ORGANIZATION AND DISORGANIZATION OF COLLAGEN

JEROME GROSS
From the Department of Medicine, Harvard Medical School, and the Massachusetts General Hospital, Boston

ABSTRACT The organization of the normal collagen molecule and fibrils is reviewed and the detection, assay, and isolation of a collagenolytic enzyme from amphibian tadpole tissue are described and its possible significance in metamorphosis is discussed

Collagen, which has been called the "excelsior of the body" because of its supposedly inert quality and passive function, has excited the interest of a diverse group of people. Its molecular structure is unique among proteins and it is readily accessible in large quantities from a great variety of animals. It is involved in the healing process, in tissue regeneration, and has been implicated, perhaps inaccurately, in a wide variety of obscure diseases. Its ubiquitous distribution throughout the animal body involves it in all growth and remodeling processes and because of its very specific structural and chemical characteristics it may be readily studied from the viewpoints of morphology, of biosynthesis, degradation, and geographic distribution as a function of time and physiologic change.

This protein is also of much interest because it provides a good model for studying morphogenesis at the molecular level. Collagen fibrils may be taken apart *in vitro* into their constituent molecules and by the proper manipulation of environmental conditions may be reconstituted in the native form as well as a variety of other structures all subject to detailed structural and physical chemical analysis.

Simultaneous advances in the study of macromolecules in solution and in the solid state by physical chemical techniques and by x-ray diffraction have in the past 15 years led to a fairly reliable characterization of the size, shape, molecular weight, and intramolcular organization of collagen and a better understanding of the way in which these molecules aggregate to form a fibril. This story has been told many times and is amply documented in a series of excellent books and review articles (Bear, 1952; Randall, 1953; Gustavson, 1956; Tunbridge, 1957; Stainsby, 1958; Harkness, 1961; Schmitt, 1959; Gross, 1961; Rich and Crick, 1961; Harrington and Von Hippel, 1961).

To summarize briefly, collagen fibrils such as those seen in Fig. 1 are composed of

