THE SEROLOGIC SPECIFICITY OF TROPOCOLLAGEN TELOPEPTIDES*

By PETER F. DAVISON,‡ Ph.D., LAWRENCE LEVINE,§ D.Sc., MAURICE P. DRAKE,‖ Ph.D., ALBERT RUBIN¶ M.D., and SUSAN BUMP‡

(From the Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139; the Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02154; and The Rogosin Laboratories, The New York Hospital, Cornell Medical Center, New York 10021)

Plates 32 and 33

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The serologic reactions occurring with sera of animals immunized with various collagen preparations have been described (1–7). Few attempts, however, have been made to locate any antigenic determinants on the tropocollagen molecule. Tropocollagen is obtained from animal tissues by selective dissolution and precipitation procedures, and it is a matter of considerable difficulty to remove noncollagenous contaminants. It is necessary, therefore, to demonstrate that any antibodies formed after immunization with tropocollagen preparations are directed toward the collagen molecule rather than other macromolecules which may occur with collagen from, for example, the ground substance.

It has been reported from these laboratories that antibodies may be produced in rabbits after immunization with purified tropocollagen preparations from calf skin. In these experiments it was shown that these antibodies were specific for the tropocollagen molecule by demonstrating the parallel loss of optical rotation and serologic activity when tropocollagen preparations were subjected to thermal denaturation (8). Furthermore, it was shown that the tropocollagen telopeptides, terminal or peripheral enzyme-labile peptides covalently bound to the molecules (9, 10), were required for complete serologic activity.

The present communication describes the tissue and species specificities of

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‡ Present address: Department of Biology, Massachusetts Institute of Technology, Cambridge, Mass. 02139.

§ Present address: Graduate Department of Biochemistry, Brandeis University, Waltham, Mass. 02154.

‖ Present address: Department of Biochemistry, University of Tennessee Medical Unit, Memphis, Tenn. 38103.

¶ Present address: New York Hospital, Cornell Medical Center, New York, N. Y. 10021.

tropocollagen preparations from calf, rat, sheep, and humans, and it attempts to localize these telopeptides on the polypeptide chains that make up the tropocollagen molecule.

Materials and Methods

Tropocollagen Preparations.—Acid-soluble tropocollagen preparations were made from skins of sheep, calves, rats, and humans, and from the swim bladders of carp (*Cyprinus carpio*) and buffalo fish (*Ictiobus bubalus*). The tropocollagens were obtained by successively washing the tissue with saline, extracting with saline and then with citrate buffers, and then selectively precipitating the tropocollagen as previously described (11). The preparations were stored as frozen precipitates until required for use. They were redissolved by dialysis against 0.05% acetic acid before subsequent dialysis to the buffers appropriate for the experiments in question.

Tropocollagen Antisera.—For production of rabbit antisera, 2.5 mg of the tropocollagen preparation in 1.0 ml of 0.05% acetic acid was mixed with an equal volume of complete Freund's adjuvant and injected intramuscularly and into the toepads of rabbits at weekly intervals for three wk. The animals were bled 6 days after each injection. After approximately 1 month, they were given a booster injection by the same procedure and bled 1 wk after the booster injection. This procedure was continued over a period of 1 yr. In general, the antibody response reached a maximum after the first booster injection. Most subsequent booster injections resulted in little change in complement fixation titers. With some rabbits, after a number of booster injections, there was even a decrease in antibody titer.

Electron Microscopy.—Tropocollagen solutions were dialyzed against 0.05% acetic acid or 0.05 M acetic acid containing 0.2% adenosine triphosphate. The precipitated segment-long-spacing aggregates of tropocollagen were negatively and positively stained with phosphotungstic acid (adjusted to pH 4.2 by KOH) and examined in a Siemens Elmiskop 1 microscope at electronic magnifications of 40,000.

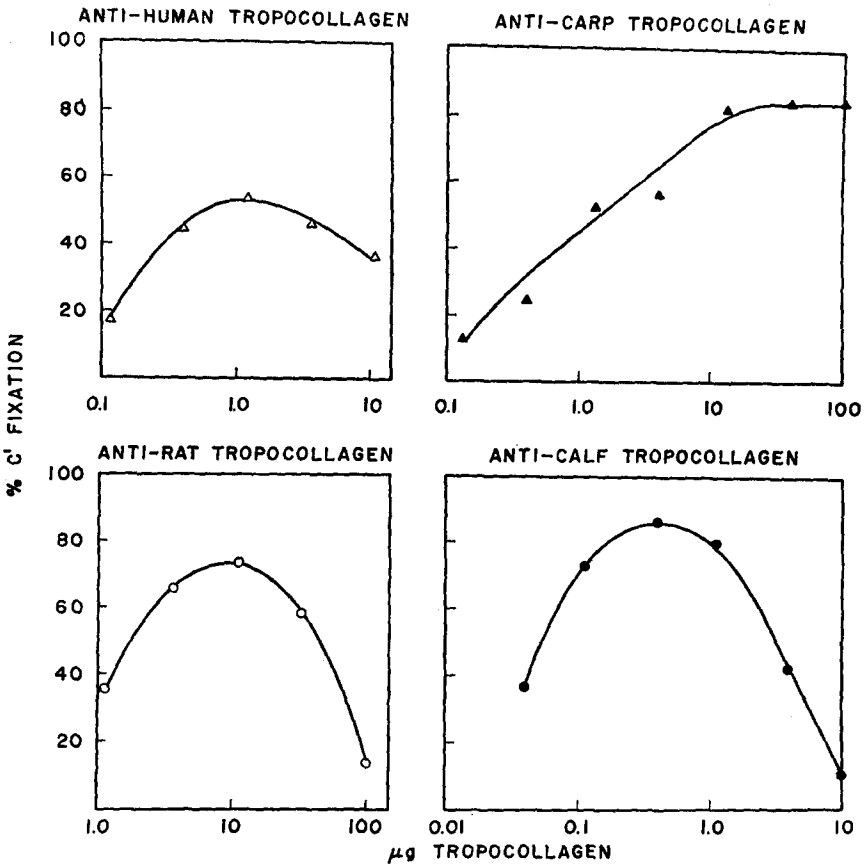
Fractionation and Characterization.—Denatured tropocollagen preparations were fractionated on a heated column of carboxymethyl cellulose (Whatman microgranular CM 32) at 44°C in pH 4.6 acetate buffer, conditions adapted from those described by Piez et al. (12). Fractionated preparations were compared by the disc electrophoresis technique of Francois and Glimcher (13). The denaturation of the tropocollagen preparations was monitored by polarimetric measurements on a Rudolph polarimeter equipped with an oscillating polarizer prism, or by viscosity measurements in Ubbelohde viscometers in a thermostat maintained at 25 ± 0.002°C.

