Reactions of Lipid-derived Malondialdehyde with Collagen*

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Malondialdehyde is a product of fatty acid oxidation (e.g. from low density lipoprotein) implicated in the damage of proteins such as collagen in the cardiovascular system (Chio, K. J., and Tappel, A. L. (1969) Biochemistry 8, 2821–2827). Its concentration is raised in diabetic subjects probably as a side effect of increased protein glycation. Collagen has enzyme-catalyzed cross-links formed between its individual molecules that are essential for maintaining the structure and flexibility of the collagen fiber. The cross-link dehydro-hydroxylysino-orleucine reacts irreversibly with 10 mM malondialdehyde at least 3 orders of magnitude faster than glucose reactions with lysine or arginine, such that there is little cross-link left after 1 h at 37 °C. Other cross-links and glycated elements of collagen are also vulnerable. Several possible products of malondialdehyde with collagen cross-links are proposed, and the potential involvement of collagenous histidine in these reactions is discussed. We have also isolated N²-(2-pyrimidyl)-1-ornithine from collagenous arginine reacted with malondialdehyde. The yields of this product were considerably higher than those from model reactions, being approximately 2 molecules/collagen molecule after 1 day at 37 °C in 10 mM malondialdehyde. Collagenous lysine-derived malondialdehyde products may have been present but were not protected from protein acid hydrolysis by standard reduction techniques, thus resulting in a multitude of fragmented products.

Glycated collagen has been shown to promote the oxidation of polysaturated fatty acids, adding to their typical fragmentation to a broad range of reactive compounds (1). The two major products are malondialdehyde (MDA)¹ and 4-hydroxynonenal (2). MDA is very reactive and reacts with nucleophilic amine groups such as lysine, arginine, and the amino termini of amino acids (3–5). It also reacts with any ketones or aldehydes from other sources, for example attached sugars or glycation products. Of particular importance is the bifunctional aldehydic property of MDA, which gives it the potential to cross-link proteins, thereby reducing their functional capacity. Significantly, it has not been detected directly by NMR at neutral pH in water, despite extensive chemical research (3–5, 8). The cross-link has always been detected as a borohydride-reduced product (3, 4), which is stable in aqueous acid (pH 1–4) solution (4, 8, 9), or when synthesized in dry, acidic ethanol (9). However, when Summerfield and Tappel (10, 11) reported the MDA cross-linking of DNA, they were unable to find a similar cross-link despite the use of the borohydride reduction protocol. The structure of these cross-links remains unknown. Interestingly, the pKₐ of MDA is 4.46, and its presence in the salt form at neutral pH suggests that the differences reported may be due to the different reaction conditions. At neutral pH, we were unable to detect the imidopropene but reported that another molecule, N-lysyl-4-methyl-2,6-dihydropyridine-3,5-dicarbalddehyde (NLMDD), may be the MDA-derived protein cross-link (see Fig. 1) and Ref. 5). The cross-link form of NLMDD with added amine-derivatives was detected in electron impact mass spectroscopy at neutral pH. Although this link is likely to be in rapid exchange with the nonlinked form (consistent with NMR data) and thus likely to be a relatively weak cross-link, the NLMD molecule is stable compared with 1,3-di(N²-lysino)propane and thus has a permanent rather than transient effect.

The major collagens in aorta and atheroma are types I and III fibrous collagens that are cross-linked enzymically to produce a stable fiber possessing a long biological half-life. Although aorta collagen contains a complex of mature and immature cross-links derived from (hydroxy)lysine and (hydroxy) lysine aldehyde, the reactions of MDA with collagen can be simplified by the use of rat tail tendon collagen. The tendon collagen is initially stabilized by oxidative deamination of the telopeptide lysines to lysine aldehyde. Lysyl oxidase (12) catalyzes this by binding to the specific sequence Hyl-Gly-His-Arg in an adjacent molecule but located opposite the telopeptide because of the quarter-staggered alignment of the molecules in the fiber (13). These modifications of the collagen fiber result in the formation of the intramolecular aldol condensation product (AcP) cross-link (see Fig. 1) and the intermolecular cross-link dehydro-hydroxylysino-orleucine (dehydro-HLNL; see Fig. 1 and Ref. 14). The latter davalent cross-link is detectable by

