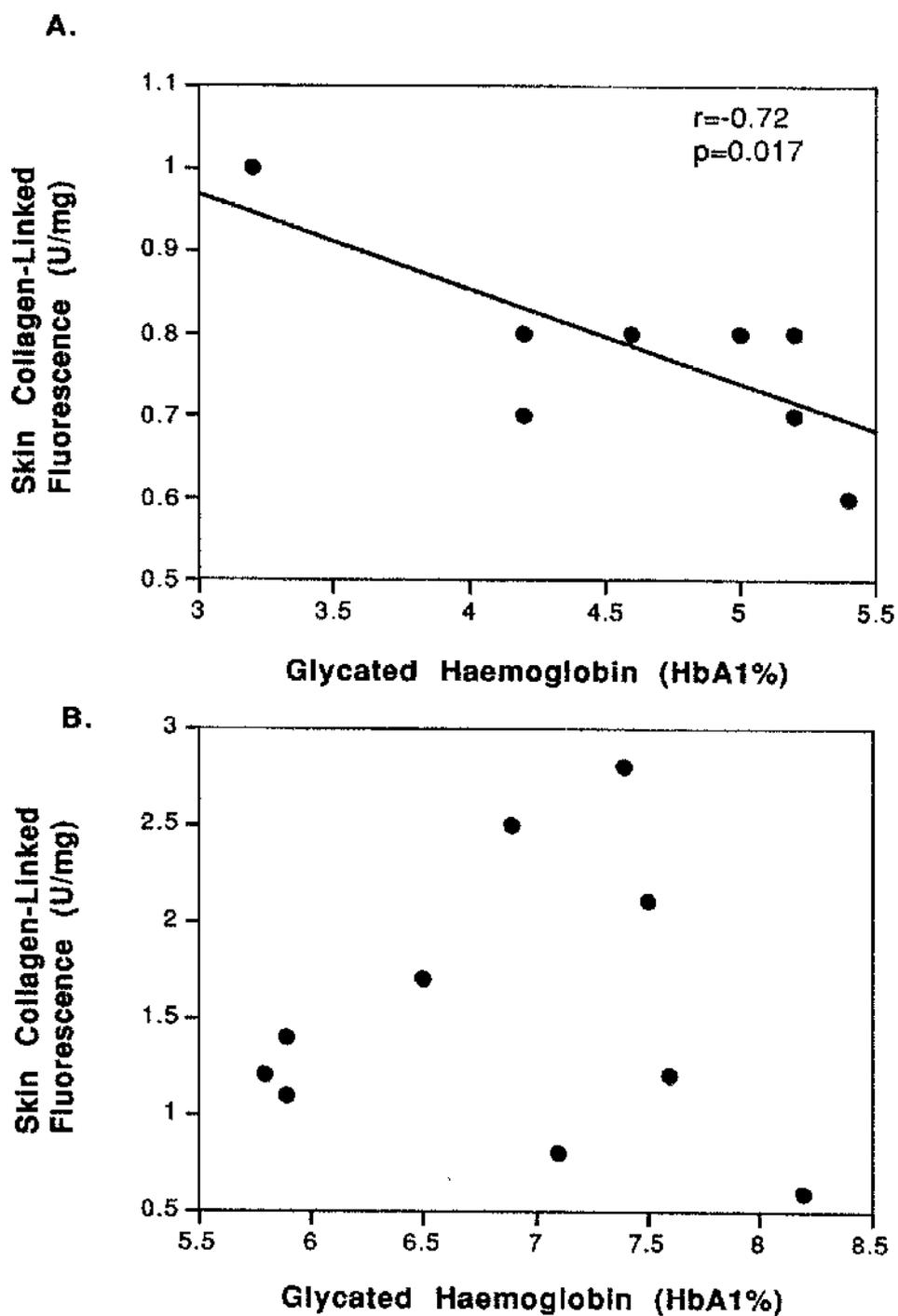


Figure 4.6
 Relationship between skin CLF and glycosylated haemoglobin in non-diabetic BB/E (A.) and diabetic BB/E rats (B).

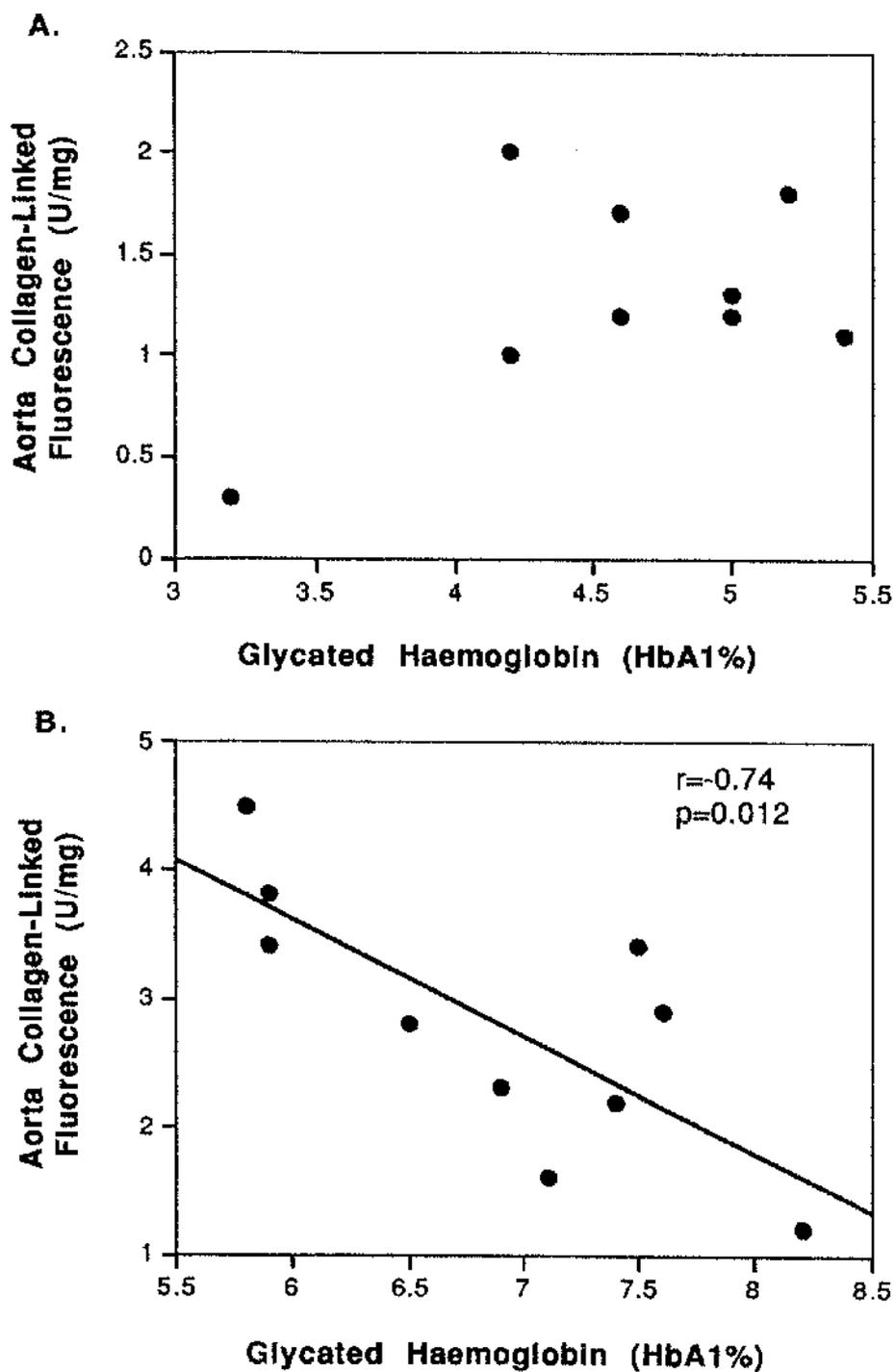


Figure 4.7
 Relationship between aortic CLF and glycosylated haemoglobin in non-diabetic (A.) and diabetic BB/E rats (B.).

4.5. Discussion.

4.5.1. Animals.

In this study, plasma glucose levels of the diabetic and non-diabetic rats were not different. This was not unexpected, as the diabetic rats were treated with insulin. However, glycosylated haemoglobin concentration, in the diabetic BB/E rats was significantly higher than in the non-diabetic rats, suggesting that glycaemic control in diabetic animals was worse than in controls. The measurement of glycosylated haemoglobin is widely used for the monitoring of glycaemic control [24,25]. Lindsay et al observed a 50% increase in HbA₁ after withdrawal of insulin for 2 days in diabetic BB/E rats (unpublished results). One reason for this is the much shorter half life of rat haemoglobin (approximately 30 days in rats compared to 120 days in humans).

Data from other studies indicate that in diabetic rats there is a considerable variation of plasma glucose concentration within a 24 hour period. This is dependent on the time of sampling relative to the feeding times and on the time of insulin administration. Consequently, a single plasma glucose values measured are of limited value.

Reported studies on AGE in alloxan or streptozotocin-diabetic rats may be misleading, since these animals are in a permanent hyperglycaemic state. This does not reflect human diabetes where plasma glucose levels change throughout the day with insulin treatment. For the diabetic BB/E rats to survive, a strict insulin regime is required. This resembles human diabetes. Thus the diabetic BB/E rats may be a better model for studying the accumulation of AGE in diabetes than the streptozotocin-diabetic rats.

4.5.2. Relationship between glycosylated haemoglobin and CLF.

In the non-diabetic rats, skin CLF correlated inversely with HbA₁. This was unexpected. An inverse relationship was also found between HbA₁ and the aortic CLF of the diabetic rat. McCance et al have suggested that the mean of serial measurements of HbA₁ is superior to a single measurement of HbA₁, since this would give a better insight into the rats glycaemic state over a period of time. They found that mean HbA₁ measurements correlated with the severity of retinopathy in diabetic patients [308]. We were unable to measure HbA₁ serially in this study. Studies by Lindsay et al (unpublished data) have shown that HbA₁ in rats changes more rapidly than in humans in response to glycaemia. Thus, the single HbA₁ measurement, performed on the day of tissue sampling, does not reflect the glycaemic control of the animal during the 5 month period of diabetes in the diabetic BB/E rats. Therefore, the observed correlations (between HbA₁ and tissue CLF) found in this study may not give a true insight into the relationship between HbA₁ and tissue CLF. Eble et al have shown that AGE formation and crosslinking continues even after the removal of glucose *in vitro* [93]. It would seem reasonable to suggest that even when the animals are under tight glycaemic control there will be occasions where the animal may be in a hyperglycaemic state. On such occasions the equilibrium between Amadori products and AGE would be affected and AGE accumulation on tissue would occur regardless of the plasma glucose concentration. It could be postulated that CLF formation proceeds independently of short term glycaemic control.

4.5.3. Tissue differences in collagen-linked fluorescence.

We were the first to investigate AGE accumulation in the spontaneously diabetic insulin-dependent BB/E rat. The results clearly demonstrate elevated CLF in the

four tissues studied in the diabetic subline. These results are comparable to those from previous studies using streptozotocin-diabetic and alloxan-diabetic rats. CLF increased in the diabetic BB/E rats when insulin was being administered to keep the animals in a good glycaemic control.

The diabetic to non-diabetic CLF ratio in the BB/E rat is comparable in skin, aorta and diaphragm. This ratio was increased in tail tendon compared to the other three tissues. This difference in AGE accumulation in tail tendon has also been observed by Soulis-Liparota et al who suggested that rat tail tendon differs from other tissues with respect to diabetes associated increase in CLF.

It is necessary to find a good animal model for the study of the effects of various therapeutic agents on the formation of AGE. AGE-related fluorescence is increased in collagen isolated the tissues of rats with streptozotocin-induced diabetes [257,258]. Brownlee et al showed that aminoguanidine reduces aorta crosslinking in diabetic animals [167]. Yagihashi et al showed that aminoguanidine reduced the fluorescence of collagenase-digested sciatic nerve from diabetic rats and demonstrated that the motor nerve conduction velocity improved after a 12 week administration of aminoguanidine. These data suggest that aminoguanidine has beneficial effects on the development of experimental diabetic neuropathy [309].

The generation of free radicals by chemicals such as STZ and alloxan which contributes to their diabetogenic action questions the suitability of these agents on these models. The role of free radicals and lipid peroxidation in the generation of AGE fluorescence have been investigated by several authors [62]. Aoki et al used vitamin E (α -tocopherol, a lipophilic antioxidant) to modify levels of oxidative stress in vivo in streptozotocin-diabetic rats. They found that there were no changes in skin CLF of diabetic rats regardless of whether

oxidative stress, assessed by the thiobarbituric acid reactant (TBAR) reaction, was modified with vitamin E supplementation in their diet. In the presence of reduced oxidative stress CLF in tail tendon was also reduced. Aoki et al also showed that the thermal rupturing time of collagen fibres from rat tail tendon was increased in the diabetic rat but reduced when the rats were supplemented with vitamin E [310]. This indicates that there is less crosslinking of collagen fibres in tail tendon in the presence of vitamin E. These findings suggest that AGE formation in skin is not affected *in vivo* by oxidative stress. However, in tail tendon, vitamin E may affect the formation of non fluorescent crosslinks or lysyl oxidase mediated crosslinking of collagen which is suggested to increase in diabetes [311,312]. AGE-related fluorescence was also found to decrease in the aorta and kidneys of streptozotocin-diabetic rats treated with aminoguanidine. The decrease in AGE-related fluorescence was followed by a concomitant decrease in mesangial expansion and albuminuria. Both are functional and structural markers of experimental diabetic nephropathy [261].

