Reports

Tropocollagen: Significance of Protease-Induced Alterations

Abstract. *Interaction properties of tropocollagen are markedly altered by treatment with pepsin. This treatment liberates terminal or near-terminal covalently bonded peptides whose amino acid composition is strikingly different from the composition of the pepsin-resistant triple-helix body of the macromolecule. Pepsin also converts most of the β-chains to α-chains. This fact indicates that the interchain link is also external to the body of the macromolecule and probably involves peptides. The role of these properties in bioregulative mechanisms is briefly discussed.*

Previous experiments in this laboratory (*1*) have shown that when soluble tropocollagen (TC) is treated with proteolytic enzymes, peptides are released and the interactions of the macromolecules are modified; at the same time the major portion of the TC macromolecule remains unchanged since the TC is still capable of forming the "segment-long-spacing" (SLS) type of ordered aggregates. Also, when TC was subjected to sonic irradiation, interaction properties were altered and structural changes at the ends of the molecules were observed by electron microscopy (*2*). It was postulated that in normal TC there are protease-labile "end structures," possibly peptide chains extending beyond the triple-helix body of the molecules, and that these structures facilitate polymerization of TC and the formation of native-type fibrils (*2*, *3*).

The peptide hypothesis was challenged by Kühn *et al.* (*4*) who claimed that highly purified TC lacks peptides scissionable by pepsin or trypsin yet forms native-type fibrils after treatment with enzyme. The results of Hodge *et al.* (*1*) were attributed to the interaction of TC with impurities. The possibility was left open that material not part of the TC macromolecule but of approximate composition and properties might, through electrostatic interaction with TC, facilitate fibrogenesis in vitro and possibly in vivo. Recently Nishihara and Miyata (*5*) published studies of the effects of proteases on soluble and insoluble collagens and found that "end regions," poor in hydroxyproline, relatively rich in tyrosine, aspartic and glutamic acids, and different in composition from that of the body of the molecule are attacked by proteases, and these end regions are responsible for polymerization and fibrogenesis. Peptide characterization of these end regions and complete amino acid analyses were not reported.

In this paper we present further studies of the products of the reaction of TC with pepsin. A prerequisite for this study was the preparation of TC of the highest purity.

Acid-soluble TC was obtained from calf skin by methods similar to that of Gallop (*6*). Salt-soluble TC was obtained from growing guinea pigs by the method of Gross (*7*). Stepwise purification employed various physical and analytical criteria. These included determinations of nitrogen, hydroxyproline, hexose, and hexosamine, and studies of optical rotation, electrophoretic mobility, viscosity, and sedimentation velocity. The amino acid composition of a typical preparation of TC from calf skin is shown in Table 1. In several instances, samples were further purified by preparative free-diffusion electrophoresis in the Hannig (*8*) apparatus.

Twice-recrystallized pepsin (Worthington) was added to solutions of TC, $pH = 2.0$, in pepsin:TC weight ratios varying from 1:25 to 1:1000. After digestion for 15 to 20 hours at 20°C the preparations were dialyzed against 25 volumes of 0.05 percent acetic acid, and the dialysates were flash-evaporated eightyfold at 45°C to 10 ml. Control experiments, pepsin without collagen and collagen without pepsin, were similarly performed. The amounts of amino acids in the hydrolyzed dialysates from the control experiments were subtracted from experimental values. These corrections were always small and became negligible at low ratios of enzyme to substrate.

The peptides dialyzed from the digest of TC and pepsin were studied by paper chromatography and analyzed for amino acid content.

In horizontal paper chromatographs [solvent butanol : acetic acid : water (4 : 1 : 5)] a characteristic pattern containing at least four peptide spots was observed after amido-black staining. Material from the controls scarcely moved from the point of application. The amino acid composition of hydrolyzed dialysate peptides is given in Table 1. These peptides are not typical of TC because they are devoid of hydroxyproline and hydroxylysine, relatively low in glycine, and high in tyrosine, aspartic acid, and glutamic acid. With but minor variations similar amino acid patterns were observed in ten analyses on five different TC preparations.

Confirming previously described results (*1*), treatment with pepsin abolishes or greatly reduces (i) the ability of acid solutions of TC to reconstitute native-type fibrils on dialysis against 0.17*M* NaCl at 4°C and (ii) the gelation or large increase in viscosity observed after several hours' dialysis against water (Fig. 1).