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¹ The abbreviations used are: MDA, malondialdehyde; NPMDD, N-propyl-4-methyl-2,6-dihydropyridine-3,5-dicarbalddehyde; LDL, low density lipoprotein; NPO, N²-(2-pyrimidyl)-1-ornithine; HLNL, hydroxylysinosorleucine; dehydro-HLNM; dehydro-histidinohydroxymerodesmosine; AcP, aldel condensation product; NLMD, N-lysyl-4-methyl-2,6-dihydropyridine-3,5-dicarbalddehyde; HHMD, histidinohydroxylysinosorleucine; DE-MALDI-TOF, delayed extraction matrix-assisted laser desorption ionization time-of-flight mass spectrometry.
borohydride reduction but in vivo subsequently matures to a nonreducible stable trivalent cross-link, histidinolhydroxylysino-
nonorleucine (14). The intermolecular cross-link dehydro-
HHMD (Fig. 1) is also commonly detected but may be an
artifact of the borohydride reduction technique (16, 17).

The low turnover of collagen can be further decreased by
additional cross-linking between the reactive amino acid side
chains and MDA or sugars. This stiffens many tissues (e.g.
arteries). With this in mind we have attempted to determine
the mechanism of reaction of MDA with collagen under physi-
ological conditions and isolate the collagen-MDA cross-links.

MATERIALS AND METHODS

Reagents

Unless otherwise specified, all chemicals were purchased from Sigma
(Poole, Dorset, UK), Fisher (Loughborough, Leicestershireshire, UK), or
Aldrich (Gilling, Dorset). [2-14C]1,3-Propanediol (3.7 MBq in 2.5 ml)
was obtained in water solvent from American Radiochemicals (Tocris
Cookson, Langford, Avon, UK), and [3H]sodium borohydride (3.7 GBq
extended to 100 mg) obtained from Nycomed Amersham (Little Chal-
font, Buckinghamshire, UK). High pressure liquid chromatography
grade solvents were all used prefiltered (0.22 μm) and obtained from
Rathburn Chemicals (Walkerburn, Peebleshire, Scotland, UK). Type I
collagen fibers were obtained from the tail tendons of 3–4-month-old
Sprague-Dawley rats following slaughter and stored in physiological
saline at −20 °C until required.

Synthesis of Malondialdehyde

[2-14C]Malondialdehyde was made from [2-14C]1,3-propanediol using
the protocol from Summerfield and Tappel (18). Nonradioactive MDA
was prepared as described previously by recrystallization from con-
trolled acidic hydrolysis of 1,3,3-tri-tetra-ethoxypropane (5).

Collagen Content

The collagen concentration was accurately determined by a
hydroxyproline assay using a continuous flow autoanalyzer (ChemLab,
Cambridge, UK).

Reduction of Collagen with Borohydride/Hydrolysis

100 mg collagen was reduced with 10 mg of sodium borohydride,
washed, reduced again with 10 mg tritiated sodium borohydride, and
hydrolyzed in 6 N HCl for 18 h. Analysis of the freeze-dried and redis-

FIG. 2. Ion exchange chromatography of acid hydrolysates from collagen (ColR*) and collagen following reaction with MDA (COLM*R*). Peaks a, reduced aldol condensation product; peaks b, 6-chloronorleucine; peaks c, hexosyl hydroxylysine; peaks d, hexosyl lysine; peak e, anhydro derivative of hexosyl lysine; peak f, reduced lysinonorleucine. Panel A, sodium borohydride-reduced native collagen. Panel B, native collagen reacted with 10 μM MDA for 1 day at 37 °C, followed by reduction with sodium borohydride.

solved acid hydrolyzate on an ion exchange column failed to reveal any
radioactive peaks, indicating that collagen cross-links are fully reduced
by the initial protocol.

Reaction of MDA with Collagen Fibers

Rat tail tendon has a well established profile of reducible enzymic
cross-links, identified following tritiated borohydride reduction, which
is shown in Fig. 2. To identify MDA products, we reacted MDA with
native collagen followed by reduction of collagen-MDA reaction prod-
ucts and existing reducible cross-links with tritiated borohydride. We
also followed the reaction of collagen and MDA by the use of synthesized
14C-labeled MDA in an attempt to isolate 14C modified cross-links.

Initial Studies

Following reduction of the MDA native collagen products with triti-
ated borohydride, we could not detect the presence of 1H-labeled cross-
links HLNL or HHMD (Fig. 2), suggesting that they might be involved
in the reaction with MDA. It was therefore decided to carry out reaction
rate studies with MDA by partially reducing collagen. We could only
detect HLNL when the partial reduction was followed by a tritiated
reduction; dehydro-HHMD is more rapidly reduced in these mild
conditions.

