surface in contact with the cell exterior, and to diffusion in the direction of the intercellular space to the exterior. For small ions the latter barrier amounts to a resistance of, at least, $10^4$ ohm $cm^2$ (*2, 3*).

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23 December 1963

## Thermal Denaturation of Collagen in the Dispersed and Solid State

Abstract. *Thermal denaturation temperature of newly reconstituted collagen fibrils from rat tail tendons is 52°C compared with 42°C for neutral solutions. This suggests that the increase in concentration of collagen within the fibril increases the stability of the individual molecules. The absence of firm intermolecular bonds in these fibrils rules out crosslinking as an explanation for increased stability. "Aging" at 37°C up to 1 year raises the shrinkage temperature of reconstituted fibrous gels by 4° to 6°C and greatly increases resistance to dissolution at high temperature. The newly formed fibrils dissolve without shrinking, whereas older gels exhibit shrinkage before dissolution. Since nearly all extractable collagen is in the form of fibrillar aggregates in tissue, it is unlikely that thermal denaturation occurs at body temperature; therefore it could not be involved as a necessary stage in collagen resorption.*

The relationship between denaturation of collagen in the solid and dissolved state has physiologic significance, since it has been proposed that denaturation of extractable collagen in vivo at physiologic temperature is a necessary stage in the catabolism of collagen, preparing it for proteolytic digestion (*1*).

It is unlikely that any appreciable amount of collagen in vivo is in molecular, or even small aggregate, dispersion in the tissues since negligible amounts of collagen are extractable at physiologic temperature and *p*H. Neutral extractable collagen is, in all probability, in the form of fibrils which dissolve in the cold, much like the newly reconstituted thermal gels (*2*).

Denaturation of collagen, as now generally visualized, is a phase transition or "melting" of the highly ordered molecule to a random state. Shrinkage of collagen in the solid state and the denaturation of dispersed molecules are considered to be different manifestations of the same phenomenon. Flory and Garrett (*3*) showed that the temperature of denaturation is concentration-dependent above a certain minimum level, and that it exhibits a smooth transition from the dispersed to the solid phase.

In an effort to shed further light on the relationship between melting in the dispersed and solid state, the denaturation temperature of dissolved collagen at neutral *p*H was compared with the temperatures of shrinkage and dissolution of reconstituted fibrils of increasing age and diminishing extractability, and also with those of the native tissue.

Freshly removed tendons from young adult rats were dissolved in 0.1*M* acetic acid in the cold; the solution so obtained was clarified by sedimentation at 100,000*g* for 1 hour and then filtered. Clear solutions of collagen (approximately 0.2 g/100 ml) were prepared by reconstitution of the lyophilized material in phosphate buffer, *p*H 7.6, Γ/20.4, at 5°C, as previously described (*4*). Opaque rigid gels composed of striated fibrils were formed from the cold neutral solutions by warming to 37°C for 10 minutes (*4*). Some gels were formed in 0.2*M* and 1*M* NaCl at *p*H 6 to 7. Collagen gels, 40 × 3 mm, prepared in this manner in hematocrit tubes under sterile conditions, were incubated at 37°C for periods ranging from 15 minutes to 1 year before use.

The temperature of shrinkage was measured by suspending the tubes in duplicate or triplicate in a water bath in which the temperature was raised at a rate of 1°C per minute. Shrinkage of gels was readily apparent and could be measured on the scale of the tube. Final denaturation (dissolution) was manifested as a loss in opacity and the rising of bubbles through the clear solution.

Samples of whole intact tendons from the same animals were freely suspended in capillary tubes. After incubation under the same conditions as the gels, measurements of the shrinkage temperature were made in the same manner.

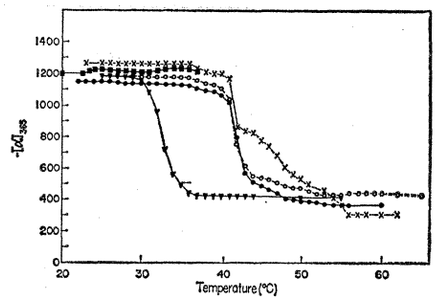


Fig. 1. Denaturation of collagen in solution measured by optical rotation. Solid triangles, acetate ionic strength, 0.15; *p*H 4.2. All other curves represent collagen in phosphate ionic strength, 0.4; *p*H 7.6 with varying concentrations of arginine. Closed circles, 0.4*M* arginine; open circles, 0.2*M* arginine; ×-×, 0.1*M* arginine; solid squares, no arginine.

Denaturation of collagen dissolved in phosphate and acetate buffers was measured by the change in optical rotation at 365 mμ again with a continuous temperature increment of 1°C per minute. Collagen concentrations, about 0.15 percent, were the same as those in the solutions from which thermally reconstituted gels were prepared. Thermal precipitation was prevented by the addition of 0.1 to 0.4*M* arginine (*5*). By comparing denaturation temperature over this range of arginine concentrations it could be shown that this agent did not greatly influence the results, nor did it affect the shrinkage and denaturation of the gels.

