THE ANTIGENICITY OF RAT COLLAGEN*

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A study of collagen was initiated several years ago in relation to work on the pathogenesis of rheumatic fever since it is generally believed that the primary injury in this disease is to collagen, the ground substance, or both (1–5). The chemistry and structure of collagen have been studied extensively (6–19) but little is known of its biological properties.[1] The studies to be reported below relate primarily to the antigenicity of collagen.

Review of Previous Work

Nageotte (20, 21) showed that when the tail tendons of the rat are placed in dilute acetic acid (1:25,000 dilution in distilled water) the collagen fibers slowly swell and over a period of time go into solution. He also demonstrated that fibers reconstituted from this acid solution by the addition of sodium chloride retain the tinctorial properties of native collagen. Wyckoff and Corey (22) demonstrated that the x-ray diffraction pattern of this reconstituted material was like that of the native tendon. Schmitt, Hall, and Jakus (9) and others (23, 24) have found that fibers reformed from dilute acid solutions either by neutralization or the addition of salt retain the characteristic striations of native collagen as observed in electron micrographs.

It is generally agreed that gelatin, a soluble derivative of collagen, is non-antigenic (25, 26). Loiseleur and Urbain (27) reported complement-fixing antibodies in the sera of rabbits immunized with an acid solution of rat tail tendons prepared as described by Nageotte. Hopps (28) demonstrated antibodies to catgut and sheep intestine; however, there were cross-reactions with sheep serum. With highly purified preparations of sheep collagen obtained from Dr. F. O. Schmitt he reports agglutination with anti-catgut rabbit serum in a dilution of 1:80; however, another sample of the highest

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[1] For a complete bibliography see Borasky, R., Guide to the Literature on Collagen, Philadelphia, United States Department of Agriculture, Eastern Regional Research Laboratory, 1950.

purity failed to agglutinate. Battista (29), employing collagen implants, was unable to find evidence of antigenicity and Waksman and Mason (30), using preparations of human and rabbit collagen alone and also coupled with sulfanilic acid and globulin, failed to demonstrate antigenicity to collagen. It is notable that of the above workers only Loiseleur and Urbain employed a soluble collagen preparation and stressed the importance of avoiding possible denaturation. Although these workers were able to demonstrate complement-fixing antibodies to their collagen preparation, they failed to show that the reaction was specifically directed toward collagen rather than toward other tissue proteins or polysaccharides.

Materials and Methods

Preparation and Purification of Collagen.—Tendons supply a relatively pure source of collagen. Tail tendons from the Whalen strain of rats were used throughout except in a single experiment in which tendons from the legs and back were employed. The tail tendons are easily obtained following removal of the skin by successively fracturing small pieces of the tail beginning at the tip and gently pulling out the tendons. Following removal, the tendons are placed immediately in cold saline and all small fragments of bone and muscle attached to the free ends are removed by scissors. The tendons are then thoroughly washed in four to six changes of cold 0.9 per cent saline, finely minced with scissors, and left in saline overnight at 4°C. The next day the minced tendons are washed with three further changes of 0.9 per cent saline, then three changes of distilled water, and placed in dilute acetic acid (1:10,000 dilution in distilled water) at pH 3.8. The washed and minced tendons obtained from six adult rats are placed in approximately 500 cc. of dilute acetic acid.[2] The preparation is stirred frequently during the next 2 to 3 days and its volume is made up gradually to 8 liters by additions of dilute acetic acid. The resulting solution is slightly viscous with a small amount of undissolved residue. After the addition of 10 gm. of filter aid, Celite No. 512,[3] per liter the solution is filtered through No. 597 Schleicher and Schüll filter paper prepared with an acid suspension of the filter aid. The water-clear, very slightly viscous filtrate, is then distributed in 250 cc. centrifuge botles, the pH adjusted to approximately 7.3 with 1/N sodium hydroxide, and left overnight at 4°C. The next day the sticky, stringy precipitate which has formed is collected by centrifugation and washed once with approximately 8 volumes of distilled water. To the clear, gel-like precipitate remaining in each bottle, 20 to 40 cc. of dilute acetic acid is added. After 24 to 48 hours the highly viscous solution is dried from the frozen state and the fluffy, white, fibrous material stored at 4°C. for use. All procedures in the preparation of the purified collagen are carried out in a cold room at 4°C. Solutions of collagen containing 1 mg. per cc. in dilute acetic acid were usually employed because more concentrated solutions are too viscous to handle easily and at concentrations of 5 to 7 mg. per cc. a gel is formed.

Immune Sera.—Immune sera were prepared by injecting rabbits intraperitoneally; intravenous injection of the collagen solution caused death from pulmonary embolism due to precipitated collagen and injections by the subcutaneous route elicited little or no antibody response. In general the following schedule for immunization was employed: After a preimmunization bleeding, rabbits were given 4.0 cc. of a 0.1 per cent solution of collagen the first 4 days of each week and allowed to rest the remaining 3. After 8 to 12 weeks test bleedings were made and at this time the sera of many of the animals showed complement-fixing antibodies in very low titer. Following a rest period of 4 to 8 weeks booster courses of injections

[2] The term dilute acetic acid as used throughout this paper refers to a dilution of 1 part of acetic acid in 10,000 parts of distilled water.

