Perkins, E. G., and Johnston, P. V. (1969), *Lipids 4*, 301.
Quayle, A. (1959), *in* Advances in Mass Spectrometry, Waldron, J. D., Ed., New York, N. Y., Pergamon, pp 365–383.
Sherman, W. R., Stewart, M. A., and Zinbo, M. (1969), *J. Biol. Chem. 244*, 5703.
Teeter, R. M. (1962), Abstracts, Tenth Annual Conference on Mass Spectrometry and Allied Topics, New Orleans, La.
Zinbo, M., and Sherman, W. R. (1970), *J. Amer. Chem. Soc. 92*, 2105.

Reversible and Irreversible Denaturation of Collagen Fibers*

Helmut Hörmann and Harald Schlebusch

ABSTRACT: The degree of denaturation of heat-shrunk rattail tendon collagen was determined by dissolving the tendons with pronase and then calculating the native portion in the resultant solution from optical rotation values. Re-formation of triple-helical structures during the dissolution process was largely prevented when the tendons were dissolved at +4°. In this way, it was possible to detect the reversibly denatured portion together with the more severely denatured collagen. The dependence of the entire denaturation process on temperature is shown in a sigmoid curve with a point of inflection coinciding with that of the curve for the temperature dependence of the shrinkage process of collagen fibers. Both methods of measurement thus indicated the same denaturation temperature. This method also permits measurement of the renaturation processes in denatured insoluble collagen. Considerable renaturation was observed up to 3 hr after denaturation. The degree of renaturation was dependent on the degree of previous denaturation. The optimal renaturation temperature was found to be 30°. Stretching the denatured fiber promoted the re-formation of native material considerably.

Native collagen fibers shrink to about one-third their original length when heated in water above 62° (Gustavson, 1956). This phenomenon is due to denaturation of collagen molecules which are covalently cross-linked within the fiber network. During denaturation the triple-helix structure of the monomers is transformed to randomly coiled peptide chains.

Many conclusive investigations have dealt with the denaturation of collagen in solution (Doty and Nishihara, 1958; Engel, 1962) and with the re-formation of dissolved triple-helix structures (Von Hippel and Harrington, 1959; Kühn *et al.*, 1964; Beier and Engel, 1966; Harrington and Rao, 1970). In contrast far less is known about the denaturation and renaturation of insoluble collagen fibers. Limited information is available here from shrinkage measurements (Weir, 1949; Verzár, 1963) and X-ray investigations (Wright and Wiederhorn, 1951). The latter show that during denaturation the low-angle pattern as well as the 2.89-Å arc disappear. After cooling and stretching only the 2.89-Å arc reappears, indicating some re-formation of triple-helix structures in a random orientation.

In order to determine the degree of denaturation of insoluble collagen fibers the principle may be useful that the triple helix of collagen is very resistant to most proteases, while denatured collagen is easily broken down (Grassmann, 1936; Grassmann *et al.*, 1937). Of special interest in this respect is pronase which dissolves all the insoluble collagen, the native as well as the denatured portion (Fujii, 1965, 1966; Drake *et al.*, 1966). At temperatures up to approximately 20°, the native portion goes into solution as collagen monomers with a triple-helix structure exhibiting a high optical rotation. Denatured collagen, on the other hand, is broken down into small peptides of low rotation. The proportion of native material thus can be measured in solution by polarimetric methods (Hörmann and Schlebusch, 1968a).

However, regarding the prolonged time required to dissolve insoluble collagen by pronase, this method yielded reproducible results only when the degree of denaturation changed little, if at all, over prolonged periods of time. Therefore, only predominantly irreversibly denatured collagen could be detected which was present after equilibrating the heat-denatured fibers at room temperature prior to pronase treatment (Hörmann and Schlebusch, 1968a).

On the other hand, there was evidence that, immediately after arresting the denaturation procedure, considerable re-formation of triple-helix structures took place. A complete description of the denaturation process thus requires the determination of reversibly denatured collagen as well. For this purpose, the enzymatic method seemed applicable only if one could prevent the re-formation of pronase-resistant triple-helix structures, at least during the dissolution process with pronase.

In this study, the problem outlined was overcome by lowering the temperature during pronase treatment to +4°. As a result, a method was developed for the determination of total denatured collagen. In addition the process of denaturation as well as that of renaturation immediately following the denaturation was recorded by measurements.

