The Deuterium Exchange of Peptide-Group Hydrogen Atoms during the Gelatin → Collagen-Fold Transition*

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Purified calfskin tropocollagen, in 6.7×10^{-3} M sodium citrate buffer, *p*H 3.2, containing 0.8% tetramethylammonium chloride, was heated 10 minutes at 55–60° and the completely gelated solution was quenched to 14°. The gelatin → collagen-fold transition was followed polarimetrically and viscosimetrically. Simultaneously, aliquots were removed from the reaction mixture and quickly lyophilized. Upon re-solution of each aliquot in D_2O at 20°, the course of exchange of peptide-group hydrogen atoms was followed at 1548 cm^{-1} using an infrared spectrophotometric technique. The increase in levorotation, $-[\alpha]^{14}_{546}$, started to level off after 24 hours at a value corresponding to recovery of 56% of the rotation in the initial native tropocollagen solution, whereas the intrinsic viscosity continued to increase after 24 hours. All peptide-group hydrogen atoms in parent gelatin at zero time were found to exchange rapidly with the same first-order rate constant of 0.16 ± 0.01 min^{-1}, in good agreement with earlier exchange data for randomly coiled poly-DL-alanine. As the gelatin → collagen-fold transition proceeded, the peptide-group hydrogen atoms separated into two classes, one in which the exchange was much slower, with rate constants of less than 1 day^{-1}. Using the same extinction coefficient for all the rapidly exchanging hydrogen atoms the rate of their disappearance was found to parallel closely the rate of gain of levorotation. This result is discussed in relation to current proposals for the reaction mechanism of the gelatin → collagen-fold transition and interpreted to indicate that interpeptide-group hydrogen bonds of the collagen triple-helix type are formed rapidly after the helical coiling of the individual peptide chains, in agreement with the mechanism proposed by P. J. Flory and E. S. Weaver (1960, *J. Am. Chem. Soc. 82*, 4518). A generalized reaction mechanism for the gelatin → collagen-fold transition is presented. Attempts to determine the absolute number of peptide-group hydrogen atoms involved in interpeptide-group hydrogen bonds during the gelatin → collagen-fold transition by measuring rates of hydrogen exchange is discussed in relation to the collagen models proposed by A. Rich and F. H. C. Crick (1958, *Recent Advan. Gelatin Glue Res. Proc. Conf. Univ. Cambridge, 1957, 20;* 1961, *J. Mol. Biol. 3*, 483) and by Ramachandran and co-workers (1961, G. N. Ramachandran, V. Sasisekharan, and Y. F. Thatkachori, *in* Central Leather Research Institute Symposium on Collagen, N. Ramanathan, ed., New York, Interscience, p. 102).

Tropocollagen, the soluble monomer of collagen, has been shown to be a relatively rigid, rod-shaped molecule having a length of approximately 3000 A, a width of 14 A, and a molecular weight of 300,000 (Doty and Nishihara, 1958; Piez *et al.*, 1963). According to the model proposed by Rich and Crick (1958, 1961), tropocollagen is made up of three chains each of which is coiled into a left-handed helix having the characteristics of the poly-L-proline II (Cowan and McGavin, 1955) and polyglycine II (Crick and Rich, 1955) helices. There is some alteration in pitch and angle of rotation of the repeat periods imposed by a gradual right-handed coiling of the three minor helices to form a superhelix. In order to accommodate the close packing of the three chains in the collagen II structure, glycine residues must be located at every third position along each chain, a requirement which is consistent with the amino acid analysis of collagen and short fragments of collagen (Grassmann *et al.*, 1960). In collagen II there are three interchain hydrogen bonds for every three-residue advance along the triple helix, *i.e.*, three hydrogen bonds for every nine residues.

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Ramachandran *et al.* (1961) have proposed a somewhat different structure. While maintaining a left-handed helical configuration of the three chains, they have distorted the collagen II structure in order to form six interchain hydrogen bonds for every nine residues. When the sequence -Gly-Pro-Hypro- occurs in one of the chains only five hydrogen bonds may be formed in the nine-residue segment of the triple helix. For a discussion of the development of these models and of the concepts and information following, see the comprehensive review by Harrington and Von Hippel (1961b).

When tropocollagen solutions are heated gradually, an abrupt change in the physicochemical properties takes place over a narrow temperature range. The intrinsic viscosity falls from about 14 dl/g to about 0.4 dl/g and the specific levorotation, $-[\alpha]_D$, decreases about 280°. The loss of viscosity and levorotation reflects the molecular transition from the rod-shaped helical structure of tropocollagen to the random coil of gelatin. The temperature of this phase transition correlates with the imino acid content of the species of collagen (Piez and Gross, 1960; Burge and Hynes, 1959).

The product of the tropocollagen → gelatin transformation consists of a mixture of single (α), double (β), and triple (γ) chains, the multiple chains being held together by cross-linkages. Except for ichthyocol gelatin, the amounts of α and β chains appear to be roughly equal and together account for all but a small fraction of the total (Harrington and Von Hippel, 1961b).

It is an early observation (Smith, 1919) that there is a time-dependent increase in levorotation when gelatin solutions are cooled. That this process reflects a trend toward the re-formation of the collagen structure is shown by the well-established fact that γ-gelatin can be made to re-form apparently normal tropocollagen which can aggregate in the segmented long-spaced form identifiable by electron microscopy (Veis and Cohen, 1960; Rice, 1960; Altgelt *et al.*, 1961; Veis *et al.*, 1962).

The gelatin → collagen-fold transition in quenched gelatin solutions is characterized by the following conditions: (a) The rate of change of $[\alpha]_D$ with time and the final value of $[\alpha]_D$ attained are essentially independent of protein concentration; (b) the reaction appears to be close to second order with respect to the amount of gelatin remaining in the unfolded configuration; (c) the rate of change of the intrinsic viscosity with time is markedly dependent on protein concentration, and (d) the reaction has a large negative-temperature coefficient (Von Hippel and Harrington, 1960).

Two mechanisms for the apparent partial re-formation of the collagen fold during the gelatin → collagen-fold transition have been suggested. Flory and Weaver (1960) pointed out that the apparent unimolecularity of the gelatin → collagen-fold transition can be reconciled with the three-strand coiled-coil model for native tropocollagen by postulating a transitory intermediate consisting of a single-chain helix, possibly of the poly-L-proline II type, the formation of this intermediate being rate determining. Three single-chain helices associate rapidly to form the collagen-fold triple-helix. Such a mechanism succeeds further in explaining the large negative-temperature coefficient of the gelatin → collagen-fold transition.

Harrington and Von Hippel (1961a,b) have proposed a mechanism for the gelatin → collagen-fold transition involving three steps: (1) A local configurational change in the pyrrolidine-rich regions of the polypeptide chain, resulting in the *local* establishment of a helical configuration of the poly-L-proline II type. This step goes to completion rapidly and is detected by the change from simple to complex collagenase kinetics (Von Hippel and Harrington, 1959). (2) Following this "nucleation" the remainder of the polypeptide chain is induced to fold into a loose poly-L-proline II type of helix. This folding is responsible for the measurable slow increase in levorotation that follows quenching. (3) Following the helical coiling a specific association of individual helices results in the yet slower increase in particle weight and viscosity.

It occurred to us that the mechanism of the gelatin → collagen-fold transition could be further elucidated by a direct experimental approach of determining the rate of formation of hydrogen bonds between peptide groups in the gelatin chains following quenching and comparing this rate with those of the increase in levorotation and viscosity. Measurement of the rate of hydrogen exchange appears to be such a direct experimental approach (A. Hvidt and S. O. Nielsen, to be published). It is the purpose of this paper to describe such experiments and the results obtained.

