Identification of a new cross-link and unique histidine adduct from bovine serum albumin incubated with malondialdehyde.

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Running title
Malondialdehyde Cross-linking of Serum Albumin and Collagen

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The abbreviations used are:
MDA, malondialdehyde ; BSA, bovine serum albumin;
β-LAA, β-lysylaminoacrolein; NPO, N-pyrimidinyl ornithine;
2EH, 2-ethylidene-histidine; 4EH, 4-ethylidene histidine;
NNL, N-(2-carboxyl,2-aminoethane)-N-methanoyl-lysine ;
OMLI, 2-Ornithinyl-4-methyl(1'-lysyl)1,3-imidazole

Key Words:-
Collagen, Serum Albumin, glycation, Malondialdehyde, cross-links.
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**Summary.**

Malondialdehyde, acetaldehyde, acrolein, and 4-hydroxynonenal are all products of fatty acid oxidation found in the fatty streaks of atherosclerotic arteries due to a lack of antioxidants and an increase in glycation products. Previously identified cross-links derived from these molecules have nearly always required more than one molecule of each type, although this is physiologically less likely than a reaction involving a single molecule. Here we provide indirect but strong evidence for a malondialdehyde-derived cross-link requiring just one malondialdehyde molecule to link arginine and lysine, giving 2-Ornithinyl-4-methyl(1ε-lysyl)1,3-imidazole, following a 4 day incubation of albumin with 8mM malondialdehyde. This cross-link was identified as its partial degradation product Nε-(2-carboxyl,2-aminoethane)-Nε-methanoyl-lysine by NMR and mass spectrometry. Analysis of plasma from treated diabetic patients revealed that one patient levels as high as 0.46%/0.67% of their lysine/arginine residues modified by this cross-link, although others had lower levels.

Alkaline hydrolysis of serum albumin also revealed two acid-labile malondialdehyde adducts of histidine in significant quantities, the isomers 4- and 2-ethylidene-histidine. These constituted up to 0.93% of the histidines in treated diabetic patients. Although collagen is readily cross-linked by malondialdehyde neither of these particular products could be found in incubations of collagen with malondialdehyde.

**Introduction.**

Glycated collagen promotes the oxidation of polyunsaturated fatty acids to a myriad of reactive aldehydes (1,2). Many of these have only one functional group, leaving the most toxic major products as 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA), the latter being the focus of this paper. These
oxidation products, mainly derived from the fats carried by lipoprotein, are implicated in the progression of atheroma by reacting with the collagen arterial wall.

Compared to many aldehydes, MDA is relatively non-reactive at neutral pH as it has a pKa of 4.46, above which it favours the enolate salt form \((3)\) stabilized by a conjugated \(\Pi\)-bond system. Resonance stabilization reduces the electrophilicity of MDA, decreasing its reactivity with protein-based amine groups such as lysine, arginine, for example, in comparison to reaction with glutaraldehyde. Thus, MDA has a half-life \(\textit{in vitro}\) with 10mM lysine of around 2 days \((3)\). However, free MDA is removed from the bloodstream much faster than this, having a half-life of approximately 2 hours in rats \((4)\), with 2-propenal adducts being detected in urine \((2)\). None the less, a significant proportion of MDA has a longer half-life \(\textit{in vivo}\), because it binds to protein amine groups mainly as a temporary and unstable imidopropene adduct, \(\beta\)-lysyl aminoacrolein \((\beta\text{-LAA})\) \((5)\). The presence of these labile MDA adducts is supported by various ways of measuring MDA, which indicate that at least 80% of MDA in tissues is bound reversibly to protein \(\textit{in vivo}\) \((5)\).

Incubation of collagenous rat tail tendon in a 10mM MDA solution for 24hr at 37°C and pH 7.4 induces structural changes in collagen, increasing both the mechanical brittleness and insolubility of the fibres, and forcing the conclusion that extensive cross-linking has occurred. We have shown that MDA and acetaldehyde can react with collagen to give a potential cross-linking dihydropyridine product \((3)\) and others \((6,7)\) have confirmed this. However, under the same conditions, 70mM MDA hardly cross-links 25mg/ml bovine serum albumin (BSA) \((8)\), and unpublished observations this lab), as demonstrated by the absence of an observable dimer band on SDS-PAGE gels after this time period. On the other hand, heavy cross-linking of BSA is observed when the reaction is carried out in the presence of acetaldehyde, but not acetaldehyde alone, suggesting synergy between the two reagents \((8)\). Several MDA cross-links have been proposed involving synergy with other reactive agents, but MDA cross-links of collagen may also involve reaction with the existing enzymatic cross-links \((3)\).

