Biochemical changes in the collagenous matrix of osteoporotic avian bone

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No detailed biochemical analysis has been carried out of the compositional changes in the collagen matrix of avian bone in relation to increased bone fragility in osteoporosis. We have shown that osteoporosis in avian bone is certainly not just a simple loss of apatite and collagen, but involves significant changes in the biochemistry of the collagen molecule and consequently in the physical properties of the fibre. The decreased mechanical strength and the change in the thermal stability can be directly related to changes in post-translational modifications,

i.e. lysine hydroxylation and the intermolecular cross-link profile. The increased hydroxylation and change in cross-linking are consistent with increased turnover of the collagen, possibly in an attempt to initiate a repair mechanism which, in fact, leads to an acceleration in the increase in fragility of the bone. Clearly there are post-translational modifications of the newly synthesized collagen in avian osteoporosis, and these changes may play a role in the pathogenesis of the disease.

INTRODUCTION

Osteoporosis in hens arises as a result of a decrease in osteoblastic structural bone formation at the onset of sexual maturity [1]. At this time the hen starts to form medullary bone, which acts as a labile reserve of calcium for shell formation and which continues to be turned over by osteoblastic and osteoclastic remodelling processes. Under normal circumstances, the supply of calcium from dietary and medullary bone sources is adequate for shell formation. Structural bone resorption continues by osteoclast-mediated processes and this may be accelerated under conditions of dietary calcium deficiency. Thus there is a continuous decline in structural bone mass during the laying period of the hen [1], but the extent of the decline appears to be related more to the length of the reproductive period than to the rate of egg laying. Osteoporosis in laying hens is characterized by a loss of mass of normally mineralized structural bone and is typified by a thinning of cortical bone and thinning and loss of connectivity of trabecular bone [1,2]. Nutritional methods for preventing or slowing this bone loss have not been identified so far and, more fundamentally, the development of genetically improved strains may be required. It is therefore important that a better understanding is achieved of the biochemical and cellular factors involved in the loss of strength and bone structure as exemplified by both the structural matrix and the mineral.

Collagen is the major structural component of bone and as such provides bone with its high tensile strength, while the mineral provides high compressive stiffness. Any defect in the collagenous structure could lead to bone fragility, and this could be caused by the changing environment of the matrix-producing cells. It has become increasingly apparent over recent years that the extracellular matrix can affect gene expression [3] and the response of cells to growth factors [4]. Many *in vitro* studies on bone resorption have been carried out using isolated cells but these do not take account of possible interactions between the cells and the extracellular matrix, and information from *in vivo* studies is crucial. The formation of new bone during growth and turnover requires the precise alignment of the collagen molecules within the fibres, which can then be immediately stabilized by

intermolecular cross-linking and thus accommodate the plate-like crystals of calcium apatite [5,6]. Changes in cytokine signals to the osteoblasts could lead to abnormal metabolism and the formation of defective fibres, and so accelerate the increase in fragility of the bone. We have shown post-translational changes in the collagen of osteoporotic women, primarily increases in lysine hydroxylation and a decrease in cross-linking [7,8]. Both of these post-translational changes are consistent with an increased fragility of the bone, and similar changes may be occurring in osteoporotic avian bones.

The stabilization of the collagen fibres occurs initially through the formation of divalent intermolecular cross-links based on enzymically produced lysine aldehydes at the N- and C-terminal domains of the molecule. The two major divalent cross-links in bone are the lysine keto-imine and the hydroxylysine keto-imine, with the latter predominating. These divalent cross-links mature to form trivalent cross-links capable of linking the microfibrils, thereby forming a network of cross-links throughout the fibre [9]. The major mature cross-links derived from the keto-imines in bone are the lysine and hydroxylysine pyridinolines (L-Pyr and HL-Pyr respectively) [10]. However, in bone there is a deficiency in the formation of the HL-Pyr from the corresponding keto-imine, suggesting the presence of additional cross-links. A possible additional cross-link is the so-called Ehrlich chromagen or 'pyrrolic' cross-link [11,12]. The structure of this proposed pyrrole has not been confirmed and its presence is based solely on the rather insensitive colour test for all pyrroles, i.e. reaction with Ehrlich reagent. Our recent studies suggest that this pyrrole may make a significant contribution to the cross-linking of human bone collagen [13].