Methods

Materials.—Throughout this work we have used the purified calfskin tropocollagen which has been previously described (Bensusan and Scanu, 1960). The acetone-dried preparation (2.0 g.) was washed with water and suspended in 200 ml of a solution of 0.8% tetramethylammonium chloride and 6.7×10^{-3} M citrate buffer, *p*H 3.2. The suspension was heated about 10 minutes at 55–60° to ensure the complete conversion of the soluble portion to form gelatin. The gelatin solution was filtered through glass wool, a No. 3 sintered-glass filter and, finally, through a No. 4 sintered-glass filter. The final solution, containing about 1.5 mg of gelatin per ml, was clear. The solution was stored for short periods at 5° until used.

Glass-distilled water was used throughout. The deuterium oxide (99.9% D_2O), obtained from the Savannah River Plant, S. Carolina, was used without further purification. Care was taken to prevent exchange with atmospheric water.

Optical-Rotation Measurements.—Measurements of optical rotation were performed using a Kreis polarimeter (Zeiss) equipped with a mercury lamp and a 546-mμ interference filter. There was no difficulty reading the optical rotation to ±0.01°. A 4-dm cell, equipped with a water jacket, was used to follow the kinetics of optical rotation. Water from a constant-temperature bath was circulated through the jacketed cell. The specific rotation of heated gelatin solutions was calculated using protein concentrations which were obtained by Kjeldahl determination. A value of 17.7% nitrogen in our collagen was used to calculate the protein concentration (Bensusan and Scanu, 1960). At temperatures above the transition temperature, calfskin gelatin had a specific rotation of −165° at 546 mμ in the solvent described (*vide infra*). This value is in excellent agreement with that which can be calculated using the value for $[\alpha]_D$ of −140° for calfskin gelatin (Doty and Nishihara, 1958) and assuming a one-term Drude equation with a λ_0 at 218 mμ (Harrington and Von Hippel, 1961b). When determinations of protein concentration had to be made in the presence of tetramethylammonium chloride, we used the polarimetric method at temperatures greater than 37°, taking $[\alpha]_{546}$ to be −165°.

Viscosity Determinations.—A specially designed constant-volume viscosimeter of the Cannon-Ubbelohde type, having an outflow time for water of 347 seconds at 14.3°, was used to determine the specific viscosity.

Deuterium-Exchange Methods.—The method used for measuring the exchange of peptide hydrogens was a modification of the techniques described by Bryan and Nielsen (1960) and Blout *et al.* (1961). The method takes advantage of the fact that the absorption peak of *N*-monosubstituted transamides at a frequency of 1550 cm^{-1} in the infrared (the amide II peak) shifts to a frequency of about 1450 cm^{-1} (Blout *et al.*, 1961) when the protium atom is replaced by a deuterium atom. Thus, by following the decrease in the amide II peak with time, the rate of the exchange of peptide hydrogens may be measured.

A Perkin-Elmer double-beam, infrared-grating spectrophotometer, Model 221, was equipped with calcium fluoride cells and fitted with thermospacers through which water at 20° was circulated. (The sample space was 0.126 mm thick.) Deuterium oxide was introduced into the reference cell. At zero time, 1 ml of D_2O, containing 70 μl of 0.2 N HCl in D_2O, was added to the approximately 8 mg of lyophilized sample. The final H_2O content of the medium was about 0.3%. After complete solution was obtained, a portion was introduced into the sample cell. Usually less than a minute had elapsed after the addition of D_2O. The recording of the decay in the amide II peak was continued on a 2.4-times-expanded transmission scale for about 30 minutes. A sample curve is given in Figure 1. The spectrum between 1600 and 1505 cm^{-1} was then recorded in duplicate.

An analog computer, with the output attached to the input of an X-Y recorder, was used to determine the kinetics of the small increase in per cent transmission

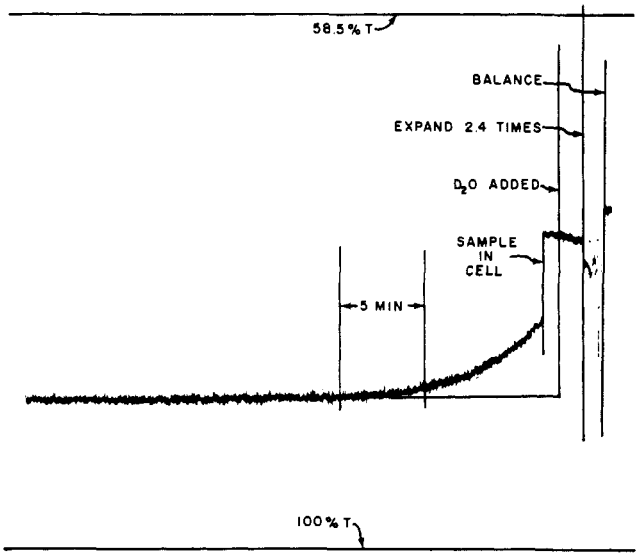

FIG. 1.—A sample curve showing the decay of the amide II band at 1548 cm^{-1} with time. The recording moves from right to left.

at 1548 cm^{-1}. We found that the best fit of our recorded curves could be obtained using a single exponential decay curve (first-order kinetics). We took advantage of the computer to determine the rate and initial amount of these rapidly exchanging hydrogens. However, a graphical solution could be used equally effectively. The semilog plots taken from three recorded curves, selected at random, are shown in Figure 2. The initial values of optical density obtained by the extrapolation back to zero time are in excellent agreement with those obtained using the computer. It should be obvious that this extrapolation back to time zero is necessary, since approximately 1 minute elapses before measurements can be made after the addition of D_2O to the lyophilized protein. The difference between the optical densities at time zero and the final constant value is directly related to the number of rapidly exchanging peptide-group hydrogen atoms. The rate constants for the rapid exchange for all experimental samples, measured during the first 30 minutes, showed a standard deviation of only 7%. Determinations of the amount of rapidly exchanging hydrogens in duplicate and triplicate samples showed a standard deviation of the distribution around the true values of $\pm 0.32 \times 10^{-2}$ OD units/1% protein solution. Since our range of values in all experiments was $2.6\text{–}6.5 \times 10^{-2}$ OD units/1% protein, the range of standard deviations was 12–5%.

The optical density of the amide II peak (1548 cm^{-1}) was calculated from the spectrum curves taken after the rapid exchange was complete (30 minutes). The difference between this optical density and that at 1530 cm^{-1}, the minimum in the spectrum which was insensitive to the extent of H-D exchange, was used as a linear measure of the amount of slowly exchanging hydrogens (not exchanged after 30 minutes). It was assumed that the zero-time sample contained no slowly exchanging hydrogens.

The Gelatin → Collagen Transition.—About 120 ml of the gelatin preparation described above (about 1.5 mg/ml) was heated 5 minutes at 55° to ensure the presence of the random-coil form. While the solution was still warm, triplicate samples of 6.0 ml were removed and lyophilized. These represented the zero-time samples in the deuterium-exchange measurements. The remaining solution was rapidly cooled to 14° in an alcohol–dry ice bath using a prestandardized thermo-

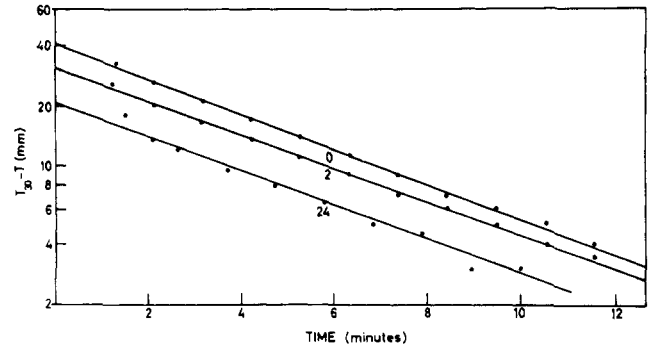

FIG. 2.—Plot of the disappearance of the amide II band as a first-order decay. $T_{30} - T$ is the difference between the per cent T after 30 minutes (assumed to be the same as that after infinite time) and an intermediate time from 0 to 30 minutes.

couple to follow the cooling in the flask. This process required about 15 seconds. The time when the solution reached the required temperature was considered as zero time for the experiment. The precooled polarimeter cell was rapidly filled and polarimeter readings were started soon thereafter. At varying intervals of time, duplicate samples of 6.0 ml of the gelatin were removed from the vessel in the bath and lyophilized for deuterium-exchange measurements. The experiment was terminated after 72 hours.