Reaction Rate of MDA with Partially Reduced Collagen

Approximately 500 mg (dry weight) of rat tail tendon was reduced by
addition of 15 mg of sodium borohydride for 1 h at 20 °C in 50 ml of 100
mM phosphate buffer, pH 7.5. The borohydride was removed from the
collagen fibers by five successive washings with 100 ml of Milli-Q water.
100-mg portions of the collagen were then reacted with 10 ml of 10
mM nonradioactive MDA in phosphate buffer for 1–96 h at 37 °C. After
washing off the MDA using Milli-Q water in the same ratio, the result-
ant glycated collagen was then reduced again using 3 mg of tritiated
sodium borohydride to give samples rColM1r*, rColM5r*, rColM24r*,
and rColM96r*. The remaining 100 mg were used as a control where no
MDA was added to give sample rColMR* (Scheme 1).

Analytical Preparations of MDA-Collagen

MDA-collagen was prepared as follows: i) 3 ml of 160 μM (0.21 GBq)
radioactive [2-14C]MDA was reacted with 100 mg of tendon collagen for
48 h in phosphate buffer at 37 °C and then with a total of 10 ml 10 mM
MDA for 24 h by adding 7 ml of 14.2 mM MDA. Excess MDA was
removed by washing, and tendons were then reduced with 10 mg of
tritiated sodium borohydride to give sample ColM*R*. ii) 100 mg of
tendon was incubated in phosphate buffer alone for 3 days before
tritiated borohydride reduction to give a standard collagen cross-link
profile ColR*.
Bulk Preparation of Collagenous-MDA Adducts

Native collagen (1 g) was treated with 100 ml of nonradioactive MDA in phosphate buffer and then with 100 mg of nonradioactive sodium borohydride in the same manner as ColM*R⁺ to give sample ColMR. This preparation was spiked with the tritium-labeled ColM*R⁺ during initial separations to allow location of ³¹C products. Once the separation was adequately characterized by ninhydrin detection and absorbance at 254 nm, the spiking was discontinued. A summary of all the in vitro sample preparations is given in Scheme 1.

Chromatographic Separations of MDA-Collagen Hydrolysate Acid Products

P₂ Gel Filtration Chromatography—Separations on sample ColMR, loaded at 50 mg/ml, were performed on a 850 × 25-mm column packed with Bio-gel P₂ and equilibrated in 10% acetic acid running at 0.5 ml/min. 5-ml fractions were collected after loading the sample, and reproducibility was checked by UV monitoring at 254 nm. The column was re-equilibrated overnight.

Ion Exchange Chromatography—A Duolite 225 cation exchange resin (600 × 12 mm) column running in a 60 °C water jacket was used to separate components by charge using a pyridine buffer system as described previously (19).

The radioactive peaks were identified for both columns by counting the collected fractions on a LKB Wallac 1219 Rack Beta scintillation counter. To locate the radioactive peaks in relation to the standard collagenous amino acids, 2-µl aliquots from 5-0 ml column fractions were spotted on Whatman No. 1 paper, and amino acid peaks were detected by spraying with ninhydrin (0.25% w/v in acetone).

Superdex Gel Filtration Chromatography—Following separation on the P₂ and then the Duolite column, the ColMR sample fractions were freeze-dried and run in 0.5-ml aliquots on an Amersham Pharmacia Biotech Superdex HR 10/30 hydrophobic interaction column in 45% acetonitrile and 0.1% trifluoroacetic acid.

Amino Acid Analysis—Aliquots of the purified, preparative samples were run on a Amersham Pharmacia Biotech Alpha Plus amino acid analyzer using a method for separating collagen and elastin cross-links (20, 21). An Amersham Pharmacia Biotech UltraPac 4B cation exchange resin (270 × 4 mm) was used with a Dionex AI 450 data collection system.

Analysis of Diabetic Human Skin—A sample of skin was obtained from a 62-year-old male insulin-dependent diabetes mellitus patient (disease duration 55 years). Excess fat was scraped from the skin prior to defatting in chloroform/methanol (2:1) overnight. The sample was then air dried, weighed, and hydrolyzed under reflux at 110 °C for 18 h in the presence of 6 N HCl (10 mg/ml). The acid was removed by rotary evaporation followed by freeze drying.

The hydrolysate was reconstituted in Milli Q water, and the hydroxyproline content was measured using an automatic hydroxyproline analyzer (ChemLab). Aliquots of the sample were then fractionated using ion exchange chromatography as described above. Fractions from successive runs were pooled, freeze dried, and characterized by amino acid analysis using the Alpha Plus analyzer (Amersham Pharmacia Biotech).

