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INTERACTIONS BETWEEN COLLAGEN CHAINS AND FIBER FORMATION

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The temperature-dependent dissociation of neutral salt-soluble collagen into its component chains was measured in 0.6–1.6 M urea solutions at pH 7.3. The temperature-dependent association of the same radioactively labeled collagen into fibers was measured in 0–0.4 M urea solutions, pH 7.3. The effect of urea on the temperature, Tm(G), for half dissociation into chains was small, and the value extrapolated to zero urea concentration was 39°C. In contrast, the effect of urea on the temperature, Tm(F), for half association into fibers was large, and the value at zero urea concentration was 30°C.

We conclude that while body temperature provides excellent conditions for the matching of collagen chains to form molecules, the conditions are not optimal for the formation of highly ordered fibers. The large effects of 0.1 M urea suggest that other factors in vivo may help to destabilize mismatched molecular association during fiber growth. Alternately this might be facilitated by parts of the extension peptides of procollagen.

In collagen fibrogenesis two steps of molecular self-assembly can be distinguished: first, the formation of collagen molecules by association and folding of three chains into each molecule and second, the association of molecules into fibers. A previous paper (1) outlined how the first association is substantially helped by special extension ("registration") peptides, which each collagen chain carries when it is synthesized as a so-called pro alpha chain, and how these extension peptides are subsequently excised, so that they are not found in normal collagen fibers. In this study we used only finished collagen molecules. Under appropriate conditions these molecules may either dissociate into single (alpha) chains or associate into collagen fibers. Both processes are reversible (2, 3, 4) and are temperature sensitive. If each process is studied as a function of temperature then the temperature for the midpoint of the transition (Tm) can be used as a characteristic of that transition. We asked what the relationships of the Tm values of these two associations are to body temperature under approximately physiological conditions. First, the relationships should be such that the sequence of associations from single alpha strands to collagen fibers would occur spontaneously. Second, Tm values close to body temperature would indicate conditions unfavorable for mismatched structures, so that the system

would have a good chance to rearrange to more stable, well-matched arrangements. In contrast, Tm values far from body temperature would suggest that additional mechanisms are involved in ensuring the detailed matching of polypeptide chains into molecules or of molecules into fibers.

We denote the first association of peptide strands to molecules by (G) and the second, of molecules to fibers, by (F). The processes have been studied before (2–12) but always under different conditions, so that they could not be compared. The technical reason is that the two processes of course overlap at body temperature under physiological conditions and interfere in each other's experimental study. Thus, while fiber formation was studied at neutral pH, the associative process to molecules has been studied at acid pH to avoid fiber formation. But this alters interactions between charged amino-acid residues and may interfere in the very processes being studied. Urea inhibits fiber formation (6). By studying both processes as a function of varying, relatively low urea concentrations at neutral pH and physiological ionic strength, we could extrapolate our results to zero urea concentration. We followed the dissociation of collagen molecules into unfolded alpha chains by monitoring the optical rotation of solutions and quantitated fiber formation by measuring the precipitation of radioactively labeled collagen. Near-equilibrium conditions were attained by using long equilibration times.

EXPERIMENTAL PROCEDURE

To obtain radioactive collagen, actively growing 100 gm laboratory rats were each injected intraperitoneally with 100 μCi ^{3}H-glycine in saline and killed 16 hr later. Rat skin, neutral salt-soluble collagen was extracted at physiological ionic strength and purified by our previously described methods (3), which ensure that fiber formation is reversible. Collagen was precipitated 3 times with KCl and dissolved in 0.01 M acetic acid followed by exhaustive dialysis into 0.067 M phosphate buffer, pH 7.3. All measurements were made in this buffer, saturated with toluene to inhibit microbial growth. Any traces of aggregated collagen were centrifuged off (Beckman type 30 angle rotor, 29 krpm, 4°C). Concentrated urea solutions were made up in the buffer just before use and added to the collagen solutions to various final urea concentrations.

Fiber formation was measured by incubating 1 ml samples containing 1 mg radioactive collagen (1,100 cpm) for 48 hr at a given temperature and then separating fibers from solution by centrifugation for 10 min at 10 krpm in a Sorvall SS-34 rotor and centrifuge; both were prewarmed to the same temperature. Radioactivity of 500 μl aliquots of supernatant was measured to better than 5% counting error in a Beckman LS-150 liquid scintillation system, using Beckman BBS-3 as solubilizer and toluene-PPO. Studies for different times of incubation showed that no further change in fiber formation occurred after 48 hr, except in samples at temperatures below 31°C, and these were measured after 72 hr incubation.