Other potential cross-links and MDA-derived adducts beyond \(\beta\text{-LAA}\) commonly require more than one MDA molecule being required to form the proposed structure, even in the presence of acetaldehyde \((3,6,7,9)\). Such a reaction would be less likely under physiological conditions since MDA is at very low serum concentrations, and even in a “glycation hotspot” environment of an atherosclerotic plaque

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would be competing with many other oxidised fatty acid or sugar products for reactive lysine. In addition, blood clearance rates of even lightly glycated proteins or erythrocytes are considerably faster than normal (11, 12). This suggests that either scavenger receptors will remove MDA-adducted proteins long before a second MDA can react to form any cross-linking structure, or that other molecules will react with bound MDA first, rather than a second MDA molecule. Therefore, physiologically important MDA cross-links are most likely to involve just one MDA molecule. It is also possible that the rapid formation of MDA cross-links only occurs in the presence of a higher concentration secondary metabolite such as acetaldehyde. Well characterised protein cross-links formed in vivo follow these patterns. For example, pentosidine and DOGDIC are derived from a single ribose or glucose molecule cross-linking lysine and arginine (13,14) while methylglyoxal-lysine dimer (MOLD) and glyoxal-lysine dimer (GOLD) are derived from two lysines, methylglyoxal or glyoxal, and a secondary metabolite, possibly acetaldehyde (15).

Previous studies employing acid hydrolysis did not reveal a cross-link involving a single MDA molecule under pseudo-physiological conditions in vitro (16). Here, we report some success in an attempt to isolate an MDA cross-link using alkaline hydrolysis of MDA reacted with both BSA and collagen.

Materials and Methods.

Reagents:- All chemicals were purchased from Sigma (Poole, Dorset, UK) or Aldrich (Gitting, Dorset, UK) unless otherwise specified. High pressure liquid chromatography grade solvents were all used pre-filtered (0.22 µm) and obtained from Rathburn Chemicals (Walkerburn, Peebleshire, Scotland, UK).

Excess fat and hair was scraped from the skin of 9-month old guinea pigs. The skin was then shredded in a Moulinette homogeniser to yield a crude collagen preparation. Fats were extracted by stirring in 1L of 2:1 chloroform:methanol overnight, the slurry centrifuged, supernatant removed, and the remaining material washed three times in methanol and freeze-dried prior to reaction with MDA. Bovine serum albumin (BSA, fat-free, 96%+) was purchased from Sigma (A-6003).

We are grateful to staff and patients at the Southmead hospital diabetic clinic (Bristol, UK) for their kind donations of diabetic plasma.
Synthesis of malondialdehyde: [2-C\textsuperscript{14}] Malondialdehyde was synthesised from [2-C\textsuperscript{14}]1,3 propanediol using the protocol from Summerfield and Tappel (17). Non-radioactive MDA was prepared as described previously by re-crystallization following controlled hydrolysis of 1,1,3,3-tetra-ethoxypropane (3).

Reaction of MDA with proteins: Reactions were carried out without prior sodium borohydride reduction and in a metal-free environment to minimise cross-link and oxidation artefacts (5,18,19), including the oxidation of MDA itself. Samples were stored frozen prior to subsequent analysis.

Fat-free BSA (15g) or crude collagen (15g) was reacted with 8mM MDA in 150ml of 100mM Chelex-treated phosphate buffer (pH 7.4) (19,20), in the presence of 0.05% sodium azide and 1mM diethylenetriaminepentaacetic acid (DTPA) under sterile conditions for 48hr at 37°C. After 48hr, another 8mM MDA was added and the reaction continued for a further 48hr.

MDA was removed from the BSA by a 3 day dialysis at 4°C against 2×5L 0.05% sodium azide in water and removed from the collagen by 5 washes of water containing 0.05% sodium azide. Control incubations of BSA in the absence of MDA for 0 or 4 days were carried out to ascertain whether BSA contains or forms non-standard amino acids under these conditions.

At the same time, 1g of BSA or collagen in 10ml buffer was reacted using 450KBq \textsuperscript{14}C\textsubscript{2} MDA.

Sample identification: Hydrolysed samples were assigned a three letter code according to their nature. The first letter denoted whether the sample was from collagen (C) or BSA (B), the second denoted whether it was a control (C), or MDA incubated sample (M), and the last denoted whether it was acid (A) or alkaline hydrolysed (L). BMLm refers to the sample derived from the alkaline hydrolysate of the 15g large scale incubation of BSA.

Alkaline hydrolysed plasma samples from five diabetic patients were labelled Pa, Pb, Pc, Pd, and Pe. Specific fractions from semi-preparative ion-exchange column separations were then appended to this to give nomenclature such as Pa 56-57.

Acid or Alkaline hydrolysis: Untreated plasma (4ml) or protein samples from in vitro work were hydrolysed at 15mg/ml in 1M barium hydroxide at 108°C for 48hr. The alkali was allowed to cool to just below boiling point, and neutralised by addition of a slight excess of cold 2M ammonium hydrogen carbonate. The barium carbonate solid was removed by centrifugation, while the excess ammonium
hydrogen carbonate and secondary ammonium carbonate product were removed by freeze-drying. For comparison, samples from in vitro incubations were also hydrolysed at 10mg/ml in 6M HCl at 110°C for 18 hr, and then freeze dried. Residues were re-dissolved in 0.1 pyridine/formate buffer (pH 2.9) at 20mg/ml prior to ion exchange chromatography.

Semi-preparative ion-exchange chromatography:- Hydrolysates derived from 100mg protein were loaded onto a 600x12mm Duolite 225 carboxymethyl polystyrene resin cation-exchange column at 60°C and employing pyridine/formate buffers to separate components by charge as described previously (21). When using radioactivity, the column fractions were counted using a LKB Wallac 1219 Rack Beta scintillation counter, and radioactive peaks located in relation to standard amino acids as described earlier (16).

