*REAPPEARANCE OF CERTAIN STRUCTURAL FEATURES OF NATIVE COLLAGEN AFTER THERMAL TRANSFORMATION**

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Communicated by Paul J. Flory and read before the Academy, April 25, 1960

The banded structure of native collagen fibrils[1] with a coiled-coil[2] arrangement of three peptide chains, and the dissolution of fibrils to macromolecules[1, 3–5] (tropocollagen or TC) with subsequent reconstitution to fibrous forms[1, 6, 7] are now well established.

Collagen fibers may shrink to as little as 20% of their original length upon heating;[8] tropocollagen solutions exhibit decreased intrinsic viscosity, decreased optical rotation, and decreased molecular weight when heated.[3, 4, 8–14] The transitions are sharp, occurring over a range of only two or three degrees centigrade. On cooling heated collagen solutions, optical rotation and viscosity gradually approach but do not reach their original values.[1, 3, 4, 8–14] Much other evidence (from wide angle X-ray diffraction,[1] infrared spectra,[13] light scattering,[4] and various physicochemical measurements[1, 3, 4, 8–14]) indicates that chilling gelatin *partially* restores the original (collagen) order of peptide chains. Information on the size and shape of regenerated particles cannot readily be obtained from wide angle X-ray, optical rotation, or infra-red measurements. Results of other measurements have been indecisive. However, the close correspondence of melting temperatures (T_m)[8, 9] for native and reverted collagen together with other very recent results[10] suggests that the crystalline structure of collagen regenerated by cooling carefully prepared gelatin must be *equivalent* to the crystalline arrangement of native collagen.

Hall[5] has developed an electron microscope technique for viewing individual macromolecules. We have applied this technique to the critical examination of particles present in collagen solutions which have been heated to effect transformation, and to material regenerated upon cooling such solutions. In particular, the structure of the latter is compared to native collagen. The results have been supplemented by those of other methods.

Tropocollagen Solutions.—Soluble collagen, or tropocollagen (TC), was prepared[15] from embryonic calf skin. It was characterized by optical rotation, intrinsic viscosity, sedimentation constant, and flow birefringence with results as follows: $[\alpha]_D^{20^\circ} = -380^\circ$; $[\eta] = 15$ dl/g; $S_{20,w} = 3.0$; particle length $L = 3000$ A. Tropocollagen was stored as concentrated (0.4% (w/v) to 0.9%) solutions in 0.05% (w/v) acetic acid at 5°C. Protein concentrations were determined by a micro-Kjeldahl method.

In Hall's technique[5] droplets of solutions are sprayed from a nebulizer onto freshly cleaved mica surfaces. Platinum is then evaporated at 10^{-4} mm Hg at 5 to 1 or 10 to 1 angles giving long shadows. Such shadows on the atomically smooth surface of mica serve to distinguish the narrow particles from metal granulation and extraneous matter.

Micrographs of calf-skin TC as shown in Figure 1 are indistinguishable from published micrographs of ichthyocol.[5] They show relatively rigid rods about 3000 A long. Whether the beaded appearance is real or an artifact of the metallic deposit

FIG. 1.—Electron micrograph of calf-skin tropocollagen. Magnification 100,000×. Untreated and sprayed at room temperature.

or of drying is not known.[5] Lengths ranging up to about 3000 A are observed.[16] Several shorter rods can be seen in Figure 1. The diameters of calf-skin TC are about 15 A, in good agreement with the published diameters of ichthyocol rods.

The thermal transformation usually was carried out by heating 0.2 to 0.4% TC solutions in 0.05% acetic acid in small test tubes in a water bath. Temperature and time of heating were varied from 50°C for 8 min to 90°C for 30 min. (After heating, optical rotation measurements showed $[\alpha]_D^{40}$ = *ca.* −120° and intrinsic viscosity about 0.3 dl/g. The ultracentrifuge pattern was also drastically changed.)[17] Usually the heated solutions were sprayed in an oven at elevated temperatures and immediately transferred to the vacuum evaporator.[18] An electron micrograph of a TC solution heated at 70°C for 10 min is shown in Figure 2. Only globules are seen. The globules are presumed to represent collapsed random coils of gelatin. The larger globules may be collapsed coils of all three polypeptide chains whereas the more predominant smaller globules may represent portions of collapsed chains. The identification of the *smaller* globules with gelatin is uncertain because of the ubiquitous presence of granules in all shadowed electron micrographs. No rods of even as much as one-tenth the length of untreated particles were found either in those areas photographed or upon scanning relatively large areas at high magnification (50,000 ×) on the fluorescent screen of the microscope.