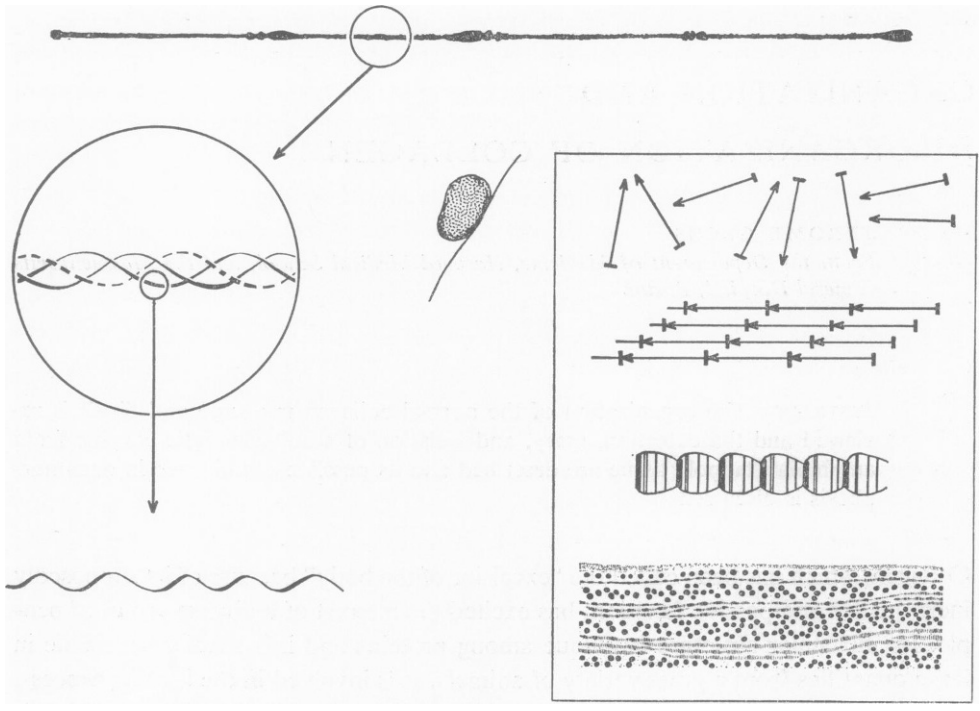

FIGURE 1 The tropocollagen molecule schematized at the top has an axial ratio of about 200:1, with an asymmetric fine structure along its length, roughly subdividing the molecule in quarters. It is compared with a 70 S ribosome on a membrane sketched to scale below it. The enlargement of the section of the molecule to the left schematizes the three polypeptide chains, one of which, the dashed line, is different in amino acid composition from the other two. A further enlargement of the section of one of the chains illustrates the helical configuration of each of the polypeptides. In the box at the right is a schematic representation of the manner in which tropocollagen units are postulated to come together overlapping each other in a staggered array by about one-quarter of the length thereby giving rise to a collagen fibril with a repeating period of about 1700 A. The fine structure within each of these periods illustrated in the fibril would be a reflection of the fine details of asymmetry of the tropocollagen units arrayed in register. The lower portion of this block illustrates the manner in which collagen fibrils are found organized in plywood-like sheets in a variety of tissues. This figure originally appeared in Organization and disorganization of extracellular substances: The collagen system, *in* Cytodifferentiation and Macromolecular Synthesis, (M. Locke, editor), New York, Academic Press Inc., 1963, 178.

long rigid rod-shaped molecules approximately 2800 A units in length and 14 A wide, depicted in Fig. 1. These molecules are composed of three polypeptide chains, one of which differs to some extent in amino acid composition from the other two. The characteristic feature of this protein is the presence of 33 per cent glycine which varies no more than ±5 per cent throughout the animal kingdom and also the presence of a large amount, approximately 25 per cent of the imino acids, proline

and hydroxyproline (for comparative biochemistry, see recent review by Gross, 1963*a*). The laboratory of Grassmann has contributed significantly in recent years to the important problem of amino acid sequence determination (see Grassmann *et al.*, 1960). Two of the amino acids, hydroxyproline and hydroxylysine, are unique to collagen among the animal proteins although they vary greatly in amount in different phyla. The probable presence of relatively large numbers of γ-glutamyl peptide linkages may play an important role in the properties and organization of collagen. These large moieties of glycine and imino acids impart to collagen its unique helical structure which has approximately the same dimensions as that of the synthetic polypeptides, polyglycine II (Crick and Rich, 1955) and polyproline II (Cowan and McGavin, 1955). The three polypeptide chains, each coiled in a left-handed helix, are wound about each other in ropelike fashion in the right-handed direction. When collagen in solution is warmed to a point above the denaturation temperature or when high concentrations of urea, guanidine, iodide, etc., are added to such solutions these rigid rods are disrupted, the superhelix unraveled, the individual chains separated, and their helical structure randomized. The resulting denatured protein is well known as gelatin, the commercial forms of which are further degraded. The peanut-shaped object seen in Fig. 1 beneath the tropocollagen particle represents a single 70 S ribosome drawn to scale. It is generally assumed that the collagen molecule is synthesized within the cell in some manner on the ribosomes, perhaps arrayed in tandem as suggested by Warner, Rich, and Hall (1962). It has been hypothesized that the molecules are then secreted into the extracellular space where they orient to form liquid crystals which condense to form the characteristic cross-striated fibrils. Others believe that they become aggregated within the cell close to the surface in the form of extremely thin fibrils which are then peeled off as such into the extracellular space where they gradually accrete more collagen molecules to thicken into fibrils (Porter and Pappas, 1959).