Despite the importance of osteoporosis in mature laying hens (up to 30% of hens suffer broken bones [14]), as far as we are aware the biochemical changes in molecular composition of the collagen in osteoporotic hens have not previously been studied. Although the initial mechanisms are likely to be different, the changing environment of the bone cells could lead to changes in the collagenous matrix that accelerate bone fragility, similar to those that we have previously reported in osteoporotic human bones [7].

Abbreviations used: HL-Pyr, hydroxylysine pyridinoline; L-Pyr, lysine pyridinoline; PTM, proximal tarsometatarsus; TBV, trabecular bone volume; HLNL, hydroxylysine norleucine; DHLNL, dihydroxylysine norleucine.

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MATERIALS AND METHODS

Hens

Pullets from the Hi-sex Brown laying strain (Ross Poultry Ltd., Inverurie, Scotland, U.K.) were reared from 1 day old in floor pens and fed on standard rearing diets. They were housed in individual battery cages at point of lay in groups that were fed on a number of different diets. The hens were maintained up to 70 weeks of age, at which point they were killed at the same point of lay when mineralization was minimal, and the bones were taken for structural studies. The bones used for the collagen studies came from birds fed on the control diet (a standard layer's diet thought to be adequate in all nutrients) or on diets obtained from it by the addition of ascorbic acid (250 mg/kg), 1,25-dihydroxyvitamin D₃ (5 mg/kg), vitamin K (10 mg/kg), NaF (2000 mg/kg), or by replacement of ground limestone with an equivalent amount of oystershell as a possible means of alleviating osteoporosis.

Source of medullary bone

End-of-lay perchery hens (72-week-old ISA Browns) were obtained from the ADAS Gleadthorpe Experimental Husbandry Farm (Mansfield, Notts., U.K.) and fed on a standard layer diet. They were reared in deep litter and transferred to a perchery system at point of lay.

Chemicals

EDTA and potassium borohydride were obtained from Sigma (Poole, Dorset, U.K.); CF1 cellulose from Whatman (Maidstone, U.K.); β -amino propionitrile fumarate from Aldrich (Poole, Dorset, U.K.); and ninhydrin and buffers for amino acid analysis from Pharmacia (Uppsala, Sweden).

Histomorphometry

The left proximal tarsometatarsus (PTM) was dissected from each bird and fixed in 10% neutral buffered formalin and processed for methyl methacrylate embedding. Undecalcified 8 μ m sections were cut on a Reichert-Jung Polycut and three sections (120 μ m apart) from each tissue block were stained with Toluidine Blue. Trabecular and medullary bone volumes were measured by point counting using an eyepiece graticule with 100 intersections according to the method of Kimmel and Jee [15].

Classification

Samples contralateral to those taken for histomorphometry were divided into five groups based on the degree of osteoporosis, as determined by trabecular bone volume (TBV) (as a percentage of total bone volume) measured as described above (Table 1). Osteoporosis occurred with each treatment to roughly the same

extent; hence the samples for collagen analysis were taken from all treatments to provide a range of extent of osteoporosis and the effect of diet was discounted.

Biomechanics

The contralateral tarsometatarsus from each bird was stored frozen prior to the mechanical studies. A 4 mm thick section from the proximal metaphysis of the tarsometatarsus was taken from immediately below the calcified tendon sheath using a Microslice 2 (Metals Research Ltd., Cambridge, U.K.) for subsequent compression testing, and the remainder was used for the three-point bending test. The loads were applied using a 10 kN load cell at a speed of 4.8 mm/min for the compression test and 4 mm/min for the three-point bending test. These conditions resulted in strain rates within the ranges observed *in vivo*. The support span for the three-point bending tests was 40 mm for all the samples, which were centrally positioned in relation to their length. Both strength tests were carried out on an Instron Materials Testing Apparatus, model 6022. The surface area of the compression section and the cross-sectional area at the break from the three-point bending test were measured with the Visual Imaging Display System (VIDS). The test loads were adjusted for the dimensions of each sample to give compression stress and three-point bending stress respectively.

