THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 247, No. 3, Issue of February 10, pp. 692–697, 1972
Printed in U.S.A.

Differential Scanning Calorimeter Studies of the Thermal Transitions of Collagen

IMPLICATIONS ON STRUCTURE AND STABILITY

(Received for publication, June 24, 1971)

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SUMMARY

Thermal transition enthalpy changes (ΔH_f) were investigated in intact and soluble collagen from a variety of sources and species utilizing differential scanning calorimetry. Results indicated that the ΔH_f determinations were not dependent on sample size, method of preparation, or sample purity. The ΔH_f values for intact collagen were virtually identical with those from tropocollagen preparations, confirming previous conclusions that the state of aggregation or extent of covalent cross-linking plays only a minor role in the thermal stability of collagen. The experimentally determined values for the enthalpy change per residue (ΔH_r) were 1055 cal per mole and appeared to be invariant in all the collagens studied. The denaturation energy per triplet averaged 3000 cal per mole, suggesting that forces other than hydrogen bonding must be contributing to denaturation enthalpy. A correlation between thermal denaturation or shrinkage temperature and total pyrrolidine content was evident. The entropy change per residue also increased with decreasing imino acid content. These results tend to support the conclusion that the tertiary superhelix structure of the collagen molecule is stabilized primarily by the steric restrictions imposed by the pyrrolidine residues.

It is now generally accepted that the structure of collagen is that of a three-stranded coiled coil held together by a systematic set of hydrogen bonds between the individual chains (1). According to the collagen I and collagen II structures proposed by Rich and Crick (1), each 3-residue-repeating element (–Gly–X–Y–) of a given chain is hydrogen bonded in a systematic manner to each of the two neighboring chains via one hydrogen bond. A structure with two hydrogen bonds has, however, been proposed and refined by Ramachandran and Karthe (2). In both types of structures the NH of the glycine amide group forms a lateral hydrogen bond with a carbonyl atom of the polypeptide backbone of an adjacent chain. The Madras structure (2) has an additional hydrogen bond formed through the NH group of the amino acid residue in position 2 of the 3-residue-repeating element.

Until recently, indirect evidence appeared to favor the model of the two-bonded type (3, 4). The nuclear magnetic resonance studies of collagen fibers by Chapman *et al.* (5) and the x-ray diffraction studies of synthetic analogues of tropocollagen by Segal, Traub, and Yonath (6) have, however, strongly supported a single-bonded structure resembling the collagen II model of Rich and Crick (1). These findings, along with those of other workers (7–9), have placed considerable doubt on the theory that hydrogen bonds alone are responsible for stabilizing the collagen molecule.

Gustavson (10) was among the first to observe that the shrinkage temperature (T_s) appeared to be directly related to the hydroxyproline content of the fibers. Subsequently other workers have shown that both T_s and T_m are linear functions of the total pyrrolidine (proline plus hydroxyproline) content (11, 12). It has subsequently been proposed that steric restrictions to rotation about the polypeptide backbone which arise from the stereochemistry of the pyrrolidine ring act to stabilize the triple helical structure by reducing the total configurational entropy change in the helix-coil thermal transition (13). If it is assumed that the denaturation of collagen in both the macroscopic and molecular state is a first order phase transition of the melting type with the free energy of unfolding $\Delta F_u = 0$, the following relationship is predicted.

$$T_m = \frac{\Delta H_r}{\Delta S_r} \quad (1)$$

where ΔH_r and ΔS_r = enthalpy and entropy per residue and T_m = thermal denaturation temperature (14). Provided there is no accompanying enthalpy change when pyrrolidine residues are introduced into the collagen chain, it follows that any increase in heat stability should be due to a fall in entropy with a rise in the content of imino acids.

Thus it is evident that direct calorimetric determination of the dependency of the enthalpy and temperature of the thermal transition on the pyrrolidine content should provide a direct method for determining the validity of current theories concerning stabilization of the collagen structures.

