

Thermal Stabilization of Collagen Fibers by Calcification*

Mineralized collagenous tissue is known to be more stable than soft collagenous tissue both mechanically and thermally. We find that the denaturation temperature of collagen in bone scanned in differential scanning calorimetry at 5°C/min is 155°C, 90°C higher than that in skin. Furthermore, when the bone is partially demineralized with citrate, a discrete intermediate denaturation temperature appears at 113°C, indicating that the mineral is retained at preferential binding sites in the collagen until it is completely leached out. It is shown by electron microscopy that these sites are located in the overlap regions of the collagen fibrils. Collagen in bovine hide can be synthetically mineralized *in vitro* by impregnation with calcium acid phosphate solution, followed by raising the pH, causing the phosphate to precipitate. Some of the collagen in this synthetically calcified tissue has an elevated denaturation temperature, 104°C. This temperature compares favorably with collagen that is tanned with chromium oxide-sulfate complexes. Calcium phosphate in synthetically mineralized hide, hydroxyapatite in bone, and chromium oxide-sulfate complexes in leather might share the same mechanism of thermal stabilization.

Key Words: Collagen, calorimetry, denaturation, hydroxyapatite, calcium phosphate, bone.

INTRODUCTION

Bone is often regarded as a fiber-reinforced composite material designed for maximum strength in its role as structural support. The fibers are bundles of collagen fibrils arranged parallel to the usual load that is borne by the particular part of the bone, while the composite matrix is the hydroxyapatite mineral. Such a structure can in principle be analyzed mechanically by the same methods used for fiber-reinforced ceramics.

An important peculiarity of bone is the complicated internal structure of the collagen fibers. In bone the mineral crystallites occur not only in and among the fibers as a matrix, but to a lesser extent (<35% in bovine tibia) within the 100-nm elementary fibrils.¹ This structure suggests an alternative view of bone as a fibrous material reinforced with mineral. The difference is not trivial to the analysis of its stability. This second view

relates better to the development of the tissue, in which mineral is deposited into the collagen, rather than vice-versa.

There is evidence that mineralization increases the thermal stability of the collagen molecules.² It was found that bone could be heated to 100°C, cooled for 24 hr and demineralized with EDTA (ethylene diamine tetraacetate) for four days with no change in either the wide- or low-angle X-ray diffraction pattern. Heating after demineralization, followed by the same annealing and EDTA treatments, on the other hand, caused the low-angle pattern to vanish, with retention of the high-angle pattern. From these observations no conclusions can be drawn about the state of the collagen molecules in bone at 100°C, since they could have been annealed by the subsequent treatments had they been denatured at that temperature. Local annealing could have occurred with loss of the long-range order associated with the regular staggered packing of the molecules in the 100-nm fibril.

Here we use differential scanning calorimetry to determine the thermodynamic parameters of denaturation of bone collagen with and without mineral. By synthesizing collagen-mineral complexes, we also attempt to clarify what specific mineralized structures might cause such stabilization of the collagen.

MATERIALS AND METHODS

Mineralization. One gram fresh bovine dermis was shredded and placed in 200 ml water equilibrated with carbon dioxide in a steel bomb equipped with a stirrer, pressure gauge, and a thermometer well. Two blocks of ice containing 5.4 mmole sodium acid phosphate and 8 mmole calcium chloride, respectively, and 1.1 mole frozen carbon dioxide were placed in the water, and the chamber was sealed quickly. The pressure rose to 12 atm at 22°C and was maintained for 17 hr, with stirring.

Under these conditions the solubility of carbon dioxide in water is 0.3 M.³ From the pK of carbonic acid (4×10^{-7} M), we calculate that the pH in the chamber was 2.9. Experiments with pH indicators in this chemical system in a glass pressure flask confirmed the low pH. From the solubility data of Gregory *et al.*⁴ for calcium acid phosphate extrapolated to pH = 2.9, we calculate that the concentration of calcium in the solution in the pressurized chamber was 0.114 M. If the wet dermis contained 1 ml solution, it comprised 3% calcium phosphate. On release of the pressure and evaporation of carbon dioxide the pH rose to 6, causing the solution to become supersaturated in calcium phosphate, which precipitated within the dermis.

