[2] Epstein, R. H., *et al.*, in *Synthesis and Structure of Macromolecules*, Cold Spring Harbor Symposia on Quantitative Biology, vol. 28 (1963), in press.

[3] Garen, A., personal communication.

[4] Gorini, L., and H. Kaufman, *Science*, **131**, 604 (1960).

[5] Davis, B. D., and E. S. Mingioli, *J. Bacteriol.*, **60**, 17 (1950).

[6] Gorini, L., *Bull. Soc. Chim. Biol. (France)*, **40**, 1939 (1958).

[7] Wollman, E. L., and F. Jacob, *Ann. Inst. Pasteur*, **95**, 641 (1958).

[8] Beckwith, J. R., *Biochim. Biophys. Acta*, **76**, 162 (1963); and contribution to Genetics Conference in Gatersleben (E. Germany), August 1963, in press.

[9] Pardee, A. B., *J. Bacteriol.*, **73**, 376 (1957).

[10] We are indebted to Dr. L. Cavalli for this information.

[11] We are indebted to Dr. M. Meselson for this information.

[12] Campbell, A., *Virology*, **14**, 22 (1961).

[13] Spotts, C. R., and R. Y. Stanier, *Nature*, **192**, 633 (1961).

[14] Spotts, C. R., *J. Gen. Microbiol.*, **28**, 347 (1962).

[15] Flaks, J. B., *et al.*, *Biochem. Biophys. Res. Comm.*, **7**, 340 (1962).

[16] Davies, J. E., unpublished results.

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*THE ANTIGENICITY OF TROPOCOLLAGEN**

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Collagen is a structural protein which constitutes about 30 per cent by weight of all protein in the mammalian body. The collagen fiber itself is relatively insoluble, but various conditions of solvent, pH, ionic environment,[1] and temperature[2] may be used to obtain soluble fractions. Physical characterization of these soluble fractions, and X-ray and electron micrograph studies on precipitates obtained under specific conditions, have shown that the native collagen fiber is built up by the highly ordered aggregation of a fundamental unit termed the tropocollagen (TC) molecule,[3-5] which is a stiff rod with a length of approximately 2800 Å and a molecular weight of about 300,000. The ~700 Å periodicity seen in electron micrographs of collagen fibrils is believed to reflect the aggregation of the tropocollagen monomers in a polarized quarter-stagger array.[6]

The internal structure of the TC molecule is characterized by three polypeptide chains wound in a triple helix.[7] The basic structural unit is the α-chain—a polypeptide strand with a molecular weight of approximately 100,000.[8] Two α-chains may be cross-linked to form a β-chain, and three to form a γ-chain.[9, 10]

The processes initiating and controlling the formation of a native-type fibril *in vivo* from TC monomers are unknown. It might proceed via the lateral aggregation of staggered monomers with a consequent progressive build-up of a filament, or it could proceed via the lateral aggregation of *protofibrils* which form initially by end-to-end polymerization of the monomers. It was demonstrated in this laboratory that sonic irradiation modifies the normal end-to-end interactions of the monomers, and it was proposed that TC molecules bear peptide "end-chains"

which by specific interaction mediate the linear polymerization of monomers into protofibrils.[11]

Hodge and Petruska[12] have recently shown that successive TC monomers in the collagen fibril overlap instead of aggregating end-to-end. It would appear, therefore, that if peptide appendages on the body of the collagen molecule underlie specificity of polymerization, these structures may not reside exclusively at the end of the molecules, and the term "end-chains" with its terminal connotation has been replaced by the term "telopeptides."[13]

Studies in this laboratory have shown that small peptide fragments can be split from TC by proteases and with their removal the aggregative properties of the TC molecules are modified without substantial alteration of the triple-helix body of the highly elongate TC macromolecule.[13-16] On the basis of these observations it was suggested that homeostatic control of TC interaction and hence of deposition and degradation of collagen fibrils may be achieved in the organism by enzymatic modification of telopeptides.[13] The telopeptides contain a large fraction of all the tyrosine in TC; their amino acid composition differs distinctly from that of bulk collagen.