Protease Treatment.—Tropocollagen preparations were digested with pepsin (1:100 by weight, or 1:8 mole/mole of enzyme:substrate) in 0.05% acetic acid (pH 3.5) at 20°C for 24 hr. Peptide fragments were removed by dialysis, and the tropocollagen was separated from the pepsin by free diffusion electrophoresis (9, 14) or by repeated precipitation of the tropocollagen by 15% KCl (15). Pronase or chymotrypsin treatment was performed at pH 7.2 in 0.1 M calcium acetate 1:100 enzyme:substrate ratio, at 20°C for 12–16 hr. The tropocollagen was recovered by KCl precipitation.

Complement (C′) Fixation.—Serologic analyses of the tropocollagen immune systems were performed according to Levine (16). The diluting buffer was 0.15 M NaCl–0.01 M Tris, pH 7.4, containing 1.5×10^{-4} Ca^{++} and 5×10^{-4} Mg^{++} and 0.1% bovine serum albumin. Because of the relative insolubility of high concentrations of tropocollagen in this buffer, stock tropocollagen preparations of around 5 mg/ml of 0.05% acetic acid were diluted to contain 10 or 100 μg of tropocollagen per ml in Tris buffer, and further diluted to contain the appropriate concentrations of antigen just prior to the addition of 1.0 ml to the 5.0 ml reaction mixtures containing the C′ and immune serum.

RESULTS

C′-fixing antibodies were present in sera of rabbits immunized with tropocollagen preparations obtained from the skins of calves, rats, and humans, and the swim bladders of carp and buffalo fish. The C′-fixing activities of the anti-

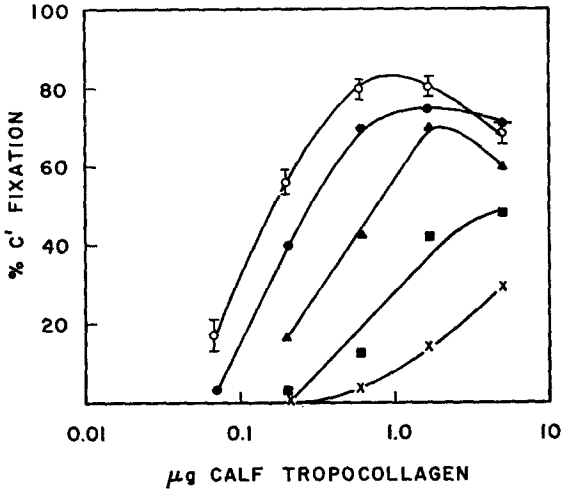


TEXT-FIG. 1. Fixation of C′ by homologous tropocollagens and rabbit anti-human tropocollagen, diluted 1/40; anti-carp tropocollagen, diluted 1/50; anti-rat tropocollagen, diluted 1/500; and anti-calf tropocollagen, diluted 1/100.

sera varied from dilutions of 1/40 with anti-human tropocollagen to 1/500 with anti-rat tropocollagen. The tropocollagen molecules are weak immunogens when compared to antigens such as bovine pancreatic ribonuclease, ovalbumin, chicken lactic dehydrogenase, or bovine serum albumin with which C′-fixing titers of 1/10,000 have usually been observed in our laboratories. The C′ fixation curves obtained with anti-human, anti-rat, anti-calf, and anti-carp tropo-

collagen, and their homologous antigens are shown in Text-fig. 1. The quantity of tropocollagen required for maximum C′ fixation varies with each system (about 0.8, 1.0, 10, and 100 μg of calf, human, rat, and carp tropocollagen, respectively). In general, early bleedings of the immunized rabbits required more antigen for maximum C′ fixation than later bleedings.