Bone. Bovine jawbone was prepared by immersing the whole bone for three minutes in 70°C water (with no apparent effect on the endotherm of the constituent collagen—vide infra), then treating with papain to remove clinging residues of tendon and finally grinding with a Wiley mill or a Spex** liquid-nitrogen mill. Most of the particles from the Wiley mill had a dimension of about 0.5 mm. It was partially demineralized by being stirred for 17 hours in 0.5 M citrate at pH from 4 to 6. Complete demineralization was achieved by stirring the particles in an excess amount of a neutral solution of 0.5 M EDTA at 4°C for 36 days, and then extracting three times overnight with water.

Collagen was determined by directly analyzing weighed DSC samples for hydroxyproline⁵, using the factor 13.5 to convert to type I collagen.

Calorimetry. Differential scanning calorimetry (DSC) was performed with either the half-gram samples in the solid-sample accessory of a Microcal scanning calorimeter (Microcal Inc., Northampton, MA) or with 20-mg samples in a differential scanning calorimeter (Perkin-Elmer Model DSC-7) at scanning rates specified in the figures. Whole bone was contained in special high-pressure steel capsules available from Perkin-Elmer.

Transmission electron microscopy was done on a Philips CM-12 instrument on unstained sections imbedded in epoxy resin mixture. We wanted to correlate the

sites of the remaining mineral crystals with respect to the faint D-bands of the unstained collagen fibrils after partial extraction with citrate. To this end, selected photographic negatives, recorded at an instrumental magnification of 28,000X, were imaged by transillumination using a light box and a Series 68 television camera (DAGE-MTI, Michigan City, IN). Ten video frames of each image were averaged into a 512×512 frame buffer of a DT2853 frame grabber (Data Translation, Marlboro, MA). In order to reduce the uneven variations in background density in the image, the averaged frames were processed by background flattening with selected defaults for bright, medium-sized objects. A fast Fourier transform of the whole frame was computed and edited using Image-Pro Plus software, version 2.1 (Media Cybernetics, Silver Spring, MD). The D-band intensities were then enhanced by increasing the intensity of the first-order maximum (at $1/D$ in the transform) by a factor of 2, before the inverse transform of the FFT was computed.

RESULTS

Collagen in bone is thermally very stable, melting irreversibly at 155°C as shown in Fig. 1. The enthalpy, determined from the area under the endotherms from four samples, is 56 ± 13 J/g. No thermal activity was observed from 20°C to the endotherm, nor from the endotherm to 180°C (above which the capsules ruptured). The collagen content of this bone was $21 \pm 1\%$, determined on the dry basis by analysis of hydroxyproline. The water content of the wet bone used for the determination of Fig. 1 was $9.8 \pm 0.7\%$. If all the water was associated with collagen, the collagen contained 34% water. The implications of this will be discussed below.

The position of the melting curve of demineralized bone (Fig. 2) is similar to that of tissue collagen, but there is a low-temperature tail, indicating low-melting species, as Danielsen also found by differential spectrophotometry.⁶ The main melting region at 63°C, with its wide shoulder on the high-temperature side, at 72°C, resembles the biphasic melting of soft-tissue collagen heated at the same rate. For skin there were two components of this endotherm, which related to the cores and sheaths of the constituent collagen fibrils.⁷ In bone collagen examined here the high-temperature shoulder is broad and is not a single Gaussian peak. Since bone collagen has more stable crosslinks than skin does, which are chemically different, it is not surprising that a distribution of stabilities is found rather than the single one in skin that was associated with thermal instability. The ratios of the areas under the resolved peaks to the total area are 0.27 at 63°C and 0.73 at 72°C.