With the development of differential scanning calorimetry, quantitative measurements of the enthalpy changes in biopolymers have become feasible. The present investigation was in-

stigated to study the thermal transitions of intact and soluble collagen from a variety of sources utilizing differential scanning calorimetry and to attempt to relate these characteristics to the structure and stability of the collagen molecule. A preliminary report of this research has been presented (15).

EXPERIMENTAL PROCEDURE

Sample Treatment—Semimembranosus and longissimus dorsi muscles were excised from beef, lamb, and pork carcasses within 30 to 45 min after exsanguination. Muscle samples were dissected free of adhering fat and visible connective tissue, frozen on Dry Ice, and stored at −40°. Rat and cod skin samples were also treated in a similar manner. The intramuscular connective tissue was isolated by a method previously described (16).

Acid-soluble collagen was prepared from the tissues after first extracting neutral salt-soluble collagen (17). The wet residues from this extraction were washed with distilled water and then extracted with 30 volumes of 0.5 M acetic acid at 4° for two periods of 24 hours each. The suspension was filtered through several layers of cheesecloth and the supernatant centrifuged at 75,000 × *g*. Solid sodium chloride was added to the supernatant fractions to a concentration of 5% and the solutions allowed to stand overnight at 4°. The collagen precipitate was collected by centrifugation, redissolved in 0.5 M acetic acid overnight, and then cleared at 75,000 × *g* for 1 hour. The preparations were further purified by dialysis against 0.02 M disodium phosphate, redissolved in 0.5 M acetic acid, and lyophilized. Samples of calf skin acid-soluble collagen and beef achilles tendon were obtained from a commercial source (Calbiochem, Los Angeles, Calif.).

The hydroxyproline content of all the collagen samples was determined with the method of Woessner (18). The hydroxyproline values were then utilized to determine collagen content with values of 13.5% hydroxyproline for mammalian collagens (18) and 8% for cod skin (19, 20).

Determination of Heat Fusion—The instrument utilized in those studies was the DuPont 900 Thermal Analyzer with the differential scanning calorimetry cell. In general, the program modes used were a heating rate of 10° per 2.54 cm, and a temperature scale of 10° per 2.54 cm or 5° per 2.54 cm. The starting temperatures were 25–30° for intact collagen and 0–10° for acid-soluble collagen. Since it has been shown that ΔH_f value is not affected by pH or salt concentration (11, 21), the determinations were made in 0.9% NaCl solution. The wet weight of intact collagen used for ΔH_f determination varied from 0.5 to to 2.5 mg, and 4 to 6 μl of 0.9% NaCl solution was added before sealing. From 0.5 to 4.0 mg were used in the case of soluble collagen, and 10 to 20 μl of 0.9% NaCl solution added.

Calibration—The ΔH_f values were calculated from the area of thermograms obtained from samples which had passed through the thermal transition. For such a transition the following relation holds

$$\Delta H = \frac{E \cdot A \cdot X \cdot Y}{M_a} \qquad (2)$$

where M is the mass of the sample, A is the area of the thermogram, X and Y are the sensitivity settings, a is the heating rate, and E is the calibration coefficient. Gallium, α-naphthylamine, palmitic and stearic acids were used to determine the calibration coefficient.

RESULTS

Fig. 1 illustrates the independence of the calibration coefficient from heating rate. Over the range investigated, no marked effect on the E value was observed. The calibration coefficient was also relatively unaffected by sample mass or packing density (Fig. 2*A*). The sample volumes investigated were 0.28 to 1.34

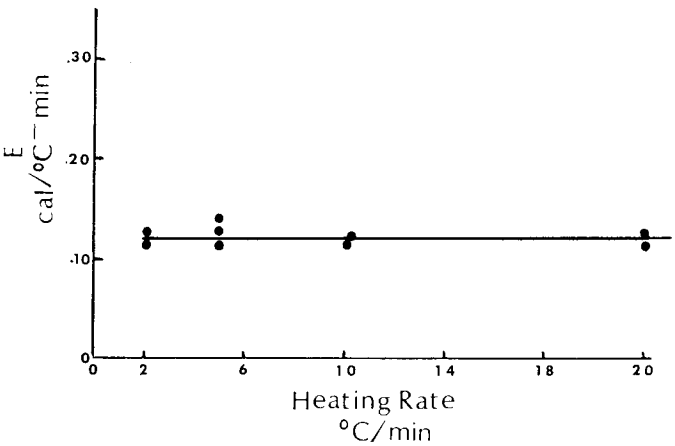

FIG. 1. Effect of heating rate on calibration coefficient (E). Palmitic acid.