All ~320mg of the protein from alkaline hydrolysis of 4ml human plasma was loaded onto the column in one run. Plasma sample fractions were freeze dried and fractions of interest analysed directly by NMR, mass spectrometry, and amino acid analysis.

Preparative ion-exchange chromatography:- Sample BMLm was loaded at 15ml/min onto a 500x50mm Duolite 225 cation-exchange column pre-equilibrated in the same 0.1M pyridine/formate buffer (pH 2.9) as the semi-preparative system. One litre of this buffer, followed by 2L at pH 3.7 (0.75M pyridine), 2L at pH 5.2 (1M pyridine), and finally 3L at pH 8.0 (2M pyridine) buffers were used to elute amino acids in a stepped gradient. Fractions of 1L were taken, and the pyridine/formate removed by repeated rotary evaporation.

Fractions of interest were dissolved in the pH 2.9 pyridine/formate buffer, and the pH re-adjusted to 2.9. These were then re-chromatographed using the smaller ion-exchange column (600x12cm- above) for improved resolution of the neutral and basic amino acids, while incorporating a small amount of radioactive sample as a tracer. Pyridine/formate was again removed by rotary evaporation, and the sample re-dissolved in 0.1% heptafluorobutyric acid for reversed phase HPLC (below).

Analytical amino acid analysis:- Aliquots from the initial protein hydrolysis samples and purified products from C18 reverse-phase column chromatography were analyzed on an Alpha Plus automatic amino acid analyser (Pharmacia, Loughborough, UK) using a standard buffer gradient with a Dionex AI 450 data collection system.
Reverse-phase column chromatography:- Radioactive peaks selected for further investigation were applied to a 250x21.5mm Spherisorb ODS1 C18 semi-preparative column at 10ml/min using a gradient increasing from 0-30% acetonitrile over 60min. Column effluent was monitored by UV at 214nm, 10ml fractions collected, and radioactive peaks identified for freeze-drying.

NMR and Mass-spectrometer analysis:- The three final fractions C1,C2 and C3 from HPLC separations of in vitro incubations were prepared in 600µl of 10% D2O, with 20µM trimethylsilylpropionic acid (TMS) as an internal NMR standard. Using water suppression by a long presaturation pulse, a series of 128 or 256-scan 1-dimensional runs were done on a 500MHz Jeol NMR spectrophotometer in neutral (pH7.4, at 70°C and 20°C) and acidic conditions (pH 3.7, 20°C). These were supplemented by 2D total correlation (mixing time 60ms, pH 7.4, 20°C) and correlated spectroscopy experiments at pH7.4/20°C for spin-system identification. Electrospray mass-spectroscopy was carried out on a Micromass instrument using acetonitrile as the carrier solvent.

The ion-exchange purified plasma sample Pa56-57 contained the most material eluting at similar position on the ion-exchange column to fraction C. It was prepared in 550µl of 10% D2O with TMS as above. Using a gradient pulse for water suppression, a 2D total correlation experiment was run with an accompanying 1D experiment on a 600MHz Bruker NME spectrophotometer in slightly acidic (mixing time 60ms, pH 5.7, at 20°C) conditions. This and several other plasma samples were subsequently analyzed using a ThermoFinnigan LCQ Classic Ion-trap mass spectrometer.

Results.

Identification of the products from reactions of MDA with BSA or Collagen.

The procedures undertaken and results outlined below are summarized in Scheme 1.

Semi-preparative ion exchange chromatography:- Fig. 1 shows the results of semi-preparative cation exchange analysis of hydrolysates of radioactive products from samples BML and CML. In addition to a major peak of unbound radioactivity (peak A), two products were reproducibly identified, peak B which was found in both collagen and BSA, while peak C was unique to BSA. Peak B was not pursued further, as it co-eluted with the bulk of the amino acids in the preparative run and no adduct of lysine, arginine, or histidine has been seen to elute so early (concurrent with ~ glycine, alanine) on an ion-exchange column.
Further separations therefore focussed on peak C, which eluted between tyrosine (fractions 46-47) and lysine (fractions 68-69), in the region where most basic amino acid-derived cross-links have been identified. Controls (not shown) showed that the previously characterised N-pyrimidinyl ornithine (NPO) from acid hydrolysis (16) eluted at fractions 48-49, and thus cannot account for any of peaks A-C.

*Amino Acid Analysis of hydrolysed proteins:* The bottom 3 traces of Fig. 2a shows a comparison between acid (BMA) and alkaline (BML) hydrolys of BSA reacted with MDA before separations plus an amino acid standard for reference (STD). Sample BML contained a new peak at 69.0 min (arrow in Fig. 2a), slightly earlier than the peak of histidine in the standard which is destroyed by the alkaline hydrolysis protocol. Histidine is seen in the acid hydrolysis sample BMA as a peak at 69.7 min. Apart from the new peak at 69.0 min, no significant differences between BML and the control BCL (data not shown) could be seen at this stage of the analysis. Furthermore, no significant differences could be observed between CML and CCL by amino acid analysis. Identification of MDA adducts from acid hydrolysis has already been done (16) and repeat amino acid analysis of samples BCA, CCA, and CMA showed no unexpected peaks. From peak integration, the new amino acid observed in sample BML at 69.0 min is equivalent to 4.25 residues per BSA molecule, assuming that its ninhydrin equivalent is the same as histidine (1.08 cf: leucine). BSA contains 17 histidines per molecule. From its elution position, this 69min peak could be the same as the radioactive peak C in Fig. 1, Peak B in Fig. 1 would be likely to elute somewhere in the 30-45min region of these amino acid traces.