[3] The filter aid, Celite No. 512, was obtained from the Johns-Manville Corporation.

were given for 2 to 4 weeks alternating with rest periods of 8 to 12 weeks. Only after 3 to 6 months of immunization were antisera of satisfactory titer obtained. A certain number of animals failed to respond satisfactorily and were discarded. Fifty cc. test bleedings were made from the ear following each course of injections and sera of satisfactory titer were pooled and stored at −73°C. Before use in complement fixation tests all antisera were inactivated by heat at 60°C. for 30 minutes. The complement fixation reaction was modified after the method described by Casals and Palacios (31). All dilutions except those of the antigen were made in veronal saline buffer containing traces of magnesium and calcium as described by Mayer and his associates (32). Antigen dilutions were made in dilute acetic acid.

Complement.—Fresh guinea pig serum stored at −73°C. was used as complement. The complement was always freshly titrated prior to each test and diluted for use in the test so that 2 units were contained in 0.5 cc.

Hemolytic System.—Washed sheep red cells and rabbit anti-sheep hemolysin[4] constituted the hemolytic system. The sheep cells were sensitized at least ½ hour before use in the test by mixing a 3 per cent suspension of packed red cells with an equal volume of hemolysin which contained 3 M.H.D. per 0.25 cc.

Complement Fixation Test.—The reaction was carried out in the following manner: 0.25 cc. of antiserum, 0.50 cc. of freshly prepared complement diluted to contain 2 units, and 0.25 cc. of antigen in serial twofold dilutions were incubated together in a water bath at 37°C. for ½ hour. The hemolytic system consisting of 0.5 cc. of sensitized sheep cells was then added and the tubes incubated again for ½ hour in a water bath at 37°C. The usual serum, antigen, and complement controls were always included. Complete hemolysis was expressed as − and absence of hemolysis as ++++, with ± to +++± indicating intermediate degrees of hemolysis. The titer was taken as the last dilution showing ++ or better fixation.

RESULTS

It was first necessary to show that the purified preparation contained collagen in a form altered little if at all from the native state and that it was essentially free from other recognizable tissue components which might be antigenic. To this end histological studies were made of the lyophilized material employing hematoxylin and eosin, Masson's connective tissue and Weigert's elastic tissue stains. These studies revealed that the purified material was of fibrous structure, that it retained the tinctorial properties of collagen, and that it was free from all cells and other recognizable tissue components. Electron micrographs of the same material dissolved in dilute acetic acid and precipitated by the addition of buffer at pH 6.0 showed reconstituted fibers of various sizes with striations characteristic of native collagen (see Figs. 1 to 3). An elementary analysis performed on a lyophilized sample yielded the following results: nitrogen, 17.25 per cent; carbon, 47.65 per cent; hydrogen, 6.72 per cent; phosphorus, 0.14 per cent; sulfur 0.82 per cent; ash, 0.50 per cent—a composition which is consistent with that of a protein.

Loofbourow and his associates (33) have suggested the possibility of assessing the purity of collagen preparations from their absorption in the ultraviolet region of the spectrum since amino acid analyses show that collagen and purified gelatin contain little or none of the aromatic amino acids, phenyl-

[4] The rabbit anti-sheep hemolysin was obtained from Parke, Davis and Company.

alanine, tyrosine, and tryptophane, which are responsible for the characteristic absorption of protein at wave lengths of 250 to 290 mμ. These workers prepared rat tail collagen according to the method of Bergmann and Stein (7) followed by reprecipitation of the material from acetic acid solutions. The ultraviolet absorption spectrum of this preparation showed no absorption maxima from 257.5 to 287.5 mμ. and resembled closely that of purified gelatin. They proposed that this purified rat tail collagen be used as a tentative standard of purity. It appeared of some interest to compare the ultraviolet absorption curve of our collagen prepared as described above, with collagen prepared according to Loofbourow and his associates. We found that the absorption curves of the two preparations were almost identical and closely resembled

TABLE I

Immunization of Rabbits with Purified Rat Collagen

Rabbit No.	Pre-immune bleeding	Antibody response following periods of immunization as indicated									
		Wk.*	Titer‡	Wk.	Titer	Wk.	Titer	Wk.	Titer	Wk.	Titer
6	—	8	1:32	11	1:128	15	1:128	19	1:512	23	1:1024
8	—	8	1:16	11	1:8	15	1:16	19	1:64	23	1:128
3-2	—	8	1:32	11	1:64	13	1:64	15	1:256	17	1:256
3-6	—	8	1:16	12	1:64	14	1:128	16	1:128	18	1:64
3-7	—	8	1:16	11	1:32	13	1:16	15	1:32	17	1:64
4-1	—	8	—	12	1:128	15	1:256	17	1:64	21	1:256

— indicates complete hemolysis or absence of fixation.