Studies of the solubility characteristics and the turnover of collagen have led to the hypothesis that collagen extractable in cold neutral solutions represents newly synthesized molecules in loose fibrillar form (Gross, 1959; Jackson and Bentley, 1960). It is worth making the point that it is the low temperature of the extracting medium which is important in the solubilizing process rather than the presence of salt since at mammalian body temperature little or no collagen may be extracted from tissues. Apparently the low temperature results in increased hydration of the loosely packed molecules which causes them to disperse. This might be expected since the hydrogen-bonding capacity of water is increased at low temperature. The temperature-dependence of non-polar bonds, as pointed out by Kauzmann in this symposium, may also play a role. This process is illustrated by the spontaneous polymerization of collagen in neutral solution to typical striated fibrils when warmed to body temperature. This phenomenon of "heat gelation" permits kinetic studies of fibril formation (Gross and Kirk, 1958; Bensusan and Hoyt, 1958;

Fessler, 1960; Wood, 1960; Cassel *et al.*, 1962). One of the characteristics of these thermally reconstituted fibrils is their ability to redissolve again upon cooling if they have been incubated at body temperature for only a few minutes (Gross, 1958). As the incubation period is lengthened, less will go back into solution on cooling until after 24 hours more than 80 per cent remains insoluble. We believe that this time-dependent decrease in solubility is a result of the formation of increasing numbers of secondary cross-links such as hydrogen, electrostatic, and non-polar bonds, resulting from increasing perfection of fit between molecules as they are jiggled about by Brownian movement. Water is forced out from between the chains, which begin to fit together like the pieces of a jigsaw puzzle.

This phenomenon has aided in understanding the pathogenesis of an experimental disease at the molecular level. In experimental lathyrism, a disease of connective tissue produced by compounds such as β-aminopropionitrile, there is progressive increase in the extractability of collagen from all the connective tissues (Levene and Gross, 1959). The extracted collagen while capable of forming typical cross-striated fibrils on warming to body temperature fails to show the above-described, time-dependent "aging" process, indicating failure to form intermolecular cross-links (Gross, 1963*b*; Hausmann, 1963). The size, shape, and stability of the lathyritic molecules are normal (Gross, 1963). There is no evidence for binding of the lathyrogenic agent; it would appear that a metabolic process is involved (Orloff and Gross, 1963). Loss of tensile strength of the whole embryo (Levene and Gross, 1959) and of individual tendons (van Heecheran and Gross, unpublished observations) is a direct function of collagen extractability.

Until recently it had been assumed that collagens extracted in cold neutral salt solutions and in acidic media were essentially the same molecules although the latter were older and more difficult to dissolve. It had been known that denatured acid-extracted collagen was composed of two separate particles as revealed by ultracentrifuge analysis (Orekhovich and Shpikiter, 1958; Doty and Nishihara, 1958). One of these had a molecular weight of about 100,000 and the other about double that figure. It was assumed that the three chains were separated into a single chain and a dimer of the other two. Several years ago it was observed by Orekhovich *et al.* (1960) and Piez *et al.* (1961) that neutral extracted collagen when denatured was composed of but a single molecular weight species, that of the individual chains. Chromatographic analysis on carboxymethylcellulose columns revealed that the denatured collagen consisted of four distinct subunits (Piez *et al.*, 1961, 1963) shown in Fig. 2, derived from three individual polypeptide chains of essentially equal molecular weight. Two of them, which have the same amino acid composition, were designated α_1 and the other whose composition differs was labeled α_2. In acid-extracted collagen there appeared to be two different dimers in addition to the α subunits, one composed of two α_1 chains bonded together and a second hybrid consisting of one α_1 and one α_2. While the former, designated β_2, and

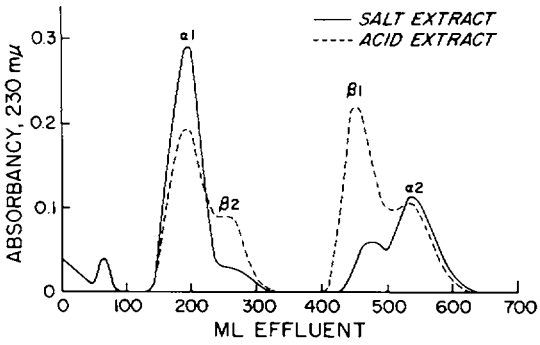

FIGURE 2 Elution patterns of denatured rat skin collagen chromatographed on CM-cellulose at 40°. Solid line, 20 mg salt-extracted collagen; dashed line, 20 mg acid-extracted collagen. A linear gradient between 0.07 and 0.17 I acetate buffer (pH 4.83) was employed. The labels refer to both patterns. This figure originally appeared in *Biochim. et Biophysica Acta*, 1961, **53**, 597.