The amount of rapidly and slowly exchanging hydrogens was determined for each lyophilized sample as described. The amount of each was calculated in per cent of the total number of secondary peptide groups assuming, all of these to contribute equally to the optical density at zero time and in gelatin to exchange completely within 30 minutes. After the exchange reaction was terminated, as much of the sample as possible was collected. The apparent *p*H was measured with a glass-calomel electrode couple and corrected to *p*D by adding 0.44 as described by Mikkelsen and Nielsen (1960). Afterwards, a 0.3-ml sample was diluted with 2.0 ml of water, the solution was heated to 55°, and the optical rotation of the diluted sample was determined using a 2-ml-capacity, 2-dm polarimeter cell. The protein concentration of each sample was then calculated assuming a $[\alpha]_{546}$ of −165°.

The entire experiment described was performed twice. The gelatin solutions were the result of two separate preparations from the same stock of dried, soluble collagen. The only real difference between the two experiments was that in the second experiment the infrared spectrophotometer was flushed with dried air to reduce the interference of water vapor. This improvement resulted in smoother spectrum curves but had no noticeable effect on the determination of the rapidly exchanging hydrogens, since the frequency used was that of an atmospheric-water transmission "window."

Preliminary experiments showed that the lyophilization and subsequent dissolution of the samples at room temperature did not result in the gain or loss of helical configuration as judged by optical rotation measurements. Table I shows the data of these experiments. The values agree within the limits of the experimental error.

Kinetics of the Gelatin → Collagen-Fold Transition.—The change in levorotation with time was followed at four temperatures between 8.2 and 14.2° following the rapid quenching of heated gelatin solutions, as described. The results were plotted on a 0 to 50- and a 0 to 500-minute time scale. The analog computer was programed for all combinations of two reactions,

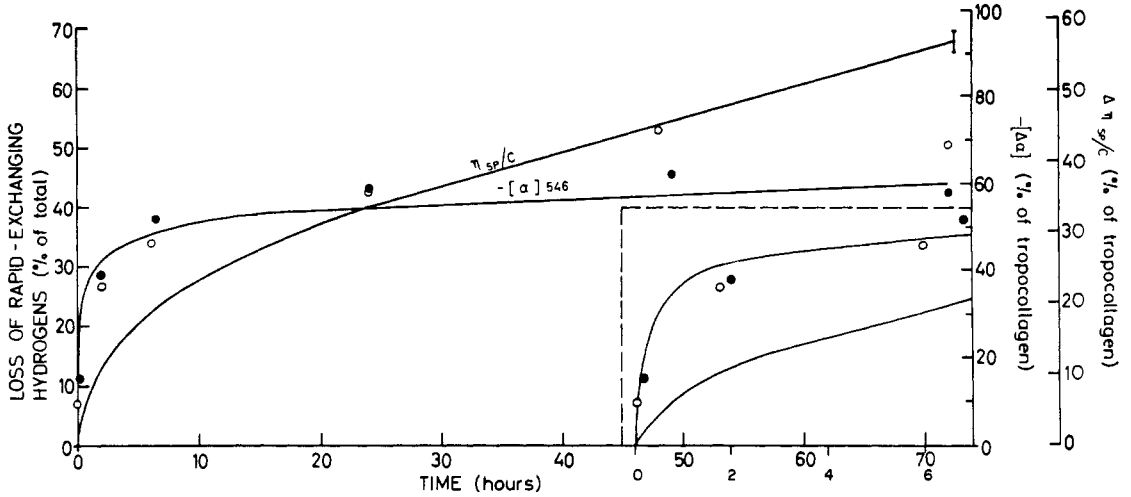

FIG. 3.—The change with time in the amount of rapidly exchanging hydrogens (experimental points) as compared with the change in the optical rotation and specific viscosity (labeled lines). The open and closed circles represent the average of duplicate determinations for two complete experiments. No line is drawn through these points.

TABLE I

THE EFFECT OF LYOPHILIZATION AND RE-SOLUTION OF THE HELICAL AND COILED FORMS OF GELATIN

Sample Type	$-[\alpha]_l$[a]	$-[\alpha]_f$[a]
Coil	165	165
Helix	280	270
Coil	164	160
Helix	340	315
Coil	164	161
Helix	272	261

[a] $-[\alpha]_l$ and $-[\alpha]_f$ are the specific rotation for the two forms of gelatin before and after lyophilization and re-solution.

both first- and second-order, consecutive and simultaneous. The final specific rotation was adjusted to $-355°$, which was the value obtained at all four temperatures. The potentiometers were varied for the initial values of the two reactions, X_0 and Y_0, and their rate constants, k_x and k_y, respectively. Without limitations on these values, we attempted to fit the experimental curves (both time scales) for the eight possible kinetic combinations. The restrictions imposed were (1) that the computer curve must not deviate from the experimental points to an extent greater than the error in reading the polarimeter, (2) that the same kinetics must fit the experimental curves at all four temperatures, and (3) that the potentiometer values must be the same for a repeated attempt to fit the experimental curves in order to demonstrate the uniqueness of the values.

RESULTS

In Figure 3 we have presented the results of duplicate experiments in which the kinetics of the increases in specific viscosity and specific rotation and the decrease in the amount of rapidly exchanging peptide hydrogens are compared. The determined amount of rapidly exchanging peptide-group hydrogen atoms of unquenched gelatin (zero time) represents the 100% value of the number of rapidly exchanging groups (0% loss). The decrease in the number of rapidly exchanging peptide-group hydrogen atoms relative to the zero-time value is plotted against time in Figure 3. For the sake of comparison the curves are normalized at zero time and at 24 hours. It is obvious that the curve relating loss of rapidly exchanging peptide hydrogens with time follows closely the curve of the increase in specific rotation.

The average values from both experiments at 0, 24, and 48 hours were: $-[\alpha]_{546}$, 165, 346, and 362; η_{sp}/C, 1.4, 5.9, and 9.0 dl/g; rapidly exchanging hydrogens, 6.06, 3.36, and 3.14 $\times 10^{-2}$ OD units/1% protein, respectively.

It was deemed necessary to determine whether or not the loss of rapidly exchanging peptide hydrogens could be accounted for by a gain in slowly exchanging peptide hydrogens rather than by a shift in the amide II band. In Figure 4 the results of the correlation between both classes of peptide hydrogens is presented. It is obvious that there is an approximately direct correlation between the loss of rapidly exchanging peptide hydrogens and the gain in slowly exchanging ones. The scatter is mainly due to the fact that our determination of the number of slowly exchanging peptide hydrogens was the less reliable of the two determinations.

The *p*D at which the H-D exchange was determined was 3.77 ± 0.02 and 3.74 ± 0.02 for the first and second experiments, respectively.

The average rate constant for the rapidly exchanging peptide hydrogens was 0.16 ± 0.01 min.$^{-1}$ The calculated half-life is 4.3 minutes. This result is in excellent agreement with the rate constant of 0.144 min^{-1} at *p*D 3.69 which was determined for the exchange of peptide hydrogens in poly-DL-alanine (Bryan and Nielsen, 1960). We have attempted to measure the rate of exchange of the slowly exchanging peptide hydrogens in samples of gelatin after storing at 5° for 24 hours prior to lyophilization. After dissolution in D_2O, *p*D 3.7, the optical density change at 1548 cm^{-1} was followed with time starting 30 minutes after adding the D_2O. We found that there was only about 10% exchange of these hydrogens in 7 hours, indicating a half-life in the order of a few days assuming only one exchange class.