The problems of free radical generation do not exist in the BB rat (table 4.1) which is one of the best animal models of human insulin-dependent diabetes currently available [313,314]. A further advantage of the diabetic BB/E rat is that, similarly to human type 1 diabetic subjects, daily insulin treatment is essential for survival. On the other hand, in chemically-induced diabetic rats, although residual endogenous secretion of insulin does not prevent hyperglycaemia, it is sufficient for survival. Consequently, investigations using either alloxan-induced or STZ diabetic rats usually do not involve the administration of exogenous insulin. Advantages and disadvantages of using the spontaneously diabetic BB/E rat as a model of insulin-dependent diabetes are listed in table 4.1.

Table 4.1

BB/E rat as a model of diabetes mellitus.

Advantages:

- (1.) A prediabetic period of 2-3 months.
 - (2.) Involvement of both genetic and immune factors in aetiology.
 - (3.) The absence of obesity.
 - (4.) The occurrence of functional and structural changes in the retina, kidneys and nerves.
-

Disadvantages:

- (1.) Increased susceptibility to infection.
 - (2.) Difficulty in animal care and breeding.
 - (3.) The need to create a sub-line of diabetes resistant (DR) BB rats as controls for experimental diabetic animals, so that any changes seen are diabetes rather than strain related.
-

Tissues which have been studied so far in the streptozotocin- diabetic rat include skin, aorta, diaphragm, tail tendon, lens crystallin, whole kidney, renal tubules and glomeruli. In these studies, protein-linked fluorescence has been the most widely used method of AGE measurement [315,316,317]. Recently, methods employing HPLC and immunoassays have been used to determine the levels of AGE [Refer to chapter 1.9]. Aminoguanidine has been used as an inhibitor of AGE formation in *in vivo* studies using rats with chemically induced diabetes. Rutin, an aldose reductase inhibitor, was also found to inhibit AGE formation [87]. Its mode of action could be different from aminoguanidine; it probably acts by reducing the levels of protein fructosylation through the sorbitol pathway [258]. D-lysine and L-arginine have also been used as inhibitors of AGE formation [265,318].

In humans, measurement of CLF in skin biopsies has been employed to show the relevance of AGE formation to diabetic complications in type I diabetes mellitus. Monnier et al observed a relationship between CLF and the severity of diabetic retinopathy [160,319]. Similarly, Dominiczak et al observed an association between retinopathy and CLF in the skin of young type I diabetic patients. Relationships between CLF in the skin and aorta could be of importance in future clinical studies using inhibitors of AGE formation as AGE in skin biopsies would reflect aortic AGE accumulation. The lack of a relationship between skin and aortic CLF in both diabetic and non-diabetic rats could be attributed to the relatively narrow range of both skin and aortic CLF values obtained from this study.

4.5.4. The use of the spontaneously diabetic BB/E rat as a animal model for the study of AGE formation.

The spontaneously diabetic BB/E rat is a good animal model for the study of both AGE formation and the late complications of diabetes. Unlike alloxan or streptozotocin-diabetic rats, the diabetic BB/E rat develops reproducible structural changes in somatic, peripheral and autonomic nerves which are characteristic of human diabetic neuropathy [320,321,322]. On the other hand, streptozotocin and alloxan have significant nephrotoxicity in addition to their toxic effect on insulin-secreting cells.

4.6. Conclusions.

- (1.) AGE measured as CLF are elevated in several tissues of the spontaneously diabetic, insulin-dependent BB/E rat compared to the non-diabetic BB/E rat. The BB/E rat seems to be a good model of AGE formation in human diabetes.

- (2.) CLF and therefore the rate of AGE formation vary in different tissues.

Chapter 5.

Collagen-linked fluorescence in human atherosclerotic plaques.

5.1 Summary.

This chapter describes:

- (1.) The different stages of atherosclerotic plaque formation.
- (2.) Measurement of CLF in human atherosclerotic plaques.
- (3.) Measurement of CLF in different human tissues.
- (4.) In vitro formation of AGE in human aorta.

5.2. Introduction.

The presence of AGE on vascular tissue is important in the pathogenesis of micro and macrovascular diabetic complications [323,324]. The vascular changes associated with AGE accumulation could also play a role in the development and progression of tissue damage associated with end stage renal disease and atherosclerosis.

It is unknown whether local changes in AGE-modified collagen occur within arteries. The aim of this study was to investigate the role of AGE in atherogenesis by measuring the concentrations of AGE-modified collagen in various forms of the atherosclerotic plaque.

5.3. Materials and methods.

5.3.1. The source of tissues.

To study the effect of patients age on skin CLF, skin (200mg) was obtained at autopsy from 21 individuals (10 males, 11 females) aged between 4-81 years. None of the individuals studied had a history of diabetes or renal failure.

In another study, samples of atherosclerotic plaque, skin, tendon, aorta and coronary artery were obtained at autopsy from 24 individuals,(15 men, 9 women), aged 55-99 years (mean 73.5 ± 9.3 yrs).

Causes of death of the 24 individuals are listed in table 5.1. Skin samples were taken from the abdominal area and were washed in 0.15M saline. Hair and any underlying adipose tissue were removed using a scalpel blade. Aorta (free of any form of atherosclerotic plaque), diaphragmatic tendon and coronary artery were also removed at the same time. Any connective or adipose tissue surrounding the tendon or coronary artery was stripped off. The samples were then washed in 0.15M saline, aliquotted into 50mg portions and stored at -20°C until analysis.

Table 5.1.
Characteristics of patients studied:

No	Initials	Sex	Age	Diagnosis at autopsy.
1	HN	F	61	Peritonitis.
2	FE	M	71	Left ventricular failure, coronary artery disease, acute myocardial infarction.
3	BL	F	73	Bronchal carcinoma, pulmonary thromboembolism, old myocardial infarction.
4	HF	M	71	Pulmonary thromboembolism, stroke.
5	AD	F	68	Metastatic cancer, pulmonary embolism, myocardial infarction.
6	WL	M	62	Respiratory failure, hepatic failure, old myocardial infarction, diventricular disease.
7	MD	F	65	Acute myocardial infarction, benign thyroid adenoma.
8	SM	M	69	Pulmonary oedema, ischaemic heart disease.
9	RG	M	75	Pulmonary thromboembolism, myocardial fibrosis, pleural fibrosis, previous cancer of colon.
10	AM	M	80	Occlusion of right coronary artery, ischaemic heart disease, left ventricular failure.
11	MW	F	86	Bronchopneumonia, urinary tract obstruction, bladder cancer.
12	AS	M	55	Cardiac failure, myocardial infarction, mitral valve disease.
13	RC	M	73	Myocardial infarction, rupture of left ventricle.
14	PM	M	71	Multiple pulmonary infarcts, pulmonary thromboemboli, deep venous thrombosis, congenital bicuspid aortic stenosis.
15	AM	M	74	Acute myocardial infarction, acute renal failure, septicaemia, congestive cardiac failure, peripheral vascular disease.
16	HG	F	89	Bronchopneumonia, endometrial cancer, breast cancer.
17	WM	M	68	Pulmonary embolism, pulmonary infarction, deep venous thrombosis, septicaemia, hepatic cirrhosis, hepatocellular cancer.
18	MW	F	79	Bronchopneumonia.
19	JD	M	74	Bronchopneumonia, oesophageal cancer, subacute obstruction.
20	CP	M	71	Myocardial infarction.
21	RK	M	84	Bronchopneumonia, coronary atherosclerosis, hepatic vascular congestion.
22	HD	F	77	Acute bronchitis, bronchopneumonia, cancer of lung.
23	JS	M	70	Hepatic cirrhosis, biventricular hypertrophy, pulmonary oedema.
24	AM	F	99	Bronchopneumonia, cardiac failure.

5.3.2. Grading the severity of atherosclerosis.

The severity of atherosclerosis was graded by examining the abdominal aorta for lesions and different forms of the atherosclerotic plaque on each patient. Each abdominal aorta was longitudinally opened posteriorly and fixed in formalin. The grading of the atheroma was a two-stage process and was carried out on a standard 10cm length of vessel between the renal arteries and the bifurcation of the aorta. Firstly, the aorta was pinned onto a flat board and the proportion of the total surface area involved by all lesions was estimated using a point-counting technique [325]. The results of this method compare favourably with those obtained by tracing and planimetry. Secondly, the total area of disease in each specimen was subdivided into the following categories and expressed in tenths: superficial plaques (fatty streaks/spots), fibrous (collagenous) plaques, complicated plaques and calcified plaques. By combining these two grading stages an overall index of severity of atheroma was obtained and graded as mild, moderate or severe. The grading process was carried out by a pathologist in ignorance of the clinical details of each case.

5.3.3. Description of different forms of plaque.

Arterial tissue with the different forms of atherosclerotic plaque was removed from affected segments of abdominal aorta. Aorta with no macroscopically visible plaques was classed as normal and used as a control in the study. The four other forms of atherosclerotic plaque were defined as follows:-

- (1) A superficial plaque (fatty streak) is a slightly raised patch of yellowish intimal thickening. Its texture was similar to that of the unaffected aortic wall. Microscopically the fatty streak shows an accumulation of smooth

muscle cells and macrophages in the intima which are loaded with lipids.

- (2.) A collagenous (fibrous) plaque is a lesion that is thicker and whiter than the superficial plaque. The fibrous tissue found in the collagenous plaque is synthesised, at least in part, by the proliferating smooth muscle cells. Smooth muscle cells have been shown capable of synthesising collagen and elastin.
- (3.) A calcified plaque is aortic tissue containing hard and brittle calcium deposits. This type of plaque may form part of an ulcerated or collagenous plaque.
- (4.) An ulcerated plaque is a result of the breakdown of the fibrous cap producing ulceration of the plaque. This type of plaque is characterised by the breached intimal layer and the presence of mural thrombi. Both ulcerated and calcified plaques produce irregularity of the luminal surface of the aorta.

5.3.4. Measurement of CLF in different tissues and different forms of the atherosclerotic plaque.

In all cases the adventitia was removed and the remaining fragments of the aorta were washed with 0.15M saline. CLF was measured in the skin, diaphragmatic tendon, coronary artery and aorta (including the four plaque types) by the method described in section 4.3.6. Briefly, bacterial collagenase was added to the tissue sample after delipidation and incubated for 24h at 37°C. Fluorescence

of the digested collagen was then measured at excitation wavelength 370nm, emission wavelength 440nm.

Collagen concentration in tissue samples was determined by the measurement of hydroxyproline using the method of Stegemann and Stalder. Tissue samples from each individual were aliquotted into three portions and analysed separately. Hydroxyproline measurement were carried out in duplicate. Emission spectra of the different forms of plaque, skin, diaphragmatic tendon and coronary artery were obtained at excitation wavelength 370nm.

5.3.5. The in vitro browning of human aorta.

Aortic tissue free of atherosclerotic plaques was obtained from an 81 year old female on autopsy. Post mortem diagnoses were: bilateral pulmonary thromboembolism, iliac deep venous thrombosis, acute myocardial infarction and primary bronchial carcinoma. The samples of aorta (35-56mg) were washed in 0.1M phosphate buffered saline, minced with dissecting scissors and incubated in sterile 10ml containers for 14 days at 37°C in the presence of 250mM G6P in 0.5M phosphate buffer pH 7.4, which had been passed through an Millex-GV filter (0.45µm). To further identify whether the CLF measured in the aorta was attributable to AGE, the samples were incubated in the presence of 200mM aminoguanidine. Control samples of aorta were incubated in phosphate buffer alone. Penicillin/ Streptomycin mixture (100IU/100µg per sample respectively) was added to prevent bacterial growth during the incubation period. Five independent samples of aorta were used in each of the different incubation conditions (control, G6P alone, G6P + aminoguanidine). On completion of the incubation period, the samples were washed thoroughly in 0.15M phosphate buffer and CLF was measured as described previously.

Fluorescence spectra generated by the incubation of aortic tissue with G6P and aminoguanidine were measured at different combinations of excitation/emission wavelengths. The following excitation/emission wavelengths were compared: 370/440nm (AGE fluorescence), 325nm/390nm (peak fluorescence) and 335/385nm (fluorescence characteristic for pentosidine).

5.3.6. Inter and intra-assay variability of CLF measurement.

In vitro browned collagen (browned with 250mg G6P over two weeks at 37°C) was subjected to the same process of digestion with bacterial collagenase as the skin sample. The between batch imprecision at fluorescence level 12.8 U/mg was 10% (coefficient of variation). The within batch precision was 10.1% and 11.0% at fluorescence levels 16.8 U/mg and 22.7 U/mg respectively.

5.3.7. Statistical analysis.

Statistical analysis included the Students t-test and Mann-Whitney test as appropriate, using Minitab software. Regression analysis (least squares method) included linear ($Y=aX+b$) and exponential models ($Y=\exp(a+bX)$). All results are expressed as SEM unless otherwise stated.

5.4. RESULTS.

5.4.1. Relationship between patients' age and skin CLF.

CLF in individuals aged between 3 and 81 years ranged from 2.5 to 31.2 U/mg respectively. Regression analysis between subjects' age and skin CLF assuming a linear model gave a correlation coefficient (r) 0.711, $p=0.003$. A higher degree of correlation was obtained ($r=0.818$, $p=0.0001$) assuming an exponential model, fig 5.2.

5.4.2. Human artery browned in vitro.

There was a high degree of correlation between fluorescence at 370/440nm, 325/390nm and at 335/385nm. The addition of G6P led to an increase in fluorescence at 370/440nm ($p=0.002$ when compared to fluorescence of the tissue incubated in the absence of G6P) and 325/395nm ($p=0.013$). There was no increase in fluorescence at wavelengths characteristic of pentosidine (335/385nm). The addition of aminoguanidine inhibited the increase in G6P-induced fluorescence at all three wavelengths measured (fig 5.3). In subsequent experiments all fluorescence measurements were carried at 370/440nm.

5.4.3. Fluorescence spectra of human tissues.

Emission fluorescence spectra of different tissues measured at excitation wavelength 370nm are shown in fig 5.4. All tissues showed a single fluorescence peak at emission wavelength 440nm. A similar fluorescence peak was observed on collagenase digests of different forms of atherosclerotic plaque (fig 5.4). Spectra obtained from the in vitro incubation of BSA with glucose showed the same fluorescent peak (fig 2.2.).

