# The Nature of Collagen in the Carrageenin Granuloma[1] (37169)

PATRICIA DEL ARENAL AND RUY PÉREZ-TAMAYO

*Departamento de Biología Celular, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, México 20, D.F., Mexico*

The injection of the sulfated polygalactan carrageenin, a high molecular weight polysaccharide obtained from *Chondrus chrispus* and other sea weeds, induces a complex reaction characterized first by inflammation and deposition of connective tissue, and later by resorption of the entire process (1–4). This biphasic reaction has been used as a model of acute inflammation (5, 6), and also to study tissue macrophages (7, 8), to examine collagen synthesis (9, 10), and more recently to analyze the mechanism of collagen breakdown and resorption (11–13). There appear to be two major possibilities to explain the peculiar biphasic evolution of the carrageenin granuloma: one, that collagen synthesized under the stimulus of the polysaccharide is unusually unstable and undergoes degradation as soon as the irritating effect of carrageenin wanes; the other possibility is that carrageenin is capable of turning on a specific mechanism of collagen degradation. Evidence has been presented in support of the existence of such a specific collagenolytic mechanism in the carrageenin granuloma (11). Furthermore, it has also been shown that such a mechanism affects both recently deposited as well as previously existing collagen (4, 14). Nevertheless, the possibility still exists that collagen synthesized in response to carrageenin may be abnormal, and for this reason it was decided to examine it directly.

*Material and Methods.* Carrageenin (Viscarin, lot No. 201588, Marine Colloids, Inc., Springfield, NJ) was prepared as a 1.0% solution in 0.15 *M* NaCl, dialyzed extensively against the same 0.15 *M* NaCl, sterilized and kept at 4° until used. Guinea pigs of both sexes weighing 400–600 g and actively gaining weight were injected in the abdominal subcutaneous tissue with 5.0 ml of the carrageenin solution; no antibiotics were used. The granulomas were observed daily and at 7 and 14 days of evolution, groups of animals were sacrificed by cardiac exsanguination under light ether anesthesia. The granulomas of each group were carefully removed and sectioned, all areas of necrosis were eliminated, and the remaining tissue was pooled, cut into small fragments (0.5 cm) and collagen was extracted in 0.5 *M* acetic acid and purified by the TCA–ethanol procedure of Gross (15). Purification was considered satisfactory when protein determinations gave the same results measured as nitrogen by nesslerization and as collagen by hydroxyproline, the latter according to Stegemann (16) as modified by Woessner (17). The skin of the same animals, as well as that from other groups of untreated and normal guinea pigs, were also extracted and purified following the same procedure. Thus, acid-soluble collagen was available from three different sources: normal guinea pig skin (NS), carrageenin granuloma during the growing or deposition period (DC), and carrageenin granuloma during the decreasing or resorbing period (RC). Identical samples of the three types of collagen were used for all the following tests.

Disc electrophoresis in polyacrylamide gels was carried out acording to Sakai and Gross (18) after dialysis of gelatinized samples for 4 hr against the upper buffer, protein determination and adjustment of the concentration to 1 mg/ml. Both upper and spacer gels were replaced by a sucrose gradient. Densitometric tracings of suitable negatives of gels fixed and stained after simultaneous electrophoretic runs of identical amounts of the three types of collagen described above

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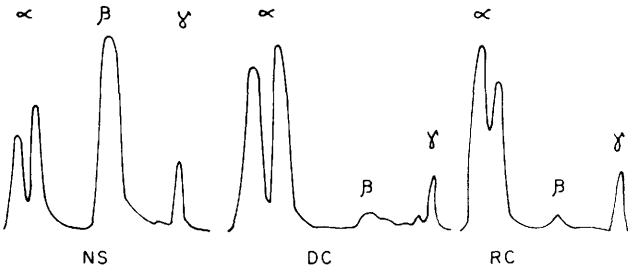


FIG. 1. Densitometric tracings of electrophoretic patterns on acrylamide gel of denatured acid-extracted collagen from normal skin (NS) and from 7-day-old (DC) and 14-day-old (RC) carrageenin granulomas induced in guinea pigs. There is a much higher proportion of α subunits and a much lower proportion of β components in both samples of granuloma collagen, compared with normal skin collagen.

were made on a Spinco Analytrol, Beckman RB, with a 0.5 mm aperture.

Enzymatic effects on the three types of collagen were tested by adding 1.5 ml of each of the following to Ostwald viscometers (flow time, 60 to 70 sec at 20°): collagen solution (2 mg/ml); 0.4 *M* NaCl in 0.05 *M* 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris) buffer (pH 7.5); 0.005 *M* $CaCl_2$ in 0.05 *M* Tris buffer (pH 7.5); and enzyme solution (trypsin, Fluka AG, Switzerland, Lot No. 562648, 1 to 6 mg/ml; bacterial collagenase, Grade B, Calbiochem, Lot No. 900606, 1 mg/ml) in 0.15 *M* NaCl. Viscosity was measured at 20° for 60 min.