These viscosity experiments merit further comment. At sufficiently high TC concentrations, large increases in viscosity may follow dialysis even after treatment with protease. However, at suitably chosen concentrations (0.03 per-

Table 1. Amino acid composition of acid-soluble tropocollagen from calf skin and of peptides liberated therefrom by treatment with pepsin (dialysate). No corrections are included for any amino-acid decomposition which might have occurred during hydrolysis.

Amino acids	Mol/100 mol amino acid	
	Acid-soluble tropocollagen	Dialysate
Glycine	33.0	16.0
Hydroxyproline	9.3	0
Proline	14.1	7.2
Hydroxylysine	1.0	0
Tyrosine	0.3	15.5
Aspartic acid	4.3	11.3
Glutamic acid	7.0	12.4
Alanine	11.2	4.0
Serine	3.2	2.8
Threonine	1.6	0.8
Valine	1.9	0.5
Methionine	0.2	0
Isoleucine	1.1	0.4
Leucine	2.2	12.6
Phenylalanine	1.5	12.0
Histidine	0.7	0.4
Lysine	2.9	0.8
Arginine	4.7	3.3

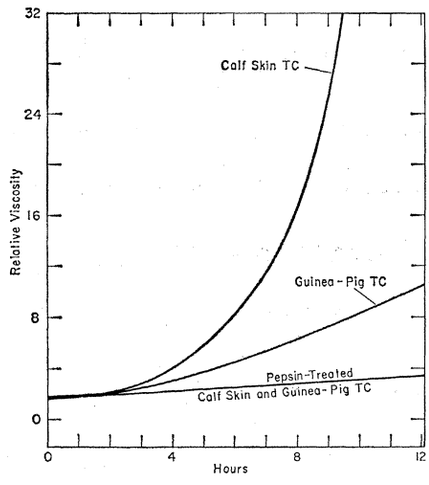

Fig. 1. Increase in relative viscosity at 4°C during continuous dialysis of tropocollagen from calf skin and from guinea-pig skin (0.03 percent in 0.05 percent acetic acid) against water before and after treatment with pepsin.

cent) of native TC and at low temperatures (*9*), characteristically large viscosity increases are observed which are almost completely abolished by protease treatment. It may be presumed that, at high concentrations of TC, either gelation or large increases of viscosity occur through both lateral and longitudinal interaction of the molecules. At low concentrations, viscosity increase is evidence of longitudinal interaction or polymerization; we believe the latter process to be mediated by terminal or near-terminal peptides.

Despite these drastic changes in interaction properties, the portion of the TC macromolecule containing the triple-helix structure and comprising about 95 percent of the total macromolecule is unaffected. This is shown by the unaltered optical rotatory properties and by the formation, after the addition of ATP, of SLS-type aggregates that have a full complement of bands in characteristic positions and stainability (as seen in electron micrographs)—a very sensitive test of the normalcy of TC structure. The inhibition by protease of the increase in viscosity is not due to interference by the combination of enzyme molecules with TC, perhaps at critical loci, because (i) the viscosity effect, unaltered by wide variation of the ratio of enzyme to TC, is still substantial at a weight ratio of 0.001 (that is, a molar ratio of 0.01), and (ii) after digestion, the pepsin, still having full activity, can be quantitatively separated by continuous free-diffusion electrophoresis. However, the TC shorn of terminal peptides and recovered separately fails to show increase of viscosity on dialysis but still gives SLS on addition of ATP.

The failure of a preparation of TC to show an increase in viscosity on dialysis is not, however, invariably due to the absence of protease-susceptible peptides. Salt-soluble TC from guinea-pig skin, preponderantly in triple-α configuration, shows little increase of viscosity on dialysis against water under our specified conditions (Fig. 1), but treatment with pepsin liberates peptides possibly in even greater amount than from acid-soluble TC. Alteration of peptide organization of triple-α TC by enzymatic action or by unidentified cofactors may have to be invoked to explain these facts.

It may be postulated that when salt-soluble, triple-α TC is converted to the acid-soluble α-β form, interbonding of α-chains may occur near the junction of the peptides with the triple-helix body of the TC macromolecule. For example, transpeptidation of the kind believed to occur in the activation of fibrin by polymerase (*10*) might simultaneously liberate a peptide and cause interbonding of two α-chains at the point of peptide scission. The terminal peptide now associated with the β-chain would be capable of interacting with neighboring TC macromolecules to produce linear aggregates of the native type.