the latter, designated β_1, sedimented at the same rate, comprising the faster moving β peak, they were clearly separated on the column, presumably as a result of difference in amino acid composition. At about the same time several groups of investigators (Altgelt *et al.*, 1961; Rice, 1960; Veis and Cohen, 1960; Grassmann *et al.*, 1961) found a small fraction of collagen accounting for no more than 10 per cent of the total soluble fraction, which appeared to be a trimer in which all the three chains were cross-linked. This they called γ. Fig. 3 illustrates the story of the subunits as described here.

It had long been known that degradation of collagen results in a dramatic fall in negative optical rotation to the low level characteristic of the amino acid residue rotation. It also had been observed that cooling gelatin resulted in a progressive increase in negative rotation to about two-thirds the level of the native material. The explanation given was that denaturation resulted in a randomization of the helical structure of the polypeptide chain and recooling caused a reformation of the local crystalline regions, thus the high negative rotation is caused by the helical structure of the polypeptide chain. The fact that there was no significant increase in viscosity of dilute gelatin solutions to parallel the mutarotation indicated that while local regions of helix reformed, the long rodlike molecules were not reconstituted. However, it has been demonstrated that the γ component, that which is triply cross-linked, not only mutarotates almost completely to the normal degree of optical rotation but that there is also a reformation of the rigid tropocollagen rods as demonstrated by both increase in viscosity and by the ability to reform striated fibrils and segment long spacings (Altgelt *et al.*, 1961; Rice, 1960). This phenomenon of reversible helix formation is of interest because of implications with regard to control of protein function, both enzymatic and structural. An excellent discussion of recent studies on this problem is to be found in the review by (Harrington and Von Hippel 1961).

With regard to the cross-links between the polypeptide chains there is good evidence to indicate that the dimers may be formed in the process of "maturation" (Martin, Piez, and Lewis, 1963). It is not yet certain whether this requires enzymatic

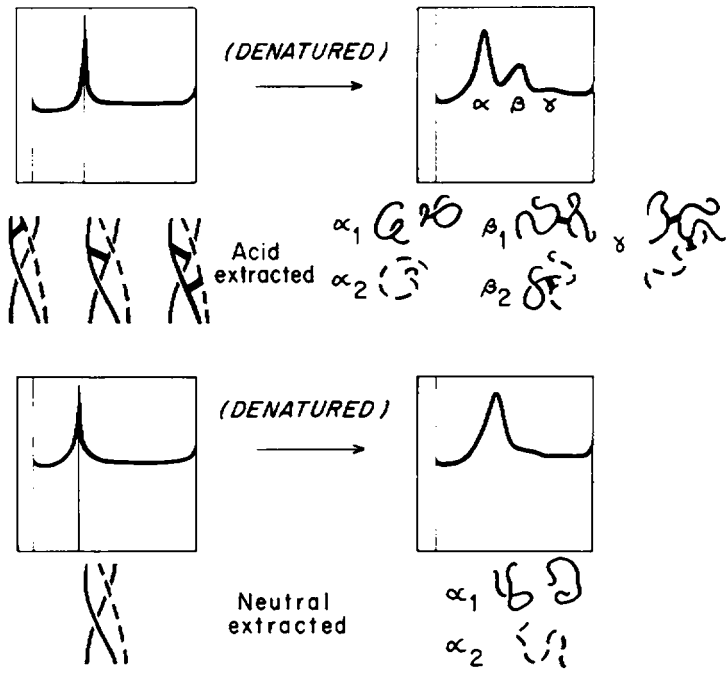

FIGURE 3 Schematic description of the dissociation of the polypeptide chains of neutral and acid-extracted collagen by thermal denaturation. The two boxes on the left illustrate the sedimentation diagrams of undenatured dissolved collagen. The boxes on the right illustrate the sedimentation diagrams of thermally denatured collagens. The diagrams underneath the boxes on the left schematize the intact structure of the collagen molecule and in the case of the acid extract, the cross-links between the several chains in 3 of the fractions. The diagrams on the right illustrate the denatured randomized polypeptide chains both cross-linked and individual.

action although it is more than likely that these cross-links are covalent in nature and perhaps are ester-like as described by Dr. Gallop in this Symposium.