Collagen biochemistry

Immediately after biomechanical testing, samples of the metaphysis and the cortical bone (from the centre of the diaphysis) from each sample were taken for biochemical analysis. The bone was defatted in chloroform/methanol (3:1, v/v) for 48 h at 4 °C, washed in methanol and then extensively washed in distilled water. Samples were powdered after cooling in liquid nitrogen using a freezer-mill (Mikro-dismembrator; Braun Biotech, Aylesbury, U.K.).

(a) Intermolecular cross-links

(i) Borohydride reduction and hydrolysis. The samples were homogenized, suspended in PBS and reduced with potassium borohydride for 1 h as described previously [16]. The sample was then washed, freeze-dried, reweighed and then hydrolysed in 6 M HCl for 24 h in a screw-top polypropylene hydrolysis tube (Sarstedt, Leicester, U.K.). The excess acid was removed by freeze-drying and the residue was dissolved in 2 ml of distilled water.

(ii) Separation of cross-links. The hydrolysates were initially separated on a CF1 cellulose column to remove the non-cross-link amino acids. The samples were assayed for the borohydride-reduced forms of the immature cross-links, i.e. hydroxy-lysiononorleucine (HLNL) and dihydroxylysiononorleucine (DHLNL), and the stable mature cross-links HL-Pyr and L-Pyr using a modified gradient on an amino acid analyser (Alpha Plus; Pharmacia, Loughborough, U.K.), as previously described [16]. The location of the cross-links on the analyser has previously been confirmed with samples of authentic cross-links prepared in this laboratory. Quantification was achieved using a ninhydrin colour reaction and their known leucine equivalents.

(iii) Pyrrolic cross-link. The putative pyrrolic cross-link was assayed by reaction with *p*-dimethylaminobenzaldehyde, the colour values being calculated using a molar absorption coefficient based on that of an *N*-methylpyrrole [11].

(b) Lysine hydroxylation

The level of lysine hydroxylation was quantified by carrying out

Table 1 Classification of bone for degree of osteoporosis

TBV is expressed as a percentage of total bone volume.

Grade	Degree of osteoporosis	TBV (%)	No. of samples
1	Normal	> 16	6
2	Mild	14–16	5
3	Moderate	11–14	5
4	Severe	8–11	12
5	Very severe	< 8	5

amino acid analysis on the Alpha Plus amino acid analyser, using the standard gradient for amino acid composition.

(c) Quantification of collagen

The amount of collagen in the tissue was determined by hydroxyproline assay of the hydrolysate [17] using a continuous-flow Autoanalyser (Chem-Lab). The resulting data were then used to calculate the cross-link and hydroxylysine values as mol per mol of collagen.

(d) Type I/type III collagen ratios

The relative proportions of type I and type III collagens in the trabecular and medullary bones were determined by SDS/PAGE of the CNBr peptide digest and quantitative scanning of the stained gel. Essentially the ratios of the two peptides $\alpha 1(I)$ CB8 and $\alpha 1(III)$ CB5 were used to quantify the ratio of types I and III, as described in detail previously [18]. In the case of the medullary bone, the increased amounts of proteoglycans were removed with guanidine hydrochloride and the remaining proteins by trypsin digestion.

(e) Calcium content

The mineral content was calculated from calcium levels determined on a Kone Specific Autoanalyser using the Raichem colorimetric assay, direct from an aliquot of bone hydrolysate.

(f) Thermal stability of collagen

Differential scanning calorimetry was used to assess the thermal stability of the collagen fibrils [19,20]. A selection of samples (nine grade 4/5 and five grade 1/2) were first decalcified in 0.5 M EDTA, pH 7.5, for 5 days and then washed in distilled water for 2 days. The collagen was heat-denatured employing a Perkin-Elmer (Beaconsfield, Bucks, U.K.) DSC-2C calorimeter fitted with intracoolers and a computer controller to scan from 5 to 100 °C at 10 °C per min. An empty pan was used as reference.