To study the reversibility of the transition, heated TC solutions were rapidly cooled to 0°C and then stored at 5°C. The solutions were sprayed at room temperature or in the cold. Figure 3 is a micrograph of particles obtained from such a solution sprayed at the same concentration (*ca.* 0.005%) as the solution used to obtain Figure 1. The presence of rods is unmistakable. Many regenerated rods are 2800–3000 A long but most are shorter. Diameters are about 15 A, the same as

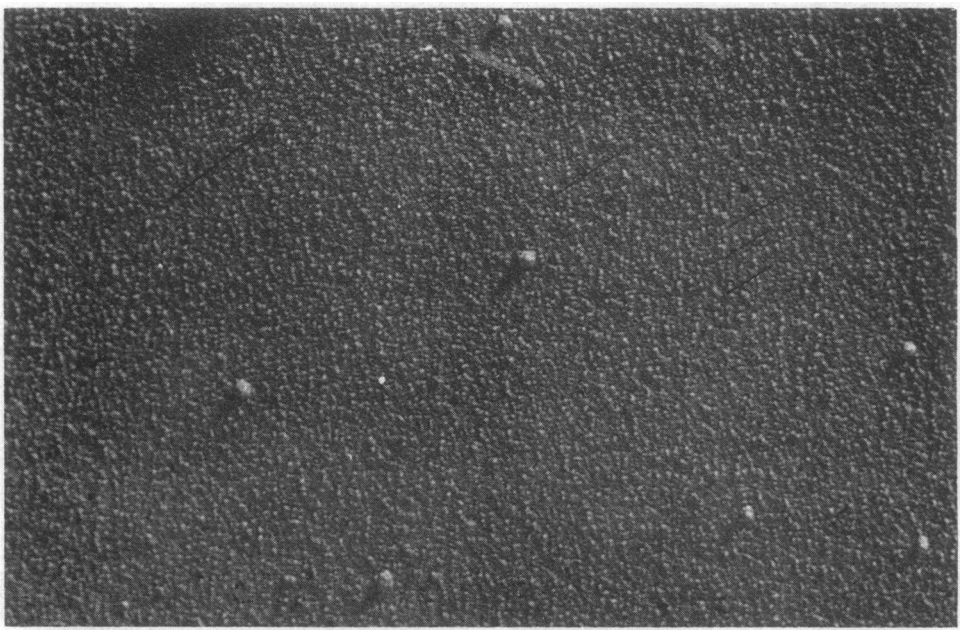

FIG. 2.—Electron micrograph of heated calf-skin tropocollagen. Magnification 100,000× Heated at 70°C for 10 min. and sprayed in an oven.

untreated TC. Regenerated rods are beaded just as native rods are, but some regenerated rods have a more irregular contour which may indicate imperfections of coiling. Significant numbers of rod-shaped particles were seen after only two hours cooling. Specific rotations of the cooled TC solutions[19] agreed with literature values.

Formation of SLS.—Schmitt *et al.*[6] have described the segment long spacing (SLS) type of fibrous aggregates which are formed by adding adenosine triphosphoric acid (ATP) to acidic tropocollagen solutions. They suggested that the tropocollagen macromolecules occur in parallel array, side chains being in register with neighbors, to give the banded pattern seen in the electron microscope. The segments do not show symmetrical striations. They interpreted this as evidence that the distribution of polar amino acids is not symmetrical about the molecular center. Additional experimental evidence[7] has been obtained recently to indicate that the SLS micrographs are indeed "molecular fingerprints" of a rather precise arrangement of polar side chains. Heretofore normal SLS has been precipitated only from native collagen solutions.[20] It appears then that the integrity of the triple helix and the side chain distribution is a prerequisite to SLS formation. Consequently, it was of interest to learn if the SLS form could be precipitated from collagen solutions which had been heated to effect transformation and subsequently cooled.