Results

Model Reactions between MDA and Nα-Acetyl-arginine/lysine

NMR Profile of a Completed Reaction—10 mM MDA was reacted with 15 mM Nα-acetyl-arginine and 9 mM Nα-acetyl-lysine, in 100 mM sodium phosphate buffer, pH 7.4, at 60 °C for 3 days, following which only a trace of the MDA remained. The mixture, MRL60, was then analyzed by NMR.

Synthesis and Acid Hydrolysis of NLMDD—10 mM Nα-acetyl-lysine and 20 mM MDA were incubated for 4 days at 80 °C to synthesize an impure mixture containing NLMDD (sample ML60). The mixture was diluted with concentrated HCl to give a 6 N HCl solution, hydrolyzed at 110 °C for 18 h, freeze-dried, taken up in the same volume of water (600 µl), pH adjusted to 7.5, and analyzed by NMR (sample ML60Hy). In subsequent investigations, propylamine was used instead of Nα-acetyl-lysine to simplify NMR spectra interpretation. Consequently, the nitrogen substituent on its equivalent MDA product to NLMDD is a propyl rather than a lysyl group, changing the abbreviation to NPMDD.

Reduction of NPMDD at Acid pH with Cyanoborohydride—The pH of an NPMDD solution MP60 (equivalent to ML60) was reduced to 2.3 using HCl and 100 mM glycine/HCl pH 2.3 buffer added. 100 mM sodium cyanoborohydride was then used as reducing agent for 2 days at 37 °C, causing a small increase in pH to 2.55. Most of the glycine buffer and reducing agent were removed by dialysis in 2 liters of water for 48 h (one change) using a 100 molecular weight cut-off SpectraPhor membrane to give sample MP60CN. The sample was then acid hydrolyzed and freeze dried for NMR analysis (sample MP60CNHy).

NMR Analysis

Samples generated by the above protocols were made up in 600 µl of 10% D₂O (100 mM phosphate, pH 7.5) for ¹H NMR analysis on a Jeol 500 MHz NMR spectrometer. Typically, 128 scans at 25 °C were taken, using a DANTE (delays alternating with nutation for tailored excitation) pulse for water peak suppression. 40 µM trimethylsilylpropionic acid was added to the sample for calibration so that concentrations could be worked out by comparison. One-dimensional NMR Peaks were grouped into spin systems by running two-dimensional total correlation NMR experiments (22) with a mixing time of 60 ms (512 rows, 16 scans) and by integrating the area under the one-dimensional peaks.

Mass Spectroscopy

The same samples used for NMR analysis were then diluted appropriately in water. Electrospray mass spectrometry was done using a Micromass instrument and delayed extraction matrix-assisted laser desorption ionization time-of-flight mass spectrometry (DE-MALDI-TOF) using a PerSpective Biosystems Voyager instrument.

RESULTS

Reaction of MDA with Partially Reduced Collagen

Dehydro-HLNL remaining after a partial sodium borohydride reduction is shown by its presence as radioactive HLNL after a second [³¹H]borohydride reduction, which shows only trace amounts of other unreduced lysine derivatives. Fig. 3 shows the effect of 10 mM malondialdehyde on this pretreated
Vulnerability of dehydro-HLNL to MDA—After partial reduction of collagen, the remaining dehydro-HLNL (detected by the tritiated HLNL peak at fractions 51–52, Fig. 3) rapidly declines such that within 1 h in 10 mM MDA at 37 °C, it is no more than 20% of its initial concentration. Within the same time period, a major product peak becomes visible at fractions 53–55. Internal ninhydrin spray controls of the elution volumes of normal collagen amino acids remained constant, confirming the reproducibility of the chromatography. Assuming then, that the half-life of dehydro-HLNL in 10 mM MDA is less than half an hour and that the MDA concentration is much larger and does not significantly change in this short time period, we can calculate a pseudo-second order rate constant \( k_1 \) as approximately \( 40 \text{ s}^{-1} \). This allows a half-life of the (unprotected) cross-link of less than 250 days at the normal steady-state physiological plasma free MDA concentrations of 50 nM (23). However, there is some confusion over what the plasma concentration really is because of the difficulty in designing a relevant test (24). Estimates rise to 1 μM or more (25–31), although most of these are likely to include protein-bound MDA on protocol examination. Free MDA concentrations higher than 50 nM would cause a concurrent decrease in the half-life of the collagen cross-link. Where it is measured, MDA concentration tends to double for diabetic subjects (27, 31, 32).