These last observations have also found expression in experimental lathyrism. We have observed that the extracted collagen in this experimental disease contains very few β components (Martin *et al.*, 1961, 1963). It would appear that there is either a failure to "mature," in other words, to form intramolecular cross-links or alternatively, cross-links already formed have been disrupted. Thus the defect in lathyrism seems to involve a failure of both inter- and intramolecular cross-linking processes. It has been shown that intramolecular cross-links in collagen may be disrupted by enzymes such as trypsin (Gross, unpublished data) and chymotrypsin (Martin, unpublished data) and by pepsin (Rubin *et al.*, 1963). How much additional damage to the molecule is done by these enzymes has not yet been ascertained. However, the gross molecular architecture appears to be intact. It is not inconceivable that in experimental lathyrism a similar enzymatic action might be responsible for the observed collagen defect.

I would now like to turn to an aspect of *in vivo* collagen metabolism, namely the removal process. It is evident that growth is not simply a matter of accretion of structural elements but involves degradation precisely timed with synthesis. It would not be possible to obtain reproducible organization of a tissue, bone for example, following essentially the same growth pattern from animal to animal unless a remarkable degree of synchronization between the two processes were maintained. It is not unlikely that at least certain congenital deformities result from improper balance between these two mechanisms. During bone growth collagen is rapidly removed in the endostial regions and produced under the periosteum. Parathormone apparently can modulate this process in a dramatic manner. Certainly in a number of diseases affecting the skeletal system the resorption process must play a significant role. In the uterus there is, again, a rapid, hormonally mediated removal of collagen during the postpartum period. In experimentally induced cirrhosis, withdrawal of the toxic factor within a certain time period results in rapid removal of newly deposited cicatrix.

The search for a collagenase in these tissues has to date been uniformly unsuccessful, leading some investigators to propose that such an enzyme does not exist in animal tissues. It has been suggested that collagen is removed by first denaturing the protein, a process which might occur naturally if the pH were lowered, since the body temperature would then denature any collagen in molecular dispersion. Subsequent removal of the denatured collagen by the ordinary proteolytic enzymes in the tissues would be expected. A major difficulty with this hypothesis is the likelihood that little or no collagen exists in molecular dispersion in normal tissues at body temperature. The "extractable" or "soluble" collagens are solubilized by experimental manipulation. The denaturation temperature of newly reconstituted fibrils is of the order of 50 to 60°C compared with 42°C for a collagen in solution at neutral pH, thus denaturation of collagen *in vivo* is unlikely.

It occurred to us that failures to detect an animal collagenase might be explained if it were present in extremely low concentrations at any one time. It also might be bound to its substrate, the collagen fibers, and thus not be available to an extracting medium until these fibers were digested. We know from the work of Gallop *et al.* (1957) that bacterial collagenase binds to collagen until digestion is complete. A possible way to circumvent these difficulties in detection would be by cultivation of tissues (capable of resorbing collagen) on a reconstituted collagen substrate in the form of an opaque gel. If the enzyme were synthesized or activated slowly in the explant it would accumulate in the medium, producing a gradually expanding area of lysis in the opaque substrate.

For culture chambers we used small plastic rings mounted on microscope slides, and also small Petri dishes. Acid-extracted, lyophilized calf skin collagen and cold neutral salt-extracted radioactive guinea pig skin collagen, both purified and dissolved in cold amphibian Tyrode's solution, were used as substrate. The sterile solu-

tions were allowed to form heavily opalescent gels in the culture chamber by warming them to 37°C for several hours (Gross and Lapiere, 1962).

