*GAMMA TROPOCOLLAGEN: A REVERSIBLY DENATURABLE COLLAGEN MACROMOLECULE**, †

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The tropocollagen (TC) macromolecule is a highly asymmetric rodlike structure with a characteristic distribution of polar groups along its length. The high degree of stereochemical order makes possible the formation of ordered aggregates of various types under appropriate environmental conditions.[1–4] The evidence from X-ray diffraction studies[5–7] indicates the presence of three-strand helical diffracting units and, indeed, a comparison of the dimensions of the TC macromolecule (*ca.* 2,800 × 14 Å) with published values of the molecular weight (in the range 300,000–380,000) strongly suggests that the TC macromolecule comprises three polypeptide chains, each containing about 1,000 amino acid residues.

The thermal denaturation of collagen has been extensively studied (see Von Hippel and Harrington,[8] Burge and Hynes[9]) by a variety of physical methods, and the partial recovery of "collagen-like structure" as deduced from optical rotatory activity[10] and X-ray diffraction behavior is a well-documented fact. However, it was not clear from any of the available evidence whether such apparent renaturation involved a meaningful reassociation of polypeptide chains to form intact TC macromolecules or a relatively nonspecific aggregation of chains to form a three-dimensional micellar network. The work of Orekhovich and Shpikiter[11] and of Doty and Nishihara[12] showed clearly that gentle thermal denaturation causes the TC macromolecule to dissociate into two easily distinguishable components, α and β; the latter tentatively suggested that, of these two components, α is a single strand and β represents two of the original three strands of the TC macromolecule bonded together by one or more covalent cross-links even in the denatured random-coil state.

In view of the considerations mentioned and particularly because of the apparently meaningful renaturation (i.e., reassociation of chains) reported for carefully annealed solutions of deoxyribonucleic acid,[13] it seemed of interest to determine whether true renaturation could be achieved in the case of collagen. The term "renaturation" as used here denotes a specific reassociation of the three component polypeptide chains to form a hydrogen-bonded helical structure having a charge profile and other properties indistinguishable from the original native TC macromolecules. Our interest was further stimulated by the work of Rice,[14] who demonstrated a reversion to rodlike structures on cooling solutions of parent gelatin and who also showed that at least some of the material could be induced to form the ordered aggregation state known as "segment long-spacing" (SLS). The examination of appropriately stained SLS in the electron microscope is probably one of the most sensitive means of detecting intact TC macromolecules, since the formation of this particular aggregation state demands a fairly specific distribution of charge.[4] In the experiments of Rice only a small fraction (~10 per cent) of the "renatured" gelatin yielded SLS, although one would have expected a higher yield of renatured macromolecules on the basis of changes in viscosity, specific optical

rotation, and other physicochemical properties of the solutions. It seemed likely, therefore, that only a fraction of the chains in the parent gelatin solutions were reassociating in a meaningful manner to produce macromolecules indistinguishable from native TC, and we accordingly set out to investigate this apparent discrepancy.

It seemed clear that one worth-while method of investigation of the renaturable fraction would be to harvest the molecules in the form of SLS-type aggregates by centrifugation and to determine their behavior in the ultracentrifuge, after further thermal denaturation, either by running at 40° or at room temperature in the presence of KCNS. If the process of renaturation really involved a reassociation of the α and β components, then isolation of these renatured molecules via SLS formation followed by subsequent denaturation should yield a pattern in the ultracentrifuge showing both of these components in the same proportions as found in denatured solutions derived from native TC. If, on the other hand, the process of renaturation involved only a certain fraction of the macromolecules which did not dissociate into one- and two-strand components on thermal denaturation, then the process of renaturation followed by SLS harvesting should result in considerable enrichment of this minor component, and its physical characteristics should be readily apparent in the ultracentrifuge. This was indeed found to be the case. When the renaturable fraction from thermally denatured solutions of calfskin collagen was harvested as SLS by precipitation with ATP and examined in the ultracentrifuge in the presence of KCNS, it was found to consist predominantly of a single component having a sedimentation coefficient higher than both α and β and a molecular weight equivalent to that of the native TC macromolecule, together with relatively small amounts of the α and β components. If the cycle of denaturation, renaturation, and harvesting in the form of SLS is carried out in serial fashion, the proportion of α and β components diminishes rapidly, with consequent enrichment of the higher-molecular-weight fraction, which we have termed the gamma (γ) component. As we shall see, the molecular weight and other physical properties of the γ component strongly suggest that while most of the three-strand TC macromolecules in an acid extract of collagen comprise one covalently bonded two-chain β component and one single-chain α component, a small fraction (5–10 per cent) of the macromolecules exist in a form in which the three constituent polypeptide chains are linked by covalent bonds, even in the denatured state, so that they behave as a single kinetic unit. This γ component exhibits completely reversible denaturation properties in the sense that renaturation involves a reformation of macromolecules indistinguishable in physical properties (including their charge profile as judged by electron microscopic examination of stained SLS-type aggregates) from the original native TC macromolecules. On the other hand, the α and β components, once dissociated from each other, show little if any meaningful reassociation under the same conditions of renaturation.[15] The present paper describes some of the properties of this gamma tropocollagen, including a preliminary study of the kinetics of renaturation.