Materials and Methods

Tail tendons of 12–18-month-old rats with a 75–80% content of collagen insoluble during denaturation were used. The tendons, about 0.12 mm thick, were prepared as described previously (Hörmann and Schlebusch, 1968a). For denatura-

* From the Max Planck-Institut für Eiweiss- und Lederforschung, Munich, Germany. *Received July 14, 1970.* A portion of this work has appeared in preliminary form (Hörmann and Schlebusch, 1968b).

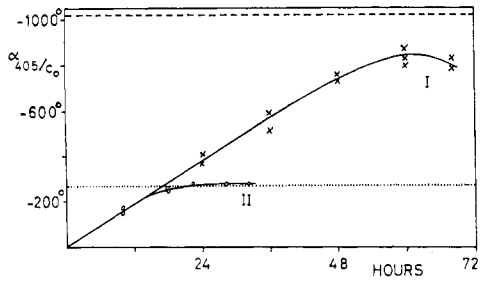

FIGURE 1: Dissolution of native (I) and denatured (II) rattail tendons by pronase at +4° as followed by measuring the optical rotation of the dissolved material. Denaturation: 60 min, 75°. Ordinate: optical rotation of the dissolved portion in relation to the concentration, c_0, of completely dissolved collagen. (----) $[\alpha]_{405}$ for native collagen. (· · · ·) $[\alpha]_{405}$ for pronase-degraded collagen.

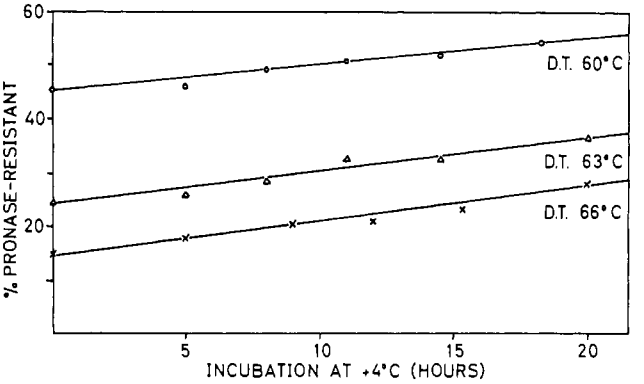

FIGURE 2: Re-formation of pronase-resistant material in heat-shrunk rattail tendons at +4°. The tendons were first heated in water for 10 min at the denaturation temperature (D.T.) indicated, then incubated in water at +4° for the time recorded on the abscissa, and finally dissolved by pronase at +4°.

tion experiments the weighed fiber was heated in 5 ml of water. After removing the heated fiber with a forceps, the proportion which had dissolved during the denaturation process was determined by measuring the nitrogen content of the remaining solution colorimetrically (Strauch, 1965). An N content of collagen of 17.6% was used as a basis for calculation.

The sample of pronase used (Serva Co., Heidelberg) showed optimal rates for dissolving rattail tendons at pH 6.5. In order to obtain reproducible results enzyme of the same batch was taken in every case. With this pronase, rattail tendons were dissolved more rapidly than indicated in earlier experiments.

The extent of denaturation of heated rattail tendons was determined by measurement of the proportion remaining native, *i.e.*, triple-helix structures present after pronase digestion. The tendons were incubated in 2 ml of pronase solution (enzyme:substrate, 1:20) containing 0.1 M $CaCl_2$ at pH 6.5 and +4° for about 60 hr with occasional stirring. After dissolution was complete, the optical rotation of the resultant solution was measured at 405 mμ with a Zeiss photoelectric polarimeter 0.005°, first at 20° and then at 40°, following a 10 min heating period. The proportion of pronase-resistant triple-helix structures θ, in per cent, of the total dissolved collagen was assumed to be linearly related to the optical rotation and was calculated by $\theta = [([\alpha]_{measured} - [\alpha]_{denatured})/([\alpha]_{nativ} - [\alpha]_{denatured})]100$; $[\alpha]_{measured} = (\alpha_{20°}/\alpha_{40°})[\alpha]_{denatured}$. A value of −830° was inserted for $[\alpha]_{native}$ and −270° for $[\alpha]_{denatured}$. The value for $[\alpha]_{native}$ includes corrections which consider a limited breakdown of native collagen during dissolution by pronase at +4°. For $[\alpha]_{denatured}$ the specific optical rotation of pronase degraded collagen is chosen.