Optical rotatory measurements were made in a Bendix automatic recording polarimeter, Series 1100, sensitive to 0.2 millidegree of arc, with less than 2 millidegree drift in 24 hr, at 540 nm. Previously degassed solutions of collagen, at the same concentrations as used in fiber formation experiments, were held in a waterjacketed cell of

5 cm path length thermostated to ±0.02°. Solution temperatures were increased from 22°C to 44°C in 0.5° steps at 30 min intervals. Continuous recording of optical rotation showed that steady values were attained during nearly all of these intervals, and model experiments showed little, if any, further change upon keeping the solutions at a given temperature. The temperatures for 50% transition, called Tm(G), were obtained from the sigmoidal plots of optical rotation vs temperature. Similarly, temperatures for 10% and 90% transition were obtained, denoted as T10(G) and T90(G), respectively.

RESULTS

The percentage of collagen which formed fibers is shown as a function of both temperature and urea concentration in Fig. 1, which is an isodimensional graph in three dimensions. Separate vertical planes correspond to separate experiments conducted at a given temperature at different urea concentrations up to 0.4 M. When no urea was present the percentage of collagen which formed fibers increased steadily with temperature to a plateau value of 82% at 35–37°C. At temperatures greater than about 39°C the yield of fibers rapidly decreased with increasing temperature, as denaturation of collagen molecules to separate alpha chains competed with fiber formation during incubation. Any fibers that did form were more resistant to denaturation than individual molecules, and at 41°C a small amount of fibers was still found.

The urea concentration which was required to suppress fiber formation increased with temperature, at first rapidly and then little. To obtain a comparable measure we define a urea concentration at any one temperature which suppresses fiber formation to 50% of the fiber formation which occurs at that temperature at zero urea concentration. This temperature is denoted as Tm(F) at that urea concentration and the results are plotted in Fig. 2 (filled circles). We similarly define higher urea concentrations which permit only 10% of those fibers to be formed which would form in the absence of urea at that temperature and, similarly, lower urea concentrations which permit 90% fiber formation. Thereby we generate the corresponding curves T10(F) and T90(F) shown in Fig. 2. The region of Fig. 2 between curves T90(F) and T10(F) therefore corresponds to the range of urea concentrations in which change of urea concentration substantially alters fiber formation at that temperature.

The other (G) transition was studied with respect to the phenomenon of collagen molecules coming apart into constituent alpha chains. This is accompanied by unfolding of the chains into so-called "random" coils and a corresponding large change in optical rotation [specific optical rotation of neutral salt-soluble collagen changes from −377° to −144° upon denaturation at 40°C, pH 3.7 (3)]. In Fig. 2 are plotted as full circles the temperatures Tm(G) at which 50% of the total change of optical rotation occurred at any given urea concentration, upon slowly warming from 22°C to 44°C (determined as described under Experimental Procedure). Similarly, temperatures for 10% and 90% of transition were obtained and are given as T10(G) and T90(G), respectively, indicated by half-filled and open circles. Each set of circles represents separate repeat experiments, and best straight lines were fitted by the method of least square deviation. By this linear extrapolation Tm(G) in the absence of urea in 0.067 M phosphate buffer, pH 7.3, is 39°C for this collagen.