Kinetics of the Gelatin → Collagen-Fold Transition.—Of the eight possible combinations of first- and second-order reactions, only a combination of simultaneous first- and second-order reactions fits the experimental curves well enough to satisfy the criteria outlined under Methods. The values of the kinetic parameters are

TABLE II
THE KINETIC PARAMETERS FOR THE INCREASE IN LEVOROTATION

Temp (°C)	X_0[a] (degrees)	Y_0[a] (degrees)	$k_x \times 10^4$ (deg^{-1} min^{-1})	$k_y \times 10^3$ (min^{-1})	$-[\alpha]_0$[b] (degrees)
8.3	173	14	7.1	2.1	168
10.3	172	16	6.5	2.0	167
12.2	164	30	6.1	2.0	161
14.2	159	34	5.3	1.9	162

[a] X_0 and Y_0 are the contributions to the increase in levorotation with rate constants k_x and k_y for the second- and first-order reactions, respectively. [b] The initial specific rotation, $-[\alpha]_0$, was calculated by subtracting the sum of X_0 plus Y_0 from 355°, the value of $-[\alpha]_\infty$.

listed in Table II. The calculated initial value of optical rotation, as listed in the last column, remains constant for all the temperatures at which measurements were made. However, there is a variation of the relative contribution of the two reactions to the increase in levorotation as the temperature was varied. For this reason, this kinetic expression may also be unworthy o serious consideration (see Discussion).

DISCUSSION

The increase in levorotation in Figure 3 starts to level off after 24 hours at a level corresponding to a recovery of 56% of the levorotation in the original, native tropocollagen solution. After 24 hours the intrinsic viscosity has increased to 33% of the intrinsic viscosity of the original tropocollagen solution and this value is nearly doubled after 72 hours. The loss of rapidly exchanging peptide-group hydrogen atoms (exchange completed within 30 minutes) is of the same order of magnitude as the number of amino acid residues helically coiled and correlates well with the increase in levorotation during the first 72 hours after quenching (Fig. 3). The number of rapidly exchanging peptide-group hydrogen atoms is calculated under the assumption that all rapidly exchanging peptide-groups make the same contribution to the recorded optical density at 1548 cm^{-1}. This assumption is only approximately valid and is discussed below. Taking the increase in levorotation to be proportional to the number of amino acid residues involved in helical coiling of the collagen type, whether single-stranded or triple-stranded (Harrington and Von Hippel, 1961a), it may be concluded that, whatever conformational processes are responsible for slowing down the peptide-group hydrogen exchange, these processes must take place simultaneously with or rapidly after the helical coiling of sections of the polypeptide chains.

Mechanisms of Hydrogen Exchange in Quenched Gelatin Solutions.—It is important to emphasize that our observations have been limited to the H-D exchange of the imide groups of the peptide backbone. It is only necessary, therefore, to confine the discussion of the results to those factors which influence the rate of exchange in these groups.

Interpeptide-group hydrogen bond formation in aqueous solutions of helical poly-α-L-glutamic acid has been found to decrease the rate of peptide-group hydrogen exchange at least 100 times relative to the corresponding rate of hydrogen exchange in randomly coiled polypeptides (Bryan and Nielsen, 1960; Blout *et al.*, 1961). The extent to which the exchange rate is decreased by interpeptide-group hydrogen-bond formation depends on the total work required to break the hydrogen bond (A. Hvidt and S. O. Nielsen, to be published). The disappearance of rapidly exchanging peptide-group hydrogen atoms and the concomitant appearance of slowly exchanging hydrogen atoms during the gelatin → collagen-fold transition (Fig. 4) might thus be ascribed to the formation of a number of stable interpeptide-group hydrogen bonds during the transition, e.g., the interchain cooperative hydrogen bonds in the collagen triple-helix. However, before this interpretation of the hydrogen-exchange results can be accepted, it is necessary to examine other possible causes of the observed loss of rapidly exchanging peptide-group hydrogen atoms.

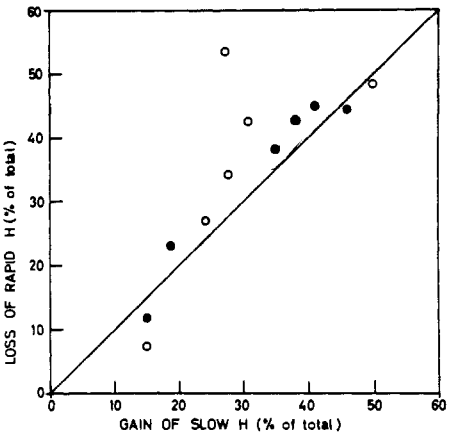

FIG. 4.—The correlation between the loss of rapidly exchanging hydrogens and the gain in slowly exchanging hydrogens during the gelatin → collagen-fold transition. The open and closed circles are from two different experiments as explained in the legend of Fig. 3.

In the quenched gelatin solutions of the present work (*p*D 3.77 and 3.74, respectively) the peptide-group hydrogen atoms were divided into two classes, one in which the exchange proceeded with a uniform rate constant 0.16 min^{-1} (exchange completed within 30 minutes) and the other in which the exchange was much slower (not exchanged after 30 minutes) with estimated rate constants less than 1 day^{-1}. The exchange data could be accounted for by varying the number of peptide-groups in these two classes and no indication was found of peptide-groups exchanging with intermediate rate constants.

The immediate molecular environment of the rapidly exchanging peptide-group hydrogen atoms in quenched gelatin solutions evidently cannot change much after quenching the initial, randomly coiled (Boedtker and Doty, 1954) gelatin solution. As the polypeptide chain in a poly-L-proline II–type helix is fully extended, it follows that all peptide-group hydrogen atoms in a single poly-L-proline II–type helix and all those hydrogen atoms not involved in interchain hydrogen bonds in a collagen II–type triple helix should exchange rapidly at approximately the same rate as observed in gelatin. Poly-L-proline II in aqueous solution is stabilized by interaction with the solvent (Steinberg *et al.*, 1960). Various schemes for hydrogen bonding of water to poly-L-proline II helices and collagen have been suggested (Harrington and Von Hippel, 1961b; Berendsen, 1962) as well as ways of packing water molecules in more or less regular arrangements around hydrophobic groups in proteins (Klotz, 1960). From what is known about such geometrical arrangements of water molecules around peptide groups exposed to bulk solvent they are expected to influence only slightly the rate of hydrogen exchange (A. Hvidt and S. O. Nielsen, to be published). Formation of labile interpeptide-group hydrogen bonds will decrease

the rate of hydrogen exchange by at most a factor equal to the fraction of peptide-groups not involved in interpeptide-group hydrogen bonding. Considering the small stability of such bonds in aqueous solutions (Schellman, 1955; Klotz and Franzen, 1960) formation of noncooperative labile interpeptide-group hydrogen bonds cannot explain the more than 1000-fold reduction in rate of hydrogen exchange observed in the present work.