Figure 4 is an electron micrograph of SLS from a TC solution that had been heated to 50°C for 10 min, quickly chilled, diluted with an equal volume of 1% ATP and stored at 5°C for 44 hr. Other SLS precipitates were formed from cooled TC solutions previously heated at 55°C for 15 min, 70°C for 25 min, and 90°C for 25 min after such solutions were cooled. The amount of SLS obtained was less than would have been formed from solutions of native collagen. After several months in the cold, the yield of SLS increases to as much as 10–15%. Much of

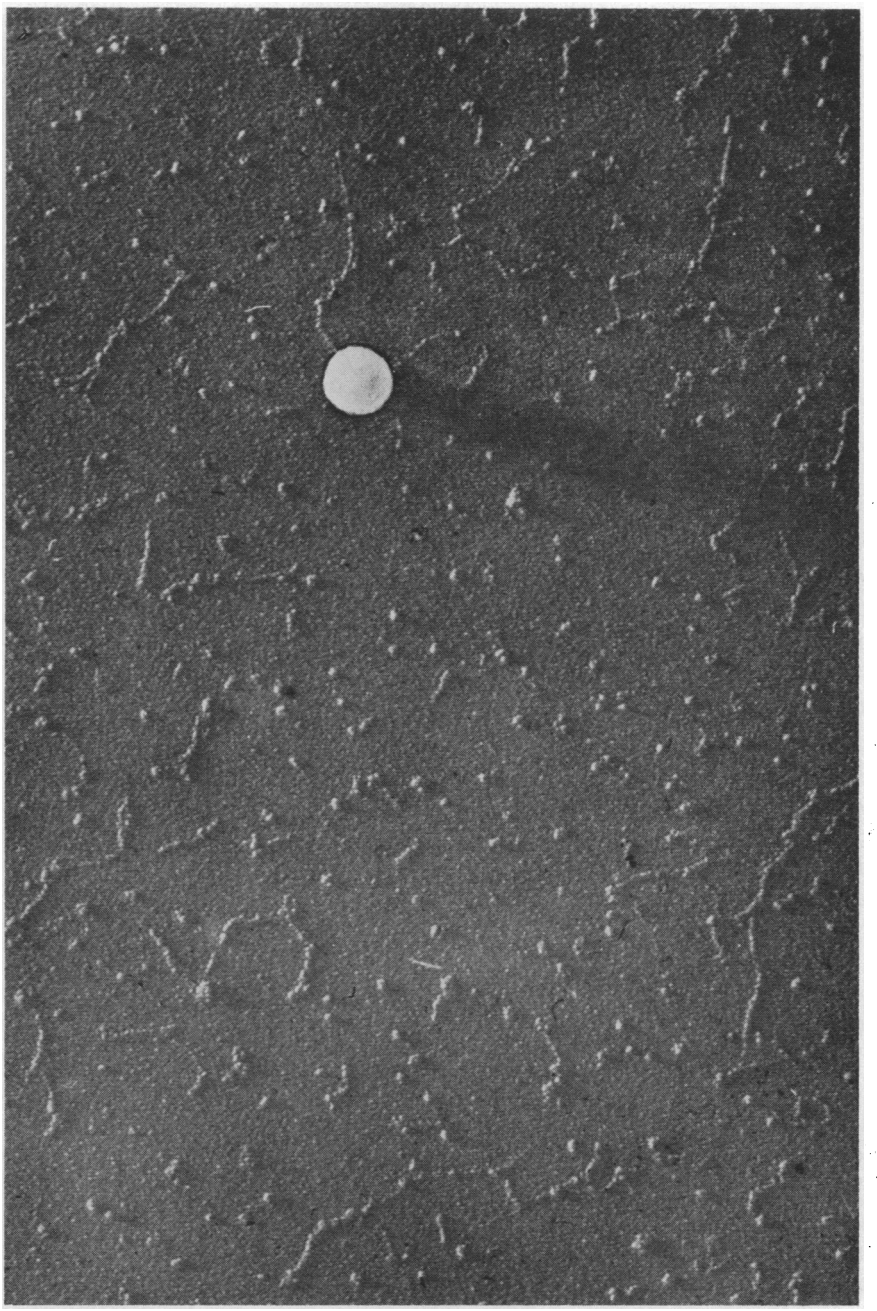

FIG. 3.—Electron micrograph of heated and cooled calf-skin tropocollagen. Magnification 100,000×. Heated at 60°C for 15 min, rapidly cooled to 0°C, and stored for 3200 hr at 5°C. Sprayed at room temperature.

the precipitate consisted of less ordered arrangements of TC particles than is characteristic of SLS particles. Nevertheless, many examples of apparently normal SLS were found. Yields of SLS were essentially independent of heating temperatures provided the latter were higher than the melting temperature.

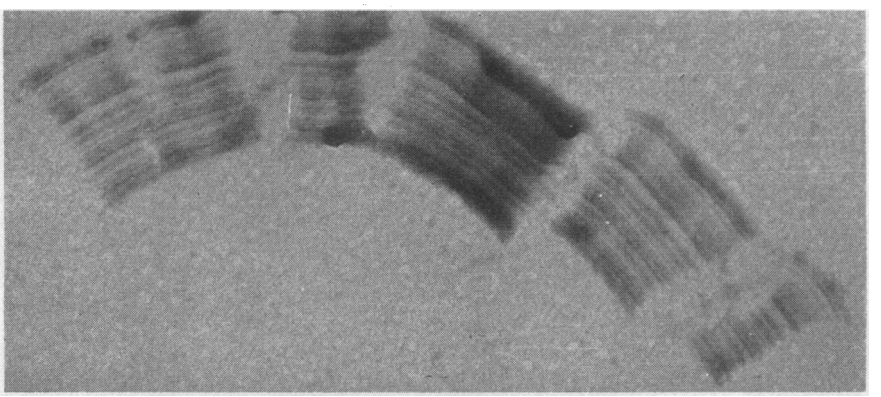

FIG. 4.—Electron micrograph of SLS. Magnification 87,000×. Prepared from calf-skin tropocollagen heated at 50°C for 10 min, chilled, and stored at 5°C for 44 hr. Stained with phosphotungstic acid.