Reconstituted collagen gels incubated at 37°C for 1 hour or less dissolved completely on cooling to 5°C, indicating the absence of firm crosslinking between molecules; the newly formed

Table 1. Temperature of shrinkage and denaturation in solid state, in degrees Celsius.

| Time of incubation | Reconstituted gels | | Intact tendons | |
|---|---|---|---|---|
| | Shrinkage* | Denaturation† | Shrinkage* | Denaturation† |
| 15 min | None | 52 | 54–58 | 59 |
| 6 hr | 45–52 | 52 | 56–58 | 58 |
| 22 hr | 49–54 | 53–55 | 56–58 | 58 |
| 48 hr | 50–53.5 | 54 | 57–59 | 59 |
| 10 days | 49–53 | 58 | | |
| 28 days | 49–55 | 57 | | |
| 375 days | 53–56 | None | | |

* Temperature at beginning and end of shrinkage. † Temperature of complete dissolution of gel or tendon.

fibrils were still loose aggregates at 37°C. With increasing time of incubation the gels grew more insoluble, and became 80 percent irreversibly aggregated in 48 hours at 37°C.

The recently formed gels, 15 minutes old, exhibited no shrinkage, but dissolved abruptly at 52°C (Table 1). Older gels exhibited shrinkage before complete dissolution. Results were the same in phosphate buffer and sodium chloride. Gels incubated at 37°C for 1 year underwent about 50 percent shrinkage at temperatures 4° to 5°C above the "younger" preparations and failed to show any visible dissolution even at 80°C after 1 hour. Small increases in shrinkage and denaturation temperature occurred after incubation periods ranging from 1 to 28 days (Table 1). Shrinkage was initiated in native tendons 6° ± 1°C higher than that for reconstituted collagen. In all cases they went into solution completely at 58° to 59°C in both phosphate and sodium chloride suspending media.

Characteristics of denaturation of collagen dissolved in dilute acid and neutral solutions (0.15 to 0.2 percent protein) are described in Fig. 1. The temperature at which one half of the total change of rotation is reached is $T_D$, and $T_M$ is the temperature at which the change in rotation ceases. The curves are identical in shape with those reported by others (*3*, *5*). The $T_D$ is lower by about 10°C for denaturation at *p*H 4.2 in acetate than at *p*H 7.6 in phosphate. The $T_M$ was much more variable and higher in the lower concentrations of arginine, in one experiment reaching a maximum of 56°C in 0.1*M* and ranging between 46° and 52°C. In the experiment reported in Fig. 1, the rotation fell appreciably more slowly after the initial rapid drop at 42°C in the sample containing 0.1*M* arginine than in those containing larger amounts of arginine. This may be an artifact caused by turbidity, since this region of the curve was accompanied by an appreciable fall in light transmission indicating the formation of aggregates. In the absence of arginine, gelation occurred at about 37°C preventing further observation. Arginine did not influence $T_D$. Unfortunately arginine is not effective in inhibiting gelation in NaCl medium, hence the necessity of working in phosphate. However, reconstituted gels and tendons incubated in NaCl with or without arginine revealed little difference in behavior compared with preparations in neutral phosphate.

There was a difference of about 21°C between the denaturation temperatures of dissolved collagen in acetate, *p*H 3.5 to 4.2, and the shrinkage temperature of the intact tissue, a value close to that reported by Doty and Nishihara (*5*).

That the denaturation temperature of collagen in solution is lower by 10°C than that of fibrils is consistent with the interpretation of Flory and Garrett (*3*) of concentration dependence of denaturation. Increases in shrinkage temperature of reconstituted collagen as compared with denaturation temperature in the dispersed state, ranged from 6°C after 15 minutes to 12°C after 1 year of incubation, a change which might be explained by slow progressive increase in the concentration of intrafibrillar protein. The high resistance of the 1-year-old gels to dissolution on heating to extreme temperatures suggests the formation of strong intermolecular bonds which maintain the overall gel fabric in spite of the collapse of molecular structure.

It is probable that the higher shrinkage temperature of the native tendons as compared to reconstituted fibers represents the mechanical restraint of the noncollagenous ground substance and perhaps circumferential fibril networks.

The shrinkage and denaturation temperatures of newly formed as well as old fibrils, as shown here, are well above the physiological range. Such reconstituted fibrils are not susceptible to digestion by the common proteases at 37°C (*6*). It seems unlikely that exposure of any of the known collagen fractions to body temperatures in vivo predisposes it to proteolytic attack. The recent detection of collagenolytic activity at neutral *p*H and physiologic temperatures in amphibian tissues, and in mammalian bone and uterus (*6*, *7*) indicates a more direct enzymatic attack in collagen resorption.

*Note added in proof.* In all experiments, denaturation temperature, $T_D$, in 0.15*M* acetate at *p*H 3.5 to 4.2, and shrinkage temperature, $T_S$, for whole tendons at neutral *p*H were consistently lower by 2° to 4°C than the values generally reported in the experimental literature. This does not in any way affect the significance of the data.

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## Regenerating Tissues from the Cockroach Leg: A System for Studying in vitro

Abstract. *Regenerating leg tissue of* Leucophaea maderae *shows considerable activity in vitro. Migrations of ameboid and fiber-like cells from the explant take place, vesicles are produced, and interaction occurs between tissues. The system described facilitates the study of these activities in vitro.*

The success of Grace (*1*) in establishing strains of cells from the larva of *Antheraea eucalypti* has sparked new interest in the culture of insect tissues. It has been difficult, however, to find tissues of paurometabolous insects that will respond to culture in vitro.

Embryos and embryonic tissues survive well in vitro (*2*), but do not continue their development over any length of time. Postembryonic tissues survive well (*3*) but, to date, differentiation has been reported to occur only in the tissues of holometabolous insects. Tissues from the regenerating leg of the nymphal cockroach *Leucophaea maderae* not only survive well, but also continue to develop in vitro, thus providing a system which can be subjected to experimental procedures.

The growth of the regenerating leg was described by Bodenstein (*4*). When the leg of a nymphal cockroach is removed at the trochanter-femoral joint,