Hydrogen exchange in protein solutions is generally characterized by a much reduced rate of exchange compared with that of randomly coiled polypeptides (Linderstrøm-Lang, 1958). As discussed elsewhere, there are reasons to believe that practically all slowly exchanging hydrogen atoms in protein solutions are located in peptide groups (A. Hvidt and S. O. Nielsen, to be published). The reason for the slow exchange rate is that the hydrogen atoms involved are removed from free contact with bulk solvent water either by being involved in strong hydrogen bonding, as e.g., in an α helix, or by being forced to occupy a predominantly hydrophobic region within the envelope of the protein molecule. In the present work two molecular structures have been visualized to explain the observed slow rate of hydrogen exchange in quenched gelatin solutions. The first is the collagen-type triple helix with cooperative interchain hydrogen bonds, and the second is the aggregates formed by lateral association of collagen-type triple helices extending over the whole or part of the individual polypeptide chains. It is unknown to what extent these aggregates will affect the rate of exchange of those peptide-group hydrogen atoms that exchange rapidly in a nonaggregated collagen-type triple helix. The rate of hydrogen exchange could conceivably be reduced 1000-fold or more by this aggregation. There is no clear indication of other molecular structures than those mentioned in quenched gelatin solutions that could account for the observed slow rate of hydrogen exchange. As a matter of fact, the number of contacts in gelatin gels are relatively few (Ferry, 1948; Harrington and Von Hippel, 1961b) and the contact points themselves behave as small crystallites, melting sharply and dissociating slowly (Boedtker and Doty, 1954; Flory and Garrett, 1958). Gelatin gels formed rapidly by cooling hot solutions develop on aging the low-angle X-ray diffraction pattern and infrared spectrum characteristic of native collagen (Smith, 1919; Ferry and Eldridge, 1949; Katz *et al.*, 1931; Bradbury and Martin, 1952).

Next it is necessary to consider how many triple helices and aggregates of these are formed as a result of the lyophilization and subsequent dissolution in D_2O. Tetramethylammonium chloride (0.8%) was included in the solutions of parent gelatin in order to facilitate the dissolution in D_2O, and the specific levorotation of a quenched gelatin solution was found to be unaffected by lyophilization and subsequent dissolution in D_2O. Although nonspecific chain interactions, e.g., aggregation of triple helices, as a result of the lyophilization are conceivable, we consider it doubtful that stereospecific interactions such as the association of single helices to produce triple helices occur under the conditions of reduced chain mobility prevailing during the lyophilization and the subsequent dissolution. We are thus led to conclude from Fig. 3 that the helical coiling of the peptide chains giving rise to the increase in levorotation in quenched gelatin solutions is followed rapidly by the formation of a considerable and proportional number of strong hydrogen bonds presumably of the type found in the collagen-type triple helix. This conclusion is in accordance with the mechanism for the gelatin → collagen-fold transition proposed by Flory and Weaver (1960) and contradicts the mechanism proposed by Harrington and Von Hippel (1961a,b). These authors proposed a stable, long-lived single-chain helix of the poly-L-proline II type as an intermediate in the gelatin → collagen-fold transition. As discussed previously, an extended single helix of the poly-L-proline II type is expected to exchange its peptide-group hydrogen atoms rapidly under the experimental conditions of this work. It should be noted that we have found that a polymer of glycine and proline with a reported glycine-proline ratio of 1:1.876 and a molecular weight of 4090 exchanges all its hydrogen atoms rapidly under these same experimental conditions. Copolymers of glycine and proline have been reported to be not completely random (Katchalski *et al.*, 1963) and it is unknown how many glycine residues are involved in the poly-L-proline II–type helix.

Recently, it has been suggested by Klotz and Frank (1962) on the basis of observed general acid and base catalysis of *N*-methylacetamide hydrogen exchange in solutions of 5 M D_2O in dioxane that it is likely that measurements of rates of protein-hydrogen exchange reflect more the state and configuration of acidic and basic side-chain groups in the local environment of the exchanging peptide groups than the overall conformation of the protein molecule. If this suggestion were correct, the interpretation of the present hydrogen-exchange data would not be valid. As they stand the results of Klotz and Frank have clearly demonstrated that measurements of protein-hydrogen exchange in predominantly nonaqueous solvents must be interpreted with great care. The reported general acid and base catalysis in peptide-hydrogen exchange (Klotz and Frank, 1962) all refer to *N*-methylacetamide dissolved in 5 M D_2O in dioxane, and the suggestion was made that general acid and base catalysis also would be of major importance in a predominantly aqueous solvent. We believe that there is no indication that side-chain groups have any catalytic influence in our system. The rate constant for the rapid exchange was remarkably uniform throughout the transformation despite the marked configurational change. The rate of the exchange in gelatin, which has many acidic and basic side chains, was also shown to be the same as that for poly-DL-alanine at the same *p*D and temperature. In addition, the difference in rate between the rapid and slow exchange in the present work was two orders of magnitude greater than the difference in exchange rate in the absence and presence of imidazole demonstrated by Klotz and Frank in their model system. Using a stopped-flow apparatus in conjunction with measurements of infrared–amide II–band absorption, no general acid and base catalysis was observed in 0.1 M aqueous buffers containing phosphate, acetate, citrate, Tris, and imidazole in the *p*D range 3–7 (S. O. Nielsen, to be published; C. V. Linderstrøm-Lang and S. O. Nielsen, to be published).

Bradbury *et al.* (1958) studied the hydrogen exchange of native and denatured collagen fibrils in thin films moistened by a D_2O atmosphere of 100% humidity. The exchange was followed by an infrared-spectrophotometric method in the 3-μ region, and three classes of groups carrying labile hydrogen atoms were distinguished on the basis of their exchange rates. In the first class, believed to consist of partially degraded material, the exchange was over after 10 minutes; in the second class, believed to comprise side chains and peptide-groups not involved in interchain hydrogen bonding, the exchange was concluded after 1 hour; and in the third class, believed to consist of peptide-groups involved as donors in interchain hydrogen bonding, the exchange required 30 days to run to com-

pletion. Recently, Englander and Von Hippel (1962), using the tritium-exchange method of Englander (1963), have briefly reported that helix formation in quenched gelatin solution monitored as a progressive increase in levorotation is accompanied by a parallel increase in the number of slowly exchangeable hydrogen atoms. Their H-T-exchange results thus are in qualitative agreement with those of the present H-D-exchange experiments.

Mechanism of the Gelatin → Collagen-Fold Transition. —The gelatin → collagen-fold transition, as followed polarimetrically, is unique in yielding a triple-stranded product in an apparent unimolecular reaction. The specific rate of increase in levorotation during the gelatin → collagen-fold transition is independent of the gelatin concentration over a wide range. At gelatin concentrations greater than 1% the specific rate is, however, dependent on gelatin concentration (Smith, 1919). An increase in the number of interchain linkages as found in β- and γ-gelatins facilitates the formation of the collagen fold (Engel, 1962b). The gelatin → collagen-fold transition does not run to completion in mixtures of α- and β-gelatin, whereas γ-gelatin rapidly and almost completely re-forms native tropocollagen (Veis and Cohen, 1960; Altgelt *et al.*, 1961). Drake and Veis (1964) have recently investigated the rate of formation of the collagen fold in α- and γ-gelatin, derived from the same source of ichthyocol, using formaldehyde as cross-linking agent. They found that the rate of rotation recovery at 15° was about 30-fold greater for the γ-gelatin than for the α-gelatin, with an apparent activation energy of -5 to -10 kcal/mole for the refolding of γ-gelatin in contrast to the -35 kcal/mole reported for ichthyocol gelatin, which is mostly in the α-form (Harrington and Von Hippel, 1961a,b). The increase in specific levorotation for both the α- and γ-gelatin was found to follow second-order kinetics with respect to the amount of gelatin remaining in the unfolded configuration, whereas Altgelt *et al.* (1961) reported the increase in specific levorotation of a γ-gelatin derived from natural sources to follow more closely first-order kinetics. Drake and Veis (1964) concluded that the role of the cross-links in γ-gelatin in securing rapid and almost complete renaturation is to keep the individual polypeptide chains in register.