The possibility that the observed 3000 A rods and the SLS from reverted gelatin represented residual undamaged TC particles was considered early in this investigation. This possibility was eliminated in the course of additional experiments. Separate heatings, at progressively higher temperatures and for longer periods, gave essentially the same results after the solutions were cooled. When heated TC solutions of higher concentration were sprayed while hot, no rods were seen in the electron microscope. Finally, optical rotation, intrinsic viscosity, and sedimentation measurements[17] of the heated TC solutions were in good agreement with published values.

Collagen Fibrils.—Ewald[21] first described the cyclic process of thermal shrinkage and re-elongation of tanned collagen fibers. More recently a thermodynamic study by dilatometry has shown crystalline regions of the re-elongated fiber to be identical with initial crystalline arrays.[8] Results of low angle X-ray diffraction investigations are contradictory since one group[1] reported partial return of the characteristic 640 A spacing upon re-elongation and another[22] failed to observe restoration of this periodicity. All experiments indicate that crystallinity disappears above the melting temperature (T_m). The characteristic T_m for tanned collagen is higher than for native fibers but the T_m can be lowered by use of diluents that are "good" solvents. With formaldehyde-tanned rat tail tendon (RTT) in trifluoroacetic acid the T_m is lowered from above 90°C to below room temperature.