* Wk. indicates the number of weeks of active immunization each animal had received prior to the test bleeding.

‡ Titer indicates the highest dilution of antigen giving ++ or better fixation with undiluted serum.

the curve described by Loofbourow and his associates; that is, with no absorption maxima from 250 to 290 mμ. The two preparations were also found to be similar serologically, having the same titer when tested by complement fixation.

In Table I the results obtained following immunization of six representative rabbits with the purified collagen are shown. It is clear from this table that dilute acetic acid solutions of the purified rat collagen induce complement-fixing antibodies in the rabbit when injected intraperitoneally. Thirty-three animals have been immunized with purified rat collagen for periods of 3 months or longer. The response to immunization was measured by testing whole serum against serial twofold dilutions of antigen for complement-fixing antibodies. By this method the sera of twelve animals reached titer levels of 1:128 to 1:1,024, thirteen reached levels of 1:8 to 1:64, and eight levels of 1:4 or less. The last eight animals were discarded as unsatisfactory. In most instances there

is some prozoning of the reaction with the higher concentrations of collagen even with those sera having a titer of 1:64 or better. It is apparent from these results that even after prolonged immunization the antibody titer fails to reach high levels, a finding which may indicate that the material is a relatively poor antigen. Efforts to increase the antibody titer by employing the Freund adjuvant technique (34) by both the subcutaneous and intraperitoneal routes have been unsuccessful.

The results of these experiments indicate that the purified material contains collagen relatively unaltered structurally from its native state and the material in the preparation is antigenic but it is not possible to state categorically that the antibody is directed toward collagen rather than toward some accompanying impurities. Since it was not practicable to prove that the collagen preparation was pure, other methods were employed to demonstrate that the antibody was really directed toward collagen. The possibility that the antigenicity of the purified collagen was due to admixture with rat serum was first considered. The results of complement fixation and precipitin tests made with rat collagen and rat serum and their respective antisera are recorded in Table II. It is clear from this table that rat collagen does not react with rabbit antiserum prepared against rat serum nor does rat serum react with anti–rat collagen rabbit serum although both react with their homologous antisera. The results of this experiment eliminate the possibility that the antigenicity of the purified rat collagen is due to the presence of small amounts of rat serum or serologically related tissue proteins.

The possibility that the antigenicity of the collagen preparation was due to admixture with a tissue polysaccharide was next considered. The purified collagen gave a faintly positive Molisch test and a faintly positive periodate-fuchsin reaction according to the method of Hotchkiss (36), indicating that the material probably contains a trace of polysaccharide. It was also found that heating collagen solutions up to 100°C. for 30 minutes at either pH 4.0 or 7.2 reduced but failed to destroy the serological activity. To test the possibility that the antigenicity of the purified collagen might be due to a polysaccharide rather than to collagen the following experiment was carried out.

300 mg. of purified collagen was added to 30 cc. of distilled water containing acetic acid in a concentration of 1 part in 10,000. With stirring in the cold at 4°C. a gel resulted containing particles of undissolved collagen. The material was then heated to 65°C. for 1 hour, resulting in a complete loss of viscosity and dissolution of the remaining undissolved particles of collagen. After cooling the solution to room temperature, 50 mg. of crystalline pepsin[5] was added, the pH was adjusted to 1.5 with HCl, and the preparation incubated for 18 hours at 37°C. The pH was then adjusted to 7.8, 50 mg. of pancreatin[6] was added together with a few drops of toluene and chloroform as preservatives, and the preparation was again incubated for 72 hours at 37°C. The material was then deproteinized by shaking with six changes of chloroform

[5] Crystallized pepsin was obtained from the Armour and Company Laboratories, Chicago.

[6] Pancreatin was obtained from Parke, Davis and Company.

TABLE II

The Serological Specificity of Rat Collagen and Rat Serum

Antisera prepared against	Type of reaction	Antigen: Purified rat collagen 1 mg./cc. diluted* 1:															
		1	2	4	8	16	32	64	128	256	512	1,024	2,048	4,096	8,192	16,384	32,768
Purified rat collagen‡	Comp. fix.	++±	++++	++++	++++	++++	++++	++++	+++±	±	−	−					
Rat serum	Comp. fix.	−	−	−	−	−	−	−	−	−	−	−					
		Antigen: Rat serum diluted§ 1:															
		1	2	4	8	16	32	64	128	256	512	1,024	2,048	4,096	8,192	16,384	32,768
Purified rat collagen	Comp. fix.		−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	Precipitin‖	±	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Rat serum	Comp. fix.									++++¶	++++	++++	++++	++++	++	±	−
	Precipitin	++++	++++	++++	++++	+++	+++	++±	++	+±	+±	+	+	±	±	−	−

− indicates complete hemolysis in complement fixation and absence of precipitate in precipitin tests or negative reactions, in this and all subsequent tables.

++++ indicates absence of hemolysis in complement fixation and maximal heavy precipitate appearing immediately in precipitin tests, in this and all subsequent tables.