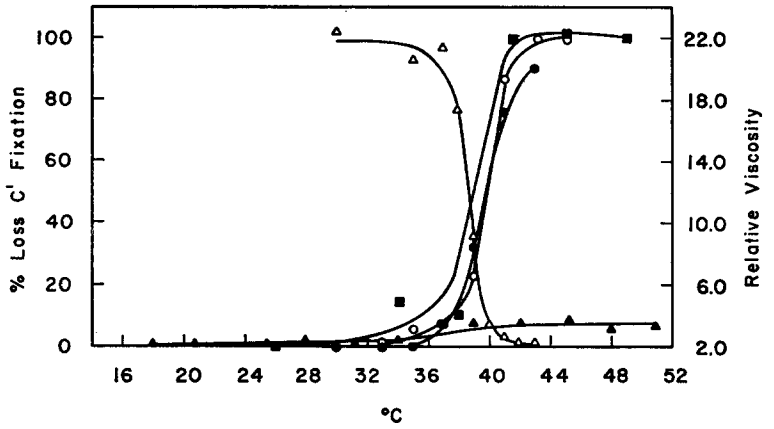
In order to identify the antibodies as anti-tropocollagen, tropocollagen preparations in 0.1 M Ca acetate (100 μg/ml) were heated at varying temperatures for 10 min, quickly chilled and diluted, and immediately assayed for C′-fixing



TEXT-FIG. 2. Fixation of C′ by calf skin tropocollagen after incubation of 100 μg/ml of 0.1 M Ca acetate at various temperatures for 10 min. After incubation, reaction mixtures were diluted to contain 5 μg/ml in Tris buffer at 0°C for serologic assay. 0°, 21°, 33°, 35°C; (○) (●) 37°C; (▲) 39°C; (■) 41°C; (×) 43°C.

activity. The data in Text-fig. 2 show the resulting C′ fixation curves after calf tropocollagen was subjected to such thermal treatment. The C′ fixation curves of calf tropocollagen incubated at 0°, 31°, 33° and 35°C are identical. After incubation at 37°, 39°, 41° and 43°C, there is a progressive decrease of C′-fixing activity. If the activities of the treated tropocollagen solutions are compared at the 0.8 μg level, a temperature profile can be obtained. The temperature profiles for calf, human, rat, and carp tropocollagens are shown in Text-fig. 3. The three mammalian tropocollagens are irreversibly denatured by 10 min heating around 41°C. Very similar temperature profiles were obtained when the optical rotation (8) or viscosity of heated and then quenched tropocollagen solutions was measured. With carp tropocollagen, however, there was a large difference between the temperature profiles measured by optical rotation and serologic activity. Whereas carp tropocollagen melted out at 32°C when meas-

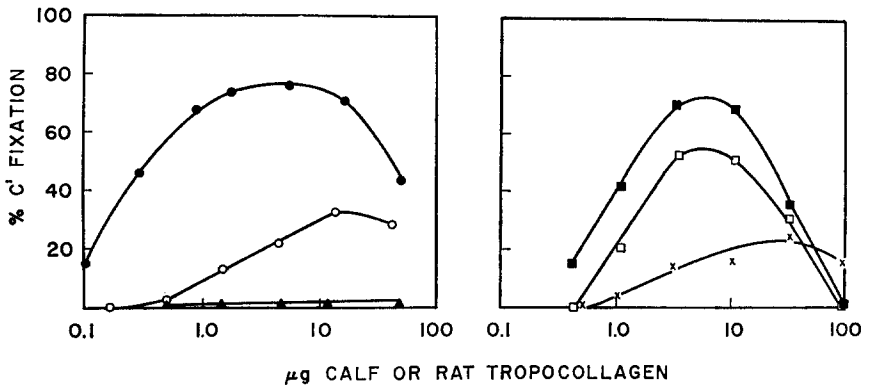
ured by optical rotation, little if any change in C′-fixing activity was observed even after heating the tropocollagen at 49°C. Thus it would appear that the antiserum provoked by mammalian tropocollagens contained antibodies directed toward the tropocollagen molecules, whereas anti-carp collagen contained antibodies directed toward material which does not readily denature, perhaps an impurity in the carp tropocollagen preparation. Since the carp tropocollagen C′ fixation reaches equivalence at about 100 μg, as little as 1% of an immunogenic impurity could conceivably account for this C′-fixing activity.



TEXT-FIG. 3. Temperature profile of calf (●), rat (○), human (■), and carp (▲) tropocollagens as measured immunologically, and rat tropocollagen measured by relative viscosity (△).

The C′-fixing activity of calf or rat tropocollagen is decreased after treatment with pepsin or Pronase (Text-fig. 4). Chromotryptic digestion also decreases the serologic activities of these tropocollagens. Whereas 1–5% of the molecule is excised and released as dialyzable telopeptides by these treatments, no changes in the optical rotation properties or the electron microscopically observed characteristics of the aggregated subunits are measurable (15). From these observations it is concluded that the antigenic determinants on the molecule measurable at these dilutions of antiserum are, or include, the telopeptides; they may be excised, or the conformation around their sites may be changed by the enzyme action.

In an attempt to assess the immunogenic and serologic properties of telopeptide-excised molecules, Pronase-treated tropocollagen was used to immunize a rabbit. Pronase-treated tropocollagen was immunogenic and these antibodies did not distinguish the native molecules from the telopeptide-excised molecule, whereas antibodies against native molecules clearly did (Table I). Although



TEXT-FIG. 4. Fixation of C′ with anti-calf tropocollagen (left side), anti-rat tropocollagen (right side), and calf tropocollagen (●), pepsin-treated calf tropocollagen (○), Pronase-treated calf tropocollagen (▲), rat tropocollagen (■), pepsin-treated rat tropocollagen (□), and Pronase-treated rat tropocollagen (×).