These gels proved to be almost completely resistant to the action of trypsin, pepsin, chymotrypsin, cathepsin C, papain, and various tissue extracts in high concentrations. Lysis of the collagen gel could easily be measured by centrifuging contents of the chamber, and determining either the released hydroxyproline in the supernatant solution or measuring the radioactivity in the case of C^{14}-labeled collagen. This latter technique has proved to be extremely sensitive and has given us a quantitative assay which permits measurement of as little as 0.01 γ of purified *Cl. histolyticum* collagenase (Lapiere, Nagai, and Gross, data to be published).

The search for a suitable biologic system led us to the frog tadpole since here a number of its tissues undergo rapid resorption during metamorphosis which can be induced at will with thyroxine. The 4 or 5 inch tail of a bullfrog tadpole resorbs completely within 14 to 16 days under the influence of this hormone. Other dramatic changes include resorption of the gills and opercular areas of the body skin and of two-thirds the length of the gut. The legs appear suddenly and grow rapidly and there are marked alterations in other structures around the head. All these morphogenetic changes should be fruitful subjects for further studies. Fig. 4 compares a normal non-

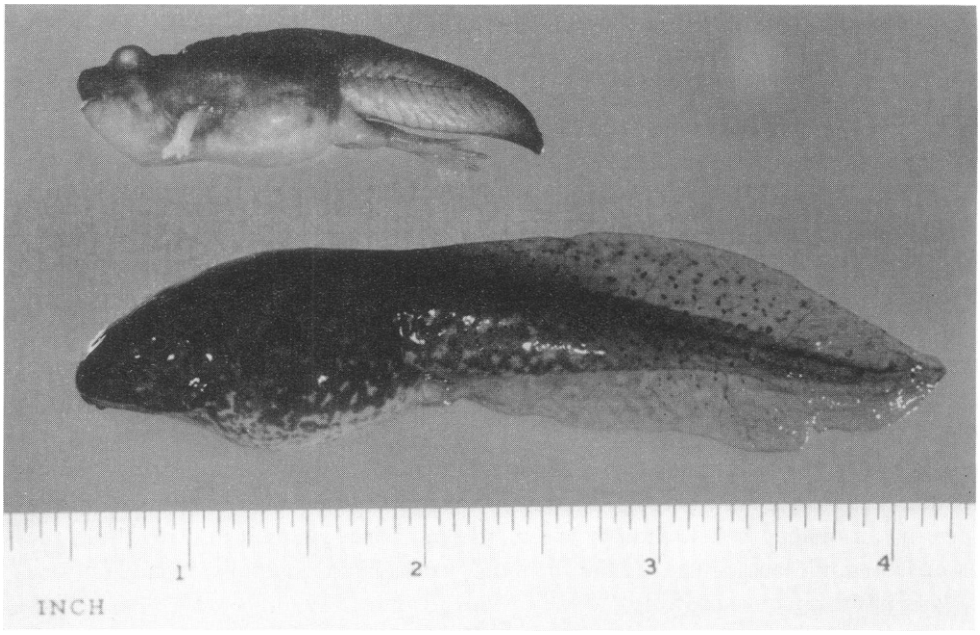

FIGURE 4 Resting tadpole (*R. catesbiana*); above, tadpole after 9 days' treatment with thyroxine. This figure originally appeared in Animal collagenase and collagen metabolism, *in* Mechanisms of Hard Tissue Destruction, (R. Sognnaes, editor), Washington, American Association for the Advancement of Science, 1963, 663.

metamorphosing bullfrog tadpole with an animal treated for 11 days with low concentrations of thyroxine in the aquarium water. Note the loss of tail fin, the shortening of the tail, the appearance of legs, and the marked changes in the shape and features of the head. Table I summarizes some of the changes in composition of