± to +++± indicates intermediate degrees of hemolysis or precipitate respectively, in this and all subsequent tables.

Blank indicates no test, in this and all subsequent tables.

* Collagen dilutions prepared in distilled water containing acetic acid in a dilution of 1 part in 10,000, pH 3.8.

‡ Precipitin tests could not be performed with collagen as this antigen is insoluble in the presence of serum.

§ Rat serum diluted 1:2 in 0.9 per cent saline and inactivated at 65° for 30 minutes. Dilutions prepared in 0.9 per cent saline.

‖ Precipitin test performed according to method of Swift, Wilson, and Lancefield (35).

¶ Complement fixation reactions with rat serum started with dilution of 1:200 and diluted twofold with highest dilution showing significant reaction (++) 1:6,400.

For details of technique see Methods.

containing a small amount of amyl alcohol. A control without collagen was included. Samples were removed at various stages in the above process for serological tests as indicated in Table III.

It is clear from Part I of Table III that the material still had serological activity after heating to 65°C. for 1 hour but had completely lost its serological activity following digestion with pepsin and pancreatin although the material still gave a positive test for polysaccharide by the periodate-fuchsin method. It is also evident from Part II of this table that the digested material failed to inhibit the fixation of complement, thus making it unlikely that polysaccharide hapten is causing the reaction.

In 1948, Bidwell and van Heyningen (37) described the preparation of a purified collagenase (k toxin) from culture filtrates of *Clostridium welchii* which apparently attacks only collagen and gelatin. Because of the high degree of substrate specificity demonstrated by this enzyme it was desirable to test its effect on the serological activity of our purified collagen. It was found that when 5 mg. portions of the lyophilized purified collagen and native rat tail collagen were each placed in 1.5 cc. volumes of a 0.2 per cent solution of collagenase[7] in veronal saline buffer pH 7.3 at 37°C., both were rapidly disintegrated whereas crystalline trypsin had little or no effect under the same conditions. To test the effect of collagenase on the serological activity of the purified collagen the following experiment was performed.

To 25 cc. of vernonal saline buffer containing 10 mg. of collagenase 25 cc. of a 0.1 per cent solution of purified collagen in dilute acetic acid was added dropwise. The pH was then adjusted to 7.3, 1 cc. of toluene added as a preservative, and the material incubated at 37°C. for 24 hours. Following dialysis against distilled water at 4°C. for 5 days, 0.1 cc. of 1:20 dilution of acetic acid was added to bring the final concentration back to 1 part in 10,000 and redissolve the collagen. This material was then used as antigen for complement fixation. As controls collagen was treated in a like manner with crystalline trypsin and M extract prepared from type 6, Group A beta hemolytic streptococci[8] was treated with both collagenase and trypsin in a similar manner. As untreated controls both collagen and type 6 M extract were carried through the same process without the addition of either enzyme. The collagen and type 6 M extract each treated by collagenase and trypsin and untreated were then used as antigens in complement fixation tests with their respective homologous antisera as indicated in Table IV. In addition to complement fixation, precipitin tests were performed with the type 6 M extract; this test could not be carried out with collagen as it is insoluble in the presence of serum.

From Table IV it is apparent that collagenase completely destroys the serological activity of the collagen whereas the serological activity of the M extract is affected little, if any, by this enzyme. On the other hand, as has

[7] The preparation of collagenase (B 5074) was obtained through the courtesy of Dr. W. E. van Heyningen and Miss Ethel Bidwell.

[8] The type 6 M extract and the homologous type 6 absorbed antiserum were obtained through the courtesy of Dr. R. C. Lancefield, The Rockefeller Institute for Medical Research.

TABLE III

PART I *Results of Complement Fixation Tests*										PART II *Results of Inhibition Tests Followed by Complement Fixation*								
Effect of heat and proteolytic enzymes on serological activity of purified rat collagen*										Tests for inhibition with treated and untreated‡ collagen (see Part I for treatment)								
Complement fixation with undiluted antiserum from rabbits immunized with purified rat collagen										Antiserum incubated with serial dilutions of treated materials followed by complement fixation with untreated collagen§ to assay inhibition by these materials								
Purified rat collagen used as antigen in complement fixation tests																		
Treatment of antigen	Dilution of antigen 1:									Dilutions of materials tested for inhibition 1:								
	1	2	4	8	16	32	64	128	256	1	2	4	8	16	32	64	128	256
Collagen 1 mg./cc., untreated	++++	++++	+++±	+++	++±	+	+	±	−	‡								
Collagen 10 mg./cc., heated at 65°C. for 1 hr.	++++	++++	++++	++++	++++	+++±	++±	+	±									
Collagen 10 mg./cc., heated at 65°C. for 1 hr., then treated with pepsin and pancreatin	±	−	−	−	−	−	−	−	−	+	+±	++	++	++	++	++	++	++
Collagen 10 mg./cc., heated at 65°C. for 1 hr., then treated with pepsin and pancreatin and deproteinized	+	±	−	−	−	−	−	−	−	+++	+++	+++	+++	++±	++±	++±	++±	++±
Control: Dilute acetic acid, heated at 65°C. for 1 hr., then treated with pepsin and pancreatin										+	+±	++	++	++	++	++	++	++
Control: Dilute acetic acid, heated at 65°C. for 1 hr., then treated with pepsin and pancreatin and deproteinized										++±	++±	++±	++±	++±	++±	++±	++±	++±

* For details of methods see text.