TABLE I

Species Specificity of Tropocollagens

Tropocollagen immune serum	Antiserum dilution	Maximum C′ fixation with tropocollagens from						
		Calf	Pronase-treated calf	Sheep	Human	Rat	Carp	Buffalo fish
Calf skin	1/100	90	0	85	0	0	0	0
Calf skin, pronase-treated	1/50	49	57	—*	0	—	0	0
Rat skin	1/500	0	—	0	0	77	0	0
Human skin	1/40	0	0	0	51	—	0	0
Carp swim bladder	1/50	0	—	0	—	—	85	88
Buffalo fish swim bladder	1/50	0	—	0	—	—	55	64

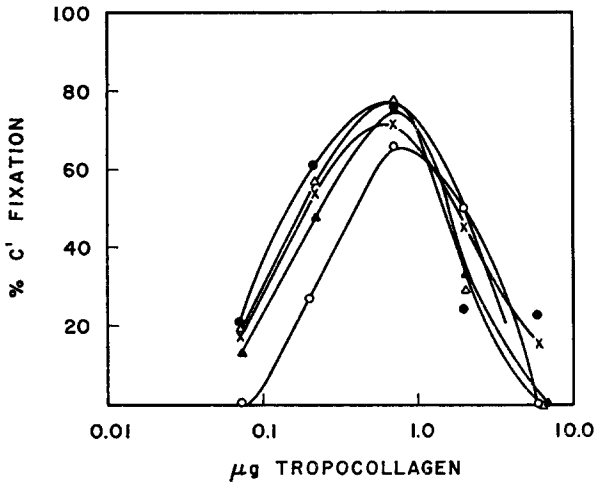
* Not analyzed.

this would suggest that the protease-resistant triple helix body of the molecule is immunogenically and serologically active, the possiblity that the activity resides in telopeptide "stumps" cannot be excluded at this time.

To assess the tissue specificity of these telopeptides, tropocollagen preparations from several tissues of a calf were tested for their C′-fixing activity with antibody produced by immunizatior with calf skin tropocollagen. Prepara-

tions were made from calf aorta, dura mater, Achilles' tendon, and sciatic nerve epineural sheaths. In each case the tropocollagen could not be distinguished from that of calf skin (Text-fig. 5). It may therefore be concluded that no tissue specificity exists with respect to the tropocollagen molecules isolated from different tissues and that the same telopeptides are present in each population of molecules in the extracts—but not necessarily on each molecule.

To assess to what degree the telopeptides from one individual may differ from those of another, antibodies produced after immunization with tropocollagen preparations from one calf were assayed against tropocollagen preparations from the skins of other calves of the same Black Angus and also Hereford

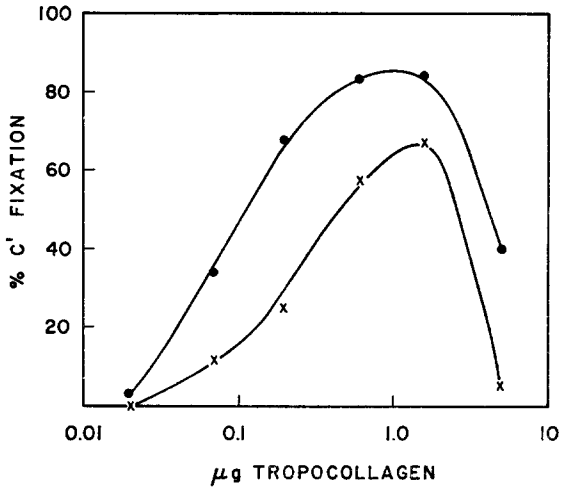


TEXT-FIG. 5. Fixation of C′ by calf tropocollagen from skin (●); sciatic nerve (○), aorta (▲); dura mater (△); and Achilles tendon (×) and anti-calf skin tropocotlagen.

strains. Identical complement-fixing curves were obtained with these tropocollagen preparations thereby, implying a lack of individual specificity in the telopeptides.

Although tropocollagen preparations from various species are reported to differ somewhat in amino acid composition (17), and in particular in the ratio of hydroxyproline to proline, it was observed that the phosphotungstic acid–stained segment-long-spacing aggregates (which have been described as the "molecular fingerprint" of tropocollagen) (18) could not be discriminated under the electron microscope (Figs. 1 and 2). The preparations from different species could, however, be distinguished by C′ fixation. The data showing the species specificities of the tropocollagens are given in Table I. At the dilutions of immune sera used to obtain C′ fixation with the homologous antigens, no cross-reaction was observed with heterologous tropocollagens of different species with

the exception of the ruminant, carp, and buffalo fish tropocollagen antisera. The buffalo fish tropocollagen did cross-react to almost the same extent as the carp tropocollagen with its own antiserum. These antibodies, however, as noted earlier, have not been identified as anti-tropocollagen. The sheep and calf tropocollagens could not be distinguished by their reaction to anti-calf tropocollagen serum, implying a close immunologic relationship between these species. With antiserum to Pronase-treated tropocollagen, species specificity was still observed (Table I). Whether this species specificity resides in the triple helix body of the molecule or in telopeptide "stumps" or in other protease-resistant telopeptides is being investigated.