Vulnerability of dehydro-HHMD to MDA—Because dehydro-HHMD may also possess a similar imine cross-link, it could be inferred that it also reacts quickly with MDA. By the end of 1 day in a reaction between native collagen and MDA (Fig. 2), it can no longer be detected. If HHMD is indeed an artifact, it could be that the MDA reacts with the aldol condensation product, thus preventing the formation of HHMD during borohydride reduction. Such a reaction would normally be faster than an imine-MDA reaction because of the reactivity of aldehydes and because MDA could react in its predominant salt-form.

Appearance of New Reducible Peaks—As time progresses in the experiment, peaks appear at fractions 57–59 and 62–63 within an hour and become larger in 5 h, reaching a maximum in 1 day, by which time peaks at around fractions 70 and 81 have also become evident, along with some small peaks in fractions 15–40 (Fig. 3). Incubation for longer periods of time results in only a minor rearrangement of the peaks from 50–85; for the most part the reaction is complete after 1 day. The number and diversity of these peaks make it unlikely that they are all derived from dehydro-HLNL, suggesting that partially reduced collagen has elements that can react with MDA to form new reducible products. From this experiment, MDA is shown to attack dehydro-HLNL in a manner at least 3 orders of magnitude faster than, for example, the reaction of glucose with amine groups in hemoglobin (33, 34) or albumin (35) to form ketoamine adducts (the initial product) in similar conditions.

Reaction of Collagen Cross-links with [2,14C]MDA and the Fate of MDA Reaction Products

Having established that the MDA reaction with collagen is more or less complete in 1 day, the second stage of the investigation was to find out the fate of the MDA. [2,14C]MDA was used in collagen incubations in preference to [1,14C]MDA because some of the reactivity in the latter compound would be lost as formic acid because of the self-cleavage reaction that MDA undergoes over time. The [2,14C]MDA will either react as MDA or as the still 100% labeled acetaldehyde when the MDA does self-cleave to a small extent. To maximize radioactive incorporation, collagen was initially reacted with the radiolabel at comparatively low MDA concentrations (160 μM) for 2 days (see “Materials and Methods”). The low concentration would also mimic the physiological condition. However, to isolate sufficient material for analysis, the MDA concentration was increased to 10 mM by nonradioactive MDA for a further day before acid hydrolysis.

Fig. 4 shows the 14C count traces from an ion exchange separation of sample ColM*R*, with the 3H traces shown in Fig. 2 given for comparison. The result is that there are only two peaks in the entire mixture that contain the MDA carbon label, namely peaks at fractions 48 and 49 and at fractions 21 and 22. Both of these appear to be marginally reducible with sodium borohydride as seen from concurrent 3H peaks. The surprising inference is that all the other reducible products in Figs. 2 and 4 are formed as a result of MDA reaction with collagen but do not actually contain MDA.

These data show that hexosyl-lysine-derived aldimine Schiff base adducts react with MDA. This is shown by the marked drop in their reduction products hexosyl-lysine and hexosylhydroxylysine seen at fraction 38 and fractions 41 and 42 (Fig. 2, c and d) and by the drop of the acid hydrolysis artifact, the anhydro derivative of these reduced products (Fig. 2, peak e). Dehydro-HHMD reacts avidly with MDA, its reduced HHMD peak at fractions 82–84 entirely disappearing in 1 day and the product possibly being the new peak close by at fractions 79–83. Dehydro-HLNL can no longer be detected as a visible peak after the same time period with MDA.

Isolation and Characterization of an MDA-Collagen Product

Preparative Molecular Weight Separations—Fig. 5 shows the 14C trace from three 100-mg (2 ml at 50 mg/ml) P2 Bio-gel runs of sample ColMr spikied with 200 μl of the ColM*R* sample at 10 mg/ml. Most of the amino acids eluted between fractions 60 and 68 as determined by a ninhydrin spray test.

The major radioactive carbon peaks were collected and freeze dried (Fig. 5, peak A, fractions 55 and 56; peak B, fractions 57–61; peak C, fractions 75–79). The major fraction A gave an observable color on a ninhydrin spray test, allowing further P2 runs to be made with confidence without the radioactive spiking. The early peak at fractions 37–42 proved to be an unidentified high molecular weight mixture that eluted at the column void volume.