In the case of neutral salt-soluble collagen, it has recently been demonstrated by ultracentrifugation[16, 17] (confirmed independently by us) and by column chromatography,[17] that most of the macromolecules give rise only to α components on thermal denaturation.[18] Presumably none of the three constituent chains is covalently linked together in this material which is thought to represent molecules

more recently synthesized than those present in the acid extracts. It seems likely, therefore, that formation of cross-links between chains to give rise to β and γ components is a phenomenon associated with "maturing" or "aging" of the TC macromolecules and occurs following the synthesis and assembly of the constituent polypeptide chains. Although we have not yet investigated the kinetics of "renaturation" in neutral salt-soluble collage, it seems likely that the rate of meaningful reassociation of three separate chains will be even less than that found for recombination of the α and β components of acid-soluble collagen. If this proves to be the case, it may be necessary to postulate some kind of "reference point mechanism" for synthesis *in vivo*. This would ensure the correct longitudinal displacement of the three chains relative to one another and thus allow them to form a helical hydrogen-bonded structure with the charge profile characteristic of the native TC macromolecule.

Materials and Methods.—Most of the work reported here was carried out on purified calfskin tropocollagen solutions[19] prepared according to the Gallop method.[20, 21] Stock solutions having TC concentrations in the range 0.1 to 0.5 gm/100 ml in 0.05 per cent acetic acid were prepared and stored at 2°C. Concentrations were usually determined in a Zeiss Laboratory Interferometer at 25°C. Since this instrument gives readings in terms of refractive index increment, the values of dn/dc had to be measured for TC in the various solvents used. The values were related to TC concentration by determining the hydroxyproline content of three different TC samples using Martin and Axelrod's[22] modification of Neumann and Logan's method.[23] The dn/dc values found for TC in 0.05 per cent acetic acid and in 0.1 M Sörensen citrate buffer of pH 3.7 were identical within the limits of error: 0.186 ± 0.003. No changes were observed when the TC solutions were heat denatured. These findings are in good agreement with Boedtker and Doty's[21] data on ichthyocol. The accuracy of the readings on the interferometer was better than ± 0.2 per cent even when the samples were kept in the cuvettes for several hours, provided the salt concentration was not too high. The presence of 1.2 M KCNS made the readings so uncertain that, in this case, the TC concentrations were determined before adding the KCNS and the values adjusted by allowing for a measured 5 per cent volume increase resulting from the addition.

Denaturation of the samples was accomplished by heating them to 60°C for 5 min, this procedure being followed either by quenching in cold water or by allowing the samples to cool slowly to room temperature. In some instances the samples were denatured in the presence of 1.2 M KCNS or 8 M urea. In these cases, the renaturation procedure consisted of extensive dialysis of the samples against 0.05 per cent, acetic acid.