The denatured fraction slowly recoiling at 16° to native collagen was measured by determination of pronase-resistant triple-helix structures in rattail tendon which, after denaturation, had been annealed at 16° for 1 hr. Pronase digestion was carried out at 20° and a value of −1020° was used for $[\alpha]_{native}$.

The values presented in this report as pronase-resistant proportions refer to that part of the collagen which remained insoluble after denaturation.

Shrinkage of tendons was measured with a ruler after heating fibers of known length for 10 min in water of different temperature.

Electron micrographs were made with the Siemens Elmiscope I (Siemens Berlin): high tension, 80 kV, electron microscopic magnification 40,000:1. The fibers were stained with a 2% solution of phosphotungstic acid (pH 4).

Results

Investigations on Methodology. The experiments of the following section were performed to show whether collagen can be dissolved at low temperature by pronase and if, during dissolution, the re-formation of pronase-resistant triple-helix structures from partly denatured collagen can be prevented.

In Figure 1, the dissolution of a native and of a denatured rattail tendon at +4° was followed by measurement of the optical rotation in solution. The fiber which previously had been denatured at 75° for 60 min, resulting in a predominantly irreversible denaturation, went into solution within 20–25 hr. The rotation of the dissolved material corresponded to that of completely degraded collagen.

The native fiber at +4° required about 60 hr to go into solution. When dissolution was complete, the optical rotation was somewhat lower than would be expected for native collagen. Prolongation of the enzyme treatment even caused a decrease of rotation. Obviously, the native collagen was slightly affected by pronase in solution. Therefore, when determining the proportion of native collagen, the optical rotation has to be measured immediately after complete dissolution. The slight breakdown during pronase treatment must be taken into account by the value for $[\alpha]_{native}$ which must be determined anew for each sample of enzyme.

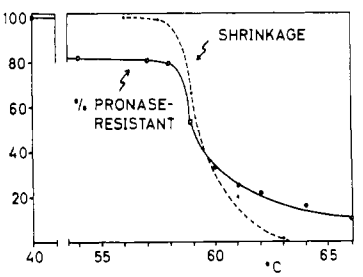

FIGURE 3: Temperature dependence of denaturation of rattail tendons. Extent of denaturation and shrinkage after 10-min heating in water. (O—O) Ordinate: per cent pronase-resistant collagen. (●---●) Change of length during shrinkage. Ordinate: $100(l - l_{min})/(l_0 - l_{min})$.

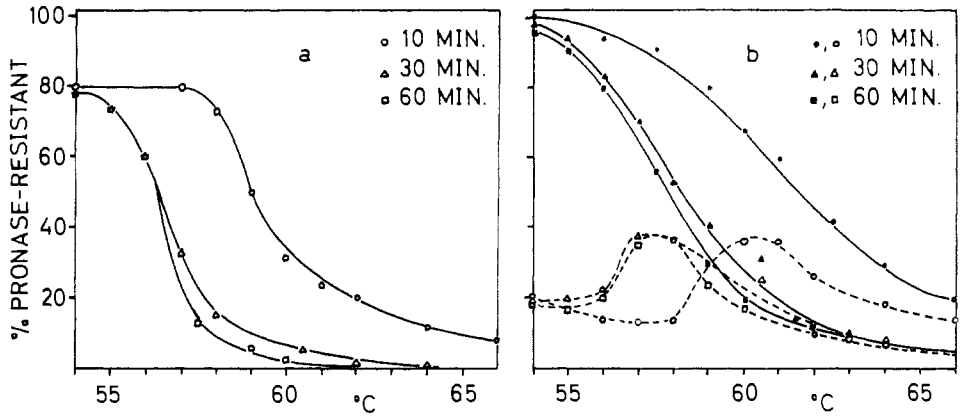

FIGURE 4: Temperature dependence of denaturation after heating rattail tendons in water for 10, 30, and 60 min. (a) Pronase-resistant material immediately after heating. (b) Different fractions of pronase-resistant collagen. (—) Resistant portion after annealing the heat-shrunk fibers for 1 hr at 16°. (----) Increase of resistant material during annealing.