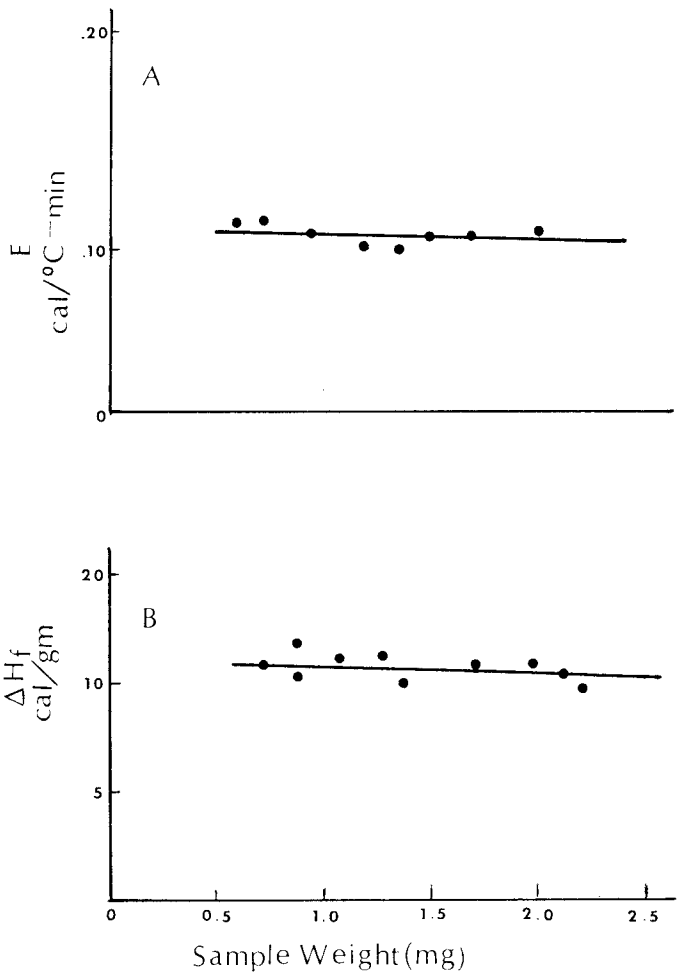

FIG. 2. Effect of sample weight. *A*, calibration coefficient (E) with stearic acid; *B*, ΔH_f of bovine longissimus dorsi muscle collagen.

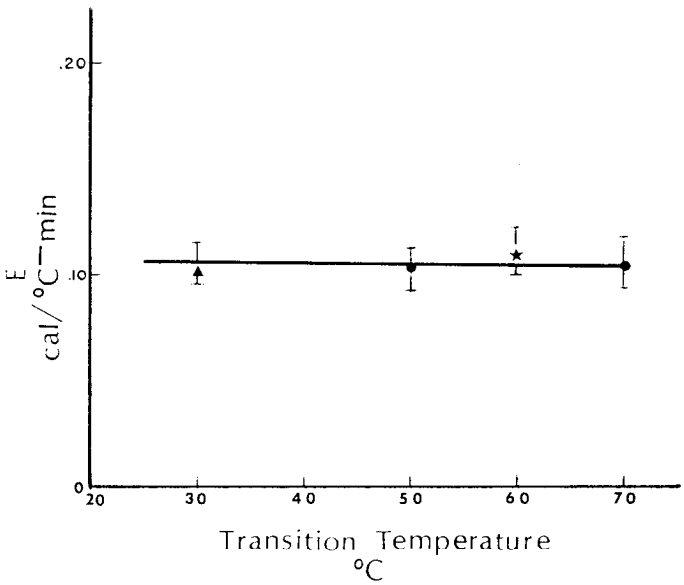

FIG. 3. Effect of transition temperature on calibration coefficient (E). ▲, gallium; ●, α-naphthylamine; ★, palmitic acid; ●, stearic acid. Mean and range.