The γ component was isolated and purified by the following procedure. Solutions of TC (0.1% in 0.05% acetic acid) were denatured as already described and allowed to cool to room temperature. ATP was then added to a final concentration of 0.3 per cent. Within a few hours the solution became turbid, indicating the formation of SLS aggregates. The precipitate was harvested 5 hr after denaturation by centrifugation in a Spinco Model L ultracentrifuge. The formation of SLS aggregates is critically dependent on the absence of salt. For this reason it was often necessary to dialyze the TC solutions extensively against dilute acetic acid. The precipitates could be redissolved in aqueous solvents of pH 3.5 and ionic strength about 0.1. In most instances, they were taken up in 0.1 M phosphate buffer of pH 3.5 and dialyzed against this solvent to remove both free and SLS-bound ATP. The compositions of the γ preparations were determined by heat denaturing a sample of the ATP precipitate dispersed in 0.05 per cent acetic acid, adding KCNS to a final concentration of 1.2 M and running it in the Spinco Model E ultracentrifuge at 59,780 rpm, together with a control sample of normal denatured TC in a wedge-window cell. The α, β, and γ peaks began to separate in about 1 hr and were well developed after 2 hr. The apparent concentrations of α, β, and γ were determined from the areas under the schlieren peaks, and their respective ratios calculated. These results were in error because of the Ogston-Johnson effect, but reasonably good values could be obtained by doing several runs at different concentrations and extrapolating both the peak areas and ratios to zero concentration. The apparent concentrations were also corrected for the radial dilution effect.

The sedimentation rates of the various components were measured in terms of the migration rates of the peaks on the schlieren patterns. In each case the An-D rotor of the Spinco Model E ultracentrifuge was run at 59,780 rpm. The sedimentation characteristics of purified γ component in the denatured state were generally determined at 25°C in 0.05 per cent acetic acid containing 1.2 *M* KCNS. In a few cases the solvent was 0.1 *M* citrate buffer of pH 3.7 containing 1.2 *M* KCNS. Several runs were also carried out at 40°C either in 0.05 per cent acetic acid containing 0.1 *M* NaCl or in 0.1 *M* citrate buffer at pH 3.7. The sedimentation rates of the γ component in the renatured rodlike form were also determined by running at 25°C in 0.05 per cent acetic acid containing 0.1 *M* NaCl. The sedimentation coefficients in each case were determined by correcting the experimental values to water at 20°C and by extrapolating to zero concentration.

Density measurements of TC and of the γ component in 0.05 per cent acetic acid plus 1.2 *M* KCNS at 25°C or in plain 0.05 per cent acetic acid at 40°C, were carried out using a 20-ml pycnometer immersed in a water bath with a temperature constancy of better than ±0.005°C. The densities were plotted versus concentration according to the equation:

$$m_{12}/v_{12} = \rho_1 + (1 - \bar{v}_2\rho_1)m_2/v_{12}$$

where m_{12} = mass of solution, m_2 = mass of solute, v_{12} = volume of solution, $\bar{v}_2$ = partial specific volume of solute, m_{12}/v_{12} = density of solution, m_2/v_{12} = concentration of solution, ρ_1 = density of solvent. The slope of this plot yields immediately $1 - \bar{v}_2\rho_1$, i.e., the term used for the determination of molecular weight using the Svedberg equation. Whenever KCNS was present, the points in the plot scattered sufficiently to cause an error of about ±3 per cent; in acetic acid alone the accuracy was ±1 per cent.

Intrinsic viscosities of the various solutions were measured in Ubbelohde viscometers, modified[24] to facilitate dilution of the solutions down to a ratio of 1:8. Shear gradients for water at 25°C were *ca.* 800 sec^{-1}, and the outflow times for this solvent were of the order of 60 sec.

The renaturation kinetics of the various γ preparations and also of control TC preparations and γ-free mixtures of α and β components were followed by monitoring changes in both viscosity and specific optical rotation. The viscometers used were of the Ostwald-Cannon-Fenske type with shear gradients of the order of 1,600 sec^{-1} for water and were immersed in a water bath maintained at 25.0 ± 0.01°C. Kinematic energy corrections were applied. Optical rotation changes were measured in a Rudolph precision polarimeter, Model 80, equipped with the Rudolph photoelectric polarimeter attachment. Use of an air-cooled mercury arc with a Bausch and Lomb monochromator permitted work in the near ultraviolet, λ = 367 mμ, with a consequent increase in accuracy as compared with measurements at wavelengths in the visible. For these studies the samples were heated to 55°C for 5 min, quenched to 25°C, and inserted into the temperature equilibrated 10-cm polarimeter microcells as rapidly as possible.