‡ Purified rat collagen 2 mg./cc. completely absorbs equal volume of antiserum (see Table V).

§ Tested with a single optimal dilution of antigen previously determined as a 1:20 dilution of a 1 mg./cc. solution in all tubes. Antiserum diluted 1:2 by use in preliminary inhibition test (30 minutes at 37°C.).

TABLE IV

The Specific Effect of Collagenase on Collagen

Antisera prepared against	Treatment of antigen	Type of reaction	Antigen: Purified rat collagen diluted 1:											
			2	4	8	16	32	64	128	256	512	1,024	2,048	4,096
Purified rat collagen	Collagen untreated control	Comp. fix.	++±	+++±	+++±	+++±	+++±	+±	−	−	−	−		
	Collagen treated with collagenase	Comp. fix.	−	−	−	−	−	−	−	−	−	−		
	Collagen treated with trypsin	Comp. fix.	±	++	+±	±	−	−	−	−	−	−		
			Antigen: Type 6 M protein extract diluted 1:											
			2	4	8	16	32	64	128	256	512	1,024	2,048	4,096
Type 6 Group A hemolytic streptococcus, Matt strain	Type 6 M extract untreated control	Comp. fix.			++++	++++	++++	++++	++++	++++	++++	++	±	−
		Precipitin			++±	++	+±	+	±	±	−	−	−	−
	Type 6 M extract treated with collagenase	Comp. fix.			++++	++++	++++	++++	++++	+±	−	−	−	−
		Precipitin			++±	++	+±	+	±	−	−	−	−	−
	Type 6 M extract treated with trypsin	Comp. fix.			−	−	−	−	−	−	−	−	−	−
		Precipitin	−	−	−	−	−	−	−	−	−	−	−	−

previously been shown (38), the serological activity of the M extract is completely destroyed by trypsin, which also reduces the serological activity of collagen. Although intact, native collagen fibers or tendon is very resistant to the action of trypsin; we, as well as others (39), have found that native collagen which has been very finely divided with scissors or precipitated from acid solution is more readily attacked by trypsin.

It seemed possible that by use of electron microscopy further and possibly more direct information might be obtained regarding the specificity of the antibody. Several different techniques were employed but the following method gave the best results.

3.0 to 5.0 cc. of a 0.5 mg. per cc. solution of purified collagen in dilute acetic acid was added dropwise through a 25 gauge needle to equal volumes of anti-rat collagen rabbit serum and normal rabbit serum, the latter serving as a control. The material was then incubated for 30 minutes at 37°C., the serum removed by centrifugation, and the precipitate washed in the cold with three changes of veronal saline buffer, pH 7.3. The precipitated collagen was then resuspended in McIlvaine's buffer containing 2.5 per cent sodium chloride, macerated, smeared on screens, dried, fixed in 1 per cent osmic acid, washed with distilled water, and examined under the electron microscope.

It was found that when collagen in solution was added to normal rabbit serum, fibers of various sizes were re-formed, an occasional one being sufficiently well formed to detect definite periodicity, as illustrated by Fig. 5. More often, however, the fibers were not sufficiently well formed to detect periodicity and were of the type shown in Fig. 4. In contrast, however, when collagen in solution was added to homologous antiserum, in addition to poorly formed fibers as occurred in the case of normal rabbit serum, another form appeared. This consisted of a globular precipitate as illustrated in Fig. 6. This globular form was never seen on repeated searches in the many preparations studied with normal rabbit serum but was invariably present and readily demonstrable in those preparations made with homologous antiserum. It is well known that when collagen is precipitated from acid solution either by neutralization or the addition of salt, fibers of various sizes are formed, depending upon the conditions, and they usually show striations characteristic of native collagen (24). It would appear, then, that in the presence of normal rabbit serum this same tendency for the collagen to form fibers is present. However, when the collagen is precipitated in the presence of homologous antiserum apparently the macromolecules of collagen in solution are rapidly coated by antibody, which prevents fiber formation, and the collagen is therefore precipitated in globular form. It is likely that the relatively poor antibody content of the sera plus the rapid and complete removal of this antibody by the addition of excess antigen accounts for the appearance in the precipitate also of poorly formed fibers as well as the globular form. It is difficult to explain this phenomenon except by the presence of antibody which is specifically directed toward collagen.

A similar type of experiment was performed, employing fibrin in place of collagen. Fibrin like collagen belongs to the fibrous class of proteins.