It is well known that collagen is at best a weak antigen. In view of the similarity of the X-ray diagrams obtained from collagens isolated from a variety of animal species, it would appear that the structure of the molecule is very similar in all species examined thus far. With this in mind and in view of the high tyrosine content of the telopeptides we were led to postulate that most if not all the antigenicity of collagen might be ascribable to the telopeptides.[13] The experiments described below substantiate this postulate.

Experimental.—Physical and chemical methods of analysis have been described previously.[13, 15]

Collagen preparation: Calf-skin collagen was prepared by a procedure detailed elsewhere.[15] In essence the procedure consisted of reprecipitating citrate-extracted, 1 per cent sodium chloride-insoluble TC until the preparation was rendered pure as judged by the criteria of amino acid analyses and electrophoretic homogeneity.

Protease treatment: The TC was digested with pepsin (1:100 by weight, or 1:8 mole/mole of enzyme:substrate) in 0.05 per cent acetic acid (pH 3.5) at 20° for 24–96 hr. Peptide fragments were removed by dialysis, and the TC was separated from the pepsin by free diffusion electrophoresis[13, 15] or by precipitation of the TC by 15 per cent KCl.[16] Pronase treatment was performed at pH 7.2 in 0.1 molar calcium acetate (at a similar enzyme:substrate ratio) at 20° for 24–96 hr. The TC was recovered by KCl precipitation as mentioned.

Antibody production: Antibodies to calf-skin collagen were prepared by injecting 2 ml of 0.35 per cent TC solution in complete Freund's adjuvant intramuscularly and into the toe pads of three rabbits. The rabbits were bled three weeks after injection. The antibody concentration was measured by complement fixation.[17]

Results.—All three rabbits produced antibodies to the injected TC. The complement-fixation curve of the reaction between TC and one antiserum is shown in Figure 1.

After pepsin digestion approximately 1 per cent of the TC molecule becomes dialyzable, and some of the β- and γ-cross-links are removed. An almost insignificant loss of optical rotation accompanies this change, and the intrinsic viscosity

is lowered only slightly, although the concentration dependence of the viscosity is markedly decreased.[15] Segment long-spacing aggregates[11] (SLS) produced by the addition of ATP and observed in the electron microscope after phosphotungstic acid staining reveal no demonstrable change in the TC molecule. After peptic digestion, however, the antigenic response is modified as shown in Figure 1.

Preliminary experiments with the pepsin-digested telopeptides gave indications that they could inhibit the TC: anti-TC complement fixation.

Pronase digestion renders approximately 5 per cent of the TC molecule dialyzable; all the β- and γ-cross-links are lost.[16] The changes in the gross physical properties of the TC are small, but the antigenic response is now completely abolished at the levels of antibody used (Fig. 1).

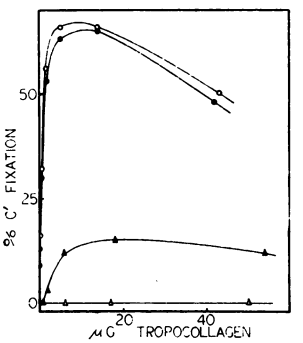

FIG. 1.—Fixation of complement by: (*a*) TC dissolved in 0.1 *M* calcium acetate pH 7.2 and treated with 1% w/w pronase for 48 hr at 20°C (△). TC was separated from the pronase by KCl precipitation and was redissolved in 0.05% acetic acid for assay. As a control, TC (○) was treated in the same manner but pronase was not added. (*b*) To a TC solution in 0.05% acetic acid 1% w/w pepsin was added. TC was precipitated immediately from the control sample (●) by KCl. For the experimental sample (▲) TC was precipitated by KCl after 48 hr reaction at 20°C. Both TC samples were redissolved and assayed as above.