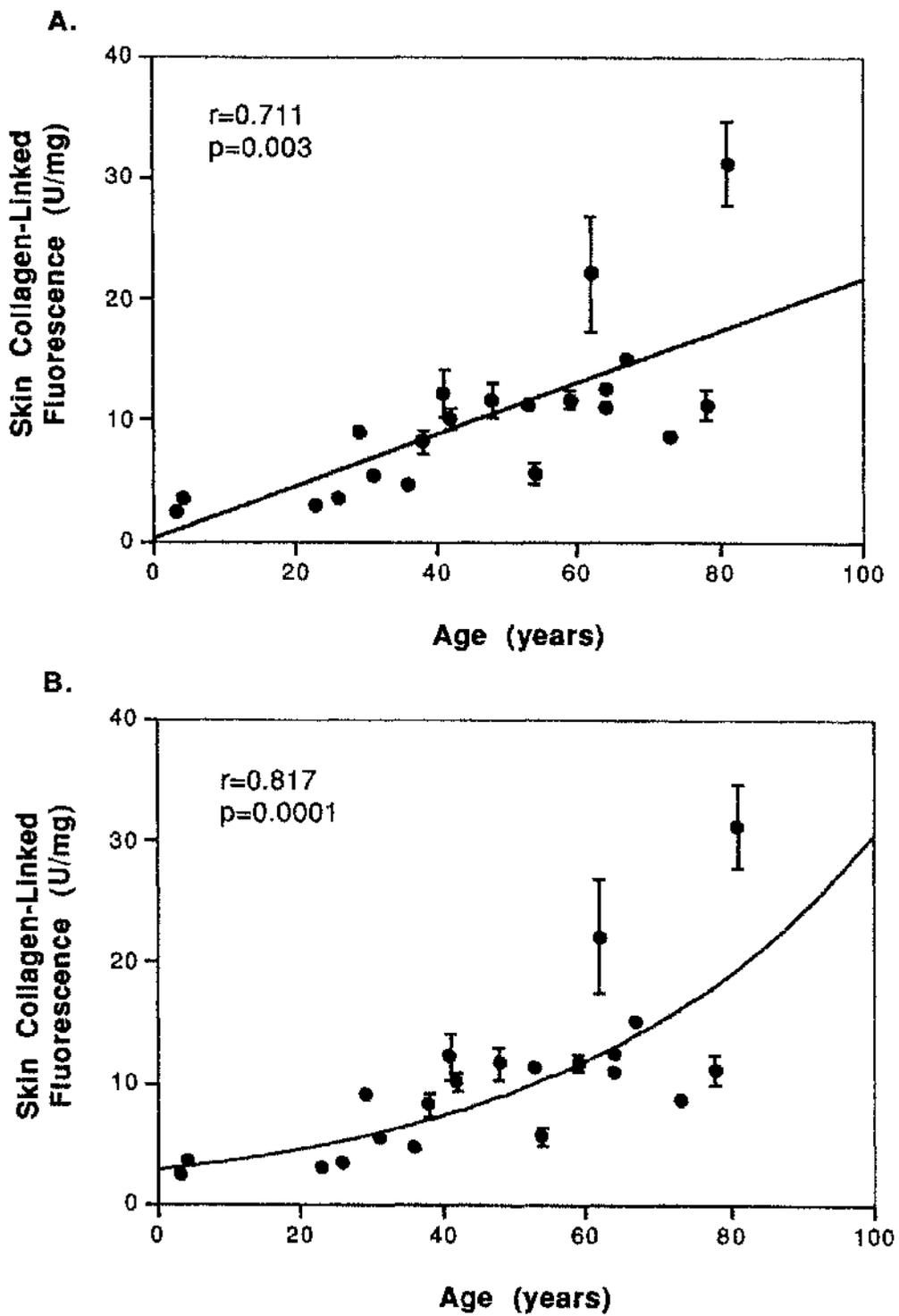


Figure 5.2

The relationship between subject's age (years) and CLF (U/mg) in human skin assuming a linear model (A.) and exponential model (B.). Error bars denote SEM.

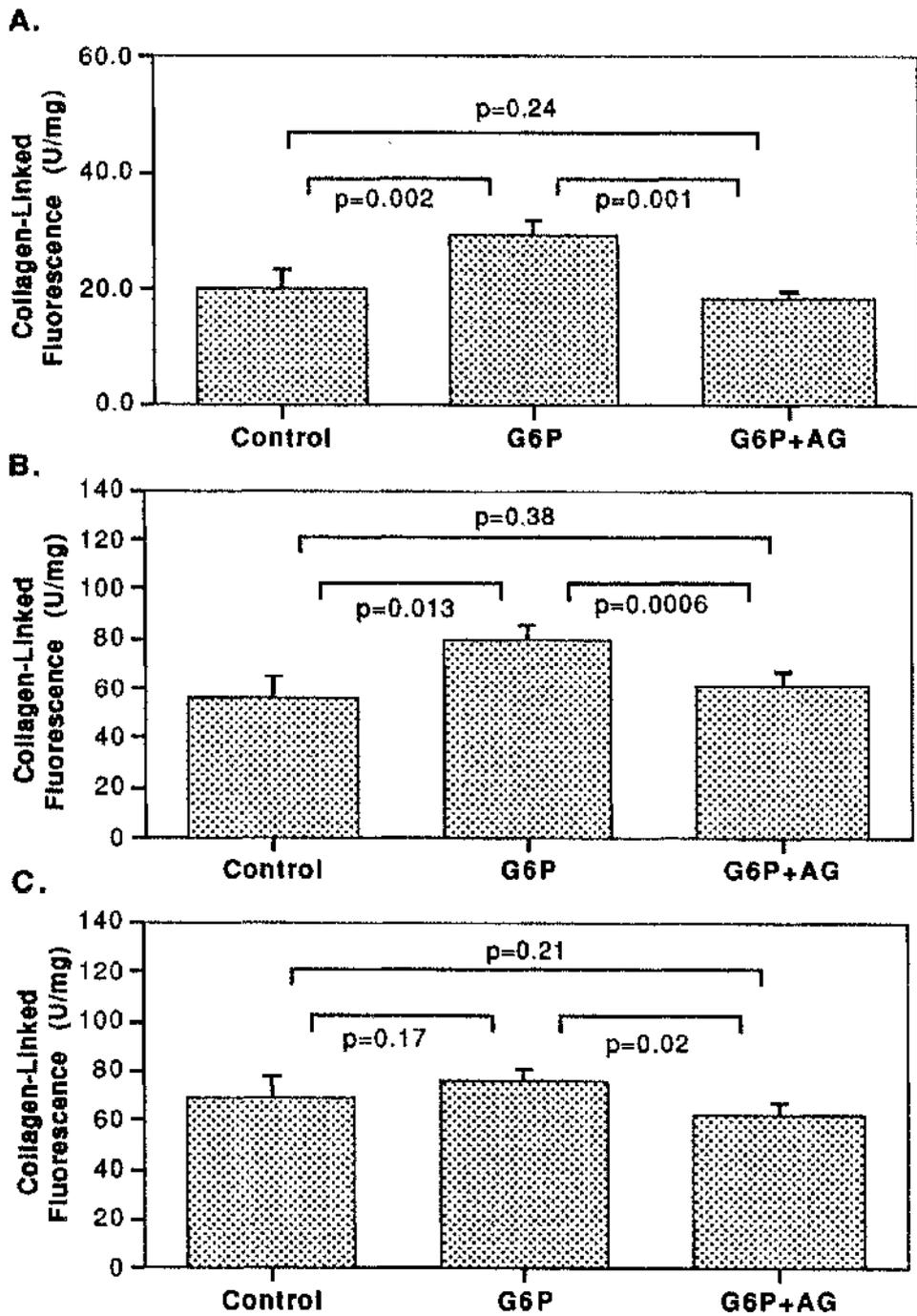


Figure 5.3

Fluorescence of human aorta incubated in the absence of G6P, 250mM G6P and in the presence of G6P and 200mM aminoguanidine (AG). (A.) Excitation/emission wavelength 370/440nm, (B.) Excitation/emission wavelength 325/390nm, (C.) Excitation/emission wavelength 335/385nm

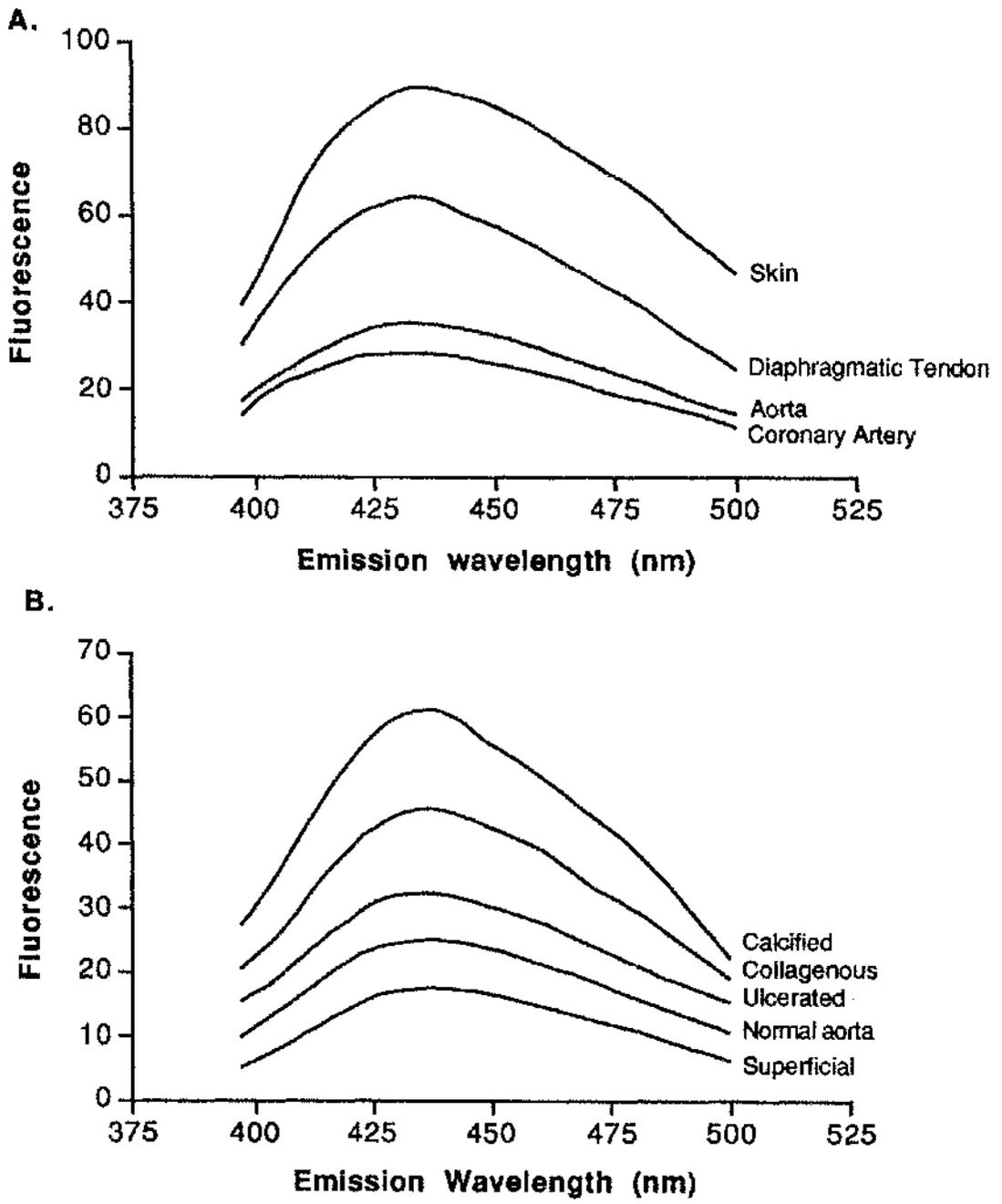


Figure 5.4

Fluorescence emission spectra of human tissues (A) and different forms of atherosclerotic plaque (B).

5.4.4. CLF in different tissues from human autopsy material.

CLF in the aorta was 27.9 ± 1.8 U/mg (mean \pm 1SEM), in the coronary arteries 25.9 ± 1.5 U/mg and in diaphragmatic tendon 47.8 ± 2.6 U/mg. All these were higher than skin CLF, 18.3 ± 1.5 U/mg (fig 5.5). There was no relationship between the subjects' age and CLF in the aorta and diaphragmatic tendon. However, there was a borderline significant relationship between skin CLF and age ($r=0.388$, $p=0.061$). Skin CLF correlated with CLF in the aorta ($r=0.467$, $p=0.025$) but not with CLF in the coronary arteries ($p=0.935$, fig 5.6).

When the subjects were separated into those with mild, moderate and severe atherosclerosis (as assessed by the numbers of lesions in the abdominal aorta, section 5.3.4.), there were no differences between the three groups in CLF measured in the skin, aorta, diaphragmatic tendon and coronary artery.