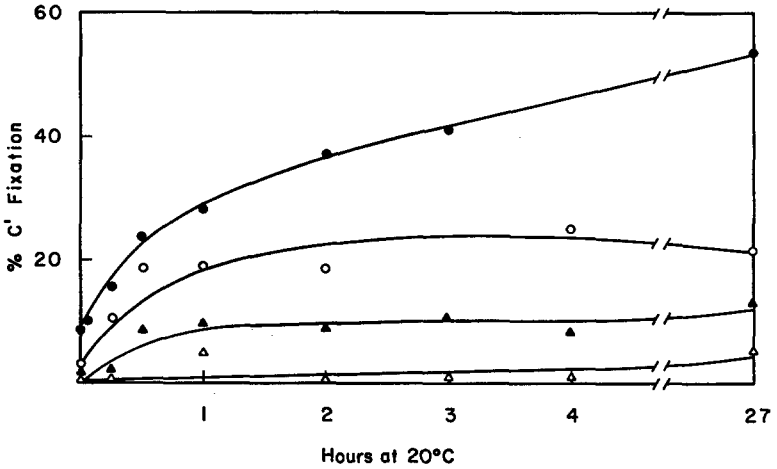


TEXT-FIG. 6. Fixation of C′ by untreated calf skin tropocollagen (●) and renatured tropocollagen after treatment at 45 for 10 min (0.3% tropocollagen/ml 0.05 M acetic acid) and incubation at 2-4°C for 48 hr. (×).

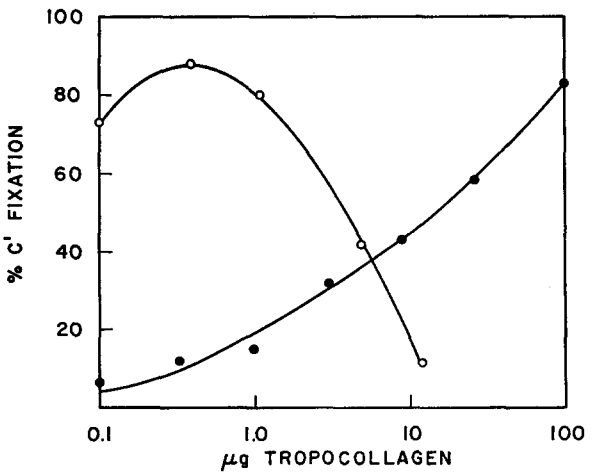
The C′-fixing property of heat-denatured tropocollagen can be restored by cooling the solution at an adquately high concentration. This point was discovered when on reassay, cooled tropocollagen solutions originally used to establish optical rotation melting curves were found to have regained most of their C′-fixing ability, whereas such recovery was not detected in the more dilute solutions used for C′ fixation assay; since the assay is performed on cooled solutions, the melting profile shown in Text-fig. 3 could not otherwise have been found. Further investigations to resolve these ambiguous results indicated that we were observing a concentration dependence for the recovery of the serologic activity; only at gelatin concentrations close to 0.3% was it possible to observe a C′-fixing ability in the recooled solutions similar to or only slightly lower than that of the original native preparation (Text-fig. 6). The time course of the

serologic renaturation as a function of tropocollagen concentration is shown in Text-fig. 7.

Heat-denatured collagen solutions (i.e. gelatins) regain up to two-thirds of their original optical rotation—an indication of ordered helical structures—on cooling. Except under selected annealing conditons, however (19), this increase

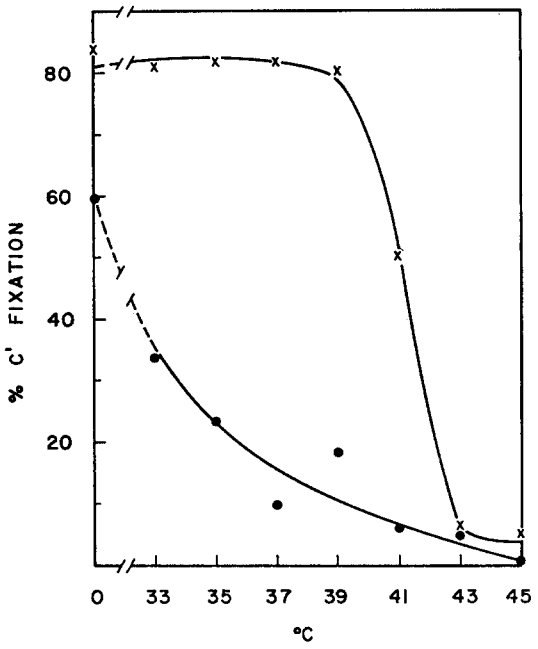


TEXT-FIG. 7. Fixation of C′ by calf skin tropocollagens after thermal denaturation at various concentrations (45°C for 3 min) and incubation of the denatured tropocollagens at 20°C for various periods of time. 0.13% (●), 0.065% (○), 0.032% (▲), and 0.016% (△) in 0.1 M Ca acetate. Maximum C′ fixed by untreated tropocollagen was 88%.



TEXT-FIG. 8. Fixation of C′ by calf skin tropocollagen (○) and a γ-enriched fraction of renatured collagen (●).

in optical rotation mostly reflects intrachain refolding and bimolecular association, and the only significant fraction of the gelatin restored to the state of the original triple helix is the triply cross-linked γ-molecules (20, 21). In independent experiments, however, with solutions fractionated and highly enriched with γ-molecules (Drake and Davison, unpublished experiments) it was found that γ-tropocollagen does not react with antibody at the low levels of protein used in these experiments (Text-fig. 8.).