Melting temperatures of the three collagen samples [0.035% in 0.5 *M* $CaCl_2$ (pH 7.0)] were followed in a Zeiss DB-1 Universal spectrophotometer equipped with an automatic temperature water bath; the wave length was set at 230 nm and absorption was monitored at 5° intervals up to 30°, and at 1° intervals from this temperature up to 42°. The samples were allowed to remain for 10 min at the set temperature before reading the absorption.

Ultracentrifugation was performed at 4° in a Beckman Model E analytical ultracentrifuge, using Schlieren optics with a phase plate angle of 70° and at a speed of 52,640 rpm; the three samples run simultaneously were of 600 μg each in 0.25 *M* sodium citrate (19). The RC sample was slightly smaller in volume to allow simultaneous visualization of the two peaks.

Antigenic specificity was tested by hemagglutination using formalinized and tanned goat red blood cells. The antibody was raised in rabbits using normal guinea pig skin collagen as antigen.[2] This antibody induces hemagglutination of collagen-coated erythrocytes at titers ranging up to 1:243. Gelatin inhibits hemagglutination of collagen-coated erythrocytes but fails to fix enough antibody to give positive direct hemagglutination.

Segment long spacing (SLS) crystallites were precipitated by dialyzing a 0.1% collagen solution in 0.05% acetic acid against a 0.3% solution of salt-free ATP on 0.05 *N* acetic acid. Dialysis was carried out at 4° for 1 to 3 hr, a drop of the turbid solution was placed on carbon-coated grids, the excess drained and the crystallites stained with phosphotungstic acid at pH 2.5. The grids were examined in a Hitachi HU-11A electron microscope.

*Results.* Disc electrophoresis in polyacrylamide gels revealed that both DC and RC contain few β components and other aggregates of molecular weight higher than α subunits. In the densitometric tracings the area corresponding to both α subunits is greater in DC (85.2%) and RC (97.4%) than in SC (35.7%) (Fig. 1). Trypsin was unable to lower the specific viscosity of the three types of collagen more than 20% of the 0 time values in 60 min, whereas bacterial collagenase rapidly eliminated practically all viscosity in the three samples (Fig. 2). The melting temperatures of the three types of

[2] The antibody was generously provided by Dr. Irmgard Montfort.

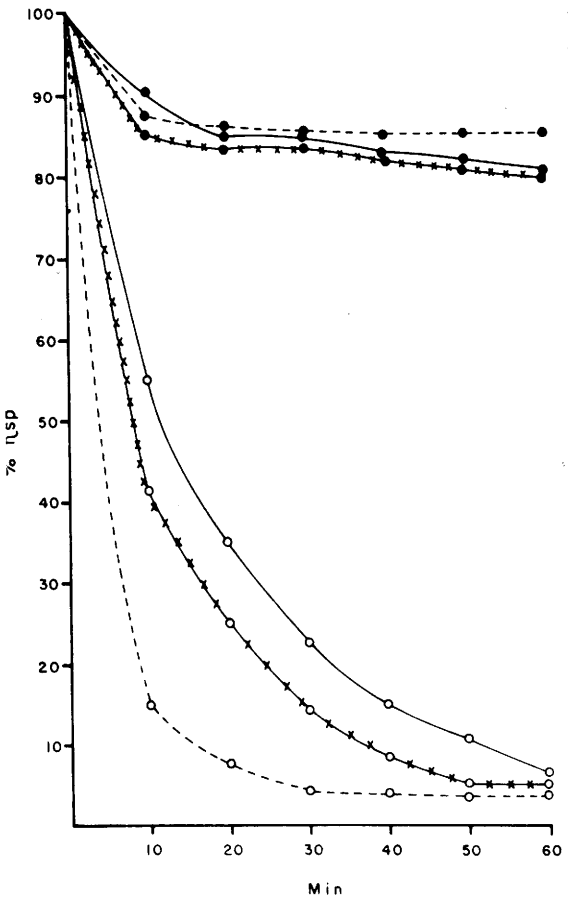


FIG. 2. Effect of trypsin (●) and of bacterial collagenase (○) on the specific viscosity of native acid-extracted collagen from normal skin (× × ×) and from 7-day-old (- - -) and 14-day-old (—) carrageenin granulomas induced in guinea pigs. Is is apparent that trypsin has little effect (no more than 20% of the original 0 time values) whereas bacterial collagenase rapidly eliminates all viscosity.

collagen were not significantly different (Fig. 3); the slopes of the curves, calculated between 32 and 35° for SC and RC, and between 33 and 36° for DC, also showed very similar values. The ultracentrifugal pattern of the three types of collagen (Fig. 4) was identical, and the sedimentation coefficients expressed in $S$ values (± 2%) were as follows: SC, 1.26; DC, 1.27; RC, 1.31.

Hemagglutination of formalinized and tanned goat red blood cells, coated with the three types of collagen studied, was positive using a 1:3 dilution of the antibody; in three experiments the last positive dilution was the same for all samples tested, 1:243. The very small amount of materials available prevented absorption and other studies of cross reactivity. Finally, SLS crystallites prepared from the three types of collagen studied revealed identical dimensions and banding patterns.