Although the high purity of the TC used in these experiments and the consistency of the analytical results strongly support the view that the peptides released by pepsin are integral parts of the TC macromolecule, more definitive evidence would be desirable and is provided by the following results. The sedimentation diagrams of thermally denatured TC were studied before and after enzymatic attack. The relative proportions by weight of α- and β-chains in a typical acid-soluble preparation of TC, determined at a concentration (0.3 percent) where the Johnston-Ogston effect is small (*11*), are 46 percent α and 54 percent β. After treatment with pepsin, which causes release of terminal peptides, and the separation of TC and pepsin by free-diffusion electrophoresis to prevent digestion of the strands after denaturation, the same preparation now has a weight ratio of 85 percent α- and 15 percent β-chains. This seems clear proof that interbonding of α-chains to form β-chains occurs outside the pepsin-resistant triple-helix part of the TC macromolecule and implies that some, at least, of the peptides released by the action of pepsin were covalently linked to the molecule.

The fact that the protease-susceptible bonds covalently linking α-chains to each other (to form β-chains) and to terminal peptides are outside the triple-helix, "collagenous," protease-resistant part of the macromolecule suggests that it is primarily the "non-collagenous," extra-helical portions of the TC macromolecule that participate in (i) biological control of fibrogenesis, through "maturation" changes of intramolecular, interchain bonding and through insolubilization and fibril stabilization by inter-TC bonding; (ii) pathological alterations in the so-called "collagen diseases"; and (iii) in aging phenomena. Immunological properties of TC may be similarly localizable to the non-collagenous peptides.

Before the bioregulative mechanisms can advantageously be investigated, it is necessary to characterize more definitively the TC macromolecule, particularly the extra-helical portions and the nature of intra- and inter-molecular bonding (*12*). Our results indicate lines along which such investigations will prove profitable (*13*).

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9. It should be noted that formation of gels containing native-type structure from saline solutions of TC incubated at elevated (about 35°C) temperatures may reflect primarily lateral interaction of charged side-chains which, when arranged in quarter-stagger, may stabilize native-type, 700 Å-axially repeating structures even in the absence of end peptides. However, to investigate the important physicochemical and biological role of these peptides or of pathological alterations such as those of lathyrism, the more critical conditions of reconstitution at near-zero temperatures must be employed.
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13. Presented in part at American Chemical Society Symposium of Molecular Biology of Collagen, 21 March 1962, Washington, D.C. Thanks are due to John H. Highberger of the United Shoe Machinery Corporation, Beverly, Massachusetts, for several samples of purified collagen used early in these studies and for numerous helpful suggestions; to Kurt Hannig of the Max-Planck-Institut für Eiweiss- und Lederforschung, Munich, Germany, who provided a chamber prototype of the Bender and Hobein free-diffusion electrophoresis apparatus; to James W. Jacques for the preparation of calf-skin collagen and to Helgard Lengyel for technical assistance. Aided by grant E-1469 from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, U.S. Public Health Service. A. L. Rubin, an established investigator of the American Heart Association, is on leave from Cornell University Medical College. Peter T. Speakman is a Sloan Foreign Postdoctoral Fellow of the School for Advanced Study, M.I.T.

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Removal of Potassium Alters b-Dimension of Muscovite

Abstract. *By treatment of muscovite with molten lithium nitrate at 300°C we have been able to remove a large portion of the interlayer potassium and observe a decrease in the apparent* b*-axis length as a function of potassium content over the range of 8.8 to 0.7 percent potassium. The* b*-axis length of Silversheen mica (a 2M₁ muscovite) decreases from 9.024 to 8.988 Å as the potassium content decreases from 8.79 to 3.31 percent. The contraction of the* b*-dimension results in an effective decrease in the size of the ditrigonal opening in the planar surfaces with a consequent increase in the d_{001} upon re-saturation with potassium.*

The fact that structural cations significantly influence the unit cell dimensions in layer silicates has been appreciated for some time. The effect of the composition of the tetrahedral and octahedral layers on the *b*-dimension of clay minerals was discussed by Brindley and MacEwan (*1*). In a recent paper proposing modifications in the accepted muscovite structure, Radoslovich (*2*) showed that tetrahedral groups can rotate quite easily to allow a fit between the tetrahedral and octahedral layers, thus producing a ditrigonal configuration of surface oxygens into which the interlayer potassium ions fit. More recently, Radoslovich (*3, 4*) and Radoslovich and Norrish (*5*) have shown both theoretically and by multiple regression analyses that the *b*-dimension in most cases is unaffected by tetrahedral composition but that the composition of the octahedral layer, and, in the case of micas, the interlayer cations, play major roles in determining the length of the *b*-axis. It was shown for a restricted range of potassium contents that the amount of potassium present as the interlayer cation in muscovite was directly related to the length of the *b*-axis.