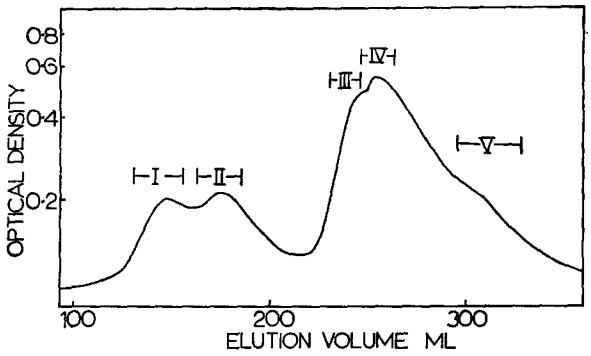
TEXT-FIG. 9. Temperature profile of C′ fixation by untreated calf skin tropocollagen (×) and renatured collagen (●).

It would appear, therefore, that a reconstitution of the antigenic sites is possible when the intermolecular aggregation of two α-chains, or possibly one α- with a β-chain, occurs and this process is facilitated at high gelatin concentrations. However, the intramolecular folding of and one α-chain or helix formation in a β-chain which occurs in dilute solution (22) does not appear to give rise to a serologically reactive site.

Confirmation that the C′-fixing ability does not pertain to a small complement of native tropocollagen molecules was obtained by further denaturation experiments. The renatured tropocollagen was melted out at 100 μg/ml and the resulting temperature profiles of the native and renatured solutions are

shown in Text-fig. 9. Whereas the native tropocollagen denatured at around 41°C, the renatured species melted out at around 33°C.

As the enzyme studies showed, the antigenic sites are associated with the small fraction of the tropocollagen molecule vulnerable to digestion by pepsin or Pronase. The intramolecular distribution of these sites was sought by frac-



TEXT-FIG. 10. Elution profile (monitored at 230 mμ) of peptide chains derived from denatured calf skin tropocollagen applied to a 5 cm high, 2 cm diameter column of Whatman CM32 at 44°C and eluted by a sodium chloride gradient. The fractions I to V were collected, dialyzed, and lyophilized for serologic tests (see Table II).

TABLE II

Serologic Activity of Renatured Fractions From Heat-Denatured Tropocollagen

Fractions	Intramolecular distribution	Maximum C′ fixation	Weight protein giving maximum C′ fixation
			μg
I	α_1	16	12
II	β_{11}	28	12
III	γ	64	20
IV	β_{12}	30	16
V	α_2	25	12
Native		91	0.6

tionation of the denatured tropocollagen preparations into the constituent α- and β-chains. Fractionation was accomplished on a heated column of carboxymethyl cellulose (Text-fig. 10) and fractions of eluent corresponding to the separate peaks were grouped, lyophilized, redissolved in a small volume, dialyzed free of buffer, and again lyophilized. This procedure was carried out as rapidly as possible and at low temperatures since prior experience showed that the reactivity of the preparations in certain instances could be readily lost, presumably from bacterial or enzyme contaminations.

It was found that each of the separated fractions of the tropocollagen reacted with the antiserum, but none achieved C′-fixing ability of the native tropocollagen (Table II). The identity of these fractions was checked by disc electrophoresis: Only fractions I and II appeared pure, the remainder containing admixtures of other components than those indicated as predominant.

Collagen has been shown to contain polymeric molecules, by both the identification of intermolecularly linked chains (15, 23, 24) and by the physical characterization of tropocollagen solutions (25). The elimination of the end-to-end polymers by controlled sonic irradiation, with the scission of the interlinking structures (probably the telopeptides) through which the polymers are formed, did not detectably change the C′-fixing ability of the solutions, but the possibility of exposing new immunogenic groups by this treatment was not tested.

DISCUSSION

These experiments demonstrate that purified tropocollagen is immunogenic and that the serologic activity is, apart from the ruminants, species specific, although the specific antisera did not distinguish between tropocollagen from animals of the same species or strains nor from the different tissues of any one animal. Similar conclusions have been reached by other investigators (4, 5, 6). On the other hand, the electron microscopic examination of the stained segment-long-spacing aggregates of precipitated collagen showed no consistent difference by which the preparations from the different species could be distinguished. Kuhn, Grassmann, and Hofmann (26) have shown that the phosphotungstic acid–stained segments of tropocollagen are binding stain to arginine residues, and to a lesser degree to lysine residues. The identity of the staining diagram for the different tropocollagen preparations implies that the distribution of basic residues along the lengths of the constituent polypeptide chains is, to the limit of the resolution of this technique, unchanged from fish to man. To what extent this homology extends to other amino acid residues remains to be discovered. Collagen may, therefore, be an excellent substrate for definitive studies on the evolutionary development of protein primary structure.

Earlier publications from this laboratory (11, 15) have reported that small segments of the native tropocollagen molecule are accessible to proteolytic enzyme attack (exempting the collagenases from disscussion), and it was postulated that the sites of proteolysis are terminal or peripheral regions of the molecule where peptide chains protrude beyond the protease-resistant triple helix body of the molecule. It was also shown that on or through these structures, which we designated as telopeptides, intermolecular and intramolecular interchain cross-links were formed, and it was postulated that enzymes act upon these telopeptide structures in the course of maturation of collagen tissue to produce the highly cross-linked structure of adult collagen. The existence of telopeptides was for some time disputed by other workers in the field, but

other laboratories have subsequently confirmed many of our findings (27, 28). For example, Bornstein and Piez (27) have recently analyzed chain-terminating peptides isolated from rat skin tropocollagen which conform perfectly to our definition of telopeptides: They are accessible to enzyme attack; are enzymically modified in the process of maturation; and through them cross-links between chains are formed.