In order to estimate to what extent reversibly denatured collagen at +4° again formed a triple-helix structure, we carried out the following experiment with partly heat-denatured collagen. Rattail tendons were first denatured for 10 min in water at 60, 63, or 66° and then incubated in water at +4°. After various periods of time, the tendons were removed from the water and were reacted with pronase at +4°. In Figure 2, the proportion of dissolved pronase-resistant triple-helix structures determined by optical rotation measurements is shown in relation to the incubation time at +4° prior to pronase treatment. The slope of the curve shows that at +4°, re-formation of triple-helix structures proceeded very slowly. In addition, the rate of renaturation appeared to be largely independent of the degree of denaturation and did not decrease during the time investigated.

Since partly denatured insoluble collagen at +4° re-forms pronase-resistant structures very slowly, there is strong evidence that, at this temperature, pronase breaks down almost all of the denatured collagen, including the reversibly denatured proportion. Only the fraction that has resisted denaturation may become solubilized as triple helices exhibiting a high optical rotation.

The amount of pronase-resistant material regenerated during the dissolution process of partly denatured collagen at +4° can be estimated if one takes into consideration that resistant material may form only in the first phase of dissolution of the denatured portion. Within 4–5 hr, all chains which have not stabilized to triple helices are probably damaged by pronase to such an extent that pronase-resistant material can no longer be formed. Figure 2 indicates that the magnitude of renaturation within this time is approximately 5%. The values presented in this paper have not been corrected for this error.

Dependence of Denaturation on Temperature. Following a heating period of 10 min in water at various temperatures, the denatured proportion of rattail tendons including the reversibly denatured fraction was determined by digesting the fibers with pronase at +4° as outlined under Methods. The amount of pronase-resistant material is plotted in Figure 3. In the figure, the change in lengths of the tendons under identical denaturation conditions is also recorded. Both curves have their point of inflection at the same temperature and thus both measurements indicate the same denaturation temperature for the fibers.

The values determined by digestion with pronase revealed that a certain amount of denatured collagen is already formed at temperatures at which the fibers as yet show no contraction. This limited denaturation decreases at lower temperatures and vanishes at 40° (Figure 3).

Denaturation curves obtained after various heating times are shown in Figure 4a. As heating proceeded, the amount of pronase-resistant, *i.e.*, triple-helical, material decreased for a given temperature and the inflection point of the curves shifted toward lower temperatures. After a denaturation period of 30 min, however, further changes became negligible. Obviously, denaturation came to a standstill.

Figure 4b shows the proportion of pronase-resistant collagen present after annealing the heat-shrunk fibers at 16° for 1 hr (see Methods). The denatured proportion calculated by subtracting these values from total insoluble fiber collagen represents material which, at 16° recoils very slowly to the triple-helical structure. Furthermore, this fraction did not change significantly after denaturation for 30 min. The rapidly renaturing fraction of heat-shrunk tendons was calculated from the total denatured material and the slowly

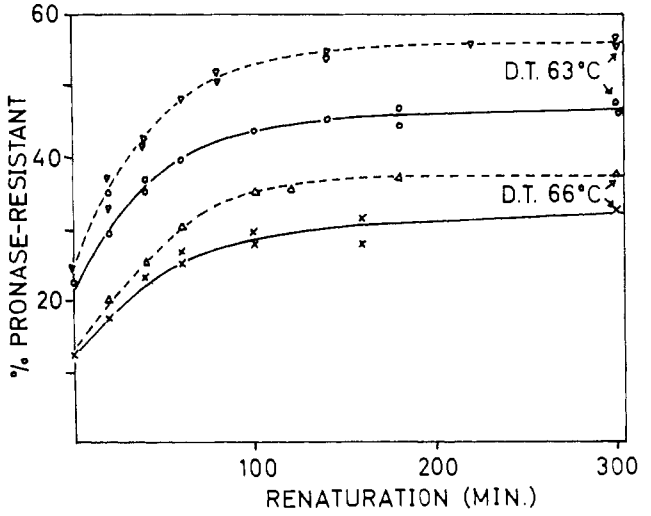

FIGURE 5: Renaturation of heat-shrunk rattail tendons. Denaturation: 10 min at 63 or 66°. Renaturation: (—) 20°, (---) 30°. Following renaturation in water for times indicated on the abscissa, the fibers were dissolved by pronase at +4°.