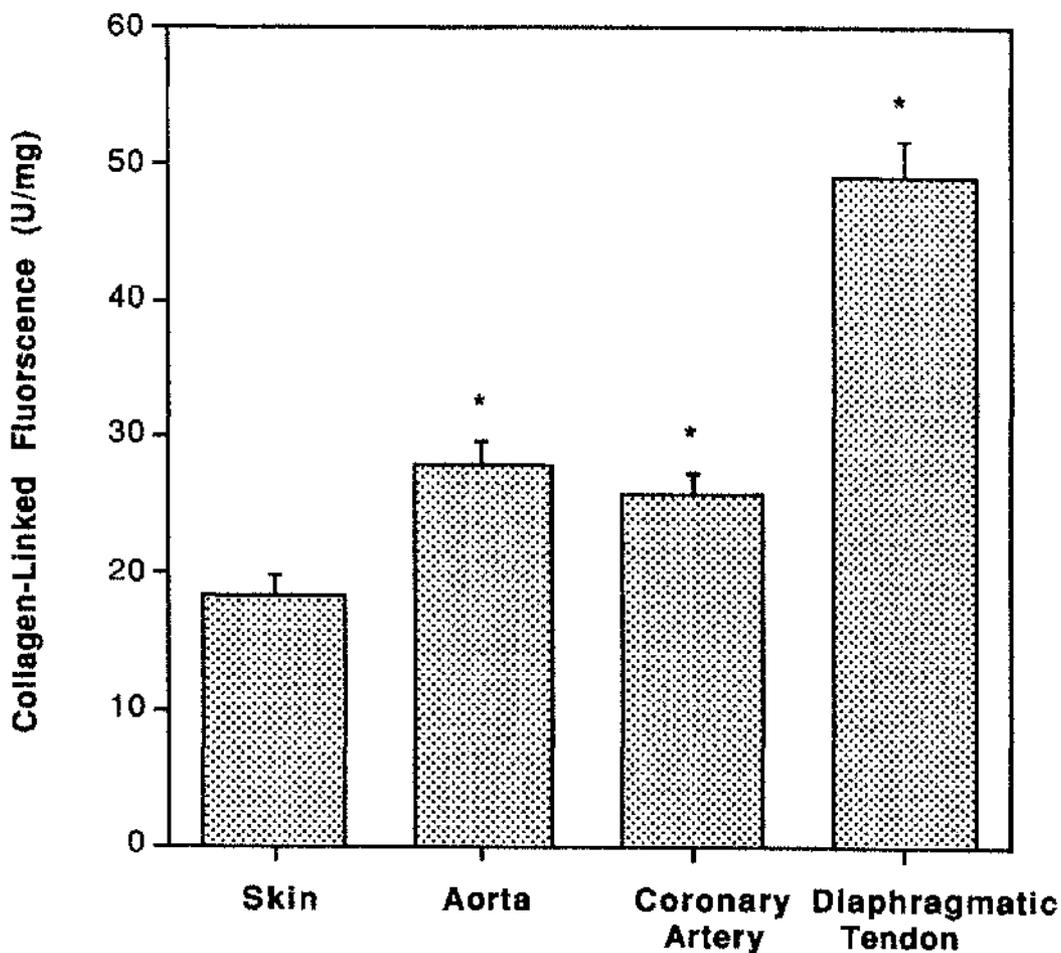


Figure 5.5

Collagen-linked fluorescence in different tissues obtained at autopsy. Fluorescence measured at excitation 370nm, emission 440nm. * Skin CLF vs other tissues CLF, all $P < 0.001$. Error bars denote SEM.

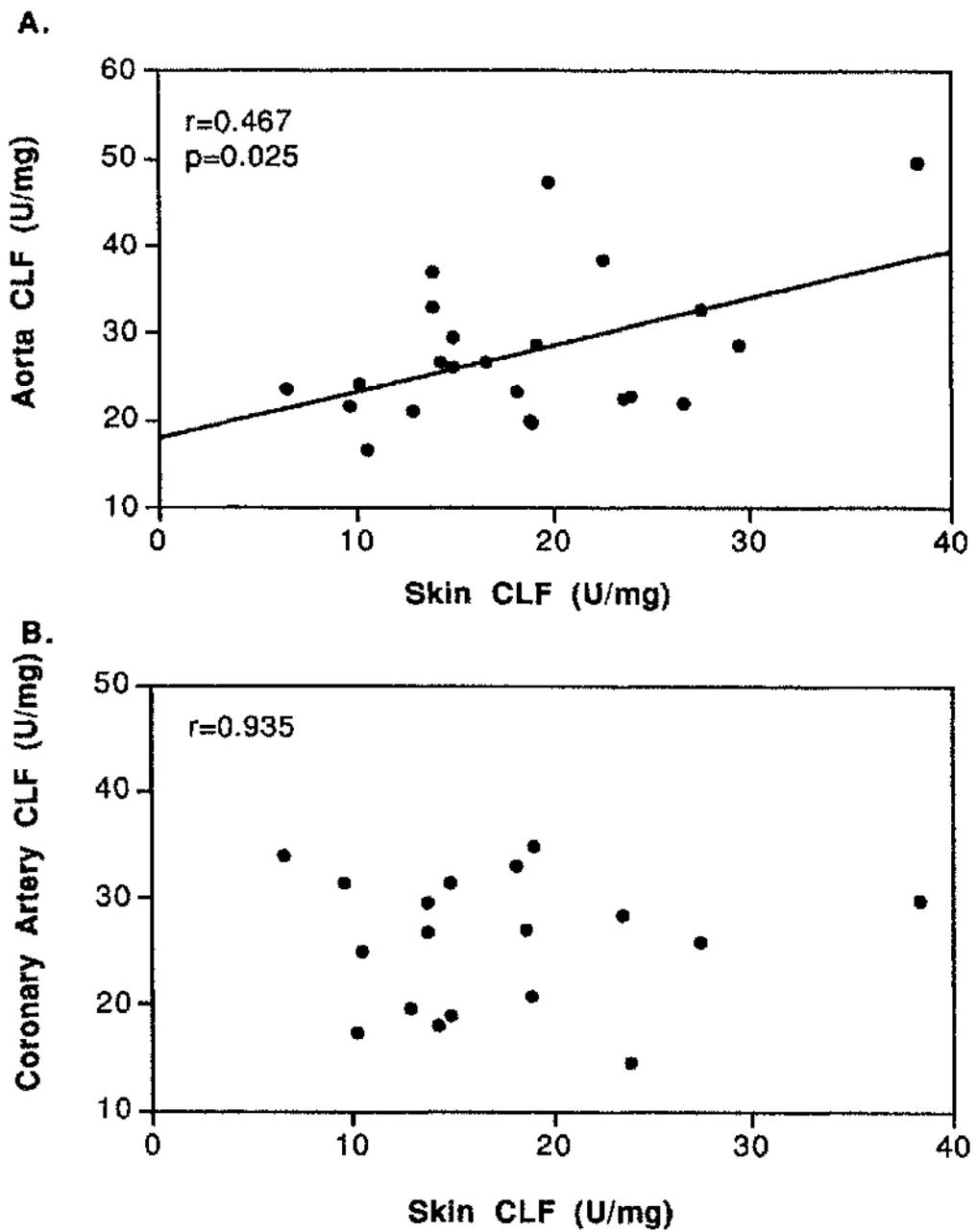


Figure 5.6

Relationship between collagen-linked fluorescence in skin and aorta (A) and between skin and coronary arteries (B). Fluorescence measured at excitation 370nm, emission 440nm.

5.4.5. CLF in different forms of the atherosclerotic plaque.

CLF was lower in areas covered by superficial plaque compared with adjacent, atheroma free segments of the arterial wall (22.2 ± 1.1 U/mg vs 27.9 ± 1.8 U/mg, $p=0.0141$), fig 5.7. AGE fluorescence in the areas covered by calcified, collagenous and ulcerated plaques was not different from that of plaque-free aorta. CLF in collagenous plaques correlated with CLF in the atheroma free regions. However, CLF in superficial, calcified and ulcerated plaques did not show this correlation (data not shown).

Individuals with mild to moderate severity of atheroma in the abdominal aorta had lower median CLF (20.0 U/mg) in the superficial atherosclerotic plaques than those with severe atheroma (22.5 U/mg, $p=0.0466$; fig 5.8).

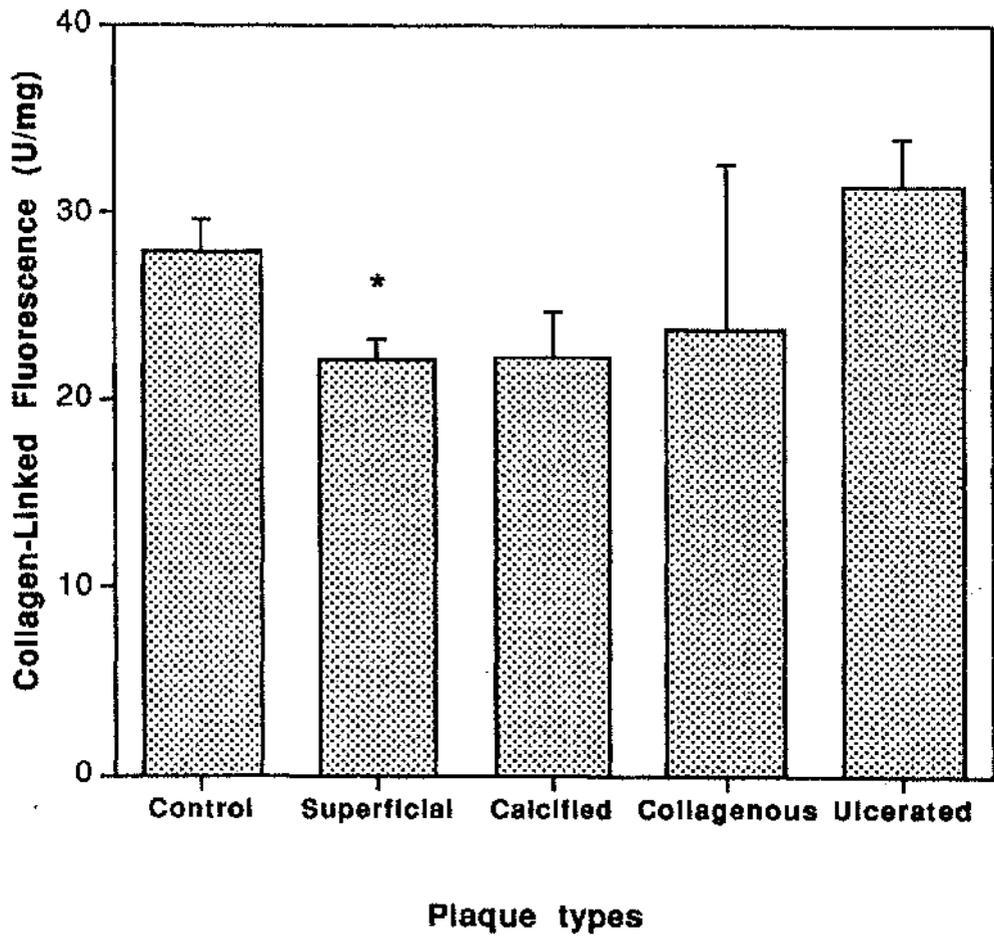


Figure 5.7

Collagen-linked fluorescence in different forms of atherosclerotic plaque in the human aorta. Excitation 370nm/emission 440nm. * Control vs superficial plaque: $p=0.0141$. Error bars denote SEM.

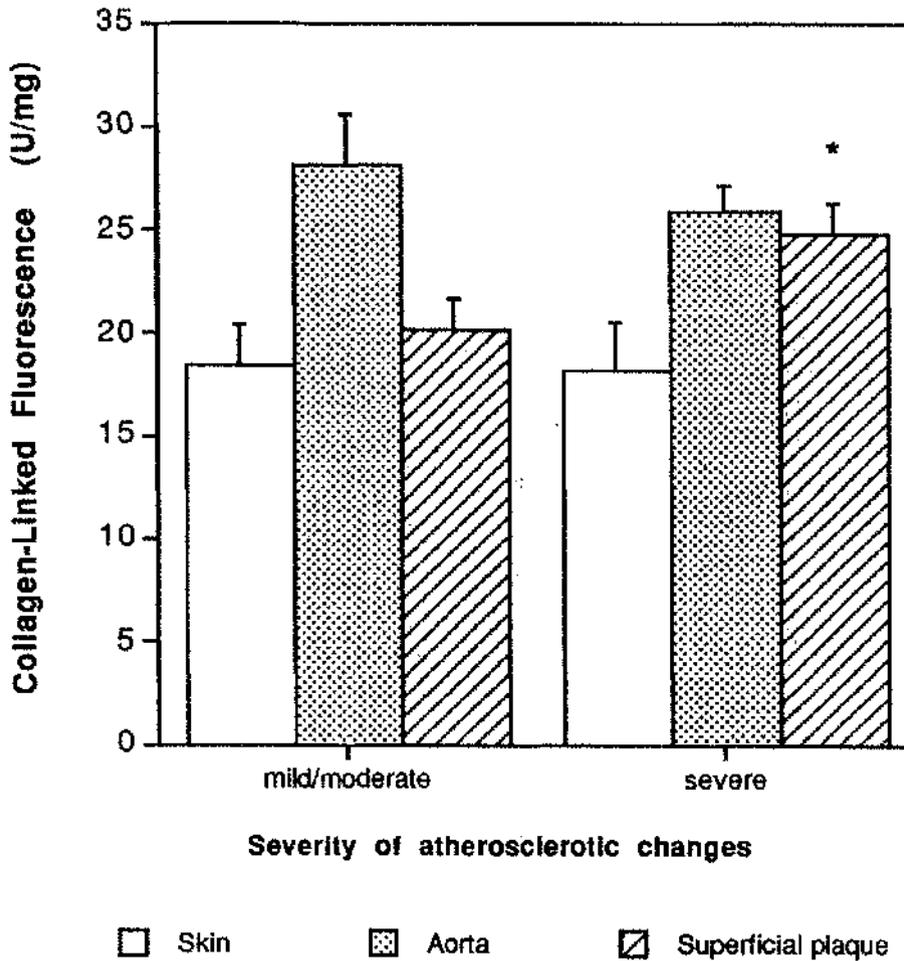


Figure 5.8

Collagen-linked fluorescence in the superficial atherosclerotic plaques in individuals with different severity of atheroma. Excitation 370nm/emission 440nm. * Superficial plaque fluorescence mild/moderate vs severe atherosclerosis, ($p=0.0466$). Error bars denote SEM.