Ion Exchange Chromatography—The three peaks (Fig. 5, peaks A, B, and C) were then run separately on the Duolite ion exchange column. Fraction A ran to give a single carbon radioactive peak, namely the peak previously observed at fractions 48–49 in Fig. 4 (fraction A48). Fraction B proved to be a mixture of the 48–49 fraction in Fig. 4 and a very small 26 fraction that can just be observed (fraction B26); this could be expected given its overlap with Fraction A in the P2 column. Fraction C eluted to give a single 14C carbon peak at fractions
acid where the side chain has 3-CH₂ groups (Scheme 1). Fig. 7 shows its NMR spectrum. One of these components (fraction C21), synonymous with the other ¹⁴C carbon peak previously seen in Fig. 4. Fraction A48, however, was not only identifiable as ninhydrin positive but also gave a single peak in the region of interest on an amino acid analysis run (Fig. 6), where many lysine derivatives elute close by but remote from any previously identified compound.

¹H NMR Spectra—Fractions from these ion exchange separations were concentrated, further purified using the Amersham Pharmacia Biotech HR10/30 Superdex column, and investigated by ¹H NMR spectroscopy. Fraction B26 was at too low a concentration for useful analysis by NMR, and no ninhydrin-positive peaks could be observed using the Amersham Pharmacia Biotech amino acid analysis system, suggesting that it was an acid breakdown fragment. Fraction C21 gave a ninhydrin-positive peak in the same position as glycine, but there were too few NMR signals for it to be big enough to be a side chain derivative from arginine or lysine. Although the NMR spectra could not be interpreted fully (data not shown), it suggested a small zwitterionic molecule derived from some acid-oxidized MDA-ammonia adduct.

Fraction A48 was present in sufficient quantities to provide useful NMR spectra. We therefore prepared a larger amount of the pure sample from a large scale batch preparation (1 g; 21 and 22 (fraction C21), synonymous with the other ¹⁴C carbon peak previously seen in Fig. 4. Fraction A48, however, was not only identifiable as ninhydrin positive but also gave a single peak in the region of interest on an amino acid analysis run (Fig. 6), where many lysine derivatives elute close by but remote from any previously identified compound.

FIG. 5. ¹⁴C trace of three P2 Bio-gel separations from ColMR spiked with ColM*R⁺.

FIG. 6. Amino acid analysis showing the elution position of molecule A (bottom trace) in comparison with standard collagen and elastin cross-links (middle trace) and collagenous amino acids (top trace). Peaks are ninhydrin-derivatized amino acids detected at 570 nm. Peak 1, hydroxyproline; peak 2, isodesmosine; peak 3, desmosine; peak 4, dihydroxylysinoorleucine; peak 5, HLN; peak 6, HHMD; I/L, isoleucine/leucine; Y, tyrosine; F, phenylalanine.

Mass Spectroscopy—Fig. 8 shows the electrospray mass spectrum using 10 nmol of material as calculated using the NMR data and a DF-MALDI-TOF mass spectrum of molecule A with 10 times the loading. A peak at molecular weight 211 is seen in both of them; the extra peaks in both spectra proved to be due to instrumental aberrations and were not reproducible. The peaks at 233 and the peaks appearing weakly at 255 in the DF-MALDI-TOF spectrum are the mono- and di-sodium derivatives of the weight 211 molecule respectively, where varying MALDI conditions gave different proportions of these quite easily. DF-MALDI-TOF experiments are shown in the absence of a matrix and were calibrated using bradykinin.
Molecule A was reduced to about one-fifth of its original 2 mM concentration, now being around 400 μM. Therefore, the original yield of molecule A from 1 g collagen/MDA reaction was a minimum of 6 μmol from a total of 3.3 μmol of collagen, giving it an incidence of approximately two per collagen molecule (10 mg/ml collagen is equivalent to 3 mM lysine and 5 mM arginine as worked out from its amino acid sequence).

Comparative Model Reactions—Fig. 9 shows the resultant NMR spectrum from the almost completed reaction between MDA, Nα-acetyl-arginine, and Nα-acetyl-lysine in sample MRL60. Characteristic peaks for NLMDD (5) are seen at a concentration of ~1.8 mM. Formic acid from the breakdown of MDA is seen at 11.1 mM (8.41 ppm), β-lysyl aminoacrolein is at 2.3 mM, and the remaining lysine and arginine are at 4.8 and 15.4 mM, respectively. However, there is one final pair of peaks at a concentration of 600 μM that exactly match those observed from molecule A. These peaks were not observed in experiments using lysine and MDA alone (5) but can be observed in experiments that use arginine and MDA alone (data not shown).