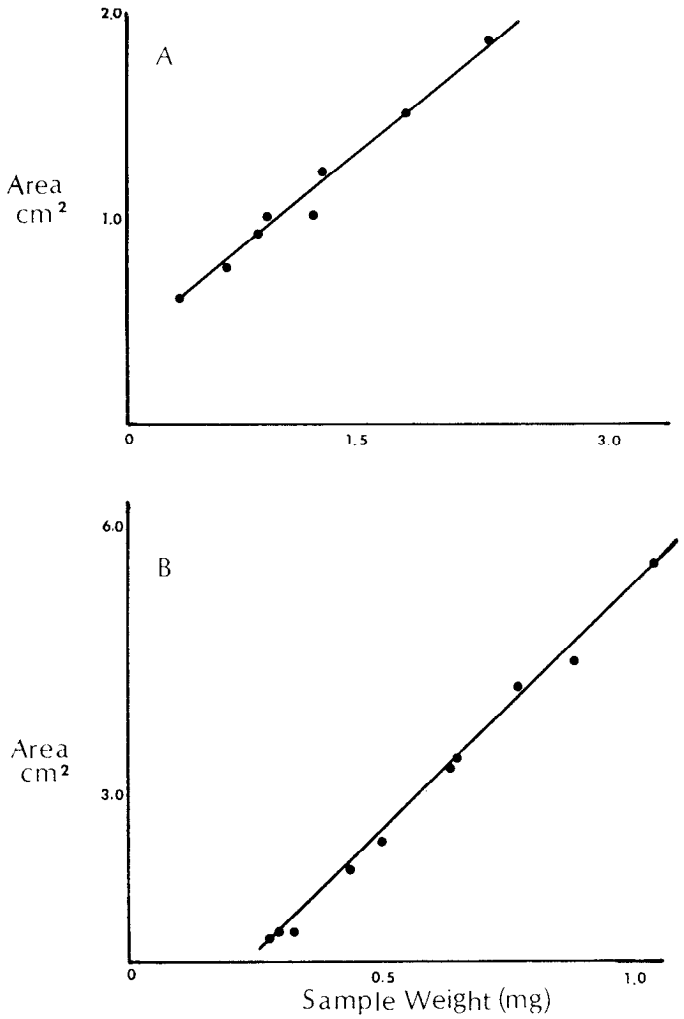

FIG. 4. Relationship between peak area and sample weight. *A*, bovine longissimus dorsi muscle collagen; *B*, palmitic acid.

mg in the case of stearic acid and 0.22 to 1.0 mg for palmitic acid with no marked variation in the E value. A plot of calibration coefficient *versus* temperature of transition for the four standards used is shown in Fig. 3. It is apparent from these data that over the temperature range of interest the E value was virtually constant. These results thus assure that the calibration coefficient is independent of the operational variables and thermal properties of the samples.

The average value of E obtained from the standards was 0.108 cal per °C^{-min}. The coefficient of variation ranged from 4.6% in the case of gallium to less than 2.8% for α-naphthylamine, with an average of 3.7% for all the standards. Fig. 4*B* shows the linear relationship observed between peak area and sample mass. Using the average E value determined in the above studies the the heats of fusion (ΔH_f) were calculated for the four standards (Table I). The close agreement with literature values is apparent.

Fig. 5*A* illustrates a typical thermogram obtained from palmitic acid and defines the location of the three transition temperature parameters evaluated in this study. The onset temperature was obtained at the point where the peak starts to develop, the extrapolated onset was derived at the intersection of the steeply rising portion of the peak to the initial base-line, and the peak temperature was taken at the point of reversal of the thermogram. A comparison of temperature values for the standards with reported literature values (Table II) reveals that the extrapolated onset temperature gives a very close estimate of the temperature of transformation. This parameter was also found to yield the best estimate of temperature of transition by Ozawa (23). The close agreement between observed and literature values also indicates that the differential scanning calorimetry method is not limited by superheating which has been reported to occur at higher heating rates in other thermal techniques (24).