The remaining amino acid traces in figure 2, shown here for easy comparison, are from purified products and will be referred to later.

*Preparative ion exchange chromatography of sample BMLm:* Fractions from the preparative ion exchange column run were evaluated by amino acid analysis. The first of two fractions eluted by the pH 5.2 buffer contained tyrosine and lysine respectively (data not shown). Since Peak C eluted between tyrosine and lysine on the analytical column (Fig. 1) these two fractions which between them should contain Peak C were freeze-dried and further separated on the semi-preparative ion exchange column to obtain larger quantities of fraction C. The fractions were spiked with radioactivity from the BML sample.

*C18 Reverse phase column chromatography:* All fractions containing Peak C from incubations BML and BMLm were loaded onto a 250x21.5mm C18 semi-prep column over two runs. Three peaks of
radioactivity were detected in peak C (Fig. 3, trace a), giving peaks C1, C2 and C3. An NPO standard (16) detected by light absorbance gave a single peak in a region distinct from C1, C2, C3 (Fig. 3, trace b). Peaks C1, C2, C3 were prepared for analysis by NMR and Mass Spectrometry.

**NMR and Mass-spectrometer analysis of peaks C1-C3**

**Peak C1:** Mass spectroscopy of this sample gave a single ion at 182.05 (M+1) molecular weight (Fig. 4, left traces). The NMR spectrum, (Fig. 5a) was almost identical to histidine, but with one of the 4H or 2H ring protons missing and a new CH-CH$_3$ spin system as identified by 2D COSY experiments (data not shown). Replacement of the 2H or 4H proton of histidine with a =CH-CH$_3$ group plus removal of a ring N-H proton gives a molecule, either 2-ethylidene-histidine (2EH) or 4-ethylidene histidine (4EH) respectively (Fig. 6), that exactly fits both NMR and Mass-spectroscopy data. The remaining 2/4H proton was observed to shift in a manner akin to a standard histidine proton when the pH was adjusted to pH3.7 (data not shown).

2EH and 4EH may be artefacts of alkaline hydrolysis of an MDA-histidine adduct rather than being a direct product from the reaction between the MDA degradation product, acetaldehyde, and histidine (fig 6C, 6D). All these structures are more stable Schiff-bases than the lysine adduct β-LAA due to additional resonance stabilisation from the double bonds in the histidine ring.

**Peak C2:** This fraction contained three sets of peaks by NMR (Fig. 5b, 7) as identified by integrals of their proton peaks. The major one was the other variant of the histidine adducts described above. The ring proton is further downfield (7.83 ppm, peak rH’ in Fig. 5b) than the molecule in Peak C1 (7.72ppm), making this likely to be 2EH and leaving the Peak C1 adduct as 4EH, although these ring proton shifts are closer together compared to unmodified histidine (8.12 and 7.14ppm). Like Peak C1, the mass spectrum of C2 showed a strong peak at 182.05 (Fig. 4). By NMR, the total yield for the both types of histidine adduct was 2.1% of available histidine before the MDA reaction started, of which 31% is the isomer seen in fraction C2, however there will inevitably have been significant losses during purification. The original amino acid compositional analysis of BML (Fig. 2) suggests that up to 25% of the histidines could have been modified.
The second set of peaks was a very small lysine impurity (~<3%) seen only when separated out by 2D NMR in Fig. 7. This lysine impurity was not a significant feature on the mass-spectrum of C2 (Fig. 4) in comparison with other peaks.

Unaccounted by either of these first two peak sets, Peak C2 contained also two mass-spec peaks at m/z of 243.91 and 262.10 (Fig 4). The final set of NMR peaks correlating to these masses in Peak C2 were observed in several groups which can be seen more clearly in the total correlation 2D spectrum of this fraction (Fig. 7). Two sets of correlated shifts with equivalent proton intensities could be identified as N\textsuperscript{-}-adducted lysine side chains. These had ppm shifts of 3.73(\alpha), 1.88(\beta), 1.49/1.42(\gamma), 1.77(\delta), 3.14(\epsilon), annotated as 2L’\alpha-\epsilon (Fig. 5), and 3.72(\alpha), 1.87(\beta), 1.38/1.32(\gamma), 1.68/1.64(\delta), 3.44/3.32(\epsilon), annotated as 2L\alpha-\epsilon. They also had a complex CH-CH\textsubscript{2} spin system (2L, 2L’ \psi,\omega, Fig. 5) with fine structure that could only be resolved by repeating the 1D spectra at 70°C (inset, Fig. 5). The coupled proton shifts of 3.98(\psi) and 3.83(\omega), from this CH-CH\textsubscript{2} spin system are a triplet and doublet at 70°C. For this to occur, there must be two isomeric forms of the molecule in slow exchange (<2s\textsuperscript{-1}) on the NMR time-scale at the lower temperature which go into fast exchange at 70°C (>10 s\textsuperscript{-1}). In other words, these two sets of peaks denoted 2L and 2L’ are two isomers of the same molecule. Finally two CH singlets were observed in the aromatic region of the spectrum, the one at 8.15ppm (2L \phi, 2L’ \phi, Fig. 5).