The molten lithium nitrate method employed by White (*6*) and Bronson *et al.* (*7*) to create a series of potassium-depleted, charge-reduced micaceous minerals offers an excellent possibility for studying the effect of gradual potassium removal from interlayer positions in a muscovite on the *b*-dimension. The migration of lithium into vacant octahedral sites (*8*) which accompanies this potassium removal should not in itself affect the *b*-dimension (*2, 5*).

The *b*-parameter is usually calculated from the (060) reflection which occurs in the region 1.50 to 1.54 Å. As noted by Brindley (*9*), however, caution should be exercised in this, since other reflections such as (331) and (332), which are often equally strong, may coincide or nearly coincide with the (060) and cause assignment of incorrect *b*-parameters. For the $2M_1$ muscovites used in this study it should be noted that the powder line inspected is a superposition of (060) and (33$\bar{1}$) (*10*) which are practical equals. They may be treated as one entity, however, because of the pseudohexagonal nature of the structure. The correct designation of the line is thus (060, 33$\bar{1}$) for the untreated muscovite; this becomes (06, 33) in the expanded mineral, since the structural layers become displaced randomly along the *a* and *b* directions (*9*).

Since the *a*-dimension is considered to be related to the *b*-dimension by the relationship $a = b/3^{1/2}$, it will be affected in the same manner as the *b*-dimension.

Two $2M_1$ muscovites, Delamica (*11*) and Silversheen mica (*12*), were treated with molten lithium nitrate for varying periods of time for the purpose of removing potassium. Figure 1*A* shows the 1.50 Å region (60 to 63 degrees 2θ Cu Kα) of the diffraction pattern of the 5 to 2 μ fraction of Delamica (*6*) for 0 and 409 hours of treatment with molten lithium nitrate (*7*). The d-spacing decreased from 1.506 to 1.500 Å as the potassium content decreased from 7.45 to 0.75 percent. The *b*-dimension was reduced from 9.038 to 9.000 Å. The latter value represents a maximum value for the reduced *b*-dimension; the presence of a very definite shoulder at about 62.2° 2θ (1.492 Å) indicates the minimum reduced *b*-dimension to be approximately 8.950 Å.

The effect of removal of potassium from the 2 to 1 μ fraction of Silversheen mica on the 1.50 Å region of the diffraction pattern is shown in Fig. 1*B* for treatment times of 0, 4, 8, 12, and 24 hours. The d-spacing decreased from 1.504 to 1.498 Å as the potassium content decreased from 8.79 to 3.31 percent; the *b*-dimension decreased from 9.024 to 8.988 Å.

The relationship between the *b*-dimension and potassium content is not a simple linear relationship as indicated by the regression analysis of Radoslovich (*4*). Our data indicate a curvilinear relationship with at least two distinct slope values.

Once the unit cell has contracted slightly, presumably by rotation of the tetrahedra, the size of the opening in the oxygen surface, initially occupied by potassium, decreases and potassium can-

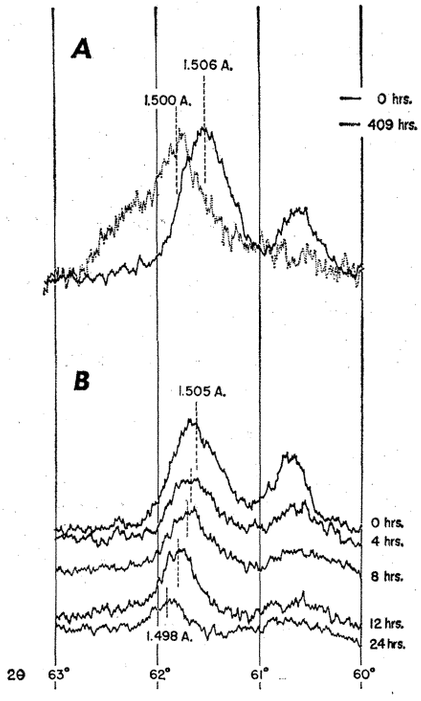

Fig. 1. Effect of removal of potassium by molten lithium nitrate treatment on the 1.50 Å region of the diffraction pattern of muscovite. *A*, Delamica (5 to 2 μ) treated for 0 (7.45 percent potassium) and 409 hours (0.75 percent potassium). *B*, Silversheen mica (2 to 1 μ) treated for 0 (8.79 percent potassium), 4, 8, 12, and 24 hours (3.31 percent potassium). Cu Kα radiation.