Comparison of cortical and medullary bone

Tibia containing medullary bone were dissected from 20 birds, and the cortical and medullary bone carefully separated. Biochemical analysis of the collagenous matrix of the samples was carried out as described above.

Effect of the level of cross-linking on bone strength

The lathyrict chick model was used to study the relative importance of collagen cross-links in providing bone strength. Fertile 15-day-old eggs (Millards, Bath, U.K.) were injected with 0, 5, 15 or 20 mg of β -aminopropionitrile fumarate in sterile PBS, pH 7.5, into the embryonic cavity. After 3 days incubation at 37 °C, the embryos were killed and the tibias removed for biochemical and biomechanical testing.

Compression testing was carried out on the Instron machine detailed above, at a test speed of 1.3 mm/min and with a 100 N load cell. The three-point bending strength of the bone was measured on a Stevens C. R. Analyser (C.R. Stevens Weighing Machines Ltd.) and the strength was taken as the force in g required to fracture the bone. The test speed was 50 mm/min, with a 5 kg load cell, and the support distance was 6 mm. The test loads were adjusted for the dimensions of each sample as described earlier. The cross-links and the mineral content of these bones (from dose groups 0 and 20 mg of β -aminopropionitrile) were measured as above.

Statistical analysis

For the comparison of normal and osteoporotic groups (unpaired), diaphysis and metaphysis (paired), and cortical and medullary (paired) bone, Student's *t* tests were used to determine the significance of any differences. Correlations between related groups of data were calculated using the linear correlation coefficient *r*. For ease of comparison, the results for grades 1 and 2 were pooled and termed normal/mildly osteoporotic (a total of 11 samples), and those for grades 4 and 5 were also pooled and termed severely osteoporotic (total of 17 samples).

RESULTS

Histomorphometry

The dietary treatments did not affect the egg production or feed intake characteristics of the hens. Osteoporosis, as determined by mean TBV, was likewise unaffected by diet, though fluoride and oystershell treatments increased the medullary bone volume. Consequently, for the present study, the dietary groupings were ignored and the bones were divided into groups based on the degree of osteoporosis as determined by TBV of the PTM according to the classification shown in Table 1.

Histological sections of typical bones with grade 1 and grade 5 osteoporosis are shown in Figure 1. The severely osteoporotic section of the PTM shows the characteristic thinning of the cortices and trabeculae. Figure 2 illustrates the mean TBV and medullary bone volume as a percentage of the total bone volume

Figure 1 Undecalcified sections of normal and osteoporotic bone

(a) Normal (grade 1) bone; (b) osteoporotic (grade 5) bone (see Table 1).

Figure 2 Mean TBV and medullary bone volume (MBV) for each grade of osteoporosis

Results are expressed as a percentage of total bone volume. See the text for further details.

for each degree of osteoporosis. As percentage TBV was used to grade the samples, this parameter decreases with increasing severity of osteoporosis. In addition, the medullary bone volume increases with severity of the disease, and as a result the sum of TBV plus medullary bone volume varies little with the degree of osteoporosis.

Biomechanics

With both strength tests we recorded a significant decrease in the strength of the bone in the severely osteoporotic group compared with the normals and mild osteoporotics. The three-point bending test on the diaphyseal (cortical) bone demonstrated a greater significant difference than the compression test on the metaphyseal (trabecular) bone (Table 2). As expected, the percentage TBV correlated well with the strength of the diaphysis ($r = 0.476$; $P < 0.001$). The amount of medullary bone did not affect the

strength of the bone even in the metaphysis where the greatest proportion of this bone is found ($r = 0.257$; not significant).

The level of mineral as a percentage of the dry weight does not appear to have any significant effect on the strength of the bone. The collagen content of the osteoporotic group was lower than that of the normal diaphyseal group, although this difference was not highly significant ($P < 0.07$) (Table 2). However, although there was no correlation of the collagen content with the breaking strength of the cortical bone, the ratio of mineral to collagen increased in the diaphysis of osteoporotic samples compared with normals, and this change was correlated with the three-point breaking stress ($r = 0.359$; $P < 0.05$).