Two steps have been demonstrated in the denaturation of soluble collagen (Engel *et al.*, 1962; Engel, 1962a,b). In the first step, which was accomplished after heating the solution of native collagen 11 minutes to slightly above its denaturation temperature, the decrease in levorotation was essentially completed but the three component chains were still connected. In the second step, completed after longer periods of heating, the component chains dissociated. The gelatin → collagen-fold transition proceeded at a higher rate and more completely in tropocollagen solutions which had been only partially denatured (step 1) than in completely denatured solutions (step 2). Partially denatured solutions also gave fibrils and segments indistinguishable in appearance and quantity from those precipitated from native solutions, whereas completely denatured solutions gave low yields of ill-defined fibrils and segments.

The present H-D-exchange results fall in line with other contraindications of the mechanism proposed by Harrington and Von Hippel. Thus it has been pointed out that their single-stranded, helical intermediate gains stability only at temperatures well below the melting temperature of native collagen (Flory, 1960). The stable configuration of proline sequences in polypeptide chains has been shown to be only partly helical in aqueous solution when the number of consecutive proline residues is less than 6 (Yaron and Berger, 1961). Harrington and Von Hippel proposed that the rate-determining step leading to the observed increase in levorotation in quenched gelatin solutions is the linear growth of helical sections along the single polypeptide chains starting from rapidly formed helical "nuclei." This helical coiling of single chains must therefore proceed with the observed large negative enthalpy of activation in contrast to the analogous *cis'-trans'* mutarotation in poly-L-proline, in which the measureably slow part of the isomerization around C(α)-C bonds proceeds with an enthalpy of activation of +20 kcal/mole. High concentrations of urea prevent the increase in levorotation of quenched gelatin solutions (Harrington and Von Hippel, 1961a) whereas urea further stabilizes poly-L-proline II helices (Fasman and Blout, 1963).

The mechanism proposed by Flory and Weaver (1960) is supported by the present H-D-exchange results. It further succeeds in explaining the observed temperature dependence of the enthalpy of activation $\Delta H^{\ddagger}$ of the gelatin → collagen-fold transition, which is suggestive of nucleated crystallizations (Flory and Weaver, 1960):

$$\Delta H^{\ddagger} = -(\text{constant})^2 \times (T_m - T)^{-1} \qquad (1)$$

where T_m is the denaturation temperature of native collagen. The mechanism provides a *rate-determining* reaction which is truly unimolecular, and therefore predicts that the levorotation should increase during the gelatin → collagen-fold transition in a manner that follows first-order kinetics not only with respect to initial gelatin concentration but apparently also with respect to the amount of gelatin (eventually to be built into the collagen fold) that remains in the unfolded configuration. This latter inference is contrary to what is generally observed; the increase in specific levorotation usually follows approximately second-order kinetics. It has also been pointed out that the mechanism proposed by Flory and Weaver predicts a concentration-dependent specific rate of the gelatin → collagen-fold transition at so low concentrations of gelatin that the lateral association of single helices to triple helices is no longer a rapid process compared with the reversion of single helices to random coils (Harrington and Von Hippel, 1961b). It is therefore interesting that Piez and Carrillo (1964) recently have shown that the specific rate of collagen-fold formation with purified preparations of rat-skin α1- or α2-gelatin varies with gelatin concentration in the range below 1.5 mg/ml. Collagen-fold formation is accompanied by only a 2-fold increase in molecular weight at a concentration of 0.24 mg/ml. At this low concentration β_{12}-gelatin forms helices without an increase in molecular weight and at a concentration-independent specific rate.

Drake and Veis (1964) have pointed out that it might be useful to consider intramolecular collagen-fold formation involving cross-linked chains. A similar proposal was briefly made by Bensusan and Nielsen (1963). It should be noted, however, that if the intramolecular formation of either double- or triple-stranded helices does not occur *after* the rate-determining step in the gelatin → collagen-fold transition, the specific rate of collagen-fold formation cannot be expected to be independent of gelatin concentration over as wide a concentration range as observed. This conclusion seems unavoidable considering the increase in molecular weight during the formation of collagen fold (Engel, 1962b). The special case of intramolecular collagen-fold formation corresponding to a gradual linear growth of triple helices is ruled out because it leads to a rate of

collagen-fold formation proportional to $T_m - T$ (Flory and Weaver, 1960).

We shall attempt to extend the mechanism proposed for the gelatin → collagen-fold transition by Flory and Weaver in order to deal also with the effects of interchain cross-linkages and with the approximate second-order kinetics of collagen-fold formation with respect to the amount of gelatin remaining in the unfolded configuration. Flory and Weaver (1960) represented the processes involved in the gelatin → collagen-fold transition as

$$C \underset{k_1}{\overset{k_1'}{\rightleftharpoons}} I \underset{(k_2)}{\overset{k_2'}{\rightleftharpoons}} (1/3)H \qquad (2)$$

where C, I, and H represent, respectively, the random coil, the proposed intermediate single helix, and the collagen-type triple helix. It is this formation of the collagen-type triple helix that is responsible for the observed conversion of rapidly exchanging peptide hydrogens to slowly exchanging ones as shown in Figure 3. It was assumed that k_2' was so much larger than k_1 that the rate of lateral association of single helices I to form the triple helix H did not influence the over-all rate of the reaction C → (1/3)H. The sections of individual polypeptide chains involved in (2) constitute only a small fraction of one α-gelatin chain. Therefore, to build a total of more than half the amino acid residues into triple helices in quenched gelatin solutions, (2) must be followed either by gradual linear growth of already formed triple helices or (2) must repeat itself a number of times coiling different sections of the chains involved. The first alternative cannot be accepted. First, if the linear growth of triple helices is very fast relative to the rate of (2), the over-all rate of triple-helix formation will follow approximately first-order kinetics with respect to the amount of gelatin still remaining in the unfolded configuration. Second, if the rate of linear growth is slow relative to (2), the rate of triple-helix formation will be proportional to $T_m - T$ and not exponential in $-(T\Delta T)^{-1}$ as observed (Flory and Weaver, 1960; Von Hippel and Wong, 1963). On the other hand, if scheme (2) is repeated a number of times to produce the final helical coiling, different sections of the chains may not be expected to form triple helices with the same ease. The respective rates of triple-helix formation might conceivably distribute themselves in such a way as to approximate second-order kinetics for the over-all rate of triple-helix formation. A 10-fold variation in rates is sufficient to reproduce the observed kinetics. The frequently observed second-order kinetics (including the present investigation) for the rate of increase of specific levorotation is found to be only approximately obeyed, and in some systems even apparent first-order kinetics are observed (Altgelt *et al.*, 1961). The variation in rate of triple-helix formation among various chain sections to be helically coiled is not inconsistent with the pronounced variation in the distribution of polar amino acid residues, and of proline and hydroxyproline along native collagen triple helices (see the review by Harrington and Von Hippel, 1961b).

The mechanism proposed for the gelatin → collagen-fold transition involving the triple-helical coiling according to scheme (2) of a large number of chain sections at somewhat different rates also affords an interpretation of some of the effects of interchain cross-links on the kinetics of the gelatin → collagen-fold transition. Thus, the almost complete reformation of the native collagen fold in quenched solutions of γ-gelatin or partially denatured collagen can be understood from the plausible assumption that the triple-helical coiling of sections of the three component chains according to (2) is preferred in the case that the section of triple-helix formed constitutes a continuation of an already existing triple helix in which all three chains are in register (*vide infra*). Once the first stable triple-helical section with the three chains in register has been formed (initiation) within a γ-gelatin molecule according to (2) (presumably within an apolar-, pro-hypro-rich region), the triple helix will grow in consecutive steps according to (2). From the arguments underlying (2) (Flory and Weaver, 1960), some of the propagation reactions (in the apolar regions), adding triple-helical sections to the growing parent helix, are predicted to proceed at rates that are fast relative to the initiation step. Other propagation reactions should, on the other hand, be much slower if they involve chain sections that are hard to coil, i.e., sections that form triple-helical coils of low inherent stability. Such hard-to-coil regions of the polypeptide chains are found in the numerous polar regions which are distributed in an irregular fashion along the length of the native tropocollagen triple helix (Schmitt *et al.*, 1955). In addition, there are reasons to believe that the polar and apolar regions of the native triple helix are in register in the sense that all three chains have polar amino acid residues within every polar region of the protofibrillar particle (Harrington and Von Hippel, 1961b). We are therefore led to suggest that the over-all rate of collagen-fold formation in quenched solutions of γ-gelatin and partially denatured collagen is determined mainly by the rate of propagation of the collagen triple-helical fold through the polar regions along the polypeptide chains.