50 mg. of purified bovine fibrinogen dissolved in 50 cc. of distilled water was converted to fibrin by the addition of 100 National Institutes of Health units of bovine thrombin. The fibrin clot was then removed on a glass stirring rod, washed in several changes of distilled water, and dissolved in 2.0 cc. of 0.1 M acetic acid. When 0.1 cc. of this fibrin solution was added to 0.9 cc. of distilled water and neutralized with 1/N NaOH a typical fibrin clot was formed. Likewise when 0.1 cc of the fibrin solution was added to 0.9 cc. of normal rabbit serum a fibrin clot was formed. However, when 0.1 cc. of the fibrin solution was added to 0.9 cc. of anti-bovine fibrinogen rabbit serum a white precipitate appeared but no clot was formed.

Again it appears as if the antibody reacts rapidly with the fibrin in solution, thus preventing the reconstitution of a fibrin clot.

It next appeared important to see whether the antibody is specifically absorbed by its homologous antigen, purified collagen, and also by the homologous native collagen. Complete absorption by the homologous antigen, the purified collagen, would be expected but absorption by native rat collagen would be helpful in indicating whether the purified collagen had been denatured or greatly altered from its native state and also whether native collagen exists in a reactive form. To this end the following experiment was performed.

To 7.0 cc. aliquots of anti-rat collagen rabbit serum equal volumes of 0.2 per cent dilute acetic acid solutions of purified rat and fish[9] collagen were added dropwise through a 25 gauge needle with constant stirring. To 13.0 cc. aliquots of the same pool of anti-rat collagen rabbit serum 5.5 gm. each (wet weight) of thoroughly washed, finely minced, native rat and fish collagen was added. Two serum controls were included, one of which was diluted twofold with dilute acetic acid to serve as a control for the serum absorbed with equal volumes of the purified collagen solutions. The above antisera were then incubated for 2 hours at 37°C. and kept overnight at 4°C. The sera were centrifuged first in the cold at 3,500 R. P. M. to remove all large particles of collagen and then recentrifuged for 90 minutes at 40,000 R. P. M. at 0°C. The results of complement fixation tests done on these sera are presented in Table V.

It is apparent from Table V that both the purified and the native rat collagens completely absorbed the antibodies from the antiserum whereas the heterologous antigens, both purified and native fish collagen, failed to reduce the titer significantly. Similar results not reported here were also obtained using as a control a purified preparation of collagen obtained from the guinea pig rather than the fish. Further work on species specificity of collagen will be reported separately.

In 1930, Nageotte and Guyon (21) raised the question of the identity of the collagen in various parts of the body. Although it is known that by ordinary histological techniques and also, in so far as this problem has been elucidated, by the electron microscope, there is no fundamental difference between the various collagens, it seemed important to test this possibility further by immunological methods. A purified preparation of collagen was, therefore, pre-

[9] The purified fish collagen, obtained from the tunic of the carp swim bladder, was prepared in exactly the same manner described above for the rat collagen.

pared from tendons obtained from the legs and back of the rat in exactly the same manner as described above for the tail tendons. This material in a concentration of 1 mg. per cc. in dilute acetic acid was used as antigen in complement fixation tests with antiserum prepared against purified collagen made from rat tail tendons using the homologous antigen as a control. By this method both preparations of collagen were found to be of identical titer and serologically indistinguishable.

TABLE V

Absorption Experiment, Rat and Fish Collagens
Complement Fixation Reactions

Antiserum prepared against purified rat collagen	Antigen: purified rat collagen 1 mg./cc. diluted 1:										
Treatment of antiserum	1	2	4	8	16	32	64	128	256	512	1,024
Antiserum unabsorbed—control	+++±	+++±	++++	++++	++++	++++	++++	+++	+	±	−
Antiserum absorbed with native rat collagen	−	−	−	−	−	−	−	−	−	−	−
Antiserum absorbed with native fish collagen	+++±	+++±	+++±	++++	++++	++++	++++	++++	+	±	−
Antiserum diluted 1:2 with 1:10,000 acetic acid—control	++	+	++	+++±	+++±	+++±	+++±	+++±	+++±	±	−
Antiserum absorbed with purified rat collagen	−	−	−	−	−	−	−	−	−	−	−
Antiserum absorbed with purified fish collagen	+±	±	+±	+++±	+++±	+++±	+++±	+++±	++	−	−

RECAPITULATION

A method has been described for preparing purified collagen from the tail tendons of rats with minimal alteration of the collagen from its native state. This material, like native collagen, is soluble in dilute acetic acid and when injected intraperitoneally into rabbits induces complement-fixing antibodies in low titer. Since it was not practicable to show that the collagen preparation was free from other extraneous tissue components which might be antigenic, other methods had to be employed to show that the antibodies were probably directed specifically toward collagen. Immunological methods were used to demonstrate that the serological activity of the purified collagen was not due

to admixture with rat serum or with a tissue polysaccharide. It was shown that a purified collagenase (k toxin of *Cl. welchii*), which apparently attacks only gelatin and collagen (37), not only caused rapid disintegration of the purified and native collagen but also completely destroyed its serological activity. Under similar conditions this enzyme was found to have little or no effect on the serological reaction of another protein, used as a control, type 6 M prepared from Group A streptococci which is known to be rapidly destroyed by trypsin (38).