In the present experiments, the fractionation studies have shown that the antigenic sites exist on both α1- (and/or α3-) and α2-chains, and the enzyme experiments show that these antigenic structures can be largely removed by enzyme treatment. In particular, the loss in serologic activity of rat tropocollagen after chymostrypsin treatment may well be related to the peptides released in this process and partially characterized by Bornstein and Piez (27) The residual C′-fixing activity could relate to structures below the points of enzyme attack, or to other peripheral or carboxy-terminal peptides. Preliminary experiments with the unfractionated dialyzable peptides released by chymotrypsin have shown that they can inhibit tropocollagen–anti-tropocollagen C′ fixation. Attempts are being made to fractionate and identify these serologically active peptides from rat skin tropocollagen. We therefore conclude that the primary immunogenic sites on the tropocollagen molecule are telopeptides, and we now postulate that these structures have changed in the course of evolution from species to species whereas the main triple helix body of the tropocollagen molecule has remained largely unaltered.

Granted that the immunologic characteristics of tropocollagen directly reflect the nature of the tropocollagen telopeptides, it is likely that immunologic techniques will provide a sensitive probe to test for modification in telopeptide structures—those that occur as a function of maturation and those that may also be presumed to occur through metabolic disturbances and disease processes in connective tissues. Thus a study of the immunologic characteristics of normal and lathyritic tropocollagen of rat skin is in progress.

The fact that C′-fixing activity is recovered in cooled gelatin solution enabled us to ascertain that C′-fixing sites occur on both α1- and α2-chains. That the recovery of serologic activity is concentration dependent carries implications about the structure at the active sites. Piez and Carillo (22) showed that intramolecular folding and helix formation occurs in β-chains at low concentrations without aggregation but such changes clearly do not reconstitute the antigenic sites from our experiments. The same authors showed that intermolecular association and helix formation can occur in solutions of α-chains but the process is markedly concentration dependent at concentrations below 1.5 mg/ml. We may deduce that the restoration of serologic activity requires the association of separated strands and perhaps the reformation of a locally triple-stranded structure from α- and β-chains before the telopeptides and/or the adjacent polypeptide backbone chains fold and adopt a conformation that the antibodies can recognize.

SUMMARY AND CONCLUSIONS

Tropocollagen preparations from carp, buffalo fish, rats, calves, sheep, and humans have been studied by electron microscopy and serologic methods. Tropocollagens from each species appeared identical by electron microscopy but they were readily distinguished (except between sheep and calves) by C′-fixation tests with rabbit antisera against the various tropocollagens. Tests with calf tropocollagen antiserum showed no distinction between tropocollagen isolated from different tissues nor between individuals of the same or different strains.

The major immunogenic sites in native tropocollagen are the telopeptides, and these are present on both α1- and α2-chains. The C′-fixing activity was lost with heat denaturation of the tropocollagen, but could be recovered in a concentration-dependent process on cooling.

The fact that pure and enzyme-treated collagen can provoke serologic reaction implies that collagenous sutures and prostheses used in surgery may lead to sensitization and rejection, a fact which may merit clinical concern.

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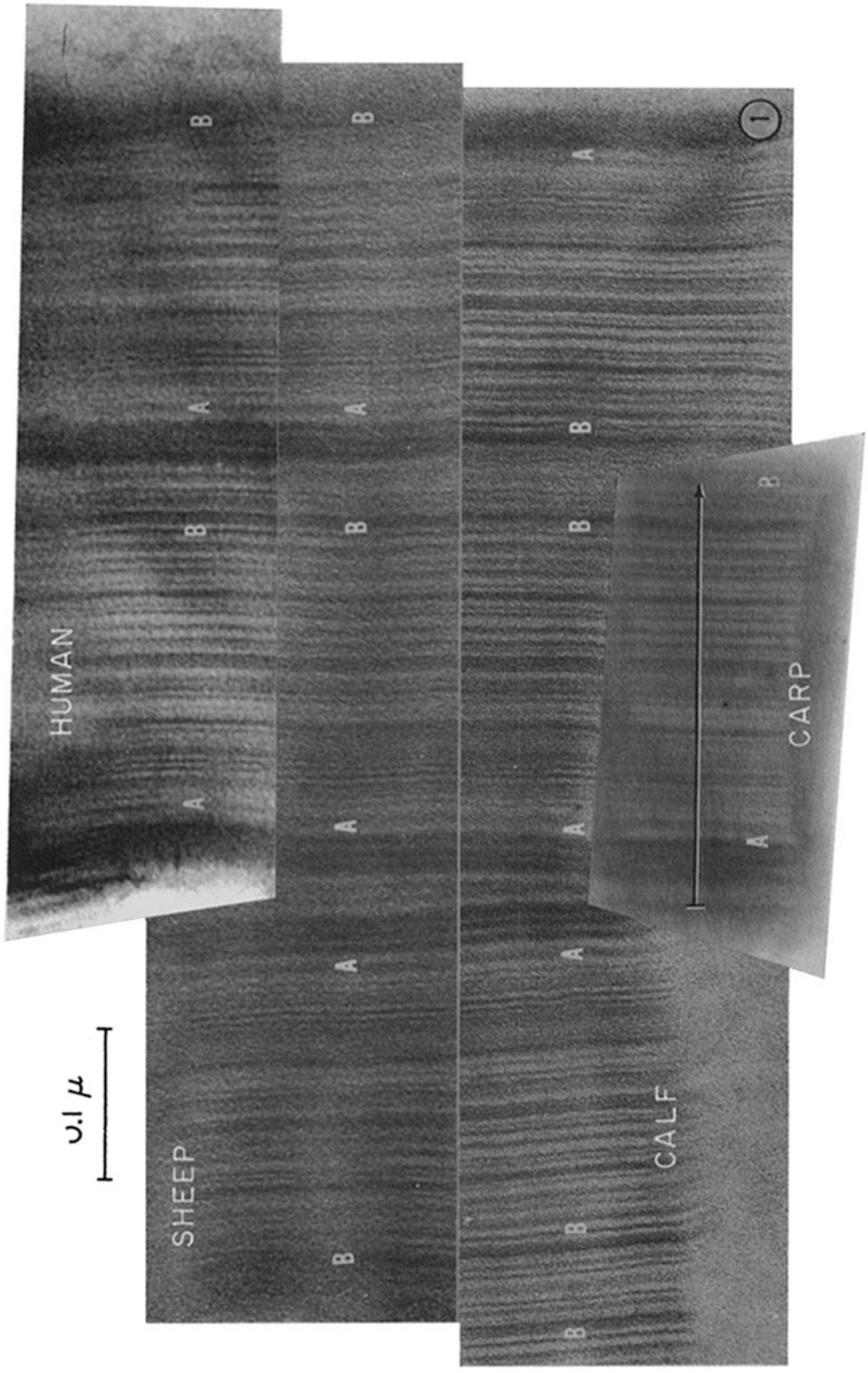
EXPLANATION OF PLATES