Molecular Structure of Molecule A—From these data it has been proven that Molecule A must be Nδ-2-pyrimidyl-L-ornithine (NPO) as shown in Fig. 10. The mass is correct for the 211 Da observed in Fig. 8, there is a -CH-CH2-CH2-CH2- section in the molecule that satisfies spin system Y in Fig. 7, and there is a ring from which the included meta- and para-protons satisfy spin system Z. Furthermore, the molecule represents a logical reaction between MDA and arginine to form a ring that could easily occur in physiological conditions. The potential isomer shown in Fig. 10 is not present because all three of its ring protons would be chemically inequivalent, giving three peaks in the aromatic region (6–8 ppm) of the NMR spectrum in Fig. 7 rather than the two as observed. From the structure of NPO, it is self-evident that its aromatic ring is resistant to borohydride reduction, or the aromatic proton peaks would not be observed.

NPO also has a resonance structure shown in Fig. 10. The carbanion in this structure would be quickly protonated in acidic solution, rendering the molecule vulnerable to hydrolysis as the two double bonds on the side of the ring are now like a pair of Schiff bases. This hydrolysis will release MDA and arginine, of which the MDA will quickly oxidize or self-cleave. Another interesting property of this resonance structure is that the para-carbon can react further as a nucleophile.

Presence of NPO in Diabetic Skin—Fractions isolated from the NPO region on the Duolite ion exchange column were shown by amino acid analysis to contain a component that eluted in the position of NPO (Fig. 11A). The identical elution characteristics of this component to that of NPO was confirmed by spiking the sample with authentic standard (Fig. 11B).

Model studies of MDA and Sodium Acetyl-lysine/propylamine

Low Incidence of NPO in Early Stage Model Incubations—No NPO could be detected by NMR when 5 mM Nα-acetyl-arginine and 3 mM Na-acetyl-lysine were reacted with 10 mM MDA for 1 day at 37 °C in 100 mM phosphate buffer, despite concentrating the sample 10 times before analysis.

Susceptibility of Reduced MDA-Na-Acetyl-lysine Products to Acid Hydrolysis—Acid hydrolysis of a product mixture from an incubation of 10 mM MDA and 10 mM Na-acetyl-lysine at 60 °C for 3 days (sample ML60) left only lysine (sample ML60Hy), destroying all the other products. Attempts at reducing the products with sodium borohydride after the MDA incubation but before hydrolysis had no effect.

Sodium Cyanoborohydride Reduction Does Not Protect MDA-Propylamine Products—Fig. 12 shows NMR spectra of a similar incubation using propylamine (MP60) with further cyanoborohydride (MP60CN) and acid treatments (MP60CNHy).
The data show that although the propylamine is always stable, the MDA/propylamine products NPMDD and β-propyl aminoacrolein have been reduced to a myriad of different compounds. The MP60CN spectrum also shows a strong cyanoborohydride signal upfield of 0.7 ppm, which is split into seven and four peaks by the two isotopes of boron (B-10 and B-11). This signal disappears on the addition of acid because of the formation of the borate ion (with effervescence of hydrogen), which does not show proton NMR signals. Both the MP60CN and MP60CNHy spectra show a large peak at 3.55 ppm that is residual glycine from the reduction buffer. Hydrolysis of sample MP60CN to give MP60CNHy shows that some molecules apart from propylamine are resistant to acid in some form but are still fragmented because there is little resemblance between the two spectra except for the propylamine signals. From this, it can be said that further identification of MDA-collagen compounds via the standard reduction hydrolysis route is likely to be fraught with difficulty.

**DISCUSSION**

Collagen clearly offers much more complex possibilities when reacted with MDA than studies on most proteins because of the presence of Schiff base cross-links that form an extra group of chemical substrates with which MDA can react. The Schiff base cross-links in native collagen are in equilibrium with the free aldehyde form. MDA must be an aggressive competitor for the ε-amino group of lysine or the free aldehyde group, thus effectively cleaving the original bond and creating another complex. The MDA-collagen cross-link complex will still contain a free reactive aldehyde capable of reacting to form different intermolecular cross-links. These new cross-links could easily of themselves account for the observed increase in mechanical brittleness and insolubility of tendon fibers, but they may also work in conjunction with NLMDD-derived cross-links (5) and any other as yet unidentified cross-link(s). The fibers therefore become virtually metabolically inert and are consequently accessible to further glycation by glucose, which in turn renders the collagen a more active oxidizing agent of LDL and results in further release of MDA. The process therefore becomes a vicious cycle resulting in further stiffening of the vascular system.