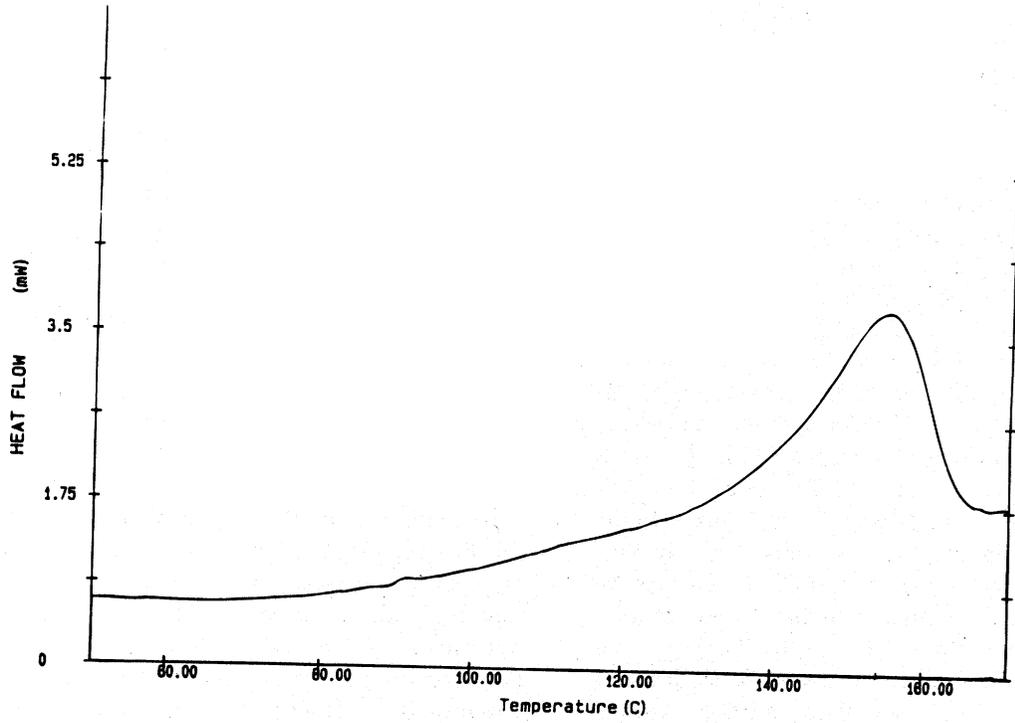


FIGURE 1 Differential scanning calorimetry of collagen in bone, scanned at 5°C/min.