Daughter ion spectra were obtained from the Mass-spectroscopy peaks initially observed at m/z 244 and 262 (M+1, Fig. 4). In the same manner that the two sets of NMR peaks, 2L and 2L’ were from the same molecule, it can be seen that the initial two peaks with masses 261.99 and 244.31 are likely to be derived from the same molecule. Only the ion ratios differ in their fragmentation patterns.

Lysine, the recognizable backbone of this new molecule in Peak C2, has a mass of 146 daltons (C\textsubscript{6}H\textsubscript{14}N\textsubscript{2}O\textsubscript{2}). To obtain the observed m/z values of 244 and 262, masses of 97 and 115 need to added to lysine respectively, along with the ionising H\textsuperscript{+}. This indicates that an extra nitrogen atom must have been added in addition to the radioactive \textsuperscript{14}C\textsubscript{2} MDA which gives the observable radioactive peak. Furthermore, there cannot have been any contribution from the reactive MDA breakdown product acetaldehyde, as that would result in methyl groups being present in the final product, whereas no methyl groups are observed in Peak C2. MDA must therefore be responsible for the CH-CH\textsubscript{2} spin systems seen at 3.98/3.83ppm, which implies that the third carbon of the MDA has been oxidised to a carboxyl group. From this, it can be
deduced that the 244 ion involves the addition of C₄H₃NO₂ to lysine, while the 262 ion fragment includes an additional water molecule. The 4th carbon is required to give the observed aromatic CH proton.

Three of the additional carbons in this lysine adduct come from MDA, so possible sources for the 4th carbon, that has a downfield proton attached (8.15/8.02 ppm depending on isomer), and the extra nitrogen were investigated. Protein main-chain nitrogen atoms are unreactive, so the nitrogen must come from a second lysine, histidine, or arginine side-chain, indicating that the MDA had cross-linked two amino acids at one point, the cross-link being damaged by alkaline hydrolysis. Of these, only arginine has both the required properties of a single carbon that could conceivably be separated from the rest of the side-chain, and the potential to form a stable cross-link via nitrogen attack on a carbon double bond, as shown in Scheme 2. This cross-link, 2-Ornithinyl-4-methyl(1ε-lysyl)1,3-imidazole (OMLI), can then rearrange and fragment to form Nε-(2-carboxy,2-aminoethane)-Nε-methanoyllysine (NNL), a stable di-amino acid which has the required mass of 261 for Peak C2 (Scheme 2). Scheme 3 shows that positive ions can be derived via chemical ionisation fragmentation pathways of this molecule to fit the fragmentation spectra of C2 in Fig. 4. The accompanying neutral ions are omitted for simplicity.

Several properties of the NMR spectra also support the conclusion that the new molecule in Peak C2 is the structure NNL. The central methanoyl group is part of an amide functional group, and slow amide rotation around the C-N bond to give cis/trans isomers can explain the two distinct structures seen by NMR at low temperature. The singlet peaks at 8.15 and 8.04 ppm can be two forms for the proton in the methanoyl group. The ratio of the two CH-CH₂ groupings could not be measured at 25 °C, but it is interesting that these groups, very close in ppm shift at 25 °C (<0.01 ppm), became averaged at 70 °C. Raising the temperature has increased the frequency of rotation enough for these peaks to report intermediate to fast exchange on the NMR time-scale, while other paired peaks, further apart in shift, remained in a slow exchange system that reports both structures (data not shown).

As mass-spectroscopy and NMR data match well, and can explain some unusual features, NNL can be assigned as the molecule in fraction C2. Working back, MDA must have cross-linked lysine specifically to arginine during incubation with protein, as only arginine can react with β-lysyl aminoacrolein to form a stable link that can breakdown to NNL on alkaline hydrolysis. Therefore, an MDA
cross-link is 2-Ornithinyl-4-methyl(1ε-lysyl)1,3-imidazole (OMLI), which is destroyed by acid hydrolysis and damaged by alkaline hydrolysis to form NNL.

**Peak C3**:- This product co-eluted with a significant pyridine impurity (Fig. 5) that could not be removed from the fractions of initial ion-exchange separation. Other than this, the main element was again an Nε-modified lysine side chain, with NMR shift positions of 3.73(α), 1.88(β), 1.41/1.34(γ), 1.72(δ), 3.53(ε), (annotated 3Lα-ε, Fig. 5) the ε-CH2 protons being noticeably further downfield-shifted than for the C2 "NNL" molecule. Like Peak C2, there was a second set of lysine-like shifts at 3.73(α), 1.87(β), unresolved(γ), 1.66(δ), 3.44/3.27(ε), (annotated 3L’α-ε, Fig. 5) except that the ratio of major:minor peaks increased from 2:1 (2L:2L’, Fig. 5) to approx. 4:1 (3L:3L’, Fig. 5) for the molecule in this fraction. A CH-CH2 system was observed at 4.16(H) and 3.91/3.93(2H) ppm (3Lω,ψ, Fig. 5) with a second CH-CH2 system at 3.97(H) and 3.82(2H) ppm (3L’ω,ψ, Fig. 5) maintaining the 4:1 integral ratio. Finally, aromatic protons (3Lφ, 3L’φ, Fig. 5), are observed at 8.19ppm and 8.23ppm at a 4:1 ratio again.