Further electron microscopical studies were performed on formaldehyde-tanned RTT. The tendon was homogenized to obtain a suspension of fibrils.[1] The phase transformation was accomplished either by heating or by replacing water with trifluoroacetic acid. No striations of resolvable periodicity could be found in the shrunken fibrils. These fibrils were thicker than striated fibrils of normal preparations and they contained more debris. However, characteristic striations returned upon re-elongation; these are revealed in the uranium shadowed fibrils of Figure 5. The return of fine structure between the major periodicities was also observed when re-elongated fibrils were stained with phosphotungstic acid. The periodicity of the major striations averaged 580 A in several dozen fibrils (versus 700 A in untreated fibrils). In addition to an apparent lower average periodicity, greater variance in periodicity from one fibril to another was observed. Upon cooling,

striations returned to every fibril observed, indicating virtual equivalence to the original state. The cycle may be repeated many times.

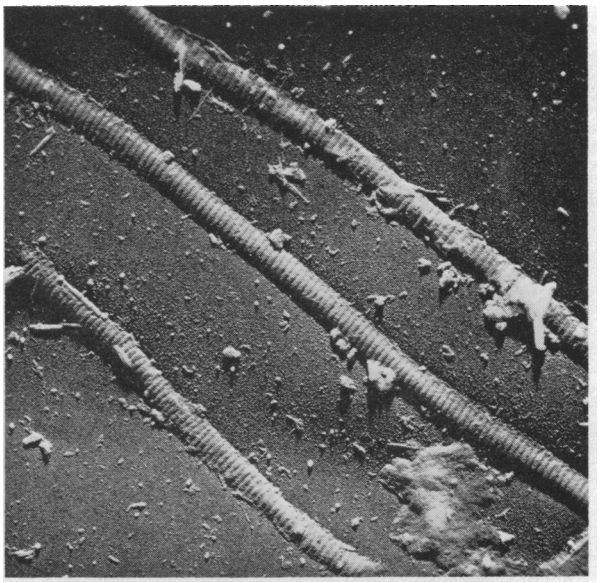

FIG. 5.—Electron micrograph of re-elongated tanned RTT fibrils. Magnification 31,700×. Uranium shadowed. Prepared from RTT previously contracted with trifluoroacetic acid, and re-elongated with water at room temperature.

Discussion.—Previous extensive studies on chilled gelatin solutions have determined that some type of aggregation takes place upon cooling. Although good agreement has been obtained on the nature of the aggregating particles, the end product of the aggregation and the mode of particle interaction have, until recently, remained elusive.

The polypeptides in hot gelatin appear to be in the form of random coils above the T_m.[4, 5, 8–10] The phase transition has been termed "melting," to emphasize the sharp conversion from helices to random coils. Curves of $[\alpha]_D$ versus time and $[\eta]$ versus time can be superimposed[3] indicating that simultaneously with the destruction of crystalline order upon melting, the helices collapse to random coils. Low angle X-ray diffraction[1] and precise dilatometric[8] studies on tanned fibers also showed that heating destroys the ordered arrangement of chains. These observations paralleled results of extensive studies on fibrous polymers. The reversibility of thermal phase transitions in fibrous polymers is well established; the reversibility with some fibrous proteins and nucleic acids is less clear.

Many methods used in the past to study the end product of aggregation of collagen failed to provide morphological information. However, recent studies lend increasing support to the view that reverted collagen is equivalent in structure to the native material.[8–10] The electron micrographs of the present study now show directly that some, at least, of the rod-like structures formed during reversion are morphologically indistinguishable from native particles.

Moreover, ordered aggregation of these has been shown to occur with formation of SLS. In these patterns tropocollagen particles are in register as evidenced by the familiar striations. The conversion of tropocollagen helices to random coils and then back to the original crystalline arrangement of polypeptide chains is evident. The striking fact that rods are present in abundance in cooled solutions, although completely absent in hot solutions (at even higher concentrations) indicates that regeneration necessarily includes formation of asymmetric particles.