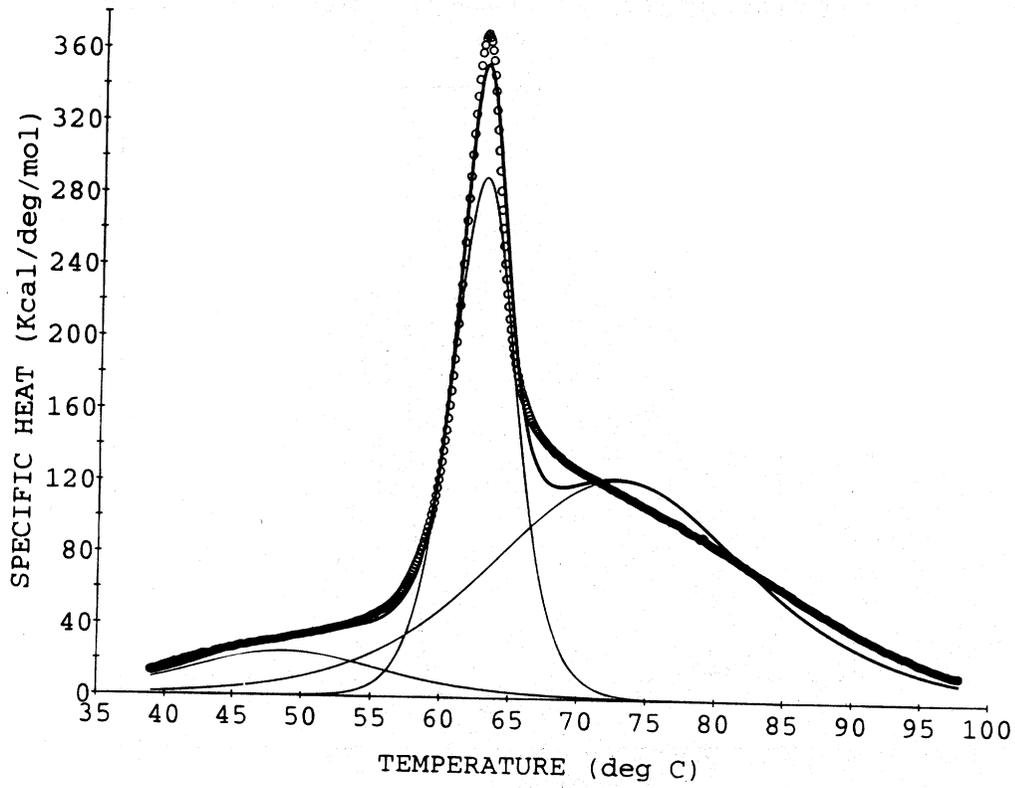


FIGURE 2 Differential scanning calorimetry of demineralized bone, scanned at 0.5°C/min. Scatter plot, experimental points; thick line, calculated; thin lines, Gaussian components.

Frequently, an added feature is a broad exotherm centered at 80°C, which is not present if the collagen has been first frozen or if the sample has been deoxygenated by flowing nitrogen immediately before the DSC scan. It seems to be due to an irreversible oxidation reaction, perhaps of a non-collagenous component.

When the tissue contains amounts of mineral intermediate between that of bone and collagen, the endotherm is still discrete, but appears at an intermediate temperature, 113°C (Fig. 3). As with the demineralized bone, there is a shoulder, here at 120°C. The enthalpy of melting was 86.0 ± 8.2 J/g. The material described by the figure was prepared by first grinding in a Wiley mill, then further reducing by hammering at 77K with a Spex mill, and finally treating with 0.5 M citrate at pH=5.8 for 17 hr. The product contained $57.4 \pm 3\%$ collagen. Two discrete melting temperatures are found for larger particles prepared by the Wiley mill alone, one at 62°C characteristic of fully demineralized tissue and another at 130°C. Inasmuch as the demineralization was unlikely to have been uniform throughout the sample, it is surprising that these peaks are discrete. We found only small but unsystematic variations of the peaks with treatment time, pH, or variation of chelate from EDTA to citrate. Small peaks are always seen at temperatures above the main transition temperature, sometimes negative as in Fig. 3. Like the main endotherm they

are discrete, but much more variable. They might be the ones expected at various temperatures because of nonuniformity in the spatial distribution of the mineral remaining after extraction with citrate.

The remnant mineral after such partial extractions takes the form of fine needles and platelets, about 50 nm long, and amorphous mineral, both associated mostly with the collagen striations. In Fig. 4b there is shown a micrograph negative of an unstained thin section of the partially demineralized bone. Remnant mineral seen as light masses is scattered among collagen fibrils. The blank spaces in the photograph, however, actually have faint striations with the D-spacing of collagen fibrils. To show these better in relation to the mineral crystals, we increased the intensities of the corresponding peaks in the (fast) Fourier transform (shown before the alteration in the inset in Fig. 4b), and then transformed the result again to obtain the image with enhanced D-bands (Fig 4a). (The inset shows the altered Fourier transform.) Here it is clear that the remnant mineral is located within the fibrils rather than between them, since it overlays the bands in the sections. This pattern is consistent with the early precipitation of mineral in nascent bone, which must be due to an increase in stability of the mineral in contact with collagen. It is also clear that the crystals are associated with the dense (light in the negative) bands of the collagen fibrils, the overlap