Since it was known that collagen reconstituted from an acid solution by neutralization or the addition of salts tends to form fibers with the characteristic striation or periodicity of native collagen it was believed that additional information regarding the specificity of the antibodies might be gained by attempting to reconstitute collagen fibrils from an acid solution of our preparation in the presence of the homologous antiserum. When collagen was precipitated by normal rabbit serum fibers were re-formed which at times were sufficiently well formed to demonstrate the characteristic periodicity of native collagen in electron micrographs; when, however, this collagen solution was precipitated by homologous rabbit antiserum another distinct form of precipitate appeared. This was globular in appearance and was never found in the many control preparations made with normal rabbit serum. The most reasonable explanation of this phenomenon is that the macromolecules of collagen in solution are rapidly coated with antibody, thus preventing fiber formation.

In support of this explanation it was found in a similar type of experiment that when an acid solution of bovine fibrin was added to normal rabbit serum or a heterologous antiserum (serum containing heterologous antibody) a fibrin clot was re-formed. When, however, the acid solution of fibrin was added to homologous anti-bovine fibrinogen rabbit serum the re-formation of a fibrin clot was prevented but a heavy immune precipitate appeared. It was also demonstrated that the antibody is completely absorbed by the homologous purified collagen as well as native rat collagen but not by similar preparations of collagen obtained from the fish and guinea pig. In this connection it is of some interest to note that although the collagens obtained from various species cannot be differentiated with certainty by histochemical methods, electron micrographs (9), x-ray diffraction patterns (13), or chemical analysis in so far as it has been studied, definite species specificity appears to be present when immunological methods are employed.

Nageotte's contention that the collagen from various parts of the body may be different was not confirmed although tested in only a single experiment. It is obvious, however, that further work is necessary before this question can be answered.

SUMMARY

A method is described for preparing purified collagen from the tail tendons of rats with minimal alteration from its native state.

This purified collagen is soluble in dilute acetic acid and when injected intraperitoneally into rabbits induces complement-fixing antibodies in low titer.

It has been demonstrated by the use of certain immunological tests, enzymatic analyses, and electron microscopy that these antibodies are probably directed specifically toward collagen rather than toward accompanying impurities such as tissue proteins or polysaccharides.

Evidence is presented suggesting that collagen exhibits species specificity.

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EXPLANATION OF PLATE 16

FIG. 1. Electron micrograph of native collagen fibers obtained from rat tail tendon. The tendon was "cleansed" with a 1.0 per cent solution of pancreatin (Parke, Davis and Company) for 5 days at 37°C. and washed in 0.9 per cent saline. A small piece of tendon was then placed in 0.9 per cent saline and teased with needles until a suspension of fine fibers was obtained. This was washed with three changes of veronal saline buffer, pH 7.3, and small portions of the final sediment were placed on electron microscope screens. The preparation was fixed with formalin, washed in distilled water, dried over phosphorus pentoxide, and shadowed with chromium. The fibers show the striated appearance which is characteristic of native collagen. The macroperiod consisting of a cross band and the following depression measure approximately 650 A. × 21,750.

FIG. 2. Electron micrograph of the margin of a droplet of purified rat collagen solution. A 0.1 per cent solution of purified rat collagen in 0.1 per cent acetic acid was sprayed onto an electron micrograph screen with an atomizer, dried, fixed with 1.0 per cent osmic acid, and washed with distilled water. The collagen in solution appears to be in part amorphous but with a network of fine filaments. × 33,900.