From the Mass-spectroscopy, the mass ions from Peak C2, m/z 244 and 262, were again observed in Peak C3 (Fig. 4). Therefore, one can conclude that Peaks C3 and C2 are the two diastereoisomers of NNL, where the chiral carbon is shown in Scheme 2. Each diastereoisomer also has a cis and trans form. Total yields of this damaged cross link NNL after purification were 0.33% of the original lysine and 0.85% of the orginal arginine. Of that, 76% is the diasteroisomer found in Peak C3. If losses of this molecule were similar to those of 2EH/4EH, the initial yields may have been an order of magnitude higher.

**Amino Acid Analysis**:- Peaks C1,C2 and C3 were analysed on the automatic amino acid analyser, (Fig. 2a, traces C1-C3) in order to locate them with respect to other amino acids and glycation products. The 2EH and 4EH adducts in Peaks C1-C2 gave a peak at 69min as seen before in sample BML, distinct from the known histidine peak location at 69.7min. 2EH and 4EH could be separated by a different protocol designed to separate collagen cross-links (Fig. 2b, C1i, C2i) (22).

Amino acid data for the NNL is harder to interpret. All remaining peaks in the 60-75min range in fractions C2 and C3 are only showing trace quantities. One would expect a NNL peak at least 50% the size of 2EH/4EH from rough quantifications by NMR and mass-spec, eluting very close to the adduct given its almost identical elution position on the semi-preparative ion exchange column. Conversely, in fractions C2 and C3 (Fig. 2a), there is an unassigned doublet peak at 41min. This doublet could be acid-degradation
product(s) of NNL, as any MDA cross-link will be acid-labile (16), and initial conditions in the amino acid analysis column are pH2.2 and 70°C. These same acid degradation products of NNL may also be responsible for peak B in Fig. 1.

Identification of alkaline hydrolysis products from human plasma.

Table 1 quantifies the 2EH/4EH calculated by amino acid analysis as found in fractions from the semi-preparative ion-exchange separations of 4ml alkaline-hydrolysed diabetic plasma samples Pa-Pe. Amino acid traces from four paired fractions are shown in Fig. 2c (Pa56-57, Pa54-55, Pb52-53, Pb54-55) where all four fractions have the 41min and histidine adduct peaks, along with some other peaks, of which the major ones will be ornithine, lysine, and/or ammonia. The 41min peak seen in fraction Pa56-57 (figure 2c), the largest observed for all the samples, represents at least 0.46%/0.67% of the total lysine/arginine residues from the Pa plasma sample should it be an NNL derivative. Unfortunately, as it is apparently an NNL degradation product, it could not be reliably quantified, although such degradation will mean its concentration will always be underestimated.

Mass-Spectroscopy traces of fractions Pa 54-55 and Pa 56-57 are considerably more complex than those of fractions C1-C3 (Fig. 4). This is expected due to the fewer separation steps, the large number of small metabolites in plasma, and the extra peaks seen in the amino acid analysis (Fig. 2c). However, the 182.1 from 2EH and/or 4EH is the largest peak in fraction Pa 54-55 while the 262.1 mass from NNL is the largest peak in fraction Pa56-57. This agrees with the amino acid analysis. While MS-MS of Pa 56-57 was done on a different instrument to the result shown for MS-MS of C2, both traces show an m/z of 244 as the major fragmentation ion of m/z 262.

To further prove that OMLI and 2EH/4EH occur in vivo, the significant similarities between the pair of 2D total correlation spectra Fig. 7 show that molecules within plasma fraction Pa56-57 (right) are essentially the same as those seen in fraction C2 (left), simply differing in their relative quantities.

Of secondary interest are the masses 357.1 and 234.1 seen in the mass-spectrum of fraction Pa56-57. The mass 357.1, while only seen as a very small peak, happens to be the right mass (356+1) for the intact cross-link. The MS-MS fragmentation pattern, while not confirming the intact cross-link structure, is
consistent with it, where the main ions can be accounted for by release of H$_2$O, OH$^-$, CO$_2$, and NH$_2$. The mass m/z 234 is consistent with a lysine adduct similar to the NNL, and is of interest because its fragmentation pattern is also similar to NNL. The 198$^+$ fragment of m/z 234 is shown in Scheme 3, while the 130$^+$ fragment could easily be a variant of the 129$^+$ fragment also shown, simply with an extra proton. The mass m/z 234 could be the 198$^+$ molecule with two extra water molecules added, the ring being open before MS-MS bombardment. These molecular masses will be further investigated in future work along with an attempted synthesis of the molecules described here.

Discussion.

The use of alkaline hydrolysis to search for MDA cross-links sensitive to acid hydrolysis revealed distinct differences between collagen and bovine serum albumin in their reaction to MDA. An alkali degradation product of a cross-link formed from MDA reacting with lysine and arginine was identified together with a new reaction product of MDA with histidine. Despite the known ability of MDA to extensively cross-link collagen neither of these new compounds was present in the alkaline hydrolysate of collagen incubated with MDA, possibly because of steric constraints imposed by the helical structure of the molecule.