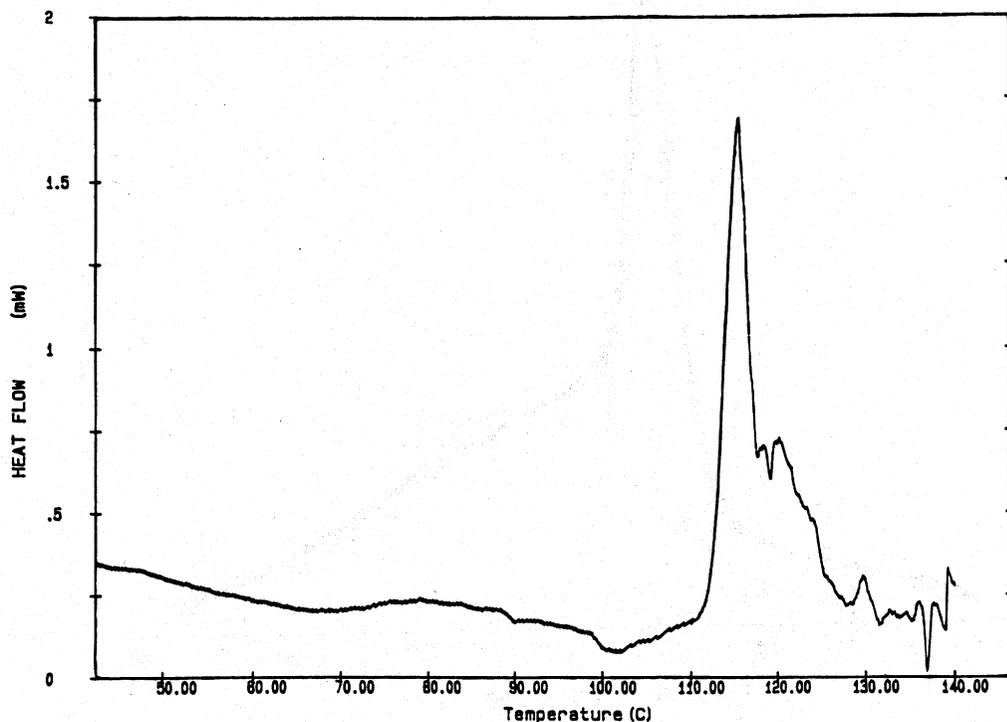


FIGURE 3 Differential scanning calorimetry of partially demineralized bone, scanned at 5°C/min. For details, see text.

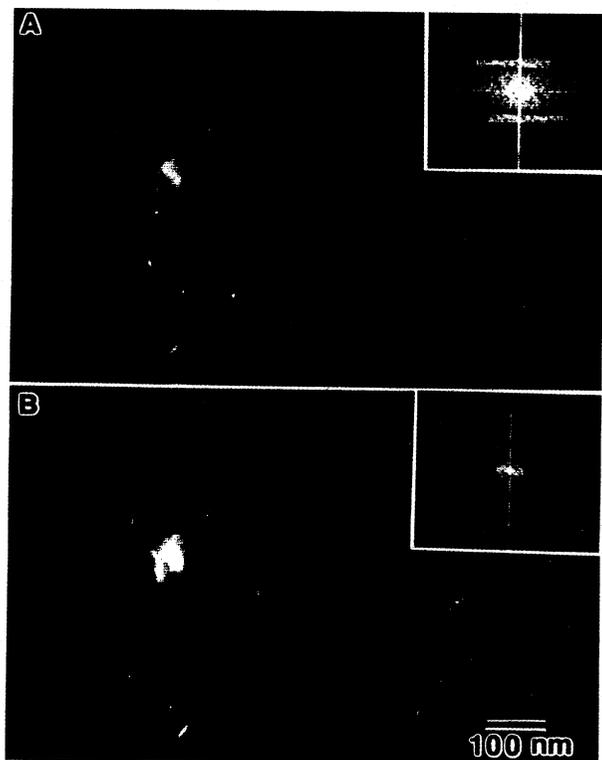


FIGURE 4 Transmission electron micrograph of an unstained section of bone partially demineralized by comminution followed by treatment with 0.5 M citrate at pH = 5.8 for 15 min. (a) The D-period bands of the fast Fourier transform of the direct image were enhanced (inset), and the transform was inverted to give the D-band enhanced image. (b) Direct image. Inset shows fast Fourier transform with prominent horizontal bands corresponding to the D-period.

regions, rather than the gaps. This differs from those in the turkey tendons examined by Traub⁸ because we are observing the mineral remnant after extraction, expected to be tenacious in the dense overlap regions, while he observed the mineral as it deposited, in the accessible gap zones of young turkeys. At later stages of development he observed plates of mineral in the overlap regions also.