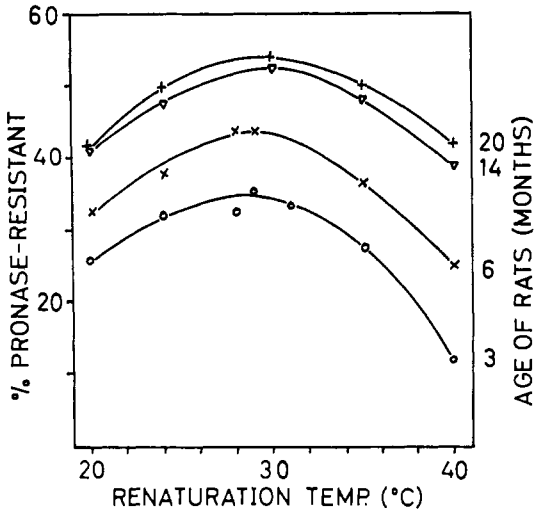

FIGURE 6: Renaturation of heat-shrunk rattail tendons as related to renaturation temperature and age of the animal. Denaturation: 10 min at 64°. Renaturation: 3 hr.

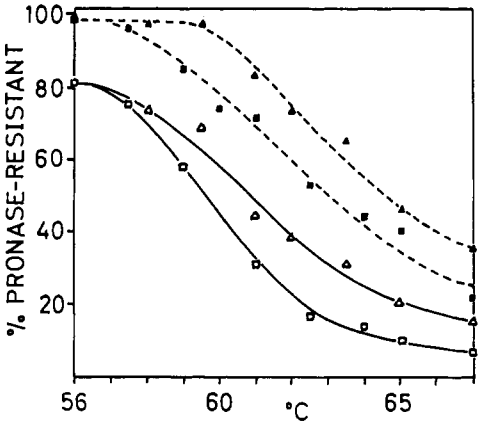

FIGURE 7: Temperature dependence of denaturation of rattail tendons fixed in a frame. Pronase-resistant material immediately after heating (—) and after subsequent annealing for 1 hr at 16° (---). Time of denaturation: 30 min (△, ▲), 60 min (□, ■).

recoiling material and was also plotted in Figure 4b. Maximum amounts are developed in the region near the denaturation temperature.

Renaturation of Collagen Fibers. The method of pronase digestion at +4° also affords one the opportunity of measuring the re-formation of triple-helix structures in partly denatured insoluble collagen. In the experiments shown in Figure 5, the rattail tendons were denatured for 10 min at 63 or 66° and subsequently incubated in water at 20 or 30°. After various periods of time, the tendons were removed and dissolved with pronase at +4° and the portion of triple-helix structures present in the dissolved material was determined by optical rotation measurements. It becomes clear from the results that collagen denatured at 63° re-formed triple helices to a greater extent than collagen denatured at 66°. At 30°, re-formation was more effective than at 20°. Within 2–3 hr, renaturation was complete for the most part. Thereafter, only a very slow increase in pronase-resistant material was observed.

Figure 6 indicates the extent of renaturation in water as related to temperature as well as to the age of the animal from which the rattail tendons were taken; 30° appears to be the optimal temperature for the re-formation of triple-helix structures from insoluble collagen. Tendons from younger animals could be renatured to a lesser degree than those from older rats. The marked decrease in the amount of native material obtained above 30° was partly due to the fact that collagen from denatured rattail tendons gradually dissolved in water above 30°.

Denaturation and Renaturation in Stretched Fibers. The solid curves in Figure 7 show the dependence of denaturation on temperature, following various denaturation periods, for rattail tendons which were fixed in a frame so that they could not change in length. Denaturation was also observed under these conditions. However, it began at a somewhat higher temperature and proceeded more slowly compared to tendons permitted to shrink. The final state of denaturation was not yet reached after 30 min. As shown by the dashed curves in Figure 7, the formation of denatured material very slowly recoiling to triple helices was also observed.

By stretching the fibers which had shrunk during the denaturation process, the renaturation to triple helices could be improved. Figure 8 shows an experiment in which the shrunk fibers were first incubated at 30°, during which some pronase-resistant material had re-formed. After 200 min, the fibers were stretched to their original length by hand. In this state, the proportion of native collagen rose considerably. The renaturation of a fiber which was stretched manually to 100% of its original length immediately following heat shrinkage is shown for comparison.

Finally, in Figure 9, the renaturation achieved after 3 hr at 30° is shown as related to the length to which the fibers were stretched after shrinkage. It becomes obvious that renaturation increases with a higher degree of stretching and that the influence of stretching on renaturation is greater with the more denatured fibers than with the less denatured. The optimum renaturation temperature was found to be the same for stretched as for unstretched fibers.