**Proposed Chemical Reactions between MDA and Collagen**

The ability of MDA to wreak havoc on enzymatic cross-links could be because of its ability to react with any available aldehyde molecule in its majority salt form (pKₐ 4.46; Fig. 1) or as NPO. Thus, it competes for the lysine aldehyde in the Schiff base equilibrium. The rest of the reactions possible in Scheme 2 are merely extensions of already known collagen chemistry, where sequences of collagens show plenty of "excess" (hydroxy)lysine close to the cross-links that can react with the extra aldehydic function group provided by the MDA, resulting in cross-linking. A similar fate could happen to the AcP intramolecular cross-link to give a quaternary (or higher) intermolecular cross-link. All of these new cross-links could be directly (not catalytically) attacked by the nearby histidine at a carbon double bond to form variants of dehydro-HHMD. These molecules would be difficult to reduce with sodium borohydride, because their Schiff base linkages are part of a conjugated II system and also require more than one reduction event to protect them from acid. Requena et al. (3) employed sodium borohydride at a high concentration of 1 M to achieve a similar reduction of the imidopropene cross-link, and thus the 25 mM or less borohydride used in these experiments is unsurprisingly inadequate. As a result, partially reduced, labile cross-links would be vulnerable to acid hydrolysis. Another facet that makes this hypothesis appealing is that it describes new cross-
Reactions of Malondialdehyde with Collagen

Three Possible Explanations for the Lack of 14C MDA Products—The surprising ability of MDA that it can react with collagen in such a way that it induces new reducible compounds in themselves do not contain MDA. Three chemical mechanisms can be considered for this reaction. The first is that the MDA carbanion has the potential to react with oxygen to generate O₂ and MDA radicals, with the subsequent oxidative damage being mainly from a free radical propagation reaction. This explanation is aided by the fact that MDA slowly oxidizes, presumably by a free radical mechanism, to malonic acid, acetic acid, and formic acid in the dark in a normal buffer solution. However, such oxidation would probably be quenched quite quickly, especially in vitro. A second conceivable mechanism of oxidation could be via catalysis of the malondialdehyde self-cleavage reaction such that one of the end carbons attaches to a primary amine group as follows (36).

\[
\text{CHO-CH₂-CHO + R-NH₂ → R-NH-CHOH-CH₂-CHO → R-NH-CHO + CH₂-CHO}
\]

**REACTION 1**

Because the radio active label on the malondialdehyde is on the carbon-2 (underlined), the reducible molecule R-NH-CHO would not register on the 14C radioactive trace in Fig. 3. Although this could make a contribution, it will be minor compared with the measurable yields of the acid-degradable β-lysol aminoacrolein and NLMDD products of lysine and MDA in model systems.

The critical problem that is hampering all these studies is the vulnerability of MDA-protein adducts to acid hydrolysis that prevents their isolation. The disproportionation of NPMDD into many reduced products on addition of sodium cyanoborohydride and the radical effects of varying the amount of reducing agent in use both highlight inherent flaws in the reduction hydrolysis approach. It also requires extra work to calculate yields, because a certain proportion of the product of interest will inevitably be destroyed by acid hydrolysis even when reduced. Enzymic hydrolysis of glycated collagen is an alternative still under development.

**Possible Roles of Histidine—**In equine liver alcohol dehydrogenase, the active site Lys-231 is preferentially glycated and believed to be catalyzed by histidine (His-348 via Thr-347) (37). The histidine is thus ideally placed to catalyze reactions on the carbon-2 side chains of collagen in significant yield rather than with the ε-NH₂ groups of lysine was surprising but is similar to the reaction we recently reported with the analogous compound glyoxal, which reacted with arginine to give an imidazolone (40). This does not eliminate the possibility that MDA also reacted with lysine to give even higher yields of NLMDD (or 1,3-di(N²-lsino)propane), which were then destroyed on acid hydrolysis. Given that NPO was back-calculated to have a concentration of 60–120 μM when isolated from 10 mg/ml collagen after only 1 day of incubation with 10 mM MDA, it was somewhat surprising that the model reaction that mirrored the conditions did not yield any observable NPO over the same time period, although NPO did appear later. We conclude that either collagen catalyzes the reaction of MDA with its own arginine molecules directly or there is some undetectable glycoxidation product generated from collagen and MDA reactions that has the same catalytic effect.

We have identified the presence of NPO in human skin from a diabetic subject. The biological significance of NPO beyond that of a potential fatty acid oxidation marker is as yet difficult to quantify. It may be prevalent in the regions of α(I) collagen next to the histidine, but we have no data on its actual location within either collagen chain. Local effects of NPO on the collagen molecule may include disruption of integrin binding to RGD sequences on collagen because of arginine modification (41), possibly affecting cellular interactions (42). The nucleophilic activity of NPO certainly needs further study.

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