5.5. Discussion.

5.5.1. Relationship between skin collagen-linked fluorescence and subjects' age.

It has been reported by many groups that the accumulation of AGE in human skin is related linearly to the patients' age and parallels changes in the solubility and digestibility of collagen. This linear relationship between CLF and patients' age has also been found in mesenteric artery [152]. In the same study, the generation of CLF appeared to accelerate in individuals ages ≥ 60 years. It is unlikely that this exponential increase is due to declining of glucose tolerance. Instead, it may be the combined result of AGE accumulation and the progressive decrease of connective tissue turnover as the patient gets older [12,326]. The exponential increase in CLF has been also been observed in the skin of non-diabetic Wistar rats [258].

5.5.2. The effect of aminoguanidine on collagen-linked fluorescence of aortic tissue browned in vitro.

The fluorescence spectra of the different tissues were consistent with those previously observed for BSA incubated with D-glucose and AGE-modified collagen (chapter 2 and chapter 3 respectively). Studies by carried out by Kohn et al and Dyer et al show comparable spectra both in vitro and in vivo [52,107].

Recently, a radioreceptor assay (RRA) using the macrophage cell line RAW 264.7 which possesses AGE receptors has been developed [162]. AGE concentrations in plasma measured using RRA were lower than those measured as CLF [164]. Different specificities of both RRA and CLF measurement would explain these differences found when measuring AGE in vivo. However, there

are disadvantages to using the radioreceptor assay: chiefly that it is assumed that the ligand used to produce the standard binding curve in the competitive binding studies, produced in vitro, is identical to AGE structures present in vivo. Though there may be common epitopes [205].

The incubation of normal aorta with G6P led to an increase in CLF characteristic of AGE (ex370nm, em440nm). This increase in fluorescence was inhibited by aminoguanidine. These results compare to those observed when incubating BSA with glucose and aminoguanidine (chapter 2). This confirms that collagen-linked fluorescence is a good marker of AGE accumulation.

AGE accumulation may also be assessed by measurement of a recently discovered fluorescent AGE (pentosidine) from human extracellular matrix with a characteristic fluorescence at excitation wavelength 335nm and emission wavelength 385nm [59,82]. Grandhee et al synthesised pentosidine by reacting ribose (pentose) with lysine and arginine in vitro [86]. The source of free ribose in vivo is unknown but it has been suggested by Dyer et al that other reactants apart from ribose such as D-glucose, fructose and ascorbate could be involved in the formation of pentosidine [58]. However, in the same study it was shown that pentosidine accounted for less than 1% of non-disulfide crosslinks in protein dimers formed during the reaction. Both of the above studies reactions were carried out at 65°C. In our study, the incubation of human aortic tissue with 250mM G6P did not result in an increase in fluorescence characteristic of pentosidine (excitation wavelength 335, emission wavelength 385nm). Reasons for the inability of G6P to produce pentosidine fluorescence are unclear and may be explained by the in vitro incubation conditions used and the fact that proteins extensively modified by pentosidine account for only small proportion of the total AGE formed.

5.5.3. Collagen-linked fluorescence in superficial atherosclerotic plaques.

The involvement of AGE in atherogenesis has previously been postulated by Cerami et al [16]. Since then a wealth of data suggesting that AGE may contribute to tissue remodelling has become available [178,179,219]. The binding of AGE to a specific membrane receptor on monocytes and macrophages induces the secretion of interleukin-1 β , cachectin/tumour necrosis factor (TNF). This in turn could lead to smooth cell proliferation and the synthesis of connective tissue elements which occur during the development of atheroma [327,328]. Recently a new class of AGE receptor (RAGE) has been characterised, and has been found to be a member of the immunoglobulin superfamily suggesting that, in addition to binding AGE, it might also serve other functions, including cell-cell recognition and binding of growth factors and cytokines [329].

The formation of AGE is a slow process, so the long half life of collagen makes it an ideal target for AGE formation. In both animal and human models, AGE have been shown to accumulate with age. The levels of AGE correlate with the severity of diabetic retinopathy [160]. The accumulation of AGE in the tissues of patients with diabetic nephropathy may be a result of increased production or decreased removal of AGE. High concentrations of serum AGE-peptides were found in patients with diabetic nephropathy, suggesting that the kidney impairment affects the clearing of circulating AGE [164,250]. The mechanisms determining tissue remodelling are similar in diabetic complications and atherosclerosis [330]. The formation of AGE on proteins in the diabetic arterial wall is best exemplified by its potential role as an accelerator of atherosclerosis, the increased stiffening of the arterial wall as well as the thickening of the capillary basement membranes, both of which occur at an accelerated rate in

diabetes. In vitro evidence shows that AGE affect cellular processes such as release of cytokines and growth factors and thus contribute to intimal proliferation through increased synthesis of collagen and migration of smooth muscle cells.

The most important finding in the present study, is that local changes in AGE concentration occur within the atherosclerotic lesion. We observed decreased CLF in areas of human aorta covered with superficial atherosclerotic plaque. This suggests that the formation of the superficial atherosclerotic plaque locally affect the AGE concentration. Early macrophage accumulation in plaques could lead to the local degradation of AGE-modified collagen and to a consequent decrease in CLF. Katsuda et al suggested that low concentrations of collagen found in the centres of atherosclerotic plaques may be due to macrophage-generated collagenase activity [331]. Cultured arterial smooth muscle cells are also known to secrete collagen-lytic enzymes. Since macrophages and smooth muscle cells are the major cell types within human atherosclerotic lesions, it is likely that these cells participate in the degradation of collagen within the lesion [332]. Such degradation of AGE-modified collagen by macrophages may stimulate macrophages to release growth factors and thus lead to the vicious cycle of tissue remodelling.

In our study, individuals with severe atherosclerosis appeared to have higher superficial plaque fluorescence than patients with mild to moderate atherosclerosis. Further studies are required to establish the significance of this. An attractive hypothesis is that initial AGE concentration in the arterial wall may be related to the extent of atherosclerosis. Initially, AGE-modified collagen within the lesion could stimulate the synthesis of tissue components by smooth muscle cells. The measurement of AGE is calculated as fluorescence units per milligram of collagen, thus the synthesis of new collagen could theoretically

lower the AGE fluorescence of atherosclerotic lesions. However, this is unlikely to have occurred in our study as collagenous and ulcerated plaques, which have the highest content of newly synthesised collagen, did not show lower levels of fluorescence.

In the present study, skin CLF correlated with aortic CLF in atheroma free regions. This indicates that the measurements of CLF on skin biopsy material reflect aortic AGE concentration and may prove useful in further clinical studies on AGE. However, neither skin nor aortic CLF correlated with CLF in the coronary arteries. This is in line with the findings of Makita et al who reported a difference between CLF and radioreceptor results in coronary arteries [164]. This lack of correlation between aorta and coronary artery could be explained by the different structure of these two types of vessels (i.e. collagen types and thickness of intima, media and adventitia).

5.6. Conclusions.

- (1.) The present study suggests that the relationship between AGE accumulation and biological age is exponential process rather linear.
- (2.) AGE concentration is affected locally by processes involved in atherosclerotic plaque formation.
- (3.) There is a relationship between AGE content of skin and atheroma free aorta. This provides a useful marker for studying the dynamics of AGE formation in vascular tissue.

CHAPTER 6.

**Advanced glycosylation endproducts in patients with renal failure.
The effect of renal transplantation on AGE levels in skin and
peritoneum.**

6.1. Summary.

This chapter describes:

- (1.) The effect of renal failure on tissue level of advanced glycosylation endproducts (AGE) in diabetic and non-diabetic patients.
- (2.) The effect of renal transplantation on AGE level in the skin and peritoneum of non-diabetic patients.
- (3.) The effect of continuous ambulatory peritoneal dialysis (CAPD) on AGE levels in the skin and peritoneum.

6.2. Introduction.

AGE have been implicated in the processes of ageing, atherogenesis and the late complications of diabetes. The removal of AGE-derived crosslinks may be impaired in patients with renal failure. On the other hand successful renal transplantation may improve the removal of such AGE adducts. Renal transplantation may be more effective than conventional haemodialysis therapy at lowering AGE levels in plasma [164]. In the present study, we have investigated AGE concentrations in the skin and peritoneal membranes of patients undergoing either CAPD or renal transplantation. The use of high glucose dialysis fluids could lead to AGE formation and to the consequent crosslinking of proteins in the peritoneal stroma. The continuous bathing of the peritoneal membrane in fluids that are both hypertonic and on initial infusion, acidic results in the peritoneal membrane becoming more prone to recurrent inflammatory episodes of either bacterial (peritonitis) or chemical (serositis) etiology. We hypothesised that the continuous use of such fluids may lead to the modification of the peritoneal membrane by AGE.

6.3. Materials and methods.

6.3.1. The effect of renal transplantation on AGE levels.

To study the effect of CAPD and renal transplantation on tissue AGE in non-diabetic patients with renal failure (section 6.3.2). We measured CLF in skin and peritoneal biopsies obtained from patients undergoing CAPD or renal transplantation.

To study the effect of diabetes mellitus, CLF was also measured in skin biopsies from non-diabetic and diabetic recipients of renal transplants (section 6.3.3). This study was designed to investigate the tissue levels of AGE in both groups of patients and to determine the effect of diabetes on the lowering capacity of renal transplantation.

6.3.2. The effect of continuous ambulatory peritoneal dialysis and renal transplantation on collagen-linked fluorescence in skin and peritoneum from non-diabetic patients with renal failure.

We have studied 38 non-diabetic patients with CRF. The aetiology of CRF was chronic glomerular nephritis (12 patients), chronic pyelonephritis (6 patients), analgesic nephropathy (2 patients), obstructive uropathy, renal dysplasia, renal calculi, Alport's syndrome, polycystic kidney disease and Henoch-Schonlein purpura (1 patient in each of the remaining groups). In remaining patients the aetiology was unknown. Patients were divided into four subgroups:

(1.) Non-diabetic patients with CRF (designated as CRF group; n=18, mean age 52.1 ± 4.3 yrs (\pm SEM)). Samples of skin and peritoneal tissue were taken at the time of Tenckhoff catheter insertion in preparation for CAPD.

(2.) Non-diabetic patients with CRF who had received transplants while on CAPD (designated as transplant group; n=16, mean age 48.2 ± 3.14 yrs). Samples were taken at the time of Tenckhoff catheter removal, 5-31 weeks (11 ± 6.5 weeks) after renal transplantation, when all patients were treated with peritoneal dialysis and had good renal function as indicated by serum creatinine concentration.

(3.) Non-diabetic patients with CRF who were treated with CAPD but had not received kidney transplants (designated as CAPD group; n=4, mean age 57.5 ± 4.6 yrs). Samples were taken when the Tenckhoff catheters were replaced due to infection or technical problems.

(4.) The control group consisted of patients with normal renal function (n=24, mean age 46.3 ± 3.65 yrs) who were undergoing surgical procedures such as elective laparotomy for a variety of reasons, or elective inguinal hernia repair. In 18 of the control patients the operation involved exposure of the peritoneum. In these patients the peritoneal samples were taken in addition to skin samples. The study protocol was approved by the local Ethical Committee.

All skin samples were obtained from the abdominal wall, at the site of incision, during general anaesthesia. Peritoneal samples were obtained from the parietal peritoneum of the anterior wall at the site of the laparotomy or from the hernia sac during hernia repair. Tissue samples were extensively washed with physiological saline and stored at -20°C until analysis. Collagen-linked fluorescence was measured in skin and peritoneal samples as described in

section 4.3.6 and expressed as fluorescence units per milligram of collagen (U/mg). All CLF measurements were carried out in triplicate.

Fluorescence spectra of skin and peritoneum were compared with AGE-modified, type I calf skin collagen. The procedure for the preparation of *in vitro* browned collagen is described in section 3.3.1.

6.3.3. Measurement of CLF in skin biopsies from the diabetic and non-diabetic recipients of renal transplants.

AGE levels were measured as CLF in diabetic and non-diabetic patients with chronic renal failure who underwent renal transplantation. Skin biopsies were taken under local anaesthetic using a punch biopsy technique. Three groups were studied:

- (1.) Diabetic patients with renal failure who underwent renal transplantation (n=10, mean age 39.0 ± 8.51 yrs).
- (2.) Non-diabetic patients with renal failure who underwent renal transplantation (n=12, mean age 38.7 ± 12.7 yrs).
- (3.) Control subjects with normal renal function (n=10, mean age 35.0 ± 3.9 yrs).

The samples of skin weighing 10mg were washed with physiological saline then scraped clean of any hair and excess adipose tissue. Samples were then stored at -20°C until CLF measurement. CLF was measured as described in section 4.3.6.

In addition to measurement of CLF, serum creatinine, glucose, serum fructosamine and lipids were also measured. This study was approved by the local Ethical Committee.

6.3.4. Measurement of plasma glucose.

Glucose was measured by a glucose oxidase method on a Beckman II glucose analyser (Beckman, Yellow Springs, Ohio, USA). Between batch imprecision was 2% (refer to 4.3.4).

6.3.5. Measurement of glycosylated haemoglobin.

The measurement of glycosylated haemoglobin represents the time-averaged glycaemia. It is unaffected by short term fluctuations in blood glucose concentrations and is a marker of glycaemic control. Blood (5ml) was collected in a fluoride/oxalate treated tube and glycosylated haemoglobin was measured as described in section 4.3.5.

6.3.6. Measurement of serum fructosamine.

Fructosamine measurements reflect average glycaemia over a shorter time period than that reflected by the measurement of HbA_{1c}. EDTA plasma is added to reagents containing carbonate buffer (0.2mmol/L, pH 10.3) and nitroblue tetrazolium/Uricase (0.48mmol/L, ≥ 2.5 kU/L), mixed at 37°C and read after exactly 10min (A1) and then again after 15min (A2) at wavelength 550nm against a reagent blank on a Cobas Bio analyser. The calibrator is also read using the same reagent blank and the fructosamine concentration (FC) is calculated as follows:-

Absorbance 550= A2-A1

$$\text{FC (umol/L)} = \frac{\text{Abs 550 (test)}}{\text{Abs550 Calibrator}} \times \text{Calibrator Concentration}$$

For samples with a fructosamine concentration greater than 1000 umol/L the samples were diluted using 0.9% saline.