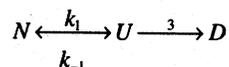
The *in vitro* mineralized product formed by release of pressure over carbon dioxide was also stable, as indicated by an unchanging endotherm from one preparation to another, but it contained only small amounts of mineral. The mineral is amorphous, not diffracting electrons in our electron microscope, nor, in previous investigations, X-rays. A typical endotherm for a preparation from shredded fresh bovine hide is shown in Fig. 5. There is a "normal" compound endotherm, with its main peak at 66°C, an elevated peak at 104°C and in addition a third, large, peak at 122°C. The DSC of the precipitate in the liquid without collagen in the pressure cell had a single endotherm at 122°C, so

only the peaks at 60°C and 104°C are due to the collagen, that at 104°C being due to collagen stabilized by the mineral. From the relative areas, we deduce that it comprises 36% of the total collagen.

The transition seen in the *in-vitro* mineralized tissue was irreversible, no peaks or valleys being observed when the heating cycle was immediately repeated. After standing overnight at room temperature, however, the sample that had been heated to 110°C renatured (Fig. 6). Judging by the enthalpy of melting, 55.6J/g, the renaturation was complete. Some of the renaturation apparently occurred at 74°C while the sample was being heated during the repeated DSC scan, since an exotherm at that temperature accompanies the endotherm at 90°C. No transitions were observed on repeated scans of unmineralized tissue, in which mineral had not been precipitated *in vitro*.

DISCUSSION

A general model for the melting transition of a protein, which seems appropriate for collagen, has recently been analyzed by Lepoch *et al.*¹⁰ When collagen molecules in their native state (N) are heated, they begin to unfold locally and reversibly at a definite temperature, absorbing heat. Because the transition is cooperative, the locally unfolded structure (U) immediately undergoes long-range irreversible melting to denatured collagen (D). The reaction is represented by



In general, the presence of reaction 3 causes the overall melting temperature to decrease. The collagen molecule can be stabilized by crosslinks, by inclusion in a rigid matrix, or by immersion in a non-aqueous environment. Stabilization here means raising the melting temperature. An alternate form of stabilization, suppression of the denaturation reaction 3 above, is never observed, although denatured crosslinked collagen does slowly renature under some conditions. On the time scale of the DSC experiment, the collagen is both unfolded and denatured; a prompt second heating scan always shows no melting or melting at a lower temperature.

The intuitive notion of stabilization by crosslinking or by rigid matrices, immobilization of the chains so they cannot move from their native configurations, is described thermodynamically by a lowered entropy of melting, since the structural order in the melted form is held to that of the native state. We have confirmed that