Collagen biochemistry

(a) Hydroxylysine

There was a small increase in the hydroxylysine content of the osteoporotic diaphyseal bone ($P < 0.08$), but a highly significant increase in that of the osteoporotic metaphyseal bone ($P < 0.001$). The level of lysine hydroxylation was significantly greater in the metaphysis than the diaphysis of osteoporotic bone (Table 2). However, on plotting hydroxylysine content against the mechanical strength, there was a significant correlation ($P < 0.05$) for the diaphyseal bone only (Figure 3).

(b) Immature cross-links

No statistically significant differences were observed in the levels of HLNL and DHLNL between the normal and osteoporotic bones. However, the DHLNL/HLNL ratios were higher for both the osteoporotic metaphyseal and diaphyseal bones compared with normal ones, consistent with an increase in hydroxylation of the collagen.

(c) Mature cross-links

No significant differences could be detected in the levels of HL-Pyr or L-Pyr between the normal and osteoporotic metaphyseal and diaphyseal bones (Table 2). The HL-Pyr/L-Pyr ratio increased with increasing levels of hydroxylation (Figure 4).

(d) Pyrrole cross-link

The levels of the pyrrole cross-link were decreased in the

Table 2 Biomechanical and biochemical parameters of severely osteoporotic (grade 4/5) and normal/mildly osteoporotic (grade 1/2) bone samples

Values are means $\pm$ S.E.M., with units of mol/mol unless otherwise indicated. Significant differences (unpaired) between grade 4/5 and grade 1/2 are indicated by * $P < 0.05$ and *** $P < 0.001$. Significant differences (paired) between the diaphysis and the metaphysis for grade 4/5 and grade 1/2 are indicated by † $P < 0.05$ and ††† $P < 0.001$. For all measurements, $n = 32$.

	Diaphysis		Metaphysis	
	Grade 1/2	Grade 4/5	Grade 1/2	Grade 4/5
Three-point bending stress (MPa)	265.4 $\pm$ 10.4	202.7 $\pm$ 6.5***	—	—
Compression stress (MPa)	—	—	28.48 $\pm$ 1.58	24.53 $\pm$ 0.97*
Mineral (%)	59.00 $\pm$ 2.20	62.54 $\pm$ 2.37	54.87 $\pm$ 2.92	57.76 $\pm$ 1.68
Collagen (%)	19.7 $\pm$ 1.1	17.34 $\pm$ 0.58	17.55 $\pm$ 0.71	16.15 $\pm$ 0.66
Mineral/collagen ratio	3.07 $\pm$ 0.18	3.62 $\pm$ 0.14*	3.19 $\pm$ 0.22	3.70 $\pm$ 0.24
DHLNL	0.527 $\pm$ 0.037	0.535 $\pm$ 0.035	0.531 $\pm$ 0.049	0.496 $\pm$ 0.052
HLNL	0.283 $\pm$ 0.019	0.267 $\pm$ 0.018	0.236 $\pm$ 0.016†††	0.214 $\pm$ 0.016
DHLNL/HLNL ratio	1.75 $\pm$ 0.20	1.84 $\pm$ 0.13	2.26 $\pm$ 0.10	2.52 $\pm$ 0.15†††
HL-Pyr	0.057 $\pm$ 0.017	0.056 $\pm$ 0.005	0.059 $\pm$ 0.003	0.061 $\pm$ 0.005
L-Pyr	0.064 $\pm$ 0.005	0.070 $\pm$ 0.004	0.062 $\pm$ 0.004	0.066 $\pm$ 0.004
HL-Pyr/L-Pyr ratio	0.919 $\pm$ 0.096	0.820 $\pm$ 0.082	0.994 $\pm$ 0.082	0.951 $\pm$ 0.082†
Pyrrole	0.362 $\pm$ 0.012	0.337 $\pm$ 0.007*	0.305 $\pm$ 0.018	0.270 $\pm$ 0.012†††
Hydroxylysine	15.37 $\pm$ 1.01	16.11 $\pm$ 0.83	16.96 $\pm$ 1.19	18.85 $\pm$ 0.88†††