The same interactions that favor triple-helix formation by adding helical sections on to already existing triple helices in γ-gelatin relative to other *intra*molecular mechanisms of triple helix formation also naturally suppress *inter*molecular triple-helix formation. These interactions are to be understood in terms of the imperfections in the secondary bonding and arrangement of the three chains at each end of a triple helix where the chains traverse the "interphase" between helix and random coil (Flory and Weaver, 1960). These imperfections give rise to a positive excess free energy of the triple helix which must be compensated in order to produce a stable triple helix from random coils. On the other hand, the excess free energy does not present much difficulty for the addition of helical sections to an already existing triple helix.

For the considerations above to be pertinent, it is necessary that, within a γ-gelatin molecule after quenching, the first triple-helix section that is formed has its three chains in register. That this is most likely the case when interchain cross-links are present is consistent with reaction scheme (2) according to which triple-helix formation is favored kinetically (and thermodynamically) in the presence of interchain cross-links quite close to the ends of the triple-helix section, H, to be formed.

Turning to the gelatin → collagen-fold transition in mixtures of α- and β-gelatin, the first triple-helix section formed along a single chain following quenching cannot in these systems be assumed to have its three component chains in register. We now further propose that the propagation of an initially formed stable triple-helix section through the polar regions of the *individual* chains will be much more difficult when the chains are not in register than in γ-gelatin. Tropocollagen consists of two $\alpha 1$ and one $\alpha 2$ chains of different amino acid composition (Piez *et al.*, 1963). The ready triple-helical coiling of the polar regions in γ-gelatin may be due in part to electrostatic interchain interactions. The growth of an initially formed triple-helix section

in mixtures of α- and β-gelatin will then stop rapidly according to the above proposition and additional triple-helix formation will occur by lateral association of single helices by scheme (2) in other more remote parts of the chains. In this way there will be randomized association of chain segments to form triple-helix sections at a specific rate that is independent of gelatin concentration according to (2). At higher gelatin concentrations this will lead to increasing molecular weight. The recovery of specific levorotation following quenching will be smaller than with γ-gelatin because the polar regions in the individual chains are largely unfolded. Drake and Veis (1964) found the specific rate of collagen-fold formation at 15° in γ-gelation and in mixtures of α- and β-gelatin, respectively, derived from the same source of ichthyocol, to have the ratio 30:1. Thus it would appear that with γ-gelatin the linear growth of triple helices in a stepwise manner according to (2) actually is able to compete efficiently with randomized association of chain segments to form stable triple-helix sections. The different character of the mechanism of triple-helix formation with γ-gelatin and mixtures of α- and β-gelatin is brought out by the remarkably different apparent enthalpies of activation $\Delta H^{\ddagger}$ in the two systems. Drake and Veis (1964) found $\Delta H^{\ddagger}_{\gamma} = -10$ kcal/mole for γ-gelatin under experimental conditions where $\Delta H^{\ddagger}_{\alpha}$ was given as approximately -34 kcal/mole for the corresponding mixture of α- and β-gelatin. The difference $\Delta H^{\ddagger}_{\alpha} - \Delta H^{\ddagger}_{\gamma}$ is almost matched by $T(\Delta S^{\ddagger}_{\alpha} - \Delta S^{\ddagger}_{\gamma})$, $\Delta S^{\ddagger}_{\alpha}$ and $\Delta S^{\ddagger}_{\gamma}$ being apparent entropies of activation obtained with $\alpha + \beta$- and γ-gelatin, respectively. These large differences in activation parameters are too large to be accounted for on the basis of decreased configurational-chain entropy following the formation of interchain cross-linkages in γ-gelatin, and indicate a different nature of the triple helix producing reactions in the two systems.

In very dilute solutions of purified preparations of rat-skin α1-, α2-, or β_{12}-gelatin, collagen-fold formation takes place in a particle with the molecular weight of β_{12}-gelatin (Piez and Carrillo, 1964). On heating of the quenched gelatins the residue optical rotation is reached at a temperature 4° below the temperature where native collagen is completely melted. The width ΔT of the melting transition is also smaller than that usually observed with quenched mixtures of α-, β-, and γ-gelatin from natural sources. The explanation advanced by Piez and Carrillo is that double helices rather than triple helices are responsible for the observed stable collagen fold. Although this proposal of double helices deserves serious consideration it should be pointed out that the melting-point depression of a double helix relative to a triple helix should be considerably larger than 4°. This melting-point depression is of the order of that observed by degrading gelatin enzymatically (Von Hippel and Wong, 1963). It is also of the order of magnitude of the increase in denaturation temperature observed after introduction of cross-linkages in tropocollagen (Veis and Drake, 1963). It would appear that the melting point depression of 4° observed by Piez and Carrillo could also be readily explained by assuming a triple-helical collagen fold. Molecular models of collagen II (*vide infra*) make it unlikely that triple helices can be formed with one antiparallel chain. A triple-helical collagen fold that forms intramolecularly in, e.g. β_{12}-gelatin, should therefore, on the average, involve a greater loss of configurational entropy than that observed for collagen-fold formation in the ordinary more concentrated solutions of mixtures of α- and β-gelatin in which chain-loop formation is not a prerequisite for the formation of triple helices.

Nature of the Collagen Fold.—Taking the measured value of a 56% recovery of specific levorotation (Fig. 3) to mean a similar fractional recovery of triple-helical collagen fold after 24 hours, it is possible to calculate the per cent of the peptide-group hydrogen atoms which would be involved in interpeptide-group hydrogen bonding. In the Rich-Crick collagen model one-third of the total number of residues have their peptide-group hydrogen atoms involved in interchain hydrogen bonding, while in the Ramachandran model approximately two-thirds are so bonded. Taking into account that there is only a 56% recovery of triple-helical structure and that there is a total of 232 proline and hydroxyproline residues per 1000 total amino acid residues in calfskin collagen (Piez and Gross, 1960), an expected loss of 24 and 45% of the rapidly exchanging peptide-group hydrogen atoms after 24 hours can be calculated for the two respective collagen models. It is assumed in the calculation that all nonrapidly exchanging peptide-group hydrogen atoms are involved in interchain hydrogen bonding of the type found in the collagen-type triple helix. The value of 45% based on the Ramachandran model is in good agreement with the figure of 42% found for the loss of rapidly exchanging peptide-group hydrogen atoms after 24 hours (Fig. 3).