Results.—Preliminary experiments had shown that solutions of acid-extracted calfskin collagen produced two main peaks, the α and β components, described by previous authors,[11, 12] with a molar ratio of 1:1, following thermal denaturation and running in the ultracentrifuge either at 40°C or at 25°C in the presence of 1.2 *M* KCNS. When the renaturation schedule described earlier was followed, it was found that the material capable of forming ordered SLS-type aggregates (presumably consisting of those molecules in which a relatively intact charge profile had been restored by renaturation), when run in the ultracentrifuge under the same conditions, showed a pattern in which the α and β components were heavily outweighed by a new component, the γ component, sedimenting at a rate considerably higher than those of the α and β components. Figure 1 shows a typical sedimentation schlieren pattern of such a preparation (upper curve) contrasted with a control solution consisting of normal TC to which some γ component had been added. After one cycle of renaturation and harvesting as SLS, the γ component is generally enriched to about 70 per cent, and after the second renaturation to about 90 per cent or more. The proportion of γ present in the solution rep-

resented by the upper curve of Figure 1 was estimated by integration to be *ca.* 80 per cent. Our results clearly show that the denaturation-renaturation-harvesting procedure already outlined results in a considerable enrichment of the

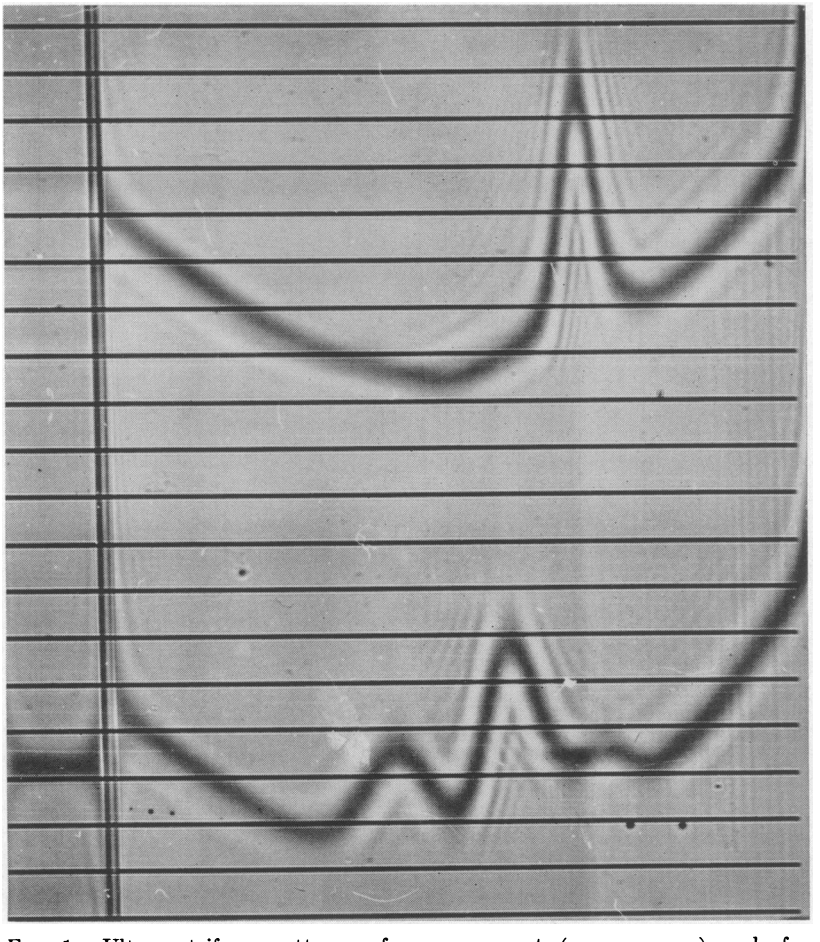

FIG. 1.—Ultracentrifuge patterns of γ component (upper curve) and, for comparison, a mixture of α, β, and γ components (lower curve).

γ component, which is normally present in acid extracts of calfskin collagen to the extent of about 8 per cent. A number of recent studies have also demonstrated the presence of a γ component in solutions of tropocollagen by ultracentrifugal[16, 17, 25] and chromatographic techniques.[17]

The sedimentation coefficients of the γ component reduced to water at 20°C are plotted in Figure 2(*a*) and (*b*). The s values determined by extrapolation to zero concentration for the α, β, and γ components are shown in Table 1, and compared with the results of several other authors.