Figure 3 Three-point bending stress and hydroxylysine content of diaphyseal bone

See the text for details.

osteoporotic diaphyseal bone compared with the normal (although the difference in the metaphysis was not statistically significant), consistent with the decreasing hydroxylysine levels (Table 2). A significant correlation ($r = 0.454$; $P < 0.01$) between the pyrrole content and the mechanical strength of the cortical bone was also obtained (Figure 5).

(e) Type I/type III collagen ratio

The proportion of type III collagen in the trabecular bone was found to be too low for an accurate determination employing the acrylamide gel technique and laser scanning of the gel, i.e. less than 5% type III collagen. Attempts to demonstrate an increase in type III collagen in the osteoporotic bone were unsuccessful for the same reason.

(f) Differential scanning calorimetry

The major transition peak during thermal denaturation of both normal and osteoporotic bone collagen occurred at the same temperature (69 °C), although the peak from the latter was lower

Figure 4 Mature cross-link ratio and hydroxylysine content of avian bone

See the text for details.

Figure 5 Three-point bending stress and pyrrole content of diaphyseal bone

See the text for details.

and broader, the height-to-width ratios being 0.95 and 1.40 for the osteoporotic and normal bones respectively (Figure 6). However, the enthalpies, calculated from the area under the peak, were found to be identical (Table 3).

In contrast to collagen in rat tail tendon [20], bone collagen revealed a second lower and broader peak with an approximate maximum at 85 °C.

Effect of cross-linking on bone strength

Embryonic bone was, as expected, found to contain only the immature cross-links, predominantly DHLNL. The level of the latter in the spent layers was 10 times that of pyridinoline, indicating its overall importance in stabilizing avian bone. As a consequence, the decrease in this particular cross-link was used to correlate with the strength of the bone. Figure 7 demonstrates that the decrease in DHLNL concentration caused by the lathyrisms is correlated with a decrease in breaking strength (r is significant at $P < 0.01$ for all three measures of strength). There was no similar correlation with calcium levels (results not shown).

Comparison of cortical and medullary bone

The collagenous matrix of the medullary bone was found to differ greatly from that of the structural cortical bone (Table 4).

Figure 6 Differential scanning calorimetry thermograms of a normal (A) and an osteoporotic (B) bone sample

The peak height/peak width ratio of osteoporotic samples was 0.948, compared with 1.392 for the normal samples ($P < 0.03$; $n = 13$).

Table 3 Thermal characteristics of normal and osteoporotic bone

Significant differences between normal and osteoporotic bone samples (unpaired) are indicated by * $P < 0.05$; ** $P < 0.01$. See the text for details.

	Normal bone	Osteoporotic bone
T_{initial} (°C)	61.8 ± 0.6	61.3 ± 0.6
T_{onset} (°C)	67.1 ± 0.6	66.7 ± 0.6
T_{max} (°C)	69.3 ± 0.6	69.3 ± 0.4
T_{final} (°C)	76.3 ± 0.4	76.3 ± 0.3
Enthalpy (J/g of collagen)		
Peak 1	14.7 ± 1.0	13.4 ± 0.7
Peak 2	26.4 ± 0.6	27.2 ± 1.4
Total	41.1 ± 1.0	41.1 ± 1.3
Peak 1		
Height (J/g of collagen)	4.5 ± 0.2	3.3 ± 0.2**
Width (°C)	3.2 ± 0.1	3.7 ± 0.2
Height/width ratio	1.4 ± 0.1	0.9 ± 0.1*

Figure 7 Three-point bending stress and DHLNL content of lathyrictic chick tibia

See the text for details.

Table 4 Cross-link content of the tibial cortical and medullary bone from end-of-lay hens

Values are means ± S.E.M., with units of mol/mol unless otherwise indicated. Significant differences between the medullary bone and the cortical bone (paired samples, $n = 20$) are indicated by ** $P < 0.01$ and *** $P < 0.001$.