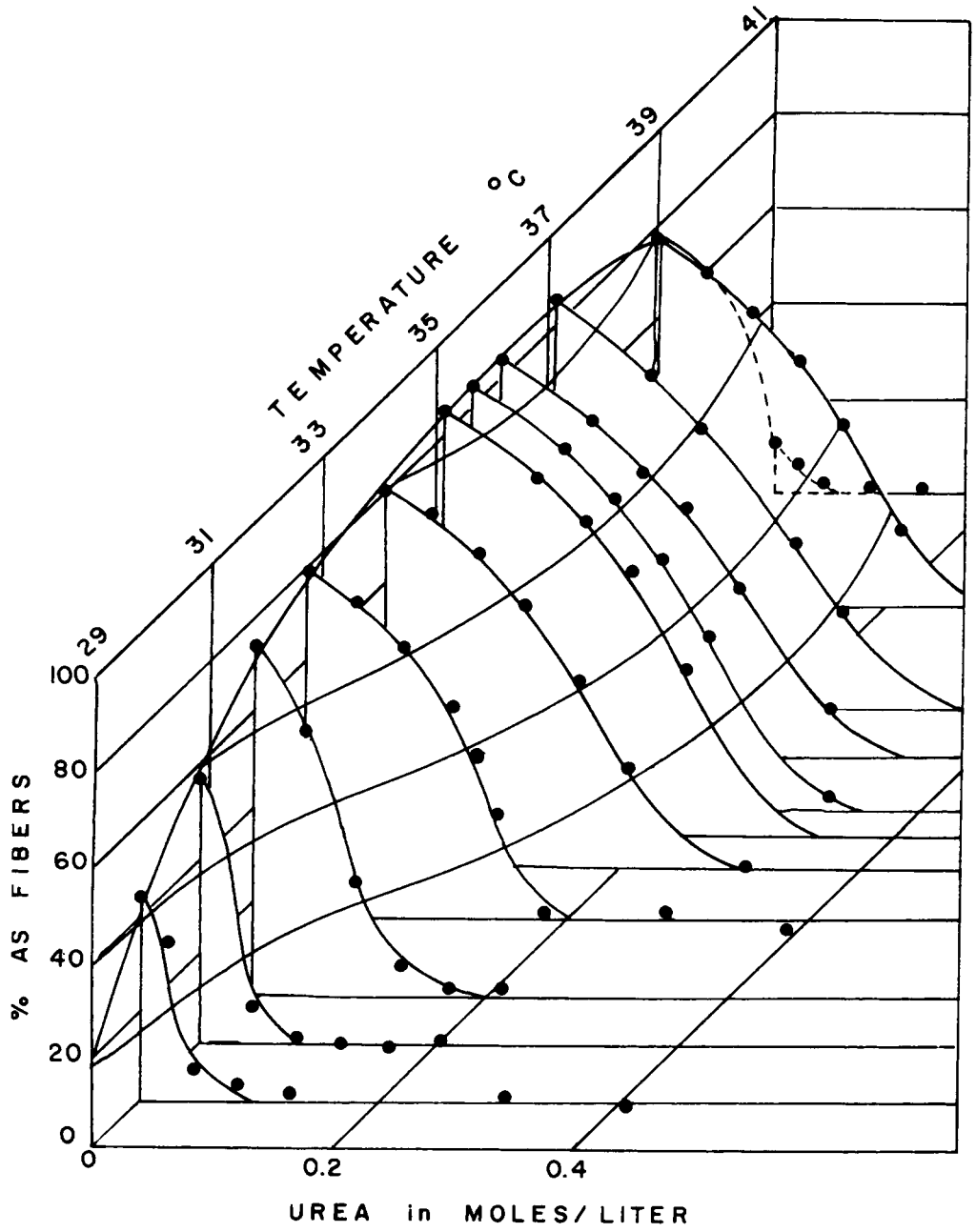

Fig. 1. Effect of urea and temperature on collagen fiber formation. This three-dimensional graph is isodimensional, with the Z axis (temperature) at 45° to the other axes as drawn on paper. Points on each separate vertical plane are data obtained at one temperature and at different urea concentrations. The percentage of collagen precipitated as fibers (Y axis) is [(cpm in precipitate)/(total cpm in sample)].