TABLE I

CHANGES IN TADPOLE TAIL FIN INDUCED BY THYROXINE

	Control	Treated
Tail length, *per cent*	100	72
Fin wet weight, *mg*	473	107
Dry weight, *per cent*	5.7	10
Collagen per fin, *mg*	2.3	1.1
DNA per fin, *mg*	0.7	0.3
Collagen/gm wet, *mg*	4.9	10.3
DNA/gm wet, *mg*	1.5	2.7
Collagen/DNA	3.3	3.8

the tail fin, attendant on the action of thyroxine for 6 days (Lapiere and Gross, 1963). Note the loss of some 50 per cent of water in this tissue with resultant doubling of dry weight. If one simply examined the collagen content per gram wet weight of fin it would appear as if the amount of collagen were increasing during the resorption process rather than the reverse. However, measurement of the collagen of the whole fin, cleanly dissected, reveals approximately 50 per cent loss in collagen content. These figures are paralleled by the changes in DNA (a reasonable measure of the number of cells).

The tadpoles are sterilized prior to explantation by adding penicillin, streptomycin, and chloramphenicol to the aquarium water. We have shown that all the bacteria indigenous to tadpole tissues are sensitive to the antibiotics and none produces collagenase. In those occasional cultures which became contaminated, collagenase activity was diminished.

Fig. 5 illustrates the lytic effect of small fragments of tadpole tail fin incubated for 24 hours at 37°. The area of lysis around the explant increases logarithmically until the entire gel is solubilized within 24 to 48 hours. This is illustrated in Fig. 6 which compares the release of radioactivity from C^{14}-labeled collagen gels by tail fin explants with that released by a low concentration of *Clostridium* collagenase diffusing from a filter paper disc. The rate of lysis of the gel is directly proportional to the area of skin used as explant. The pH of the culture medium (Tyrode's solution supplemented with amino acids and vitamins) remains between 7.4 and 8.0. As much as 80 per cent of the degraded collagen substrate became dialyzable as peptides and free amino acids; however, little or no free hydroxyproline appeared.