PLATE 32

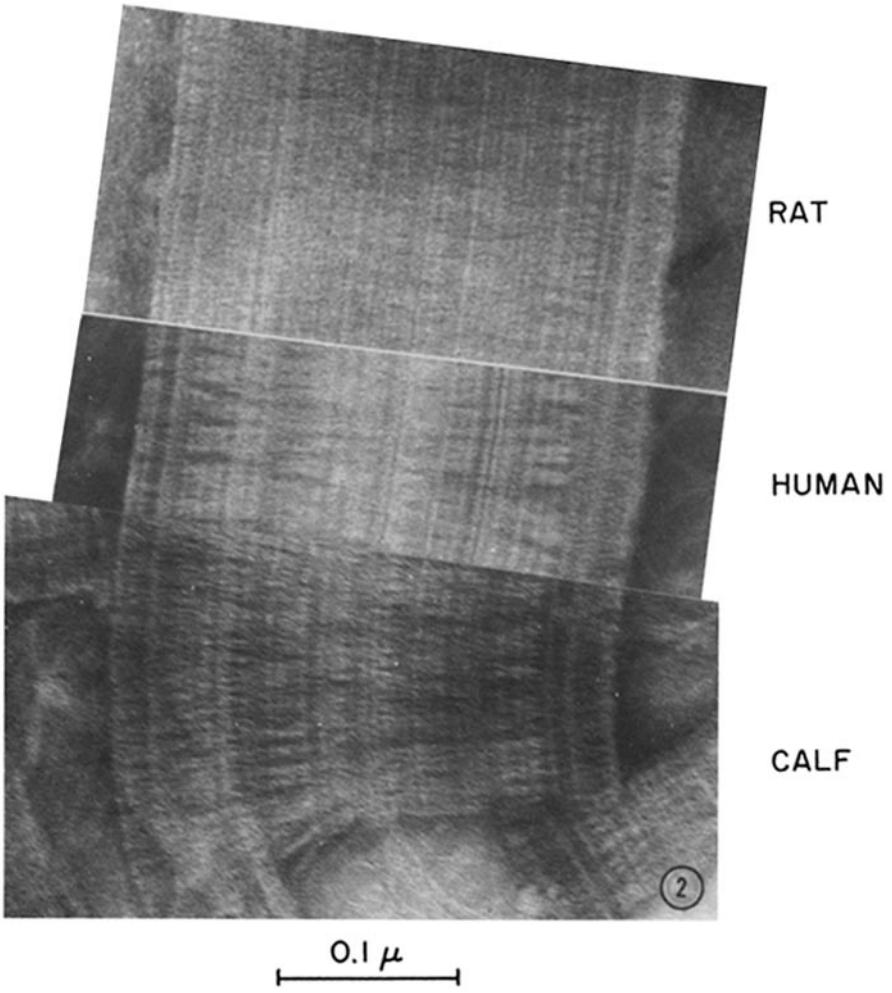
FIG. 1. Positively stained segment-long-spacing aggregates of tropocollagens from human, sheep, calf, and carp tissues. The arrow indicates the length of a molecule and the uptake of phosphotungstic acid reflects the distribution of basic aminoacid residues along the molecule. The single segment from carp with the ends marked A and B is juxtaposed to head-to-head, tail-to-tail, and head-to-tail blocks of segments from the other species; note particularly that the right hand junctions in the sheep and calf pictures are not comparable, one segment being reversed.



(Davison et al.: Antigenicity of tropocollagen)

PLATE 33

FIG. 2. Negatively stained (phosphotungstic acid pH 7) segments of rat, human, and calf tropocollagen juxtaposed to show the strong resemblance in dimensions and distribution of stained regions. Although the similarity is less obvious than in Fig. 1, the differences between the preparations are no more than those seen between different grids prepared from any one tropocollagen sample.



(Davison et al.: Antigenicity of tropocollagen)