*The alkali degraded cross-link:* The identification of the alkali partial degradation product N$^\varepsilon$-(2-carboxy-1,2-aminoethane)-N$^\varepsilon$-methanoyl-lysine (NNL) is consistent with an MDA cross-link formed by reacting one molecule of MDA with lysine and arginine to give the original cross-link 2-Ornithinyl-4-methyl (1$^\varepsilon$-lysyl)1,3-imidazole (OMLI, Scheme 2).

NNL was not isolated from collagen alkaline hydrolysis, but some cross-linking must have occurred to cause the collagen stiffening that follows MDA incubation (16). Our earlier work using tritiated borohydride reduction techniques (16) showed that the enzymatic cross-links in collagen reacted strongly with MDA which could account for extra cross-linking. OMLI is also likely to be mainly an intra-protein cross-link as incubation between MDA and BSA does not result in significant dimerisation of BSA (8). Cross-linking possibilities for MDA also exist between DNA bases and may result in molecules related to OMLI.
Histidine Adducts:- While lysine and arginine have frequently been implicated in glycation cross-linking, there have been relatively few reports on histidine based cross-linking and adducts. One related molecule, Carnosine, or β-alanyl histidine, is found in brain and long-lived tissues at concentrations up to 20mM (23), where it has been suggested to have anti-ageing and cardioprotective effect. However, its primary mechanism of action is in dispute, as it apparently scavenges aldehydes and glycation products in vitro and in vivo by reacting with them (24) as well as chelate copper to inhibit glycoxidation (25). 4-HNE also reacts with histidine to form an adduct (26), and recent evidence showed that the presence of histidine can block the formation of the stable 4-HNE-induced cross-link between two lysine molecules (27). We provide evidence here that significant amounts of protein-bound histidine can also react with MDA and probably other aldehydes to form adducts. Again, type I collagen-based histidine does not appear to react, implying that the fibrillar structure of this collagen protects histidine from glycation while other, exposed histidines such as those in BSA or carnosine can act as a ‘sponge’ for aldehydes. This collagen effect may also be partly because two of the already small number of collagenous histidines, about seven per chain depending on collagen type, are involved in formation of the mature collagen cross-link histidino-hydroxylysino-norleucine by an spontaneous reaction with a proximal immature cross-link, dehydrohydroxylysino-norleucine (28).

In vivo, the results show that five diabetic patients had between 0.22% and 0.93% of their histidine modified to either 2EH or 4EH, demonstrating that this is a potentially useful biomarker of lipid-derived chemical modification of proteins in diabetes. Further controls involving measurement of patient lipid status need to be done to verify this. There is no obvious reason why acetaldehyde or other aldehydes should not react with the histidine ring if MDA can.

Reaction mechanisms:- Similar molecules such as acrolein or 4-HNE cannot form cross-links related to OMLI. Reaction patterns for these molecules show that histidine, lysine, and cysteine, and by inference arginine, preferentially react with the C3 double bond to form Michael adducts; lysine can subsequently react with the aldehyde group (2,27,29-33). Unlike MDA, there is then no possibility of ring closure to give a stable aromatic ring-based crosslink.
One can construct schemes where any aldehyde can prevent OMLI (or other MDA-derived cross-link) formation before the second nitrogen of the arginine closes the ring simply by reacting with the precursor first (figure 8), ironically acting as a cross-link inhibitor (34). Only a potentially unlikely second reaction with MDA opens the possibility of cross-linking again, probably via a dihydropyridine-type structure previously reported (3,6,8). A variety of synthetic experiments are being carried out to investigate the potential reaction mechanism proposed in Scheme 2. In particular, it will be important to investigate whether the *in vivo* metabolite responsible for the formation of 2EH and 4EH is likely to be MDA or acetaldehyde.

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Figure Legends

Scheme 1  Overview of separation protocols

Thick lines show procedures undertaken to generate and purify the components from MDA reactions and human plasma. Thin lines link these procedures to diagrams from sample analysis shown later in the paper. AAA – amino acid analysis, M/S – Mass spectroscopy, (dns) – data not shown. See the section on sample identification for other abbreviations.

Figure 1  Cation exchange analysis of products recovered from hydrolysates BML and CML.

Fractions containing 14C MDA from CML (open circles) and BML (triangles) are detected by scintillation counting. A second 96-hour alkaline hydrolysis was done on the CML sample (closed circles) to confirm that fraction C did not occur in collagen. Fractions A-C are labelled as described in the text, while the letters ILFYKR show the elution positions of those amino acids. All other standard amino acids and hydroxyproline elute in fractions 18-29.

Figure 2  Amino acid analysis of purified protein hydrolysates and isolated products.

Fig 2a Analyses from BMA4 and BML4 incubations. These are shown together with analyses of the radioactive components C1-C3 separated out later by HPLC (fig 3). The letters CVMILYFHK refer to standard amino acids; Or and Hyls are ornithine and hydroxylysine respectively.

Fig 2b Analysis of 5:1 (C1i) and 1:5 (C2i) mixture ratios of C1:C2 show that they can be resolved using a modified gradient (20).