Electron Microscopy. Rattail tendons were denatured and renatured under different conditions and examined under the electron microscope (Figure 10). The typical cross-striation of the collagen fibrils became very diffuse after heating the tendons 5 min at 62°. Storage at 30° redeveloped distinct although not very clear cross-striation. After a 10-min denaturation period no cross-striation was present at all. Subsequently renatured fibrils showed only cross-striations over certain sections when stretched.

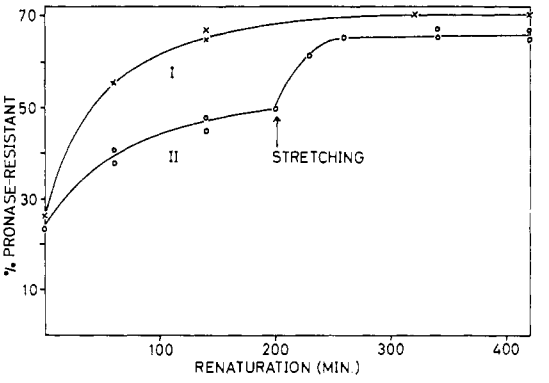

FIGURE 8: Renaturation of heat-shrunk rattail tendons after stretching by hand to 100% of their original length immediately after denaturation (I) or after 200 min renaturation in an unstretched state (II). Denaturation: 10 min at 64°. Renaturation: 30°.

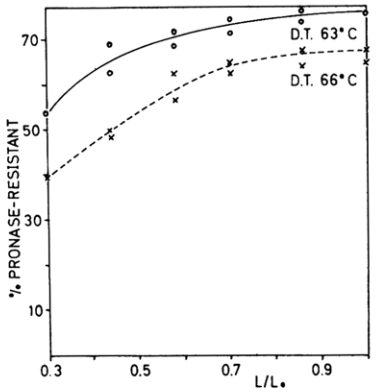

FIGURE 9: Renaturation of heat-shrunk rattail tendons as related to the extent of stretching immediately after shrinking. Denaturation: 10 min at 63 or 66°. Renaturation: 3 hr at 30°.

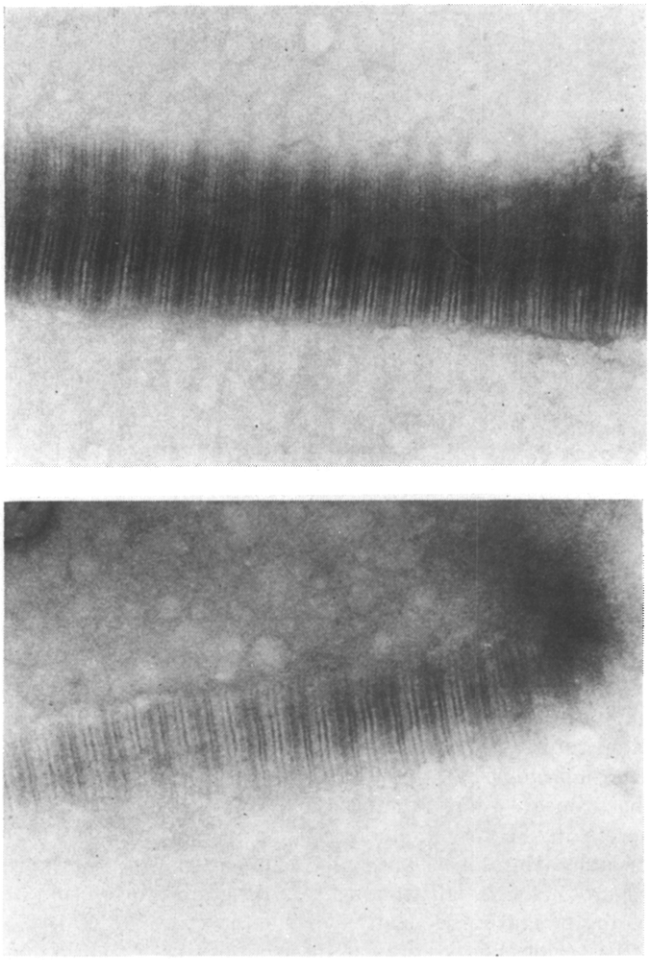

FIGURE 10: Electron micrographs of collagen fibrils taken from rattail tendons which had been denatured and renatured under different conditions. (a, top) Denatured 5 min at 62°, renatured 3 hr at 30°. (b, bottom) Denatured 10 min at 62°, renatured 3 hr at 30° after stretching to 100% of the original length.