A typical thermogram obtained from bovine tendon collagen is shown in Fig. 5*B*. The thermal shrinkage temperature (T_s) obtained from the extrapolated onset temperature was 62.5° (Table III), which agrees well with the value of 62° reported for bovine tendon by Mohr and Bendall (29). Similar to the data found for the standard compounds (Table II), the extrapolated onset temperature values for intact collagen showed the closest

TABLE I

Heat of fusion[a] (ΔH_f) *of calibration standards*

	ΔH_f Observed	ΔH_f Literature
	cal./g	
Gallium		
Mean	19.72	19.10[b]
S.E.	1.77	
α-Naphthylamine		
Mean	22.12	22.34[b]
S.E.	0.52	
Palmitic acid		
Mean	50.43	51.20[c]
S.E.	0.99	
Stearic acid		
Mean	48.13	47.50[c]
S.E.	0.91	

[a] Mean and standard error of six determinations.
[b] *Handbook of Chemistry and Physics* (22).
[c] Ozawa (23).

agreement with T_s values reported in the literature. In the case of the tropocollagen samples, however, it is apparent that the onset temperature more closely resembled the denaturation temperature (T_m) values as determined by the conventional optical rotation or viscosity measurements. The bovine muscle collagen samples had T_s values somewhat higher than that reported for bovine tendon collagen, but agreed well with the value of 65–67° reported for bovine skin collagen (10). The recently reported T_s value of 64.5° for collagen from bovine sternomandibular muscle (29) is very close to the onset temperature values for bovine muscle collagen determined in the present study. The T_m of tropocollagen from bovine semimembranosus is in accord with reported values for other mammalian collagens, indicating that the organizational structure of muscle collagen in probably very similar to that of the commonly utilized skin and tendon. In any case, it is apparent that the heat absorption data does represent the thermal changes occurring at the temperatures associated with thermal shrinkage or thermal denaturation.

The correlation independently shown by both Burge and Hynes (11) and Piez and Gross (12) between total pyrrolidine content and thermal denaturation (T_m) or shrinkage (T_s) temperature is also evident in the collagens from the present study with the exception of intact rat skin (Table III).

Fig. 2*B* shows a plot of sample size *versus* ΔH_f. The slight downward slope is probably due to failure to attain equilibrium during the melting process with larger samples (32). This had very little effect, however, on the ΔH_f determination over the range of 0.5 to 2.0 mg of collagen. The linear relationship between area of the thermogram and sample mass was also extant for the collagen samples, as was shown for the reference compounds (Fig. 4*A*). Table IV lists the data obtained from samples utilized immediately for calorimetry and that from samples stored for 1 week at 4° before analysis. It is apparent from these results that sample storage did not affect the T_s or ΔH_f values, giving further assurance that these values did not arise as artifacts due to sample preparation and handling.

Data shown in Table IV also illustrate the reproducibility obtained in replicate ΔH_f determinations, and points out the apparent animal to animal invariability in the heat of fusion measurement. Further, these data indicate that the determination of ΔH_f appears to be independent of sample purity. The collagen content of the intramuscular connective tissues, from the bovine longissimus dorsi varied from 70 to 90% with an average value of 85% (17), and yet the ΔH_f values were all in very close agreement. Similar results have been reported by Rigby (27), who found that the thermal characteristics of native collagens were unaltered by the contaminating proteins and polysaccharides often associated with collagenous tissues. Privalov and Tiktopulo (21) have also shown that repeated purification of tropocollagen extracts did not change the values for the total heat of the transition.