Although the figure for the loss of rapidly exchanging peptide-group hydrogen atoms predicted on the basis of the Rich-Crick model is some 40% lower than the experimentally found values in Figure 3, we do not feel that the Rich-Crick model should be dismissed on the basis of the present hydrogen-exchange data, which are quantitatively inconclusive mainly on two accounts: (1) It is unknown how many aggregates of laterally associated triple-helical sections are present in the quenched gelatin solutions after lyophilization and subsequent dissolution in D_2O. As already mentioned, lateral association of triple helices may increase the number of slowly exchanging peptide-group hydrogen atoms beyond the number involved in interchain hydrogen bonding. (2) The use of the same molar extinction coefficient for all peptide groups at 1548 cm^{-1}, as has been done in Figures 3 and 4, is not strictly correct. As a rule an infrared-vibration band suffers changes in the total oscillator strength, center frequency, and band shape when the molecular environment of the oscillator is changed. Hydrogen bonding is of special importance but no general theory is as yet available for calculating the effects on absorption bands of the interactions of an infrared oscillator with its molecular environment. For a recent review see the paper by Thompson (1963). Bradbury *et al.* (1958) compared infrared-absorption spectra from films of native and heat-denatured collagen and found the amide II band to be broadened and displaced toward lower frequencies following denaturation. Thus the equality between the gain of slowly exchangeable hydrogen atoms and the loss of rapidly exchangeable hydrogen atoms during the gelatin → collagen-fold transition (Fig. 4) is possibly coincidental. The same remark can be applied to the finding of Bradbury *et al.* (1958) that the number of very slowly exchanging hydrogen atoms in rat-tendon collagen appears to be roughly equal to the number of interchain hydrogen bonds in the Rich-Crick collagen model. These authors assumed proportionality to exist between the number of –CONH– groups present and infrared-band intensities in the 3-μ region. It should, however, be noted that if the variation of molar extinction coefficients at 1548 cm^{-1} among the peptide groups during the gelatin → collagen-fold transition is taken into account in Figure 3, the only effect will be to replace the scale on the left with another equidistant scale measuring the loss of rapidly exchang-

ing hydrogen atoms which does not affect the basic conclusion of the results.

Formation of triple helices during the gelatin → collagen-fold transition with one chain axis running antiparallel to the other two has been considered (Bensusan and Nielsen, 1963). In polyglycine II such an arrangement with one inverted chain is very likely possible (Crick and Rich, 1955) without violating the standard distances given by Corey and Pauling (1953). Wire models of collagen II, made in this laboratory, have demonstrated that one whole chain in native collagen cannot be inverted without either diminishing the number of interchain hydrogen bonds or violating the standard Corey-Pauling atomic distances.

Information about the stabilization of the collagen fold in aqueous solution can be obtained by considering the exchange mechanism of the slowly exchanging peptide-group hydrogen atoms. Preliminary experiments during the present investigation indicate that the exchange rate of the slowly exchanging peptide-group hydrogen atoms observed is considerably less than 1 day^{-1}. Under similar experimental conditions (D_2O solvent, pD 3.75, 20°) gelatin exchanges all its peptide-group hydrogen atoms with a rate constant of 0.16 min^{-1}. Therefore the involvement of a peptide-group hydrogen atom in interchain hydrogen bonds of the triple-helical collagen-fold type decreases its rate of exchange by a factor of approximately 1000. This figure is in rough agreement with the exchange of Bradbury *et al.* (1958). It has been argued elsewhere (A. Hvidt and S. O. Nielsen, to be published) that rates of slow hydrogen exchange in protein solutions can always be correlated with macromolecular transconformational changes taking place in order for the slow exchange to occur. This statement applied to collagen is equivalent to neglecting the rate of exchange of interchain hydrogen-bonded hydrogen atoms in a triple helix that never is allowed to alter its conformation. For collagen this exchange mechanism involving transconformational changes can actually be deduced also from another reasoning. Bradbury *et al.* (1958) found the complete deuteration of peptide groups in collagen films exposed to D_2O of 100% humidity to require 30 days whereas the back exchange of the fully deuterated collagen film following re-exposure to H_2O lasted less than 24 hours. This difference in exchange rates was interpreted as being owing to the lower vibrational-energy levels of bonded D_2O compared with H_2O. The kinetic isotope effect of 30 exceeds, however, the H-D isotope effects which have been reported for a number of simple reactions (Bigeleisen and Wolfsberg, 1958) including hydrogen exchange in aqueous solutions of *N*-methylacetamide and poly-DL-alanine (Nielsen, 1960; Bryan and Nielsen, 1960). Rather, the large-isotope effect is to be interpreted in terms of a temporary local "denaturation" of the triple helix in order for the exchange to proceed with a rate constant of approximately 0.16 min^{-1} (A. Hvidt and S. O. Nielsen, to be published). The large-isotope effect is consistent with the considerable length of the triple helix that must be distorted in order to expose a "buried" hydrogen atom to bulk solvent.

The reaction sequence producing exchange of the slowly exchanging hydrogen atoms involved in interchain hydrogen bonding can be written:

$$\mathrm{H} \underset{k_4'}{\overset{k_4}{\rightleftharpoons}} \text{intermediate} \xrightarrow{k_3} \text{exchange} \qquad (3)$$

The over-all rate of exchange R is given by

$$R = k[\mathrm{H}] = k_3[\mathrm{I}] = [\mathrm{H}]\frac{k_3 k_4}{k_3 + k_4'} \qquad (4)$$

where $k_3 \leq 0.16\ min^{-1}$, and $k/k_3 \geq 0.001$ by experiment. It follows from (4) that the equilibrium constant $k_4/k_4' \geq 0.001$. The intermediate in (3), responsible for the slow hydrogen exchange, is thus only moderately unstable ($\Delta f \approx 4$–5 kcal/mole) relative to the undistorted triple helix. This fact limits the candidates for the intermediate in (3), ruling out uncoiling of the triple helix from the ends and very likely also the lateral separation of the three individual helices in a section of the triple helix. Instead, the intermediate must be regarded as the product of the least-endergonic transconformational reaction that exposes an interchain hydrogen-bonded hydrogen atom to bulk solvent. Whatever its nature may be, it has fewer interchain hydrogen bonds than H. It may possibly be a section of the triple helix in which all hydrogen bonds between two of the chains are broken while all other interchain hydrogen bonds are intact. Such a distorted triple helix would have almost the same hydrodynamic rigidity as an intact, fully hydrogen-bonded triple helix, and its only moderate instability should not affect the hydrodynamic behavior of tropocollagen.

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Hydrophobic Interactions in Proteins: Conformation Changes in Bovine Serum Albumin below *p*H 5*

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The effect of *p*H, temperature, and partial pressure on the solubility of butane and pentane in solutions of bovine serum albumin has been studied. The binding of alkanes to bovine serum albumin is very sensitive to the conformation of the protein. The so-called *F* form, produced, in 0.15 M NaCl, in a single step around *p*H 4.1 marked by sharp but small changes in $[\alpha]$ and $[\eta]$, binds only one-fourth as much butane and one-fifth as much pentane as native bovine serum albumin. In perchlorate solutions, the solubility reduction, like the changes in $[\alpha]$, occurs in two stages. The temperature dependence of the solubility indicates that butane is bound directly to some of the hydrophobic regions of bovine serum albumin, and the pressure dependence indicates that these regions are large. A variety of considerations leads to the conclusion that the binding sites are inside the bovine serum albumin molecule (i.e., most, if not all, the apolar side chains of these regions are not in contact with solvent). These data support Foster's model of bovine serum albumin: these regions, formed by the interaction between the hydrophobic surfaces of several substructures, would be disrupted when these substructures, without much internal rearrangement, separate to make the *F* form.

The role of hydrophobic interactions in proteins has come under increasing scrutiny in recent years, from a variety of theoretical and experimental points of view. In this laboratory the emphasis has been on studies of the solubility of the shorter (C_2 to C_5) *n*-alkanes in protein solutions as perhaps the most direct way of investigating these interactions. We found that the binding of ethane, propane, and butane to BSA[1] and human hemoglobin was quite strong (Wishnia, 1962); observed enthalpies, and, for dodecylsulfate micelles (Wishnia, 1963a), entropies also, agreed with theoretical predictions (Kauzmann, 1959; Némethy and Scheraga, 1962). Moreover, the early studies of BSA, hemoglobin, and lysozyme (Wishnia, 1962) suggested that the specific behavior of these proteins was a reflection of their characteristic structural features, so that alkane-binding provided a new means to investigate such features. Subsequent work on hemoglobin and myoglobin, on ribonuclease (A. Wishnia and T. W. Pinder,

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[1] Abbreviations used in this work: BSA, bovine serum albumin; SDS, sodium dodecylsulfate.