6.3.7. Enzymatic determination of total cholesterol.

Total cholesterol was measured using a reagent kit from Boehringer Mannheim, (Meylan, SA, France) based on the cholesterol kinetic CHOD-PAP method [333]. EDTA (ethylenediamine-tetraacetic acid) plasma was incubated with a buffered enzyme reagent containing cholesterol ester hydrolase (CEH) and cholesterol oxidase (CO). Esterified cholesterol is converted to free cholesterol which is then converted to cholest-4-en-3one and hydrogen peroxide by cholesterol oxidase. The produced hydrogen peroxide becomes a substrate for peroxidase (POD) and reacts with 4 aminophenazone and phenol to produce a red chromophore (4-(p-benzoquinone-monoimino)-phenazone). This is measured photometrically at 500nm on a centrifugal analyser (Cobas Bio).

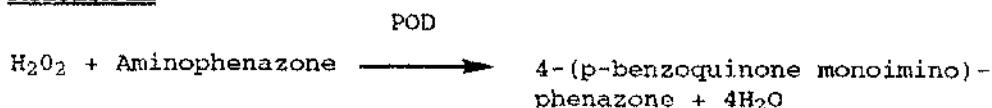
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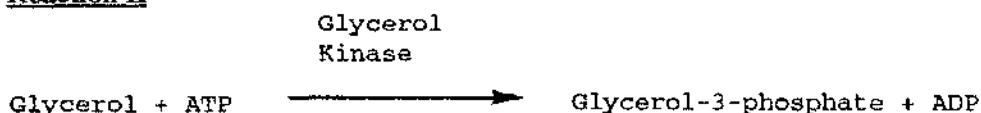
6.3.8. Enzymatic determination of triglyceride.

Measurement of triglyceride was carried out by the modified method of Bucolo and David in which triglycerides are hydrolysed enzymatically to glycerol and free fatty acids by a lipase reaction [334]. Glycerol produced from the triglyceride in the presence of adenosine triphosphate (ATP) is converted to glycerol-3-phosphate and adenosine diphosphate (ADP) by glycerol kinase. The ADP in the presence of phosphoenolpyruvate is then used as a substrate by pyruvate kinase to produce ATP and pyruvate. The pyruvate is further reduced with $\text{NADH} + \text{H}^+$ to produce lactate and NAD. The decrease in $\text{NADH} + \text{H}^+$ is proportional to the total glycerol concentration.

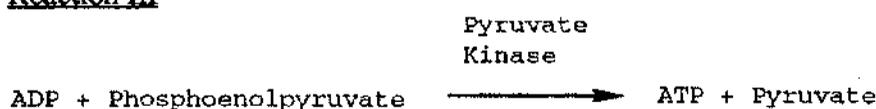
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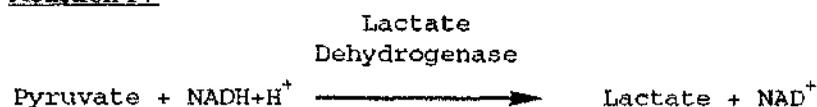
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Reaction III



Reaction IV



The reactions were carried out on the Cobas Bio centrifugal analyser (Roche) using the Merckotest Triglyceride kit (E. Merck, Darmstadt, Germany). All reactions were conducted at 37°C and $\text{NADH} + \text{H}^+$ absorbance was measured at 340nm. Samples with concentration greater than 5.0 mmol/L were diluted with saline and reanalysed.

6.3.9. Quantitation of lipoproteins by flotation ultracentrifugation using a tabletop centrifuge.

Flotation centrifugation separates the plasma lipoproteins according to their densities such that the concentrations of VLDL (very low density lipoprotein), LDL (low density lipoprotein) and HDL (high density lipoproteins) can be calculated.

Blood was taken from patients in 7ml EDTA vacutainers then centrifuged at 1000rpm on a Sorvall centrifuge for 5min. Plasma was separated, transferred (2ml) to a quick seal polyallomer belltop centrifuge tube (Beckman) and slowly overlayers with a NaCl solution of density=1.006 g/l leaving a space at the top of the tube for sealing. The density of the NaCl solution was determined using a Digital Densitometer (Paar Scientific) and adjusted to $d=1.006\text{g/l}$ with either distilled water or additional NaCl. The NaCl solution (0.195M) of specific density was made with 0.34nM EDTA and 1ml of 1N NaOH and made up to 1.13 litres with distilled water.

The polyallomer tubes were sealed with metal caps which were heated using a Beckman heat sealer. The tubes were then placed in a TL-100.3 rotor and centrifuged in a TL-100 ultracentrifuge at 39,000g at 4°C for 2.5 hours. At completion of centrifugation, the tubes were placed in a slicer, the tubes were sliced and the top fraction containing the VLDL was transferred quantitatively using a syringe into a 1ml volumetric flask. The bottom fraction containing the LDL and HDL was recovered quantitatively into a 3ml volumetric flask. During the centrifugation of EDTA plasma, NaCl solution ($d=1.006$) was added and during the separation of the fractions the volume of the bottom and top fractions were adjusted to 3ml and 1ml respectively.

The measurements of cholesterol in the top and bottom fractions were carried out using the CHOD-PAP method. Measurement of HDL was performed by precipitating out the LDL using a heparin/manganese dichloride method. Sample of the bottom fraction (25 μ l) was added to 0.5ml Heparin/MnCl₂ (7140 USP/ml/2.1M) in an 1.5ml Eppendorf tube and centrifuged for 15min at 10,000g in a microfuge (Beckman). The supernatant was then decanted into a Cobas Bio sample cup and total cholesterol was measured using the CHOD-PAP method. Quality control samples (Corning, setting value of 4.3 mmol/L) were used.

To compensate for the dilution of both fractions measured, cholesterol content in the top fraction was multiplied by 0.5 to obtain serum VLDL cholesterol concentration, measured HDL cholesterol by 1.5 and the measurement of the bottom fraction (LDL + HDL) by 1.5.

Calculations:

VLDL-cholesterol = total plasma cholesterol - bottom fraction cholesterol
(LDL + HDL).

LDL-cholesterol = bottom fraction cholesterol - HDL cholesterol.

6.3.10. Statistical analysis.

Unpaired Students t-test and the Mann-Whitney test were used for the comparison of samples as appropriate. Regression analysis using a linear model was used for determining correlations between studied variables. Minitab software (version 8) was used throughout the study.

6.4. Results.

6.4.1. The effect of CAPD and renal transplantation on CLF in the skin and peritoneum.

Patient characteristics:

There were no significant differences in age between the four groups of patients studied. Plasma glucose concentration (mean \pm SEM) in the CRF group was 5.3 ± 0.23 mmol/L, in the transplant group 5.5 ± 0.46 mmol/L, in the CAPD group 5.4 ± 0.41 mmol/L group and in the control group: 5.7 ± 0.33 mmol/L. None of the patients was classified as diabetic.

Serum creatinine in the CRF group was 868.1 ± 62.3 mmol/L, in the transplant group 123.6 ± 10.7 mmol/L, in the CAPD group 800 ± 184 mmol/L and in the control group 84.3 ± 2.4 mmol/L. Serum creatinine in the transplant group was significantly lower than both the CRF and CAPD groups but higher than in the control group. There was no relationship between CLF of skin or peritoneum and either plasma glucose or serum creatinine concentrations in all groups studied.

6.4.2. Emission spectra of skin, peritoneum and in vitro glycated collagen.

The emission spectra of in vitro browned collagen were measured at excitation 370nm and were similar to the emission spectra of skin and peritoneum (fig 6.1).

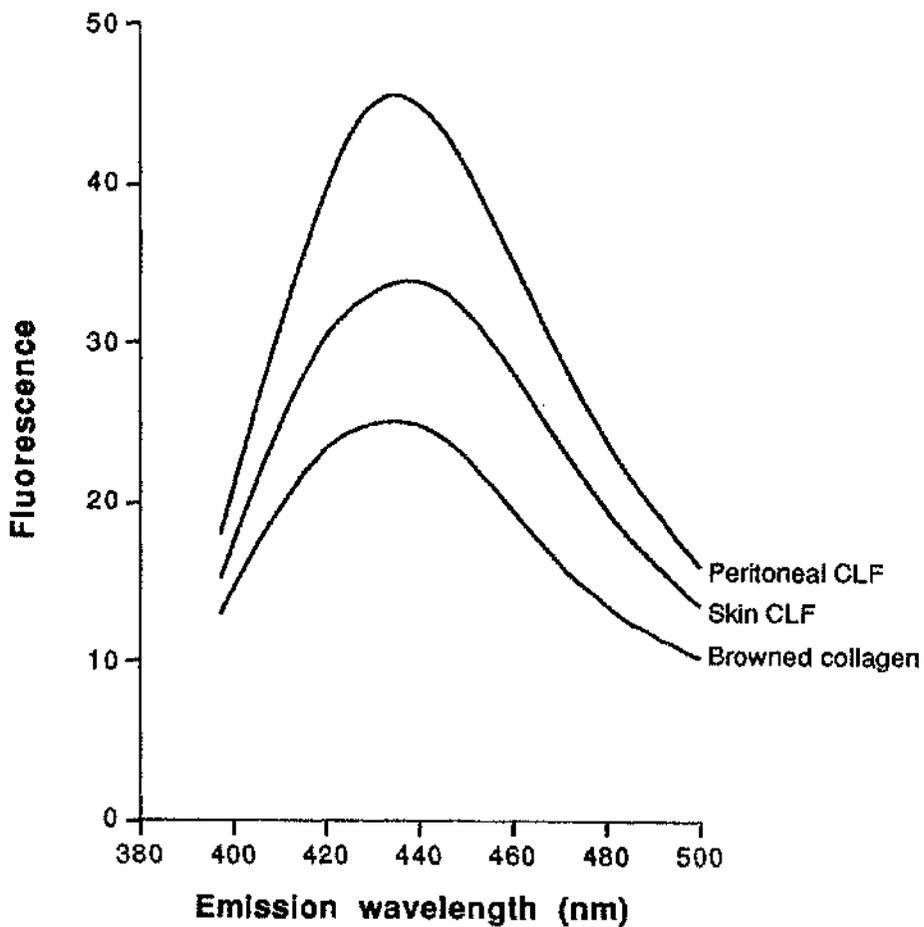


Figure 6.1

Emission spectra of browned collagen, skin and peritoneum. (Excitation wavelength 370nm).

6.4.3. Collagen-linked fluorescence in skin biopsies of CRF, CAPD, transplant and control groups.

Skin CLF in the CRF group (20.9 ± 2.02 U/mg; \pm SEM) and the CAPD group (22.2 ± 6.23 U/mg) were both significantly higher compared to the control group (8.52 ± 1.08 U/mg, $p < 0.001$) but were not different ($p = 0.814$) from each other. Skin CLF in the CRF group was higher compared to the transplant group (10.7 ± 2.4 U/mg, $p = 0.003$). Skin CLF in the CAPD group was also higher than transplant group (borderline significance, $p = 0.06$). Skin CLF in the control and transplant groups were not different (fig 6.2).

6.4.4. Collagen-linked fluorescence in peritoneal biopsies of CRF, CAPD, transplant and control groups.

Peritoneal CLF in the CRF (30.5 ± 5.64 U/mg) and CAPD (47.4 ± 9.66 U/mg) groups were both higher than the control group (16.1 ± 2.25 U/mg, $p = 0.031$ and $p < 0.001$ respectively) but were not different ($p = 0.164$) from each other. Peritoneal CLF of the CAPD group was higher than the transplant group (19.4 ± 3.66 U/mg, $p = 0.0045$).

Peritoneal CLF of the CRF group was not different from the transplant group ($p = 0.11$). Peritoneal CLF of the control and transplant groups were not different ($p = 0.45$; fig 6.2).

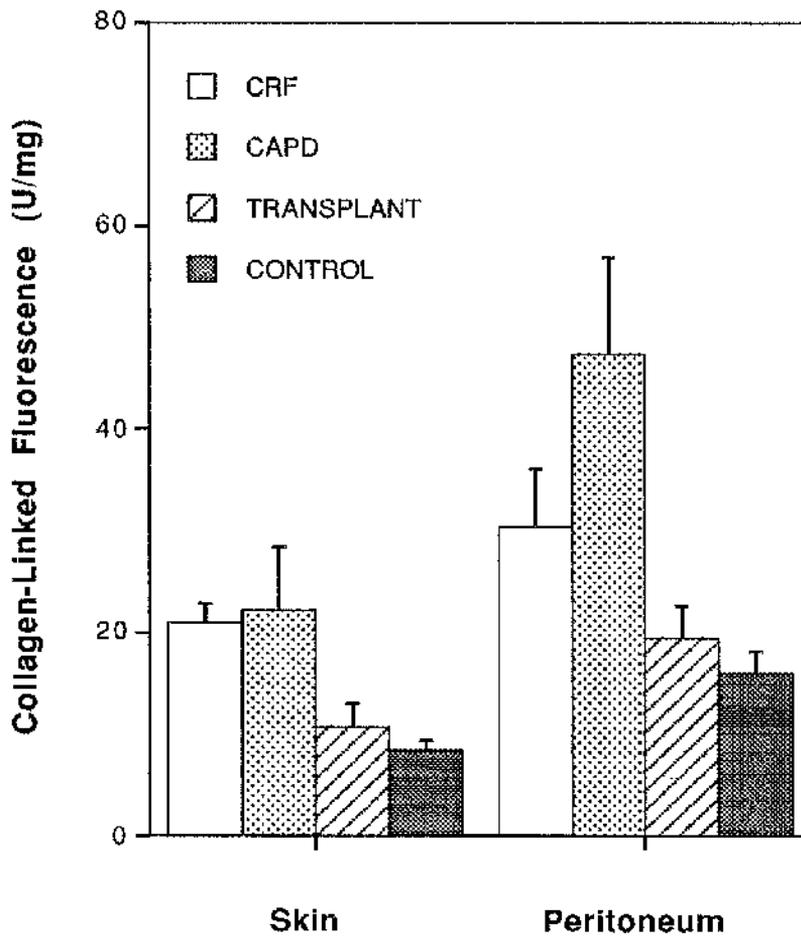


Figure 6.2

Collagen-linked fluorescence (CLF) in the skin and peritoneum of patients before commencement of CAPD (CRF group), during CAPD (CAPD group), after renal transplantation (transplant group) and in the control group. Excitation wavelength 370nm, emission wavelength 440nm (mean \pm SEM U/mg). See text (6.4.3) for description of statistical significance between groups.