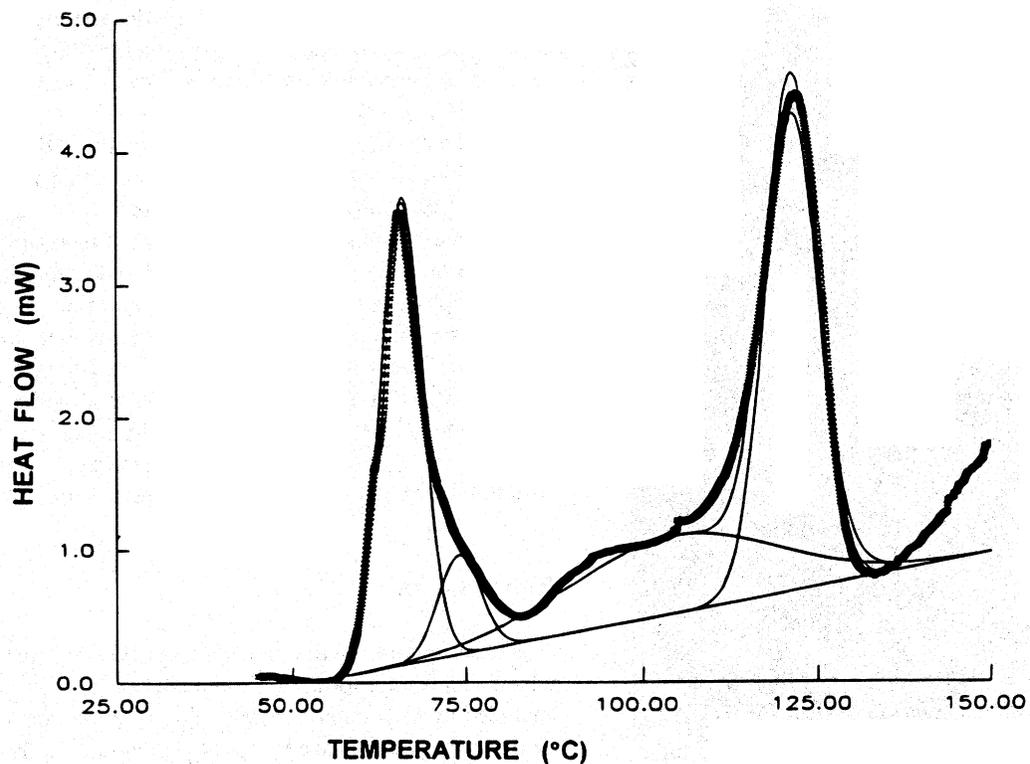


FIGURE 5 Differential scanning calorimetry of hide mineralized by precipitating calcium phosphate *in situ*. The mineral precipitated when carbonic acid was removed on lowering the pressure, as described in the text. Scan rate 5°C/min.