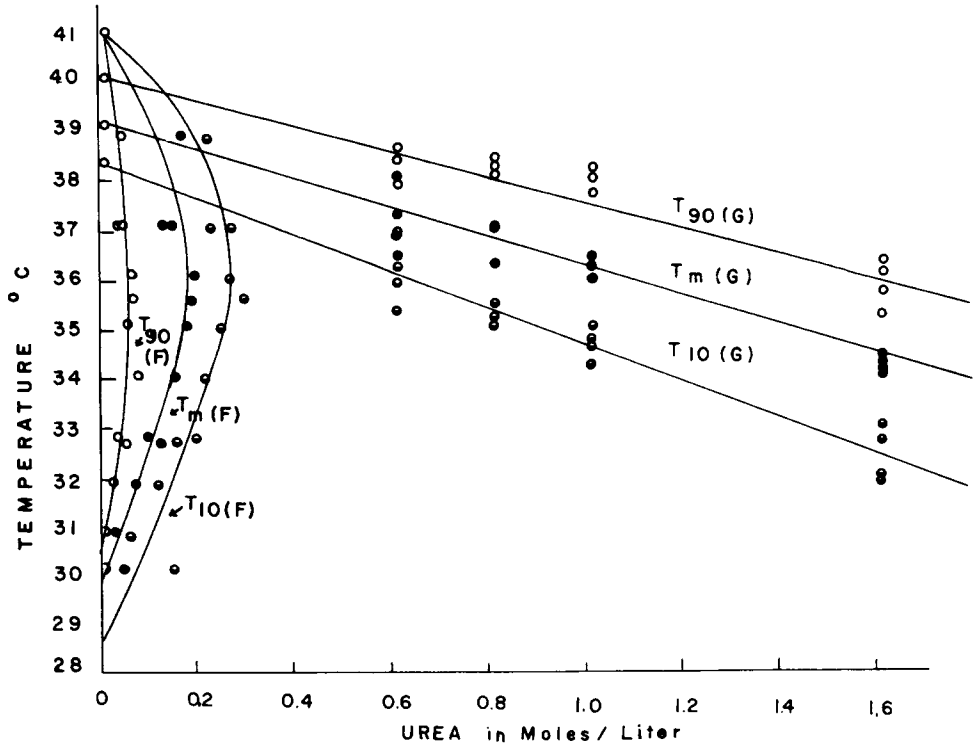

Fig. 2. Effect of urea and temperature on the transitions from collagen to component chains and from collagen to fibers. The temperatures at which half the transition from collagen to gelatin occurred, Tm(G) (●), was determined polarimetrically in solutions 0.6–1.6 M with respect to urea. The corresponding temperatures for 10% and 90% transitions are shown as T10(G) (⊖), and T90(G)(○), respectively. The temperatures for 50% of the transition from collagen in solution to fibers to occur at a given urea concentration, Tm(F) (●), was determined as described in the text. Corresponding temperatures for 10% and 90% of these transitions are denoted by T10(F)(⊖), and T90(F) (○), respectively. Each (●) symbol represents a separate experiment. Best straight lines were fitted by least square deviation.

DISCUSSION

Both transitions were measured in one direction only, but the parameters desired are equilibirum values. For the transition from molecules in solution to fibers we showed previously that this technique gives the same equilibrium values when approached from opposite directions – that is, that fibers dissolve when cooled from some higher temperature to an intermediate one (3, 4). The reassociation of separate collagen strands to helical collagen molecules, judged to be native by various criteria, has also been demonstrated (8–12). The reassociation is, however, complicated because due to the trimolecular nature of the reaction the kinetics either are exceedingly slow at low concentrations or, at higher concentrations, substantial mismatching occurs so that subsequent annealing cycles are required. For these reasons we did not attempt to measure the (G) transition by approach from both directions. The midpoint transition temperatures Tm(G)

obtained by such denaturation studies may be higher than the true Tm values, but the difference is unlikely to be greater than 1°. The effect upon Tm(G) of changing urea concentrations from 0.6 M to 1.6 M is relatively small and tends to support the linear extrapolation to zero urea concentration. In the range of urea concentrations used for polarimetry the solutions remained perfectly clear – that is, no fibers whatever were formed; their scattering effect would have been readily detected, as control experiments at lower urea concentrations showed.

We conclude that Tm(G) is 39°C, possibly slightly lower. Thus it is close to or slightly above the body temperature, consistent with spontaneous association of collagen chains into triple molecules in vivo. This means that there would be ample opportunity for mismatched regions of lower stability to dissociate and reform in proper alignment. As reviewed elsewhere (1) the biosynthetic assembly mechanism makes use of two auxiliary devices: (1) the pro alpha chains are brought into mutal register at the amino ends by interactions between the extension peptides and (2) postribosomal hydroxylation of the pro alpha chains greatly alters the Tm(G) of the collagen portion of the chains from 25°C in the absence of hydroxylation to about 41°C after complete hydroxylation (13).

The latter value was determined from denaturation experiments conducted on chick bone procollagen in 5–20% sucrose gradients in the presence of 1 mg Triton X-100 per ml. Either the differences of experimental materials and conditions explain the discrepancy with the value obtained here, or there is some stabilizing influence in the procollagen; certainly the amino ends of the procollagen chains are held together by the disulfide-linked registration peptides.

Maximal fiber formation spontaneously occurs at body temperature, and this is several degrees higher than the Tm(F) at zero urea concentration, which we determine as approximately 30°C. This suggests little opportunity for rearrangement of mismatched molecules. It fits in with the empirical observations that to obtain well-striated, native-type fibers from solutions of highly purified, neutral, salt-soluble collagen it is best to warm the solutions to only about 30°C. The substantial effects on fiber formation of urea concentrations of the order of 0.1 M is in sharp contrast to the trivial effect this has on the stability of the triple helical structure, as far as Tm(G) is an indication of this. This corroborates the suggestion of Gross (6) that other materials in the environment of collagen molecules could greatly affect the fiber forming process. The Tm(F) might thereby be brought closer to body temperature. It suggests that other agents may indirectly promote the correct association of collagen molecules (by destabilizing incorrect associations), although the final pattern must of course be governed by the stereospecific mutual affinities between the collagen molecules.

An intriguing possibility would be that the last regions of the registration peptides of procollagen, which are removed in several steps (14), play such a role and indirectly facilitate optimal fiber formation. It is known that they must be removed for optimal alignment, but this could occur on the growing fiber itself.

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