Fig 2c Analyses from selected ion-exchange chromatography fractions of plasma samples Pa and Pb. NNL and 2EH/4EH are defined later in figs 4-7.
Figure 3  C18 Reverse-phase chromatography of fraction C isolated from BMLm.

Peaks (C1,C2,C3) are shown from sample BML4 as detected by radiation counting (trace a) and absorbance at 214nm (trace b). The peak in trace c shows the elution time of N-pyrimidinyl ornithine (NPO) on the same timescale as trace b.

Figure 4  Electrospray mass spectra of Peaks C1,C2,C3 (left) and Peaks Pa54-57 (right).

The spectra also shows daughter ion (chemical ionization) spectra from the main masses in the C2 and Pa56-57 spectra. Extra ion masses are listed for the small daughter ion peaks of the C2 m/z 262 peak.

Figure 5  NMR spectra of fractions C1,C2,C3 at 25°C, pH 7.4 in 90% H2O, 10%D2O.

Peaks denoting 2/4-ethylidene histidine from fractions C1 (4EH) and C2 (2EH) correspond to the non-prefixed Greek annotations in Fig. 6.

Peaks denoting the two steroisomers of NNL shown in Scheme 2 are seen in fractions C2 and C3 and correspond to Greek annotations prefixed by 2L or 3L. Each stereoisomer of NNL has two observable forms, depending on whether the amide bond shown in Scheme 2 is cis or trans, the minor form being annotated with an apostrophe.

Between 4.4 and 4.8 ppm, fractions C1 and C2 are shown from NMR runs done at the different temperatures shown to separate the ψ proton from bulk water.

An inset of sample C2 from 3.65 to 4.05 ppm demonstrates the fast-exchange averaging of the cis and trans forms (2L and 2L’) of NNL as temperature is increased. Peaks separated by a bigger ppm shift remained in slow to intermediate exchange. For the aromatic region of the spectrum (7.6-8.3ppm), Peak Q is probably the ring proton from NNL (Scheme 2), but cannot be confidently assigned.

The singlet peak at 3.35 ppm in all of C1-C3 is a small unidentified impurity.
All NMR peaks from the fractions are shown apart from water, the standard, and the two other pyridine impurity peaks in fraction C3.

Figure 6 The two isomers of ethylidene-histidine (2EH, 4EH).

The Greek proton annotations are assigned in Fig. 5. Also shown are two possible precursor structures prior to alkaline hydrolysis (see discussion), which yield the observed adduct with release of a formate ion.

Figure 7 2D NMR total correlation spectra of peak C2 (left) and fraction Pa56-57 (right).

Assignments from Peak C2 are given for the cis/trans forms from one isomer of NNL (2L,2L’,2Lψ,2L’ψ) and also show one isomer of the histidine adduct (α’,β1’,β2’). Identical assignments could be made for fraction Pa56-57, where there are several additional peaks due to incomplete purification. The gradient suppression affected peaks near the water peak much more in the Pa56-57 spectrum, resulting in weaker α’ to β1’/β2’ correlations.

Scheme 2 Proposed mechanism for the formation of the MDA cross-link by reaction with lysine and arginine, followed by its partial degradation by alkaline hydrolysis to form NNL.

The damaged cross-link, Nε-(2-carboxy,2-aminoethane)-Nε-methanoyl-lysine, (NNL) was obtained in two forms with mass ions 243 and 261. The intact precursor is 2-ornithyl-4-methyl (1ε-lysyl) 1,3-imidazole. (designated crosslink).

Protons with Greek annotations are assigned by NMR in figure 5.

* This bond causes the cis/trans isomerisation denoted by the apostrophe or lack of it in figures 5 and 6. The major (non apostrophe) form is likely to be the trans form, but the two are not yet distinguished.

** This bond is a chiral centre that becomes racemic during the alkaline hydrolysis, giving the two steroisomers of NNL, denoted 2L and 3L. It is unknown which stereoisomer denotes each set of assignments.
Scheme 3  The partial fragmentation pathways of NNL during mass spectrometry.

Table 1

Amino acid analysis of pyridine column fractions derived from hydrolysed diabetic plasma.

| Patient | Age/Sex | Type  | BP    | HbA1C (%) | Treatment/complications       | Plasma conc. (µM) |
|---------|---------|-------|-------|-----------|------------------------------|-------------------|
| Pa      | 46/F    | I-17y | 110/70| 12.5      | Actrapid+insulatard /Hypertension | 136.5             |
| Pb      | 61/F    | II-22y| 138/70| 11.8      | Insulin+metaformin /None       | 33.2              |
| Pc      | 77/M    | II-22y| 146/86| 11.7      | Mix30 /None                   | 82.9              |
| Pd      | 50/F    | II-23y| 170/84| 12.3      | Insulin /Maculopathy          | 72                |
| Pe      | 76/M    | II-13y| 174/86| 11.3      | Insulin /Renal impairment     | 41.3              |

Plasma concentrations of the histidine adduct are in µM and are approximate, and are calculated from amino acid analysis runs of fractions 50-57 from the semi-preparative ion-exchange column.
Fig 6

4-EH

2-EH

? Alkaline Hydrolysis
Scheme 3
Identification of a new cross-link and unique histidine adduct from bovine serum albumin incubated with malondialdehyde

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