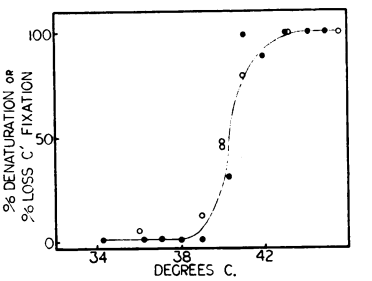

FIG. 2.—Temperature profile of TC denaturation in 0.1 *M* calcium acetate pH 7.2 as measured by optical rotation and complement fixation. Optical rotation was measured at 365 mμ on 0.25% TC solutions. For complement fixation 0.1 ml TC (0.35%) was added to 3.5 ml preheated solvent. After 30 min incubation at the requisite temperature 0.1 ml was diluted into 10 ml of chilled buffer and analyzed for serological activity. The values shown were obtained according to the formula: Per cent change = $[(X)_{Native} - (X)_T]/[(X)_{Native} - (X)_{Denatured}] \times 100$, where $(X)_T$ refers to the 30-min value at the specified temperature T, and $(X)_{Native}$ and $(X)_{Denatured}$ refer to the values of native and completely denatured samples, respectively.

To demonstrate that protease attack and the immunological response are directed against the TC molecule and not against a contaminant in the preparation, the complement fixation of denatured TC was studied. Figure 2 presents data on the optical rotation and the antigenic reactivity of our TC preparations heated in 0.1 *M* calcium acetate pH 7.2. The parallel between the loss of optical rotation and the loss of antigenic activity clearly demonstrates that the antibody reaction is dependent upon the integrity of the intact TC molecule. The requirement for that integrity was also demonstrated by the finding that collagenase treatment completely abolishes the serological activity of TC.

Discussion.—The results of the immunological experiments clearly show that an antigenic response is provoked by native TC molecules. The TC:anti-TC three-

dimensional lattice requisite for complement fixation is dependent on both the triple helix and the presence of telopeptides. If the triple helix is altered, for example, by collagenase or thermal treatment, the structure requisite for the three-dimensional aggregation is effected, and complement fixation is lost. The specificity of the TC: anti-TC reaction, however, resides in the telopeptides.

The serological activity is diminished after protease treatment, the loss being greater after treatment with pronase which removes more peptide material than pepsin. In both cases, SLS aggregates of the digested TC appear normal under electron microscopic examination, and there is no physical evidence that the triple-helix body of the molecule has been altered. It is concluded that very small parts of the tropocollagen molecule in situations vulnerable to the action of proteases are the sites responsible for antigenicity.

There is no conclusive evidence yet that the triple helix is continuous throughout the 2800 Å length of the TC molecule; hence it might be supposed that the cross-links which are subject to protease attack could be situated in short regions of disorder in the body of the helix. This possibility would appear unlikely, however, since pronase digests completely any denatured α-strand,[16] and one would expect pronase to split the TC molecule at any site where the triple helix is interrupted. The remarkable stability of TC to pronase treatment seems a reflection of the integrity of the triple-helix structure throughout the 2800 Å of its length. On this assumption we suggest that the cross-links and the antigenic sites are probably situated external to the triple-helix body. Since sonic irradiation or protease treatment modifies the inherent linear aggregative tendency of the TC molecules, at least some of the telopeptide structures must be terminal in the macromolecule. The tendency of TC to form a staggered array with overlap[12] may imply that other structures, perhaps complementary to the terminal ones, are situated at points along the length of the helix; they may be attached to it by ester-type linkages.[18, 19]

It should be emphasized that TC solutions are not homogeneous; they contain molecules with both triple-α, $\alpha\beta$-, and γ-chains in the triple helix. No evidence yet shows that the antigenic property is common to all these TC variants. If it is feasible to differentiate immunologically between them, it may be possible to confirm or deny the postulated maturation sequence α to β to γ chains.[20]

The hypothesis that *in vivo* control of collagen fibrogenesis is mediated by enzymatic modification of telopeptides remains to be proved. However, the localization of antigenic activity in telopeptide structures provides a probe by which we anticipate it will be possible to investigate collagen fibrogenesis, its homeostatic control, and pathological defects in these control mechanisms.