It is also apparent from these studies that the state of aggregation of the collagenous proteins has little if any effect on the experimentally determined heats of fusion. It can be seen that the ΔH_f values of intact and soluble collagen from rat skin, bovine semimembranosus muscle, and cod skin were virtually identical (Table V). These findings are in accord with those of Flory and Garrett (14), who were able to show that the fiber shrinkage phenomenon is simply a macroscopic manifestation of the dilute-solution collagen → gelatin transition. Privalov and Tiktopulo (21) also concluded that the thermodynamic char-

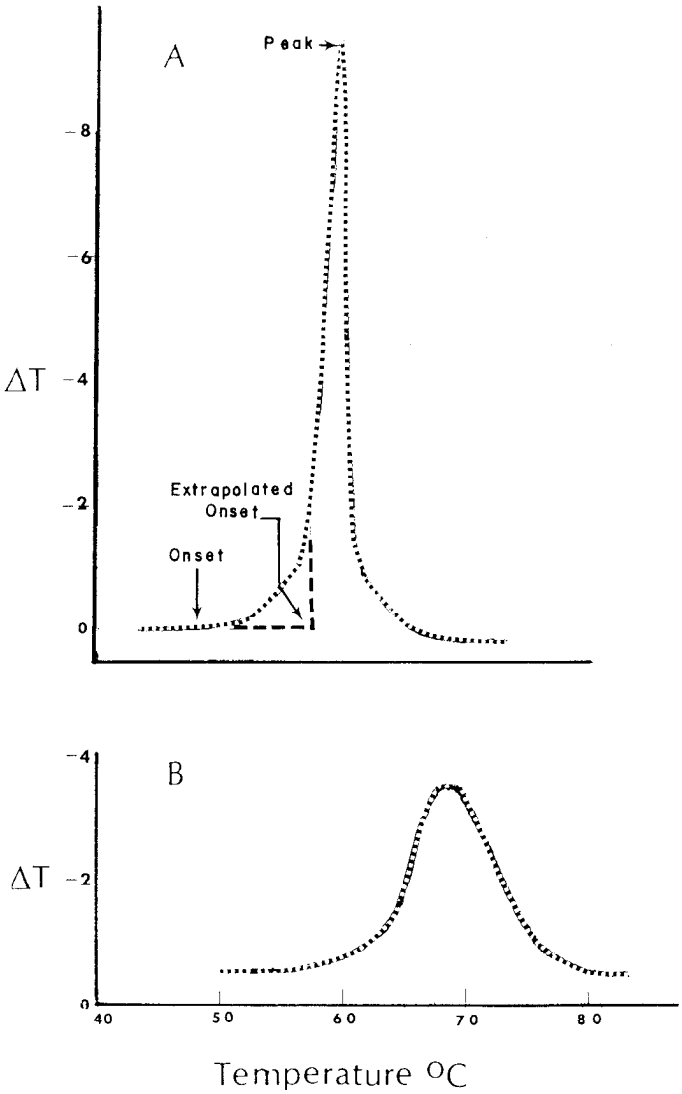

FIG. 5. Differential scanning calorimetry thermogram of palmitic acid (*A*) and bovine tendon collagen (*B*).

TABLE II

Transition temperatures[a] of calibration standards

	Extrapolated			Literature values
	Onset	Onset	Peak	
Gallium				
Mean	30.2°	30.7°	33.2°	29.8°[b]
S.E.	0.3	0.3	0.5	
α-Naphthylamine				
Mean	42.7	49.8	51.4	50.0 [b]
S.E.	0.6	0.3	0.3	
Palmitic				
Mean	50.8	61.8	65.0	62.0 [c]
S.E.	1.2	0.3	0.7	
Stearic				
Mean	55.0	68.2	71.2	69.0 [c]
S.E.	1.0	0.5	0.7	

[a] Mean and standard error of six determinations.
[b] *Handbook of Chemistry and Physics* (22).
[c] Ozawa (23).