Part of the evidence that renatured macromolecules of the γ component resemble the original native TC macromolecules in physical and chemical properties was provided by measurements of intrinsic viscosity. The values of $[\eta]$, measured at 25°C in 0.05 per cent acetic acid plus 0.1 M NaCl and in 0.1 M citrate buffer

TABLE 1

SEDIMENTATION COEFFICIENTS OF VARIOUS FRACTIONS OF DENATURED CALFSKIN COLLAGEN

Authors	Condition	α	β	γ	(δ)
Doty and Nishihara[12]	1.2 *M* KCNS	3.85	5.4	...	...
Piez, Weiss, and Lewis[29]	2 *M* KCNS	3.73	5.29	...	...
	40° (No KCNS)	3.24	4.41	...	...
Veis, Anesey, and Cohen[30]	40° (No KCNS	3.21	...	(4.57)[31]	6.24
Present authors	1.2 *M* KCNS	3.4	4.6	5.9	7.1[33]
	40° (No KCNS)	3.2	4.2	5.5	...

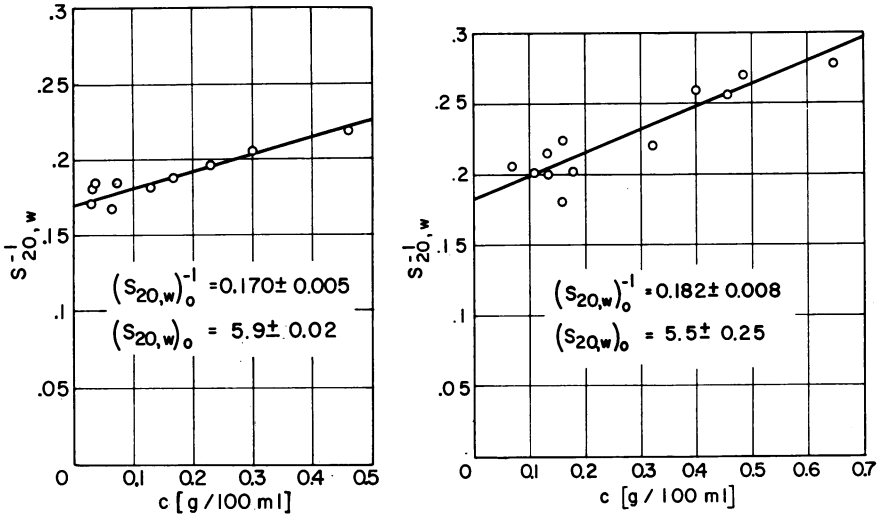

FIG. 2.—$S_{20,w}^{-1}$ values of γ component (in 0.05 per cent acetic acid) calculated from runs done at (*a*) 25°C in presence of 1.2 *M* KCNS; and (*b*) 40°C without KCNS but in 0.1 per cent NaCl.

of pH 3.7 for γ, native TC, and TC following treatment with trypsin at pH 7.5 were 11.2, 14.0, and 11.8 *dl*/gm, respectively. While trypsin does not attack the main helical portion of the TC macromolecule,[26] it does bring about a depolymerization of linear dimers and higher oligomers, which were found to be present even in extensively centrifuged solutions, with a consequent fall in $[\eta]$ to values close to 12 *dl*/gm. This value is therefore probably close to that which would be found for a truly monomeric solution of TC. In view of this, the correspondence between the intrinsic viscosities of γ and trypsinized TC shows clearly that renaturation of γ results in a virtually complete restoration of the asymmetry of the macromolecules.

Preliminary ultracentrifuge measurements by the Archibald technique on preparations from which the greater fraction of α and β components had been removed indicated a molecular weight for γ substantially the same as that of tropocollagen, *ca.* 350,000.