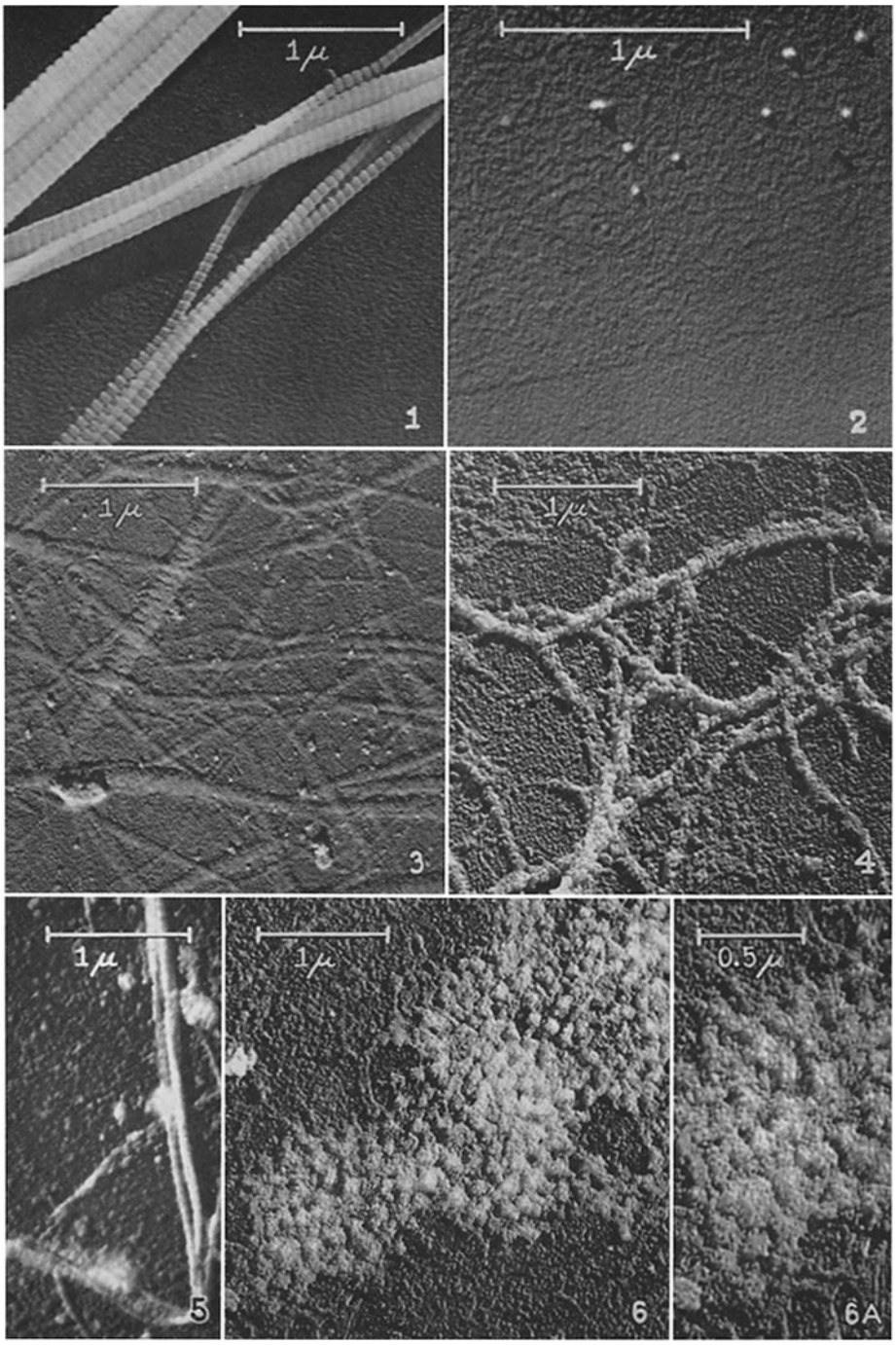
FIG. 3. Electron micrograph of purified rat collagen precipitated from 0.1 per cent acetic acid. A 0.1 per cent solution of purified rat collagen in 0.1 per cent acetic acid was adsorbed on an electron micrograph screen which was then immediately immersed in Eimer and Amend standard buffer, pH 6.0. The preparation was then cleansed with a solution of 0.1 per cent trypsin for 3 hours at 37°C., washed in distilled water, dried, fixed with 1.0 per cent osmic acid, washed again in distilled water, and shadowed with chromium. The reconstituted fibers are of varying size and show the characteristic periodicity of native collagen. × 21,400.

FIG. 4. Electron micrograph of purified rat collagen precipitated from 0.1 per cent acetic acid solution by normal rabbit serum. Three cc. of a 0.05 per cent solution of purified rat collagen in 0.1 per cent acetic acid was added dropwise through a 25 gauge needle with constant stirring to an equal volume of normal rabbit serum. The material was then incubated at 37°C. for 30 minutes, the serum removed by centrifugation, and the sediment washed with three changes of veronal saline buffer, pH 7.3. The precipitated collagen was then resuspended in McIlvaine's buffer, pH 6.6 containing 2.5 per cent sodium chloride, macerated, smeared on screens, dried, fixed in 1.0 per cent osmic acid, washed with distilled water, and shadowed with chromium. The reconstituted collagen showed poor fiber formation in that the fibers were not sufficiently well formed to detect periodicity but were the type usually seen in preparations precipitated in normal rabbit serum. × 19,600.

FIG. 5. Electron micrograph of purified collagen reconstituted from a 0.1 per cent acetic acid solution by normal rabbit serum in the same manner as Fig. 4. The reconstituted collagen fibers in this preparation were unusually well formed demonstrating the characteristic striated appearance. × 19,600.

FIG. 6. Electron micrograph of purified collagen precipitated from a 0.1 per cent acetic acid solution by homologous anti-rat collagen rabbit serum. Except for the use of homologous rabbit antiserum in place of normal rabbit serum the preparation of the material was the same as for Fig. 4. In addition to a few poorly formed fibers similar to those seen in Fig. 4, the precipitated collagen appears in a globular form without definite fiber formation. × 17,800.

FIG. 6 A. Enlargement of Fig. 6. The globular masses appear to be made up of macromolecular units. × 28,500.



(Watson *et al.*: Antigenicity of rat collagen)