TABLE III

Imino acid content[a] and transition temperatures[b] of intact and soluble collagen

Source	Total pyrrolidine	Extrapolated			Literature values
		Onset	Onset	Peak	
Intact collagen					
Bovine semimembranosus	223[c]	64.2 ± 0.9°	68.0 ± 0.9°	70.6 ± 0.6°	
Bovine longissimus dorsi	222[d]	64.4 ± 0.3	67.2 ± 0.2	71.3 ± 0.2	
Ovine longissimus dorsi	218[d]	63.3 ± 0.9	66.4 ± 1.1	71.5 ± 1.3	
Rat skin	217[e]	51.1 ± 0.4	58.2 ± 0.5	68.3 ± 0.4	59.0[f]
Bovine tendon	211[g]	58.1 ± 0.5	62.5 ± 0.3	67.7 ± 0.4	62.0[h]
Cod skin	155[i]	31.9 ± 0.6	39.2 ± 0.3	50.6 ± 0.5	40.0[j]
Tropocollagen					
Calf skin	232[i]	40.5 ± 0.2	48.7 ± 0.3	50.6 ± 0.2	39.0[k]
Bovine semimembranosus	223[e]	39.0 ± 0.4	43.0 ± 0.1	51.0 ± 1.0	
Rat skin	217[e]	37.5 ± 0.8	44.2 ± 1.4	56.2 ± 2.3	38.0[l]

[a] Residues per 1000 residues.
[b] Mean ± standard error.
[c] McClain *et al.* (17).
[d] McClain *et al.* (25).
[e] Gross and Piez (26).
[f] Rigby (27).
[g] Piez and Likins (28).
[h] Mohr and Bendall (29).
[i] Piez and Gross (12).
[j] Harrington and Von Hipple (30).
[k] Von Hipple (4).
[l] Piez and Carrillo (31).

TABLE IV

Effect of storage on transition temperature (T_s) and heat of fusion (ΔH_f)[a]

Sample	No storage		1-wk storage	
	T_s[b]	ΔH_f	T_s[b]	ΔH_f
		cal/g		*cal/g*
1	67.0°	11.48	68.2°	11.59
2	68.2	10.74	66.9	10.60
3	67.5	10.94	67.0	11.84
4	67.1	11.96	67.1	10.40
5	66.8	11.32	67.0	11.40
Mean	67.3	11.29	67.2	11.16
S.E.	0.3	0.21	0.2	0.28

[a] Mean of four determinations per sample. Collagen from bovine longissimus dorsi muscles.
[b] Extrapolated onset temperature.

TABLE V

Enthalpy values[a] for intact and soluble collagen

Source	ΔH_f[b]	ΔH_r
	cal/g	*cal/mole%*
Intact collagen		
Bovine semimembranosus	11.08 ± 0.2	1019
Bovine longissimus dorsi	11.01 ± 0.5	1013
Ovine longissimus dorsi	10.82 ± 0.8	996
Rat skin	11.77 ± 1.5	1083
Bovine tendon	10.77 ± 0.8	991
Cod skin	12.50 ± 1.2	1150
Tropocollagen		
Calf skin	11.57 ± 0.9	1065
Bovine semimembranosus	11.76 ± 0.2	1070
Rat skin	11.98 ± 1.2	1102

[a] ΔH_f = enthalpy of denaturation or shrinkage; ΔH_r = enthalpy per residue.
[b] Mean of six or more determinations ± standard error.

acteristics of aggregated tropocollagen solutions were no different than those in which aggregates were fully removed by centrifugation. In addition, the solubility of bovine longissimus dorsi collagen in 0.45 M acetic acid was over 5 times as great as that from the semimembranosus (33), and yet there was no significant variation in ΔH_f values between the two muscle collagens, indicating that intra-, or intermolecular cross-linking, or both, probably plays only a minor role in the thermal stability characteristics of collagen.

Assuming that the average amino acid residue weight of tropocollagen M equals 92 g per mole (34), the mean value of denaturation or fusion enthalpy per mole of amino acid residue $\Delta H_r = M \cdot \Delta H_f$ can be obtained. From the data shown in Table V it can be seen that ΔH_r for all the collagens investigated in this study were similar and agree very well with values reported in the literature (35–37).

DISCUSSION

The enthalpy values determined by differential scanning calorimetry in the present study are in good agreement with the value of $\Delta H_r = 1200$ reported by Flory and Spurr (35) based on the relationship between tensile strength and the equilibrium melting temperature of rat tail tendon, and the value of $\Delta H_r = 1150$ determined by Kuntzel and Doehner (37) from their calorimetric studies on bovine hide powder. Harrington and Rao (3) applying statistical mechanical theories to known thermal denaturation data, derived values of $\Delta H_r = 1165$ cal per mole for rat skin and 1185 for cod skin.