The present study identifies cooled gelatin particles with native tropocollagen rods, but it does not give direct information about the mechanism of regeneration. A transient, intermediate, single helix similar to a poly-L-proline type II has been

postulated[9, 10] as the first step. If this is correct, then the presence, shortly after cooling, of rods (presumably triple helices) suggests that the single helices "zipper up" with ease for some combinations of polypeptides. Many random coils and single helices do not quickly revert to asymmetric rods under the conditions used. Excessive degradation caused by hydrolysis of peptide bonds during heating should be expected to prevent complete reversion.[9] However, the yield of SLS after very long cooling remains at 10–15% roughly independent of the wide range of heating times and temperatures. Since peptide hydrolysis should vary drastically over the range of conditions employed, it would appear that factors other than degradation limit the proportion of material which reappears as SLS. Length distribution measurements on reverted TC rods after varying heating and cooling periods are being carried out. Such studies may bear on the mechanism of regeneration.

The demonstration of what appear to be normal TC particles in cooled gelatin readily explains many results of other studies. For example, higher optical rotation and intrinsic viscosity and evidence of original crystallinity by X-ray diffraction are to be expected according to the electron microscopic observation. Most of the solutions used were too dilute to gel at 5°C. A few observations were made on particles present in gelatin gels (0.8% protein). Large numbers of rods were seen in the electron microscope. The ridigity measurements[12] on gelatin gels may reflect the presence of long rods.

The return of striations to re-elongated tanned RTT fibrils corroborates the study on collagen solutions. The periodicity (580 A) observed is in good agreement with Bear's[1] original low angle measurements on re-elongated fibers. However, we have noted greater variance in periodicity among such fibrils than native RTT exhibits. The variance may explain the failure of Wright and Wiederhorn[22] to observe the return of the low angle X-ray patterns for a completely random periodicity from fibril to fibril would cancel out the diffraction pattern.[23] The electron microscopical observations suggest that RTT fibrils, crosslinked with formaldehyde, are converted from a state of alternating regions of order and disorder to one of complete disorder upon heating above the T_m. More debris was observed in these preparations, indicating that crosslinking did not succeed in uniting all polypeptide chains. The similarities between this fibrous system and those of synthetic fibrous polymers are obvious.[8]

We have recently extended this study to include collagen from sources other than young calf skin and also to nucleic acids. Thus, asymmetric rods appear upon cooling solutions of RTT, carp swim bladder (ichthyocol), guinea pig corium, earthworm cuticle collagen, and even commercial gelatin (Knox) in which the collagen was previously transformed by heating. Solutions of yeast ribonucleic acid (RNA) and RNA isolated from tobacco mosaic virus (TMV) when heated and cooled give narrow, long fibers.[24]

During the preparation of the manuscript for this paper evidence of reversible transformation of both collagen and deoxyribonucleic acid was published by two laboratories. Veis and Cohen[25] found fibrils with striations of about 610 A periodicity in the heat precipitates from concentrated gelatin gels. The gels were prepared from carefully fractionated bovine gelatin and stored at 4°C. Marmur and Lane[26] and Doty *et al.*[27] reported the renaturation of separated strands of bac-

terial deoxyribonucleic acid. Along with other evidence, they showed electron micrographs of long fibrils (taken by Prof. C. Hall) of "renatured" DNA.

From our studies and these recent results of others, it would appear that the reversibility of helix $\rightleftarrows$ random coil transitions is a general phenomenon of anianisometric macromolecular helices. The reversibility can be demonstrated for helices consisting of a single macromolecular chain (RNA), of two chains (DNA), or of three chains (TC).

This investigation was initiated and stimulated through discussions with Dr. Paul J. Flory. Appreciation is extended to Mr. M. D. Maser for assistance throughout this study, to Mrs. A. Brady and Mr. R. E. Kerwin for certain technical assistance, and to Dr. A. Buzzell, Department of Biophysics, University of Pittsburgh, for preparations of RNA isolated from TMV.

* This study was supported in part by Research Grant A2809 from the National Institute of Arthritis and Metabolic Diseases, U.S. Public Health Service.

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[16] Hall and Doty[5] have noted that spraying in the cold was necessary in order to get close correlation between length distributions for ichthyocol measured by electron microscopy and lengths obtained by flow birefringence and other physical chemical methods. Only a few sprayings of calf-skin TC were performed in the cold and a length distribution has not been measured. Therefore the sharpness of the length distribution plot is not known as yet for calf-skin collagen.