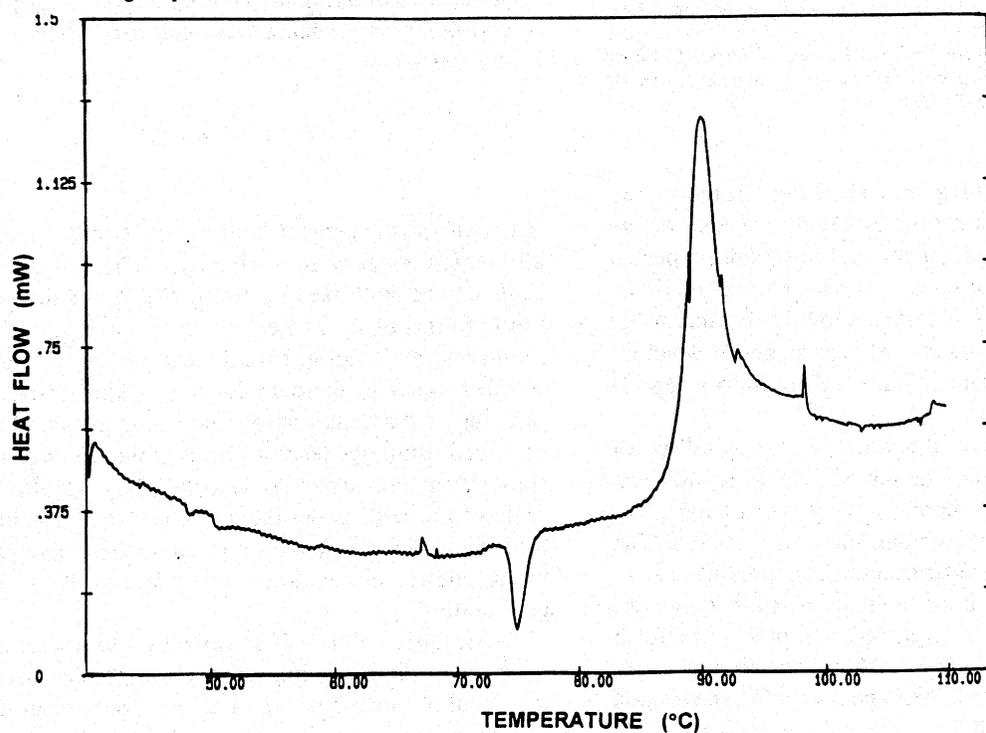


FIGURE 6 Second scan of sample in Fig. 5 after standing at room temperature for 17 hours, showing that the sample became renatured. Renaturation under these conditions was not observed in hide that had not been treated with calcium phosphate. Scan rate 5°C/min.

order persists at high temperatures in collagen cross-linked with glutaraldehyde, showing the persistence of fibrils with crossbands above the melting point, but with shortened D-spacing due to the unfolded molecules.¹¹ These forms do not revert to the native ones on cooling.

The movements of collagen molecules are constrained in bone. When it has been calcined at temperatures below 500°C, bone retains a replica of the collagen fibrils that it contained, even with the cross-banding pattern, showing the intimate earlier association between them and the mineral, which must therefore exert tight restraints on the molecules of the protein.¹² Although collagen in bone melts at a temperature higher than that observed in any other stabilized form, 155°C, the enthalpy of melting is similar to that of soft-tissue collagen, 56 J/g. From the relation $T = \Delta H^\circ / \Delta S^\circ$ the high melting temperature requires that the entropy of melting of collagen in bone must be decreased well below that of the collagen without mineral. This is probably due to the physical constraint of the mineral on the collagen molecules.

Even when the collagen has been completely demineralized it still contains a larger high-melting (ca. 70°C) component than found in skin collagen fibers. The ratios of the areas under the resolved peaks to the total area are 0.27 at 63°C and 0.73 at 72°C. In skin collagen the high-temperature component had about the same area as the one at 62°C. The lack of swelling of bone collagen in acid and the immobility of the protein chains determined from NMR line shapes¹² would lead us to expect a large crosslinked component. In skin collagen this component is due to thermally sensitive crosslinks⁷.

When the bone is only partially demineralized a discrete endothermic peak appears at 113°C. Both the discreteness of the feature and the invariability of its temperature are curious and point to the possibility of tenacious intrafibrillar mineral at a particular site in the collagen fibril. Fig. 4 shows that the mineral does tend to associate with the light striations of the unstained fibrils, which here are due to the dense overlap domains of the fibrils. Hydroxyapatite crystals are found preferably in the gap regions of the striation pattern of bone collagen, and Traub¹⁴ showed how the crystals are nucleated first in these regions and later are projected into the overlap regions. Since the overlap regions are less accessible to the external solutions used to mineralize and demineralize, they should be the last to demineralize.

High melting temperatures are also observed when animal skin is tanned with chromium oxides. This high stability is only found if the mineral is allowed to age, with increase in size of chromium oxide-sulfate complexes. We suggest that the effects of hydroxyapatite in bone and of

chromium-oxide complexes in leather are similar and that they result from similar mechanisms of stabilization of collagen, these mechanisms being different from those attendant upon the stabilization from covalent crosslinks.

In our remineralization experiments we attempted to precipitate calcium phosphate *in situ* in collagen without using large concentrations of calcium and phosphate salts, which can denature collagen. Instead, we took advantage of the pH-sensitivity of the solubility of the precipitate by adding enough salt to saturate the solution containing carbon dioxide at 12 atm, then releasing the pressure allowing the carbon dioxide to escape and the pH to rise. This technique was invented by Tung¹⁴ to mineralize tooth surfaces. It allowed little mineral to actually reach and precipitate in the hide, so the effect of the mineral on the stability of the collagen was not great (Fig 5). The experiments demonstrated that synthetic calcification of collagen can increase its stability, and that the proteins of bone are not absolutely essential.

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