Data from the present investigation indicate that denaturation enthalpy is virtually invariant throughout the collagens studied. Similar conclusions have been reached by both Burge and Hynes (11) utilizing intrinsic viscosity measurements and Harrington and Rao (3) utilizing empirical considerations. Privalov and Tiktopulo (21), utilizing direct calorimetric measurements have, however, reported an increase in both ΔH_r and ΔS_r with increasing imino acid content. The reason for the discrepancy between the latter results and those of the current study is not readily apparent. However, in our hands tropocollagen samples obtained by rigorous purification have been found to vary from 85

to 96% collagen based on hydroxyproline analysis, suggesting that estimates of collagen concentration based on the Lowry method might lead to spurious results. In any case the basic conclusions reached by the Russian workers and the present authors is not at variance. The energy per triplet calculated from ΔH_r yields values from 2973 to 3450 cal per mole in the present study, and the data from Privalov and Tiktopulo (21) reveal values of 4590, 3720, 2730, and 2400 cal per mole for the triplet energy from rat, pike, cod, and whiting skin. These values are much too high to be consistent with current knowledge of hydrogen bond energy. Since the work confirming the one hydrogen bond model appears to be conclusive, one can only interpret the above results by concluding that forces other than hydrogen bonding must be contributing to enthalpy. Cooper (7) and Privalov (9) advocate a re-evaluation of the earlier concepts of macromolecular stabilization by structural organization of the solvent around the molecule, while Segal (8) has proposed residue-specific interactions such as prolyl-prolyl attractions.

Although the triplet energy values are much too high to be accounted for by hydrogen bonding alone, it should be mentioned here that the constancy of the ΔH_f values do tend to support a one hydrogen bond-type model. In both the Rich and Crick (1) and the Madras models (2), the residues in position three are not properly oriented to allow intramolecular hydrogen bonding. Since the nitrogen atom of a pyrrolidine ring does not possess the necessary hydrogen atom, hydrogen bonding cannot occur whenever a proline or hydroxyproline residue is located in position 2. Thus insertion of an imino acid residue in position 2 of the triplet should result in one less hydrogen bond causing a decrease in ΔH_r (3). Conversely, in the one hydrogen-bonded model one would expect the enthalpy to remain constant, as was the case in the present study.

Since present results indicate that ΔH_r appears to be independent of pyrrolidine content, it follows from Equation 1 that the increase in heat stability can be accounted for by a decrease in entropy of denaturation with a rise in the content of imino acids. This conclusion has received support from the work of Harrington and Sela (13). These workers found that the synthetic polymers, poly-L-proline and polyhydroxyproline, appear to exist in solution in the characteristic collagen left-handed helical conformation, and do not appear to undergo configurational changes in aqueous solutions at temperatures up to 90°. These structures are devoid of peptide hydrogen bonds and are apparently stabilized by steric restrictions to a rotation about the peptide backbone. This concept serves as the basis for the hypothesis that $\Delta S_r = 0$ for pyrrolidine residues.

Flory and Garrett (14) have suggested that the direct correlation between T_m or T_s and the total imino acid content might manifest itself through the entropy term, since the insertion of a pyrrolidine ring into the polypeptide chain should decrease the configurational entropy of the random coil form and thus decrease ΔS_r. The ΔS_r values for calf skin, bovine muscle, rat skin, and cod skin from the present study were 3.3, 3.4, 3.8, and 3.9, respectively; values which are compatible with the above concepts. In contrast, the results of Privalov (9) are unexplicable on this basis.

Present results do not exclude the possibility that intermolecular forces make a significant contribution to collagen stability. Carver and Blout (38) have previously suggested that interactions other than hydrogen bonding and prolyl steric restrictions are needed to give rise to super coiling in the collagen fold. However, the results of this study do tend to support the conclusion that the tertiary superhelix structure of the collagens studied is stabilized primarily by the steric restrictions imposed by the pyrrolidine residues.

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