	Cortical bone	Medullary bone
DHLNL	0.420 ± 0.029	0.721 ± 0.079**
HLNL	0.314 ± 0.028	0.165 ± 0.019***
DHLNL/HLNL ratio	1.394 ± 0.060	4.618 ± 0.249***
HL-Pyr	0.090 ± 0.007	0.094 ± 0.009
L-Pyr	0.119 ± 0.011	0.029 ± 0.005***
HL-Pyr/L-Pyr ratio	0.799 ± 0.037	2.704 ± 0.358***
Hydroxylysine	17.33 ± 0.70	28.93 ± 0.77***

The levels of the immature cross-link DHLNL were significantly higher in the medullary bone, while its mature product was only slightly increased, consistent with a higher turnover rate for medullary bone. The increased overall hydroxylation obviously

results in the decrease in HLNL and its mature product, L-Pyr. The levels of the pyrrole cross-link are low in the medullary bone compared with the cortical bone (results not shown).

As in the case of cortical bone, type III collagen could not be detected in medullary bone by SDS/PAGE, even after removal of the high proportion of non-collagenous proteins and glycosaminoglycans.

DISCUSSION

The mechanical studies confirmed a significant weakening of both the cortical and trabecular bones in osteoporosis. The apparently greater relative loss of strength of the cortical bone suggests a greater responsiveness compared with the trabecular bone, but in reality we cannot compare differences in the sensitivities of the three-point bending and compression tests used for the cortical and trabecular bone respectively.

It is generally agreed, and our results confirm, that the contribution of the medullary bone to the mechanical strength of the bone is small. Even though the increase in the volume of the medullary bone is large (Figure 2) there is little connectivity between the medullary bone nodules, such that it has little resistance to bending or compressive stress. Although not contributing to bone strength, it is interesting to note that the medullary bone revealed several differences in its collagenous matrix when compared with cortical bone from the same hens. The increase in hydroxylysine and corresponding increases in the DHLNL/HLNL and HL-Pyr/L-Pyr ratios indicate that medullary bone consists of a more immature matrix, consistent with a higher turnover of the collagenous matrix. The resulting different physical and chemical properties of medullary bone and cortical bone reflect the differing demands made upon the them.

Analysis of the biomechanical data revealed that there was no correlation between either of the major components of the bone, i.e. the level of mineral or the content of the collagenous framework, and the strength of the tissue. This suggests that the loss of strength could be due solely to an overall loss of bone. However, we have noted a correlation between breaking strength and lysine hydroxylation, which indicates that the changes that we have observed in post-translational modifications (lysine hydroxylation and cross-linking) may play a role in the loss of strength of the collagen. Increased lysine hydroxylation in some forms of the heritable disease osteogenesis imperfecta due to slower triple helix formation is believed to be responsible for the formation of thin collagen fibrils [21]. An increase in hydroxylation of the lysine residues in 'osteoporotic' collagen could similarly lead to thinner fibrils, and an increase in the proportion of these newly synthesized fibres would reduce the strength of the bone. In the case of osteoporosis the increase in hydroxylation could be due to several mechanisms, e.g. decreased rate of formation of the triple helix or increased lysine hydroxylase levels, or it may possibly involve chaperones in stabilizing triple helix formation.

Lathyrictic agents are known to inhibit the cross-linking of collagen, resulting in a loss of strength of bone and other collagenous tissues. We have shown that a 10% decrease in the DHLNL cross-links in young avian bone is sufficient to produce a 15% decrease in mechanical strength (Figure 7). Studies on mature bone [22] have shown that a decrease in pyridinoline also leads to a reduction in strength, but the level of DHLNL was not considered. However, we found the overall levels of the cross-links were not decreased in the osteoporotic hens, but the hydroxylation of the lysine altered the type of cross-links present in favour of DHLNL and HL-Pyr with a consequent decrease in the putative pyrrole cross-link. We have found a good negative

correlation for the decrease in the pyrrolic cross-link with increasing hydroxylation of lysine and the mechanical strength of the bone (Figure 5). Although the structure of the pyrrole has not yet been confirmed, owing to its inherent instability during isolation procedures, it has been proposed that it is formed from the hydroxylysine keto-imine and allysine [12]. Thus any increase in the hydroxylation of telopeptide lysine would lower the amount of allysine available, and consequently the amount of the pyrrole.