[17] Additional confirmatory evidence of the reversible transition of collagen solutions in dilute acetic acid has been obtained with the ultracentrifuge. Unheated TC solutions show a hypersharp sedimentation peak with $S_{20,w} = 3.0 \pm 0.13$. Heated TC solutions, if centrifuged above 30°C immediately after the transition, exhibit a single normal schlieren peak with $S_{20,w} = 3.4$. Upon cooling, two peaks are formed. The slower peak is a hypersharp one ($S_{20,w} = 2.75$) characteristic of unheated collagen (when allowance is made for the Johnston-Ogston effect), while for the broader, more diffuse, leading peak $S_{20,w} = 3.31$, which identifies it with randomly coiled gelatin.

[18] No rods were observed in micrographs of TC solutions heated as low as 50°C for 8 min and sprayed in an oven; however, a few short rods were occasionally found after spraying at room temperature. Here much higher concentrations of TC (0.075%) were required to yield rods. The time and temperature schedule for the first appearance of rods is under study.

[19] After 15 hr a limit of mutarotation was reached with $[\alpha]_D^{15°} = -280°$. A separate measurement of heated TC solution in pH 3.7 citrate buffer (0.1 *M* citric acid plus 0.05 *M* sodium ci-

trate) indicated that after 1000 hr at 5°C the intrinsic viscosity was about 6 dl/g in fair agreement with a recently reported value of 8 dl/g for chilled ichthyocol gelatin.[10]

[20] In contrast to reconstitution of native type fibrils, SLS precipitates are more structurally uniform and nearly quantitative. SLS aggregates were formed in excess of 98% yield from the untreated TC preparation used in the present experiments. Modification of ϵ-lysine groups (e.g., by complete acetylation) greatly reduces amounts of SLS formed. Some unusual forms of SLS have been obtained from sonically ruptured tropocollagen (see Hodge, A. J., and F. O. Schmitt, these PROCEEDINGS, **44**, 418 [1958]).

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[23] However, at least one other laboratory has demonstrated the return of striations by low angle X-ray diffraction. A. Diokio obtained maxima at 495 A, 251 A, and 165 A for re-elongated tanned RTT. (Personal communication from L. Mandelkern, U.S. Bureau of Standards.)

[24] We have obtained electron micrographs of RNA (from TMV and yeast) sprayed in neutral volatile buffers before heating, while hot, and after cooling. Before heating, strands up to 10,000 A long are present; solutions sprayed in an oven show only globules; after cooling strands up to several thousand Angstroms long are found in large amounts and strands 10,000 A long are also present. These observations are obviously similar to the collagen experiments. They confirm the conclusions of Doty *et al.* (these PROCEEDINGS, **45**, 482 [1959]) concerning RNA helix-coil transitions except for present indications of relatively long helical regions (1000 to 10,000 A).

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*CHEMICAL AND ENZYMATIC SYNTHESIS OF CARBAMYL PHOSPHATE**

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Communicated July 25, 1960

The mechanisms of chemical as well as enzymatic synthesis of carbamyl phosphate (CAP) pose a number of problems which invite closer exploration. This paper is more in the manner of an exposition of these problems and a statement of their scope and implication than an attempt to solve them definitely. The first part will deal with the chemical synthesis of CAP from cyanate and phosphate, and its decomposition which recently we recognized to be largely due to elimination of phosphate rather than to hydrolysis. The second part will deal with biochemical synthesis which, in the relatively simple bacterial system discussed here, goes by way of a carbamate phosphokinase transferring the terminal phosphate of ATP to carbamate.

The animal tissue process of CAP synthesis is a more complex reaction involving additional energy input and cofactors, and although we will not deal here with this process experimentally, we will discuss some of its facets in comparison with the simpler process in bacteria. The chemical synthesis of CAP has been discussed in the context of a pre-biological synthesis of an energy-rich phosphate carrier, and some consideration will be given to this proposition.

Methods and Preparations.—CAP was synthesized and isolated as the lithium