Velocity runs in the ultracentrifuge also illustrated the effects of proteases on TC and γ under various conditions. Treatment of TC or renatured γ with trypsin and pepsin at room temperature and appropriate pH values caused no noticeable change in sedimentation characteristics, strongly indicating that renaturation of γ results in complete restoration of the helical structure characteristic of the native TC macromolecule. The specific optical rotation of purified renatured γ also

supports this conclusion. As expected, collagenase treatment under conditions similar to those used for tryptic treatment rapidly digested both TC and γ. Denatured γ, as with denatured TC samples, when incubated with trypsin or pepsin at room temperature for 1–2 hr, was digested readily as could be seen from the replacement of the sharp α, β, and γ peaks by broad humps.

Doty and Nishihara[12] reported that incubation of denatured TC at pH 12 resulted in a diminution of the β peak. Heat treatment did likewise. In our experience, this alkaline treatment results in damage to both denatured γ and TC, and incubation of the samples at 70°C in 0.05 per cent acetic acid for 2–6 hr causes broadening of the α, β, and γ peaks to rather shallow humps. Heating in the presence of KCNS gave comparable results in 15–70 min. It is still not clear to what extent these effects can be ascribed to breakage of the covalent bonds linking chains in the β and γ components on the one hand or to hydrolysis of the constituent chains on the other.

Preliminary results on the kinetics of renaturation of γ, TC, and of γ-free parent gelatin (i.e., gelatin consisting only of α and β components) have already been reported.[25] The process of renaturation was followed by monitoring changes in specific optical rotation and in relative viscosity. These properties were measured before thermal denaturation and at intervals of 2–5 min following quenching of the samples to 25°C. The results obtained for a number of samples are illustrated in Figures 3 and 4. It is clear from the data that the rates of recovery of both

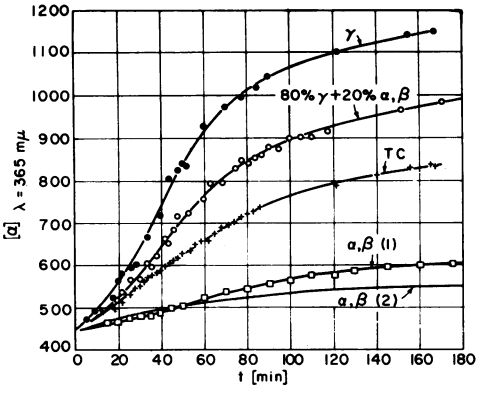

FIG. 3.—Optical rotation recovery of gelatins containing differing proportions of γ component. Concentrations were in the range of 0.05 to 0.3 per cent.

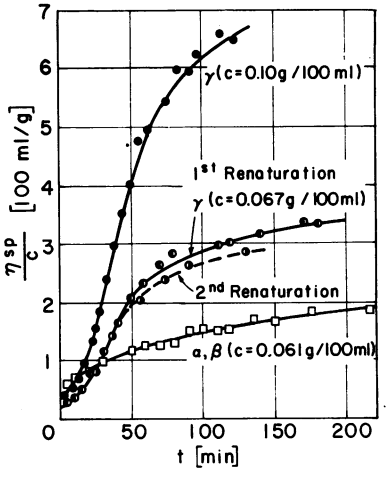

FIG. 4.—Recovery of viscosity of gelatins containing differing proportions and concentrations of γ component.

optical rotation and viscosity are strongly dependent on the proportion of γ component in the sample and hence that there exists a strong facilitative effect in the case of the γ component for rapid restoration of the polyproline-like helical structure of the individual strands primarily responsible for the optical rotatory changes. Presumably the presence of covalent cross-links, acting as nucleation centers for the formation of three-strand helical structure, between the three constituent chains of the γ component, allows a rapid and meaningful reconstitution of the

native macromolecule. In contrast to this, the recovery of optical rotation and viscosity was much slower in γ-free preparations containing only the α and β components, and a meaningful reassociation of chains to form molecules having an intact charge profile (as judged by the ability to form ordered SLS structures) was not observed. It remains uncertain in this case to what extent the recovery in optical rotation and viscosity can be attributed to partial reformation of helical structure in single strands on the one hand or to a relatively nonspecific micellization (perhaps involving short regions of three chains hydrogen-bonded together in a collagen-like helical structure) on the other. The results seem to be in accord with Ramachandran's proposal[27] based on energetic grounds that the formation of a three-residue-per-turn helix in single strands should be facilitated when at the same time three strands are able to associate to form a major helix.