*Discussion.* In this work we have assumed that an intrinsic instability of the collagen molecule synthesized in response to carrageenin and/or an increased susceptibility of such collagen to the collagenolytic mechanism active in the granuloma could be correlated with a change in one or more of the several collagen properties examined. Our studies have failed to support the possibility that collagen degradation and resorption in the carrageenin granuloma is due, at least partly, to an intrinsic abnormality of the molecule. Thus, a series of comparative tests probing different properties of the collagen molecule such as subunit composition, susceptibility to nonspecific and specific proteolytic enzymes, melting temperature, ultracentrifugal sedimentation velocity, immunologic specificity, and finally formation of SLS crystallites, all point to the conclusion that acid-soluble collagen deposited in the carrageenin granuloma is not qualitatively different from that present in normal skin. Of course, subtler differences not detectable by any of the procedures employed might still be present and account for the peculiar behavior of collagen in the carrageenin granuloma. Recent studies (20, 21) have established some differences in the reducible intermolecular crosslinks of *insoluble* collagens from mature dermis and young dermal scar tissue of the guinea pig. On the other hand, in a comparative study of the *in vitro* effect of a pure collagenase obtained from the carrageenin granuloma on acid-soluble collagens extracted from the same source and from normal skin[3] no significant differences have been observed. Our observations are limited to the acid-soluble fraction of collagen which has been shown to be the bulk of recently deposited collagen (2).

[3] A. Pardo and R. Pérez-Tamayo, unpublished data.

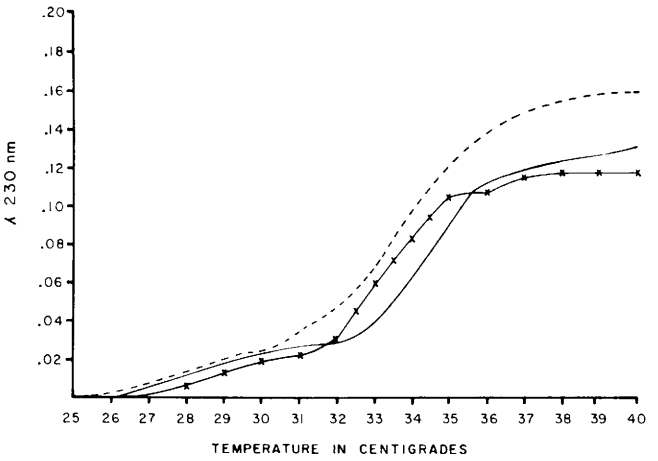


FIG. 3. Denaturation temperature curves of the three samples of collagen, identified as in Fig. 2.

Collagen in carrageenin granulomas *in vivo* may still be more susceptible to specific collagenolytic mechanisms than normal skin collagen not because of any intrinsic qualitative difference in the molecule but because it is recently deposited and, as such, it is still poor in $\beta$ components and other aggregates of higher molecular weight. Our results indicate a greater concentration of $\alpha$ subunits in collagen extracted from carrageenin granulomas, when compared with extracts of normal skin. Of course, the prevalence of $\alpha$ subunits in recently deposited collagen of whatever origin is a well-known fact (22). There is indirect evidence, however, suggesting that insoluble polymeric collagen (23) may be more resistant to specific animal collagenases than solubilized collagen molecules (24). Other observations (2, 25) also suggest that disaggregation and/or solubilization of insoluble collagen fibrils down to intact monomers may be a necessary or facilitating step in the normal mechanism of collagen degradation *in vivo,* prior to specific enzymatic cleavage of the molecule. If this was the case, then it may be speculated that recently deposited collagen would be more susceptible to degradation simply because it is easier to solubilize than older, more tightly packed and covalently linked molecules.

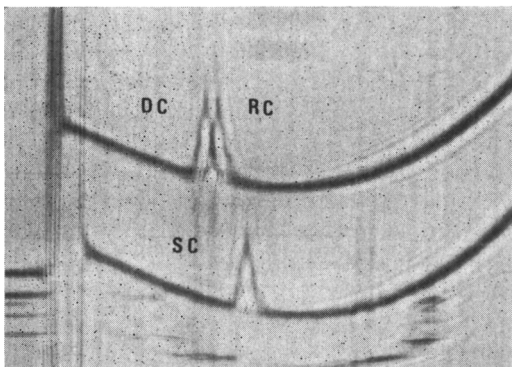


FIG. 4. Sedimentation velocity pattern of the three collagen samples. Sedimentation from left to right. The Schlieren photograph was taken with a phase plate angle of 70° after 171 min at 52,640 rpm. The separation of DC and RC is due to the use of slightly different volumes.

*Summary.* A comparative survey of several properties of acid-soluble collagens extracted from normal skin and carrageenin granulomas in guinea pigs has failed to reveal any qualitative difference that might explain the peculiar susceptibility of granuloma-derived collagen to degradation and resorption. The only quantitative difference found was a higher concentration of $\alpha$ subunits and a lower concentration of $\beta$ subunits in carrageenin granuloma collagen, compared with normal skin collagen. It is suggested that this difference might explain, at least in part, the ready degradation of collagen in the carrageenin granuloma.

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