Discussion

The investigations described demonstrated for the first time that two states of denatured material must be discerned in heat-shrunk fibers of insoluble collagen: one which easily returns to the triple-helix structure, and another which, under given experimental conditions, remains in a disordered state. The latter increases with rising temperature and increasing time of the denaturation process. However, there is no sharp distinction between the two fractions, because the capability of re-forming triple helices depends on the reaction conditions as shown in the renaturation experiments of the present report.

The detection of two fractions of denatured insoluble collagen provides evidence for a two-step mechanism of the denaturation process. First, the peptide chains indeed may become disarrayed, but they still are so well fixed in their original relative position to each other that they easily return to the triple-helix structure. In the course of prolonged denaturation, this state becomes more disordered, so that the formation of triple helices within the cross-linked lattice becomes progressively more difficult.

Previously, two steps were discerned by Engel (1962) in the denaturation of dissolved collagen. The first step was characterized by a drastic change of optical rotation due to disarrangement of the triple helix. Subsequently a gradual decrease of molecular weight was observed which indicated dissociation of the peptide chains.

In this study, a new method using pronase digestion was developed by which, for the first time, total denaturation of insoluble collagen became accessible to measurement. In order to detect the reversibly denatured portion as well, re-formation of triple-helix structures was slowed down by working at low temperatures. As a result, the denatured portion could be almost completely degraded by pronase.

Very slow re-formation of triple helices from disarrayed collagen peptide chains at low temperature was expected from earlier results on the renaturation of collagen in solution. Beier and Engel (1966) found the optimal temperature for the renaturation of native collagen monomers from completely separated peptide chains in aqueous citrate buffer to be 26°. At a lower temperature, *e.g.*, at 10° triple-helix structures developed in an incomplete form (see also Hauschka and Harrington, 1970a,b). High molecular aggregates appeared which also might contain single-stranded coils of the polyproline II type. The aggregates were affected by enzymes to a large extent. Their coiled structures including the triple-helical sections showed less stability than native collagen. At 10° less than 10% of the entire material was converted into native collagen within a period of 100 hr.

The enzymatic method employed revealed a sigmoid temperature dependence of total denaturation. The point of inflection coincided with that of the shrinkage curve measured after identical denaturation times (Figure 3). The fact that both methods show the same denaturation temperature indicates that they both measure the same process. Therefore, the coincidence also constitutes proof for the usefulness of the enzymatic method developed in this work. When only the denatured proportion recoiling slowly was determined, a different point of inflection was observed (Figure 4b).

However, the enzymatic method detected more details of the denaturation process than the measurement of shrinkage. It was found that part of the collagen already becomes denatured at temperatures at which the fibers do not yet shrink. Below 40°, where dissolved collagen is also stable, no denaturation was observed at all. It seems reasonable to assume that this more easily denatured portion involves molecules on the surface of the fibers. These molecules are not stabilized as well by the interaction with neighboring molecules as are molecules within the fibers, and are thus more easily affected by denaturing influences. The denatura-

tion below the shrinkage temperature is completely reversible as shown by comparison with the temperature dependence of the amount of denatured material in fibers annealed at 16° after heating (Figure 4).

With increasing denaturation time, denaturation proceeds at a temperature below the shrinkage temperature, but only to a limiting value (Figure 4). As early as (Weir) 1949, it was observed that the shrinkage point shifts toward lower temperatures after prolonged heating. Denaturation of the molecules on the surface probably leads to an increasing sensitivity of the adjacent layers within the fiber. Heating shortly below the shrinkage temperature, therefore, results in a gradual denaturation of the total fiber. However, if the temperature is lower, the stabilizing lattice forces within the fiber limit the progress of denaturation. Consequently an equilibrium is established at a certain level.

At temperatures above the shrinkage point, a larger portion of native collagen is indicated by the pronase-digestion procedure than by the shrinkage curve. Probably, part of the denatured molecules in the cross-linked lattice are very easily renatured even at +4° and are not degraded by pronase. Possibly they represent the γ molecules.