The specific optical rotation of renatured γ preparations was usually about $-1110°$ at $\lambda = 367$ mμ as compared with $-1330°$ for native TC preparations. At least part of the discrepancy between these values can be attributed to contamination of the γ preparations with α and β components. On denaturation the value of $[\alpha]$ falls to $-460°$ for both γ and TC. Thus the transition from native TC to parent gelatin is accompanied by a change in $[\alpha]$ of 870°. The recovery of purified γ at 25°C is about $-650°$ within 3 hr. In contrast, the recovery of γ-free parent gelatin at 25°C is very slow (see Fig. 3). After this preparation stood for several weeks at 0–5°C, the $[\alpha]$ values for these preparations were about $-900°$, representing a recovery of about $-440°$.

The recovery of specific optical rotation for the γ component was independent of concentration, and the curves for two successive renaturations were almost perfectly superposable (Fig. 5), clearly demonstrating the high degree of reversibility exhibited by this material toward denaturation.

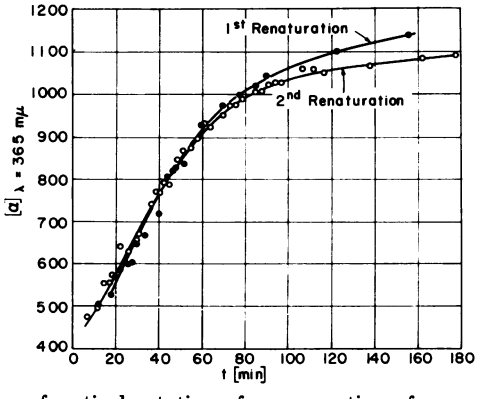

FIG. 5.—Recovery of optical rotation of a preparation of γ component for two consecutiverenaturations.

In general, the viscosity recovery of the various samples paralleled their behavior with respect to optical rotation. Thus, the increase in viscosity of denatured γ preparations was much more rapid than was the case for denatured TC and γ-free parent gelatin. The reduced specific viscosity of TC dropped from 23 (native TC) to 0.45 on denaturation and increased slowly on standing at 25°C. Even in the case of γ the viscosity recovery was relatively slow, the value of

η_{sp}/C rising to *ca.* 7 three hours after denaturation and to *ca.* 11 after several days (Fig. 4). The original value of 17.5 shown by this sample after harvesting, i.e., before the second denaturation, was not reached. Unlike the situation with respect to optical rotation recovery, the viscosity recovery of γ exhibited some degree of concentration dependence, the renaturation rates being slower at lower concentrations. However, there was no evident relation between the concentration dependence of the reduced specific viscosities due to nonideal behavior of undenatured TC or γ on the one hand and the corresponding renaturation curves on the other. Also, after two consecutive denaturations, the viscosity recovery curves began to level off slightly earlier (Fig. 4).

The kinetic measurements were analyzed by plotting the log of the recovery rate versus time. The results for the various samples are illustrated in Figures 6 and 7

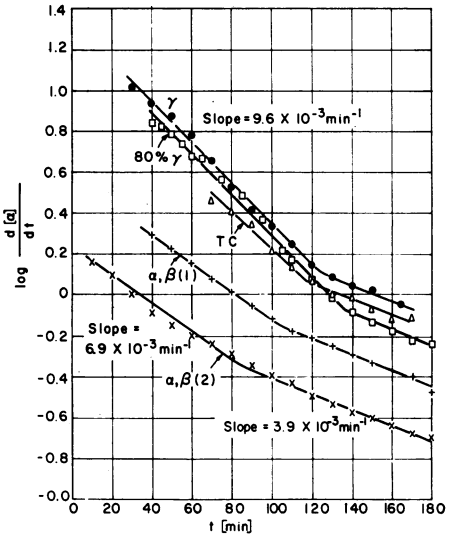

FIG. 6.—Kinetics of recovery in terms of optical rotation according to first-order reaction formula ($d[\alpha]/dt$ = const − kt) for samples of differing proportions of γ component.