6.4.5. The relationship between patients' age and collagen-linked fluorescence in skin and peritoneal biopsies.

In the CRF group skin CLF correlated with patients' age ($r=0.618$, $p=0.006$; fig 6.5), but no correlation was observed between peritoneal CLF and age ($r=0.22$, $p=0.466$; fig 6.3). By contrast, in the control group, both skin CLF ($r=0.548$, $p=0.007$) and peritoneal CLF ($r=0.508$, $p=0.044$) correlated with patients' age. In the transplant group, peritoneal CLF ($r=0.525$, $p=0.44$) but not skin CLF ($r=0.136$, $p=0.615$) correlated with patients' age.

6.4.6. The relationship between skin CLF and peritoneal CLF.

In both the CRF ($r=0.6455$, $p=0.017$) and transplant ($r=0.519$, $p=0.047$) groups, skin and peritoneal CLF correlated well with each other (fig 6.4 a-b) but there was no relationship between skin and peritoneal CLF in the control group (fig 6.4 c).

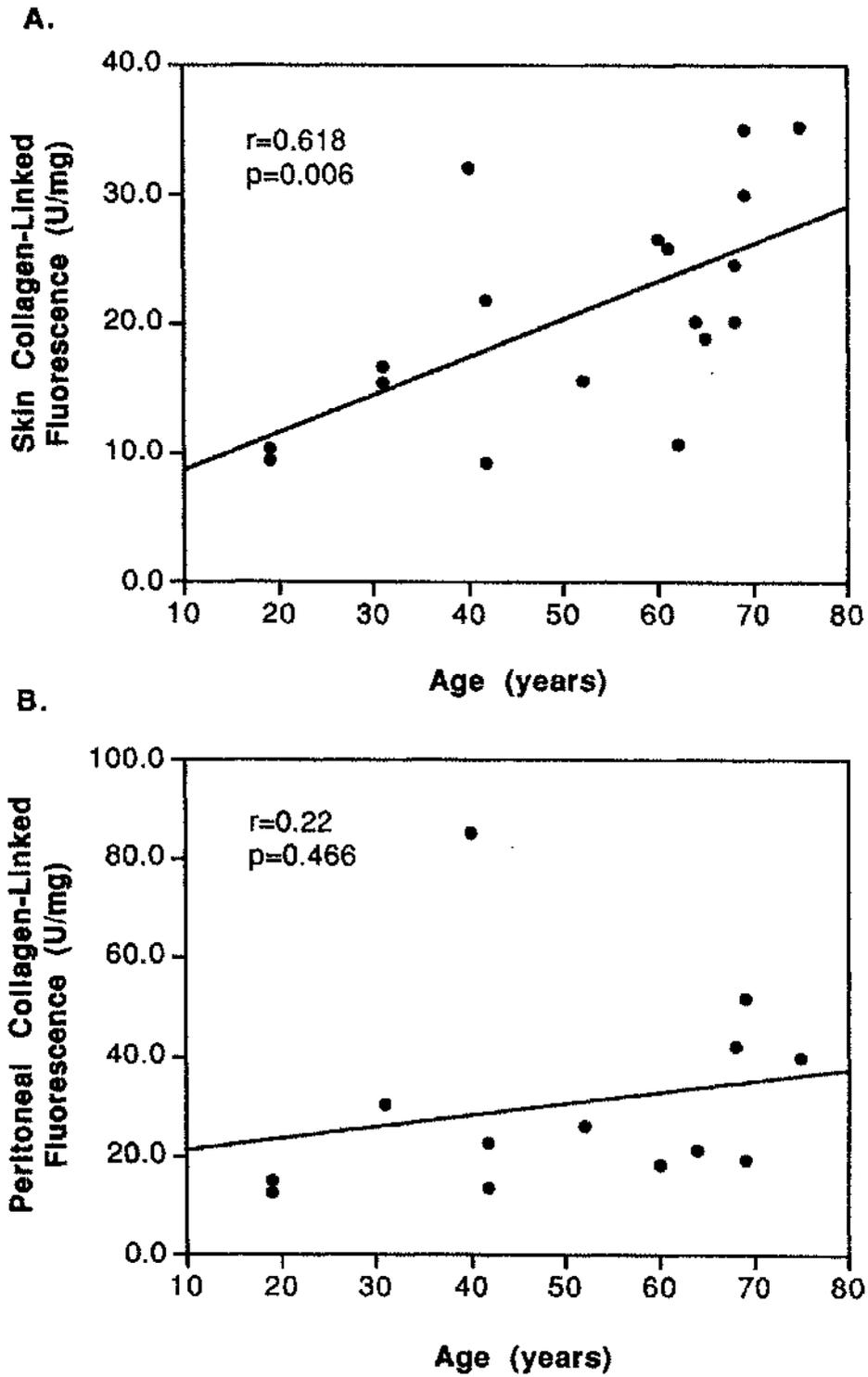


Figure 6.3

Relationship between skin (A) and peritoneal (B) collagen-linked fluorescence and age of patients with CRF (CRF group).

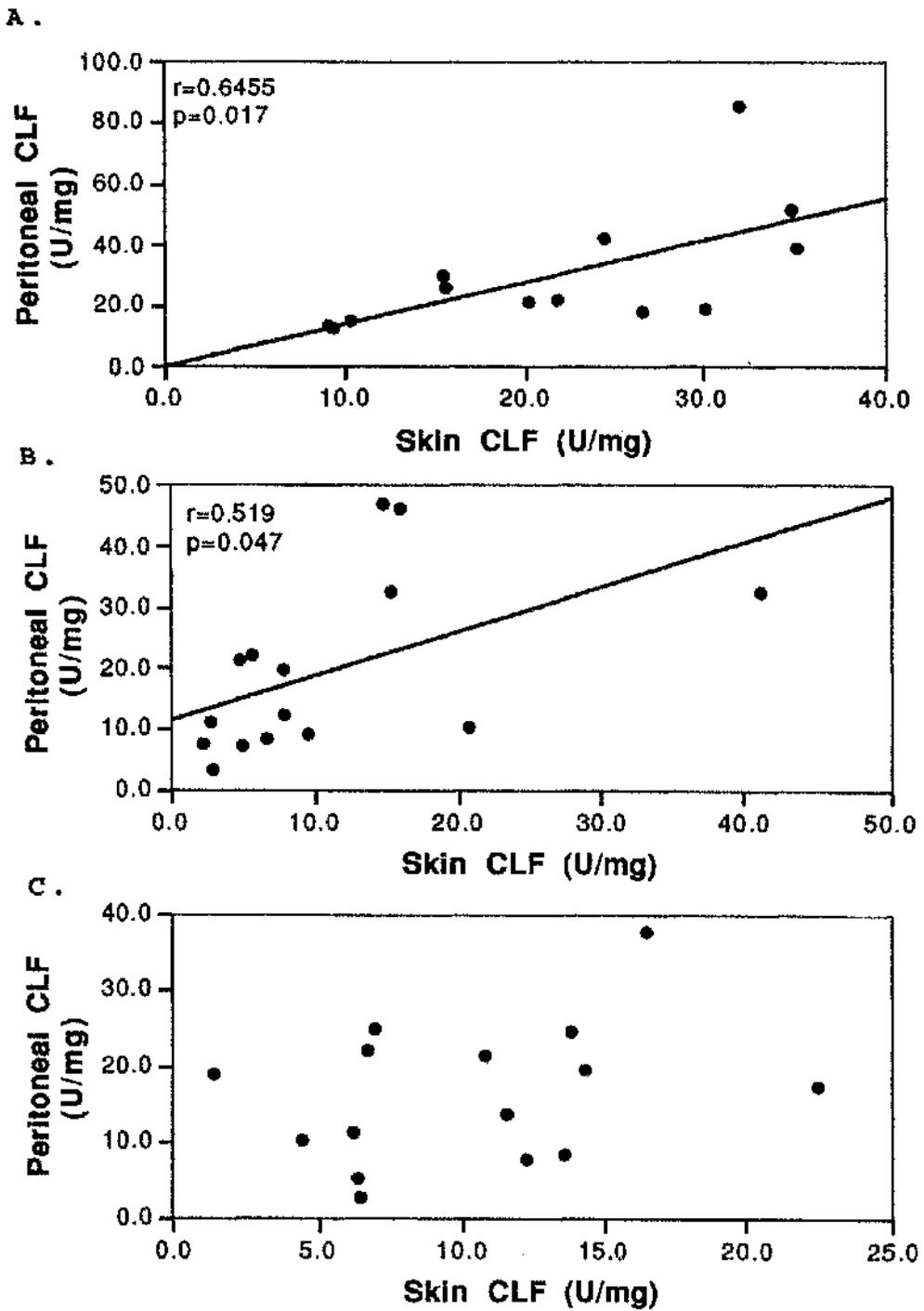


Figure 6.4

Relationship between collagen-linked fluorescence in the skin and peritoneum;

(A) CRF group, (B) transplant group, (C) control group.

6.4.7. AGE levels in the skin of diabetic and non-diabetic recipients of kidney transplants.

Patient characteristics.

The control group had a normal biochemistry profile (table 6.5). There were no significant difference in age (years) between the three groups studied. When compared to the control group, creatinine levels were found to be higher in the recipients of renal transplants (non-diabetic and diabetic group). The glycated haemoglobin (HbA₁) in the diabetic group was significantly higher than both control and non-diabetic groups (Table 6.5).

Table 6.5.

Biochemical characteristics of the controls, non-diabetic and diabetic recipients of renal transplants (**p<0.001, Non-diabetic vs diabetic).

	Diabetic mean \pm 1SD n=10	Non-diabetic mean \pm 1SD n=12	Controls mean \pm 1SD n=10
Urea (mmol/L)	11.0 \pm 5.67	7.9 \pm 2.7	5.4 \pm 2.1
Creatinine (umol/L)	155 \pm 84	136 \pm 50	81 \pm 10
LDL (mmol/L)	4.0 \pm 0.6	4.2 \pm 0.5	4.0 \pm 0.7
HDL (mmol/L)	1.4 \pm 0.6	1.4 \pm 0.3	1.4 \pm 0.1
vLDL (mmol/L)	0.6 \pm 0.4	0.8 \pm 0.6	0.4 \pm 0.2
Triglycerides (mmol/L)	1.7 \pm 0.7	1.7 \pm 0.7	1.0 \pm 0.4
Cholesterol (mmol/L)	5.8 \pm 0.6	6.3 \pm 1.4	4.4 \pm 1.2
HbA ₁ (%)	11.9 \pm 1.6	6.6 \pm 1.4 **	6.1 \pm 0.6

6.4.8. Collagen-linked fluorescence in skin biopsies from diabetic and non-diabetic recipients of renal transplants.

Skin CLF in the diabetic recipients of kidney transplants (mean \pm SEM U/mg, 14.1 ± 2.6 U/mg) was significantly higher than the control group (6.2 ± 0.64 U/mg, $p=0.026$) but was not different from skin CLF of the non-diabetic recipients of kidney transplants (8.7 ± 1.1 U/mg, $p=0.083$, bfig 6.6.). No correlations were observed between the patients' age and skin CLF in the 3 groups. Neither were there correlations between skin CLF and any of the other biochemical parameters listed in table 6.5.

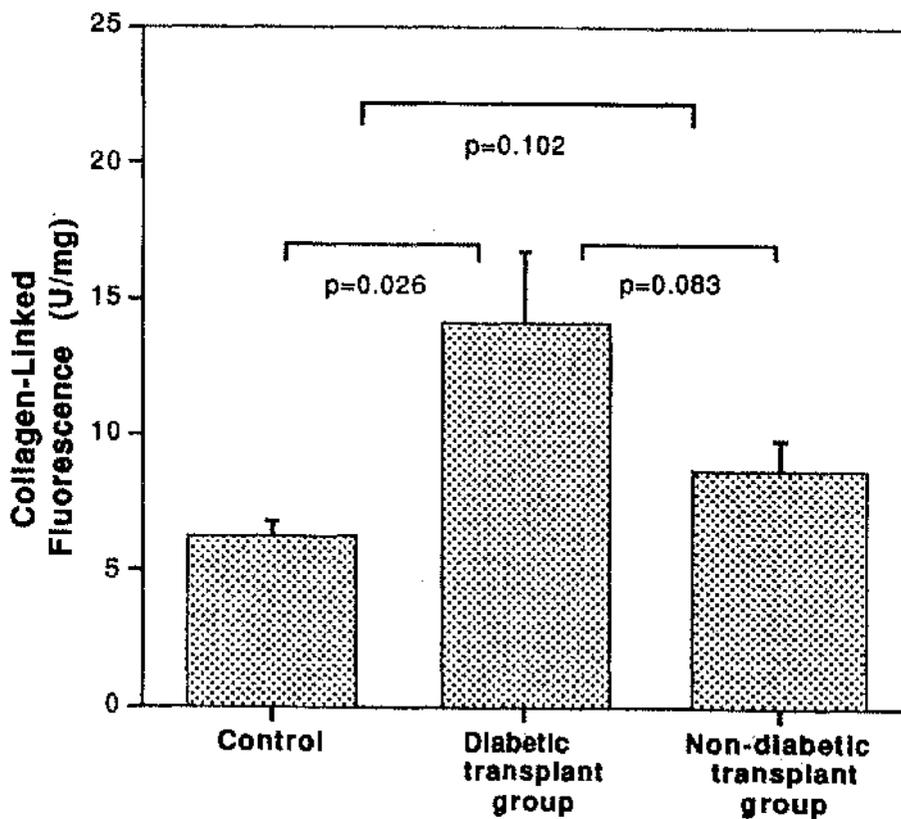


Figure 6.6

Collagen-linked fluorescence in the skin of normal subjects, diabetic and non-diabetic recipients of renal transplants. Error bars denote SEM.