Summary.—Antibodies to purified calf-skin tropocollagen may be produced in rabbits. Complement fixation by antigen-antibody reaction is dependent upon the native structure of the tropocollagen molecules. The serological reaction can be reduced or abolished by protease attack which leaves the triple-helix body of the molecule undamaged. It is concluded that the antigenic response and the protease attack are directed against peptide appendages external to the triple-helix body of the tropocollagen macromolecule.

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[1] Orekhovitch, V. N., A. A. Tustanowski, K. D. Orekhovitch, and N. E. Plotnikova, *Biokhimiya*, **13**, 55 (1948).

[2] Veis, A., J. Anesey, and J. Cohen, *J. Am. Leather Chemists' Assoc.*, **55**, 548 (1960).

[3] Gross, J., J. H. Highberger, and F. O. Schmitt, these PROCEEDINGS, **40**, 679 (1954).

[4] Boedtker, H., and P. Doty, *J. Am. Chem. Soc.*, **78**, 4267 (1956).

[5] Hall, C. B., and P. Doty, *J. Am. Chem. Soc.*, **80**, 1269 (1958).

[6] Schmitt, F. O., J. Gross, and J. H. Highberger, *Exptl. Cell Res., Suppl.*, **3**, 329 (1955).

[7] Ramachandran, G. N., in *Aspects of Protein Structure* (New York: Academic Press, 1963), p. 39.

[8] Piez, K. A., E. Weiss, and M. S. Lewis, *J. Biol. Chem.*, **235**, 1987 (1960).

[9] Grassmann, W., K. Hannig, and J. Engel, *Z. Physiol. Chem.*, **324**, 284 (1961).

[10] Altgelt, K., A. J. Hodge, and F. O. Schmitt, these PROCEEDINGS, **47**, 1914 (1961).

[11] Hodge, A. J., and F. O. Schmitt, these PROCEEDINGS, **44**, 418 (1958).

[12] Hodge, A. J., and J. A. Petruska, in *Aspects of Protein Structure* (New York: Academic Press, 1963), p. 289.

[13] Rubin, A. L., D. Pfahl, P. T. Speakman, P. F. Davison, and F. O. Schmitt, *Science*, **139**, 37 (1963).

[14] Hodge, A. J., J. H. Highberger, G. G. J. Deffner, and F. O. Schmitt, these PROCEEDINGS, **46**, 197 (1960).

[15] Rubin, A. L., M. P. Drake, P. F. Davison, D. Pfahl, P. T. Speakman, and F. O. Schmitt, in preparation.

[16] Drake, M. P., P. F. Davison, S. Bump, A. L. Rubin, and F. O. Schmitt, in preparation.

[17] Wasserman, E., and L. Levine, *J. Immunol.*, **87**, 290 (1961).

[18] Gallop, P. M., S. Seifter, and E. Meilman, *Nature*, **183**, 1659 (1959).

[19] Hörmann, H., *Leder*, **11**, 173 (1960).

[20] Orekhovitch, V. N., in *Procollagens, Their Chemical Structures, Properties, and Biological Role* (Moscow, 1952).

*INHIBITION OF CARBOXYDISMUTASE BY IODOACETAMIDE**

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The initial step in the fixation of carbon dioxide by autotrophic organisms is the reaction of carbon dioxide with RuDP to give 3-phosphoglyceric acid.[1, 2] This reaction is catalyzed by carboxydismutase, which is inhibited by parachloromercuribenzoate,[3] suggesting that SH groups are required for its activity. In order to define the function of these groups more precisely, we have investigated the irreversible inhibition of the enzyme by iodoacetamide.

Experimental.—Enzyme: Crude carboxydismutase was prepared from isolated spinach chloroplasts as previously described.[4] It was purified by fractional ammonium sulfate precipitation, followed by repeated gel filtration on Sephadex G-200.[5] The enzyme was stored as a precipitate