For the first time, re-formation of triple helices within the shrunk fibers could be measured. Considerable renaturation was observed at 20° or at 30° up to 3 hr following denaturation. Maximal refolding in water was achieved at 30°, which is near the optimum for renaturation of collagen in solution, namely 26°. On the other hand, the very slow renaturation observed at +4° did not show any decrease in rate over prolonged time. Therefore, considerable renaturation may take place during long storage of shrunk fibers in the cold.

Re-formation of triple-helix structures does not imply that the molecules also attain their original relative position to each other. As electron microscopic investigations show, this is the case only after very short denaturation periods just above the denaturation temperature (5 min, 62°). However, the restoration of the original lattice is promoted by stretching the fibers, so that cross-striations can be partly achieved even after more intense denaturation.

By stretching the fibers, the triple-helix structure of the monomers in the lattice is also stabilized. The denaturation temperature rises if the fibers are prevented from contracting and the re-formation of pronase-resistant structures is also promoted by stretching. Under these conditions, *i.e.*, when the fibers are stretched, part of the collagen which is usually irreversibly denatured can be renatured again. On the other hand, denaturation cannot be prevented entirely, even in the stretched state. Obviously, certain covalent bonds are always dissociated during heat denaturation, so that chains of molecules are no longer fixed so rigidly and become disarrayed, even in the stretched state.

Finally, it should be mentioned that in addition to completely preserved or re-formed molecules, denatured insoluble collagen may contain structures in which the triple helix has formed only to a limited extent. The question arises as to whether or not the method used detects those structures as pronase resistant. Portions of the collagen molecules where the triple helix is not developed over the entire length possess less stability than intact collagen molecules, although in some cases, the denaturation temperature is not very much lower than in undamaged rodlike molecules (Stark and Kühn, 1968). Since native collagen is already markedly affected by pronase at +4° during the long reaction period, it may be assumed that sections with the triple-helix structure are more susceptible and are therefore broken down to a high extent. However, further work is necessary in order to substantiate this point.

Acknowledgment

We are indebted to Professor Dr. W. Grassmann for his helpful discussions and for his interest in these investigations. Electron micrographs were kindly performed by Mrs. H. Wiedemann.

References

Beier, G., and Engel, J. (1966), *Biochemistry 5*, 2744.
Doty, P., and Nishihara, T. (1958), *in* Recent Advances in Gelatin Glue Research, Stainsby, G., Ed., London, Pergamon, p 92.
Drake, M. P., Davison, P. F., Bump, S., and Schmitt, F. O. (1966), *Biochemistry 5*, 301.
Engel, J. (1962), *Arch. Biochem. Biophys. 97*, 150.
Fujii, T. (1965), U. S. Patent 3.314.861 (filed Nov 18, 1965).
Fujii, T. (1966), *Bull. Soc. Sci. Photogr. Jap. 16*, 30.
Grassmann, W. (1936), *Kolloid-Z. 77*, 205.
Grassmann, W., Janicki, J., and Schneider, F. (1937), *in* Stiasny Festschrft, Gustavson, K. H., Ed., Darmstadt, Roether, p 37.
Gustavson, K. H. (1956), The Chemistry and Reactivity of Collagen, New York, N. Y., Academic Press, p 211.
Harrington, W. F., and Rao, N. V. (1970), *Biochemistry 9*, 3714.
Hauschka, P. V., and Harrington, W. F. (1970a), *Biochemistry 9*, 3745.
Hauschka, P. V., and Harrington, W. F. (1970b), *Biochemistry 9*, 3754.
Hörmann, H., and Schlebusch, H. (1968a), *Z. Physiol. Chem. 349*, 179.
Hörmann, H., and Schlebusch, H. (1968b), *Leder 19*, 222.
Kühn, K., Engel, J., Zimmermann, B., and Grassmann, W. (1964), *Arch. Biochem. Biophys. 105*, 387.
Stark, M., and Kühn, K. (1968), *Eur. J. Biochem. 6*, 534.
Strauch, L. (1965), *Z. Klin. Chem. 3*, 165.
Verzár, F. (1963), *Z. Physiol. Chem. 335*, 38.
Von Hippel, P. H., and Harrington, W. F. (1959), *Biochim. Biophys. Acta 44*, 427.
Weir, C. E. (1949), *J. Amer. Leather Chem. Assoc. 44*, 108.
Wright, B. A., and Wiederhorn, N. M. (1951), *J. Polym. Sci. 7*, 105.