At the present time one can only speculate on the apparent importance of the pyrrolic cross-link, since our attempts to locate its position in the fibre by isolating the peptide-bound pyrrole have been unsuccessful. We have, however, shown that the ratio of the pyridinoline to the pyrrole content of human bone is different at the N- and C-termini of the collagen molecule [23], suggesting a different function for each of these cross-links. It is possible that the pyrrole, in contrast to the pyridinolines, could act as an interfibrillar cross-link. As such the loss of the pyrrole cross-link would have a dramatic effect on the mechanical properties of the bone. Clearly further investigations on the role of these cross-links are warranted.

The rate of turnover of avian bone collagen is unknown, but it is clear from the present results that during turnover of collagen in old osteoporotic hens the newly synthesized collagen is different from that in the non-osteoporotic bone, and is sufficiently different to alter the properties of the bone. The primary change in the degree of lysine hydroxylation is correlated with decreased breaking strength of the bone, which in turn is significantly correlated with a decrease in the pyrrolic cross-link.

We have previously used differential scanning calorimetry to detect differences in the interactions of collagen molecules and fibres due to structural differences and changes in the environment [19,20,24]. A rapid absorption of energy, i.e. an endothermic transition, occurs on denaturation of the collagen fibre, and the area under the peak gives the total heat involved in the transition, known as the enthalpy of denaturation. Dehydration of the fibre and the presence of some salts can increase both the denaturation temperature and the enthalpy. A reduction in enthalpy without a change in denaturation temperature can be interpreted as a proportion of the molecules being denatured, for example in damaged tendon [19]. In the present studies the denaturation temperature and the enthalpy are unaffected in osteoporosis, but the shape of the thermogram is altered, indicating a change in the osteoporotic collagen. We suggest that there is an increased variability in the thermal stability of the population of collagen molecules. More detailed studies are being carried out, but in the context of the present paper the differential scanning calorimetry studies reported provide additional evidence for a change in the nature of the collagenous matrix in osteoporotic bone.

The renewed high levels of lysine hydroxylation in osteoporotic bone in the present study could be an attempt at a repair mechanism. High levels of lysine hydroxylation occur in rapidly growing foetal bone and skin, but this decreases rapidly with increasing age as the activity of the enzyme lysine hydroxylase decreases [25]. Transient increases in hydroxylation occur in collagen during bone fracture and in dermal wound healing [26] and other fibrotic situations [27]. The newly synthesized collagen of osteoporotic bone presumably has a higher rate of turnover, or possibly a higher lysine hydroxylase activity, than that of normal structural bone. Further studies on the levels of activity of lysine hydroxylase and the overall turnover of bone collagen are required to confirm this suggestion. In this context we remain intrigued by the possibility of an additional lysine hydroxylase that is confined to the control of hydroxylation of the telopeptide

lysines. The differences in the extent of hydroxylation of the N- and C-termini of bone collagen [23], and the recent report of increased hydroxylation being confined to the telopeptide lysine in tendon hypertrophy [28], lend support to the original proposal for two lysine hydroxylases [29].

The changes in the collagenous matrix of osteoporotic avian bone are similar to those reported in human osteoporosis [7,30]. Although the aetiologies of avian and human osteoporoses differ, the underlying mechanisms of change in the matrix may be similar. Whether or not a genetic solution to osteoporosis in chickens is to be sought, further studies into the normal and osteoporotic bone matrix would, in light of the results presented here, be of considerable value.

In conclusion, it is clear that the newly synthesized collagenous framework of the bone of osteoporotic end-of-lay battery hens is biochemically modified in a manner consistent with a decrease in the mechanical strength of the bone, thus increasing the incidence of broken bones in these flocks.

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