6.5. Discussion.

6.5.1. The effect of CAPD and kidney transplantation on skin and peritoneal CLF in non-diabetic patients with renal failure.

Non-diabetic patients with renal disease have high levels of circulating protein-bound AGE. It is thought that a large portion of these circulating AGE-modified peptides are formed from the catabolism of AGE-modified tissue proteins, though a small fraction may form from the reaction of glucose with serum proteins. When glomerular filtration is impaired by renal failure, high concentrations of circulating AGE-peptides in the plasma result [250].

Using a macrophage an AGE radioreceptor assay, Makita et al have shown that there is a correlation between serum AGE-peptide levels and creatinine clearance [164]. In the same study, AGE levels in serum were shown to decrease in non-diabetic and diabetic patients with end stage renal failure (ESRD) who were treated with haemodialysis, though levels remained higher than the control group. Makita has also shown that diabetic patients with ESRD requiring haemodialysis had higher levels of AGE in arterial collagen than diabetic patients without renal disease. Diabetic patients who had received transplants 2-9 years previously had lower serum AGE concentrations than diabetic patients treated with haemodialysis. In two renal transplant recipients who were studied longitudinally, kidney transplantation restored serum AGE to levels comparable to the control group within 4 days.

Pentosidine levels are also increased in diabetic and non-diabetic patients with ESRD. Hricik et al have shown that plasma pentosidine concentrations in

patients with ESRD fell within 4 months after successful kidney transplants [171].

Our study is the first to provide data on tissue AGE levels in patients treated with CAPD and kidney transplantation. In patients with CRF, tissue AGE were increased when compared to the control group. Skin AGE levels were lower in the recipients of kidney transplants when compared to the non-transplanted CRF patients and CAPD group but this was of borderline significance in the latter group ($p=0.06$). This suggests that kidney transplantation, apart from lowering AGE-peptides in serum, as shown by Makita et al, may also restore tissue AGE concentrations towards normal. The cause of increased tissue CLF in non-diabetic patients with CRF probably reflects decreased removal of AGE-modified proteins. Restoration of renal function following transplantation may explain the decrease in the concentration of small AGE-peptides (<10kDa) in serum observed by other authors, but a decrease in tissue AGE observed in this study suggests the existence of as yet an unknown phenomenon.

We observed no difference between skin CLF in the CRF and CAPD groups, suggesting that peritoneal dialysis is not as efficient at removing tissue AGE as renal transplantation.

AGE crosslinks in tissue collagen are chemically stable, so dissociation of these within a short time is improbable. However since AGE-peptides may be deposited in tissues, one could speculate that two fractions of AGE exist : one consists of AGE present on structural, intact collagen molecules and the other of AGE-protein fragments derived from plasma, which would have been secondarily deposited within the tissue. These fragments would remain in equilibrium with serum AGE and could be cleared from tissues following restoration of renal function. The AGE-specific receptor found on macrophages

probably involved in degradation and removal of AGE-modified proteins [96,163]. Clearly if stimulation of AGE-related macrophage responses was associated with the presence of non-covalently bound AGE-peptide fragments in tissues, then the decrease in AGE-peptide fragments would be of benefit.

During CAPD, peritoneal collagen is exposed to high glucose dialysis fluid. We also measured CLF in peritoneal collagen and observed that peritoneal CLF is elevated by a factor of two in patients with CRF on CAPD when compared to the control group.

Peritoneal CLF in the CAPD group was higher than in the control and transplant groups. Thus it might be that dialysis fluid induced AGE formation takes place in peritoneal collagen.

It has been suggested that high glucose concentration used in dialysis fluids could lead to the formation of peritoneal AGE, and as a consequence, contribute to ultra-filtration failure [249]. Lamb et al have observed, that compared to serum levels, there was an increased amount of glycated albumin in the dialysate from patients treated with CAPD. They however have not been able to determine if this represents preferential transport or intra-peritoneal glycation [335]. A more recent study by the same group has shown that *in vitro* formation of AGE can occur in dialysis fluids, but dialysate removed from patients on CAPD have been shown to contain factors that may modulate AGE formation, at least *in vitro* [336]. In our study, CLF was observed to be higher in the peritoneum of patients on CAPD when compared to the CRF group but this did not reach statistical significance. Further studies are required to confirm this.

6.5.2. AGE and diabetic nephropathy.

Individuals with diabetes mellitus are at a 3-4 fold increased risk of myocardial infarction. A similar increase in the risk of atherosclerotic events occurs in patients with renal disease. The mechanism of this phenomenon remains to be established. Numerous studies have suggested that the high glucose level found in diabetes is the common denominator in the of the late complications of diabetes. Monnier et al observed an association between collagen-linked fluorescence and the severity of retinopathy, nephropathy, arterial stiffness and joint stiffness in patient's with diabetes and suggested that AGE may serve as an index of the severity of the diabetic complications [160,337,338].

We measured AGE as collagen-linked fluorescence (6.4.2) in diabetic and non-diabetic recipients of renal transplants. No significant differences in total cholesterol, triglycerides, LDL, HDL, vLDL levels were found between the control group and the diabetic or non-diabetic recipients of renal transplants. No relationship was observed between CLF and patients age in all three groups. This was not unexpected as the ages of the patients were in a relatively narrow band.

Glycosylated haemoglobin was higher in diabetic patients who had renal transplants compared to the non-diabetic and control group. Williams and Siegal have shown that glycosylated proteins are preferentially transported into the renal glomerulus [339]. The glomerular mesangial cell is involved in the development of diabetic nephropathy, which is characterised by glomerular hypertrophy, mesangial matrix accumulation and a thickened glomerular basement membrane. Cohen and Ziyadeh have shown that Amadori products can inhibit mesangial cell growth and stimulate type IV collagen synthesis. The glycosylated protein-induced decreases in cell proliferation and increase in collagen production are prevented by monoclonal antibodies against glycosylated epitopes [340].

In the present study the skin CLF in the non-diabetic transplant recipients was not higher than the control group. Skin CLF in the diabetic transplant recipients was higher than the control group but was not significantly different to the non-diabetic renal transplant recipients. Studies have consistently shown that AGE levels are higher in non-diabetic and diabetic patients with renal failure requiring haemodialysis [65,158,168]. The fact that in our study the levels of AGE in the skin of non-diabetic recipients of renal transplants were similar to that of the control group would suggest that the transplanted kidney affects in an unknown fashion, the removal of AGE from the skin.

It has been shown that renal mesangial cells cultured in the presence of AGE-albumin express extracellular matrix components (EMC). Yang et al have shown in mice that in vivo administration of AGE-modified mouse serum albumin (AGE-mSA), induced increased EMC gene expression and glomerular hypertrophy similar to that found in experimental diabetic nephropathy [341]. Co-administration of aminoguanidine with AGE-mSA reduced the glomerular hypertrophy observed in mice treated with AGE-mSA alone.

AGE may play a role in the development of cardiovascular disease in both the non-diabetic and diabetic recipients of renal transplantation. Higher AGE levels in diabetic transplant recipients may adversely affect tissue remodelling processes, vascular permeability and the diabetes-related abnormalities in vascular responsiveness to endothelial derived relaxing factor (nitric oxide). Vascular tone and regional blood flow are mediated in part by nitric oxide (NO), a radical species produced enzymatically by endothelial cells. NO can rapidly traverse the subendothelial space and induce smooth muscle relaxation and vasodilatation. In addition to its role in regulating vascular tone, NO exerts antiproliferative effects on different cell types [342]. Protein bound AGE can inactivate NO via a direct chemical reaction [343]. Hogan et al observed that

AGE functionally inactivate the antiproliferative effects of NO in model cell culture systems that include a fibroblast cell line, vascular smooth muscle and mesangial cells [344]. The inactivation of endothelial cell-derived NO by AGE may represent a pathway in the development of vascular disease that accompanies diabetes mellitus.

In diabetic patients with ESRD the elevation of AGE in tissue and plasma could be the result of two mechanisms. Firstly, AGE in tissue increases in direct proportion to ambient glucose concentrations which in turn generates an increased flux of AGE-protein degradation products (AGE-peptides). Secondly, there is decreased elimination of these products as renal function deteriorates. In non-diabetic patients with ESRD the increase in tissue and plasma AGE is a result of defective AGE removal. A newly transplanted kidney appears to be effective at removing tissue and serum AGE. Diabetic patients have the problems of increased formation of AGE (when compared to non-diabetic subjects) to contend with, other strategies including chemical inhibition of AGE formation must be developed to improve life expectancy in this group of patients.

6.6. Conclusions.

- (1.) Tissue AGE are increased in non-diabetic patients with CRF.
- (2.) In non-diabetic renal transplant recipients tissue AGE levels were lower than in non-transplanted CRF patients. Restoration of renal function leads to a fall in tissue AGE. This is the first study to show that transplantation lowers tissue AGE.
- (3.) CAPD employing high glucose dialysis fluid has a minimal effect on the levels of both skin and peritoneal AGE. Further studies are needed to confirm this.
- (3.) Diabetic renal transplant recipients have higher skin AGE levels than control group. Diabetes contributes to the increased AGE levels observed after renal transplantation.

Chapter 7.

Perspectives.

Over the past decade a number of studies pointed to the association of AGE with various biological and pathological phenomena. The nonenzymatic glycosylation of proteins and the subsequent formation of AGE has been shown to occur in vivo. Yet the isolation and elucidation of AGE structures has remained difficult. Substances such as FFI, pyrraline and pentosidine, are thought to be AGE present in vivo, but are not recognised by AGE-antibody [165,345]. The origin and the nature of the immunologically reactive material is not yet known. Further studies are needed to identify and characterise the immunologically reactive substances present in vivo.

Many studies have concentrated on the effects of AGE formation on the function of structural extracellular matrix proteins. The recent discovery of AGE-peptides (<10kDa) in plasma adds a new dimension to this. The structure of these AGE-peptides and their role in diabetic complications are at present unknown. Recent experiments have shown that the injection of AGE-peptides into animals over several months reproduces pathological sequelae similar to that observed in diabetic animals.

It is important to ask to what degree do the formation and accumulation of AGE contribute to atherosclerosis and the late complications of diabetes in relation to other known mechanisms, which include oxidation of lipids and proteins, abnormalities of the aldose reductase pathway and other risk factors that contribute to the risk of atherogenesis. The recent discovery that AGE can accumulate on low density lipoproteins (both lipid and apoprotein moieties) and that LDL isolated from diabetic patients shows a strong relationship between the

AGE content and the levels of oxidised LDL may suggest that advanced glycosylation plays a role in lipid oxidation in vivo.

The characterisation of receptors for AGE-modified proteins on macrophages, endothelial cells, fibroblasts, mesangial and smooth muscle cells have opened up new fields for the study of the role of AGE-modified proteins in connective tissue homeostasis, vascular permeability, and the cell proliferation. It must be noted that the extrapolation of results obtained from in vitro to the in vivo situation must be done with caution, since other contributing factors cannot be accounted for.

Two recent reports provide new evidence that AGE may be involved in the development of Alzheimer's disease [346,347]. When amyloid β protein, the precursor of Alzheimer's senile plaques, is modified by AGE, an accelerated aggregation of soluble amyloid protein occurs. Furthermore Alzheimer's plaques have three times more immunoreactive AGE than does the normal brain. Although this does not establish a causal role for AGE in Alzheimer's disease, further investigation will define the exact relationship between AGE accumulation and Alzheimer's disease. More proteins that can accumulate AGE are being characterised [348].

The evidence obtained thus far points to a broad based mechanism by which AGE-modified proteins can contribute to disease processes. Drugs such as aminoguanidine are being developed to enable the inhibition of AGE formation in vivo. Administration of aminoguanidine to human subjects resulted in lowering of AGE-haemoglobin (Hb-AGE) a 30 day study period. Hb-AGE should prove useful in clinical studies aimed in the evaluation of pharmacologic agents that inhibit the Maillard reaction in vivo.

We have demonstrated that fluorescence used as a marker of AGE formation is reliable. Though it has to be stressed that samples used for measuring AGE concentration should be homogenous such as collagen extract rather than samples of plasma where there may be interfering substances. We have also shown that oxidative processes affect the early stages of the Maillard reaction in vitro rather than the late stages. In addition, we have also demonstrated that both oxidation of LDL and modification of extracellular proteins such as collagen by AGE can affect their interactions. In addition we have also shown that there are local differences in AGE concentration in atherosclerotic plaques. Both the interaction of LDL with collagen and local differences in AGE concentration could have relevance in foam cell formation and the release of growth promoting substances, both of which are signals for atherogenesis.

The elevated levels of serum AGE-peptides seen in both non-diabetic and diabetic patients with end stage renal disease reported in other studies has great relevance. We have shown that tissue AGE levels are elevated in both these populations, and in that tissue AGE levels are decreased after kidney transplantation in the case of non-diabetic patients with end stage renal disease. This is the first study to show that tissue levels of AGE can be decreased. It does raise the question as to what the tissue components of AGE are, and how they are decreased after transplantation? An attractive hypothesis would be that they are AGE-peptide fragments that are bound and accumulate on the structural and extracellular components of tissue. Further research is needed to isolate and characterise serum AGE-peptides. We must investigate its role in disease processes such as renal failure and determine as to whether serum AGE-peptides are a causative factor or just a marker of disease.

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APPENDIX.

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