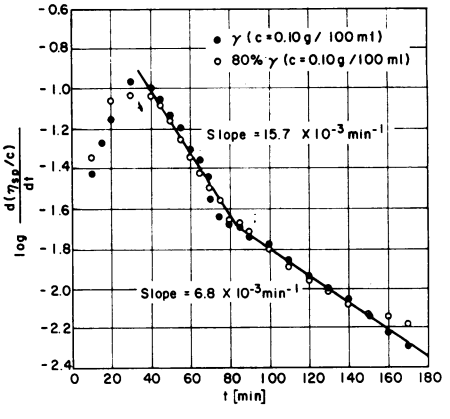

FIG. 7.—Kinetics of recovery in terms of reduced viscosity according to first-order reaction formula [d (η_{sp}/c)$/dt$ = const′ − kt] for two γ preparations.

and indicate first-order kinetics for recovery of both optical rotation and viscosity for all samples. In no case was second- or third-order kinetics observed. The steep slope in Figure 7 obviously reflects a first-order reaction comparable to the fast optical rotation recovery of the γ component in Figure 6. The maximum at 30 min. and the preceding positive slope of the curve in Figure 7 can be explained at least partially in terms of nonideality of the gelatin solutions.[28] The results clearly show that in the case of the γ component the recovery of optical rotation, reflecting a restoration of the helical structure, is accompanied by a comparable recovery of molecular asymmetry, as is to be expected for the case of a real renaturation of the TC macromolecule. In contrast to this, the slopes were much lower for control preparations of TC and for γ-free preparations of the α and β components. In the case of relatively impure preparations of γ, the plots were

bimodal, the initial slopes being approximately parallel with those for pure γ and then later following those for the γ-free preparations.

Summary.—A fraction of calfskin collagen is described which is capable of renaturation after thermal denaturation. The evidence presently available indicates that this fraction has a molecular weight similar to that of native tropocollagen and, like the latter, consists of a three-strand helix (hence the designation γ, consistent with one-strand α and two-strand β components). Even in the denatured state, γ behaves as a kinetic unit because of strong, probably covalent, bonds between the three chains. The γ-type tropocollagen probably represents a more mature form than the all-α or α–β forms.

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[19] We are greatly indebted to J. H. Highberger of the United Shoe Machinery Corporation, Beverly, Massachusetts, for supplying the collagen solutions used during the earlier phases of these investigations.

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[33] In some preparations a component heavier than γ appeared with the sedimentation constant indicated.

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*THE MECHANISMS FOR CHYMOTRYPSIN**

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From kinetic, degradative, and isolation studies and the establishment of virtual reactions the following facts are known about the mechanism of chymotrypsin action: (1) the reactions catalyzed by chymotrypsin occur in two steps. The first step involves the acylation of the enzyme with loss of HX and the second step involves solvolysis of the acyl enzyme by water or other appropriate nucleophile,[1-6]

$$\text{En}\cdot\text{RCOX} \underset{+\text{HX}}{\overset{-\text{HX}}{\rightleftharpoons}} \text{RCOEn} \underset{-\text{H}_2\text{O}}{\overset{+\text{H}_2\text{O}}{\rightleftharpoons}} \text{RCOOH}\cdot\text{En}. \qquad (1)$$

(2) The deacylation and acylation steps require a basic group of the enzyme to be present in the nonprotonated form. (3) The pKa values for the basic group, as determined kinetically, are different in the acylation and deacylation steps. For acylation $pK_{app} = 6.6\text{–}6.7$[2, 4] while for deacylation $pK_{app} = 7.2\text{–}7.4$.[2, 3, 4, 7] (4) From the inability to assign these pKa values to other functional groups as mercapto (there are none) or amino (they may all be blocked without loss of activity),[8-10] etc., and from the knowledge that the pKa values of protein and peptide bound histidine groups fall in this range,[11] and on the basis of other chemical evidence[12-14] the essential basic group is most logically an imidazolyl group of histidine. (5) From the pH-rate profiles for chymotrypsin catalysis there can be detected the involvement of no other basic groups of pKa below the maximum pH operationally allowed (12.5).[20] (6) When employing acetyl derivatives as substrates in which X is a good leaving group (as *p*-nitrophenyl acetate), the rate constant of acylation exceeds that of deacylation and since $pK_{deacylation} > pK_{acylation}$, a stable acetyl enzyme can be isolated at moderately acid pH values.[15] (7) The hydroxyl group