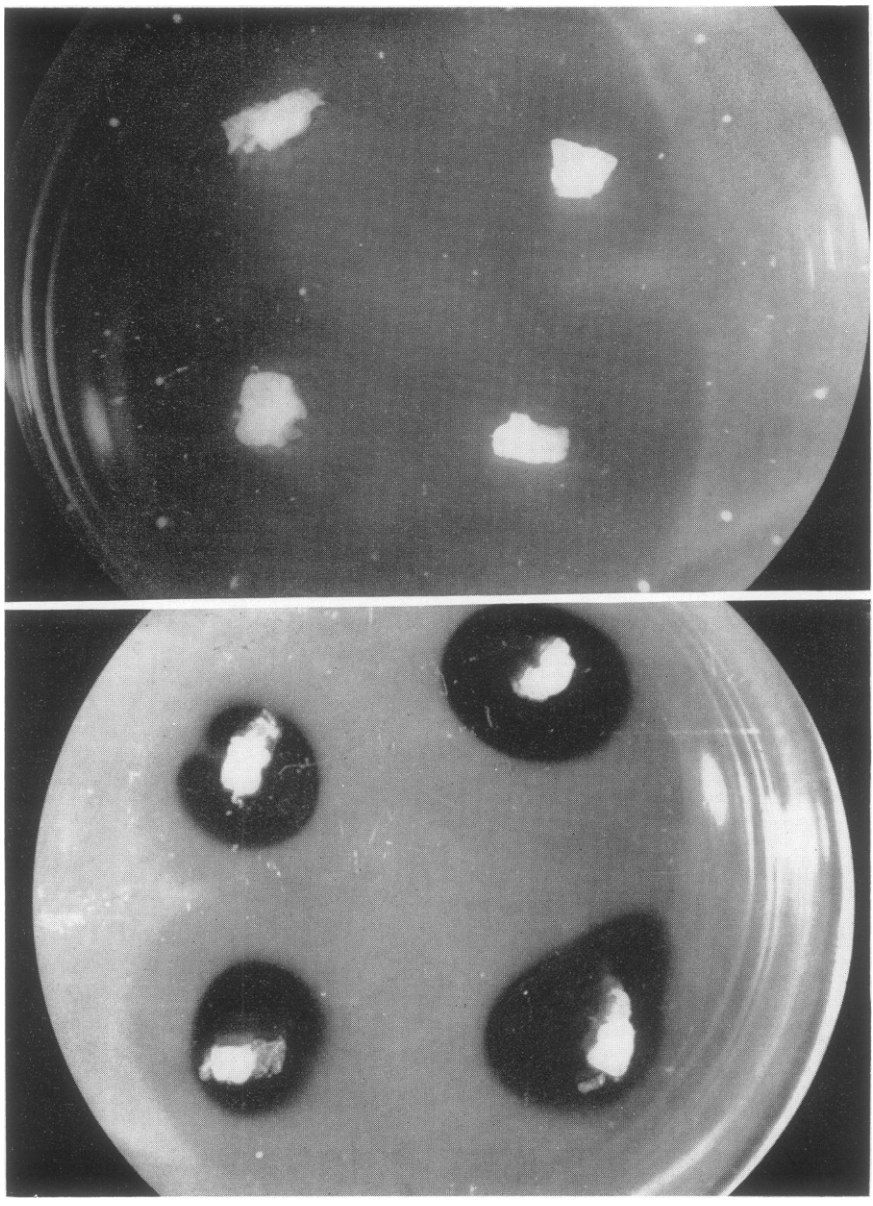

FIGURE 5 Four tadpole fin explants on reconstituted calf skin collagen gel before and after incubation for 24 hours at 37°C. This figure originally appeared in Animal collagenase and collagen metabolism, *in* Mechanisms of Hard Tissue Destruction, (R. Sognnaes, editor), Washington, American Association for the Advancement of Science, 1963, 663.

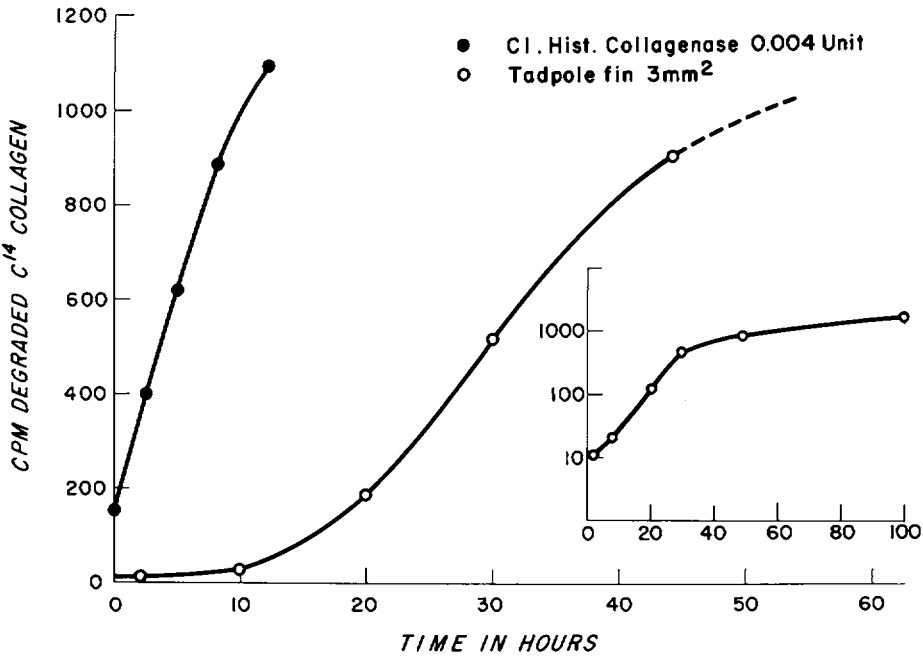

FIGURE 6 Comparison of lytic activity of bacterial collagenase and tadpole tissue as a function of time. Insert is logarithmic plot of radioactivity release in the tissue culture. This figure originally appeared in *Proc. Nat. Acad. Sc.*, 1962, **48**, 1018.

(See Table II.) The rate of release of collagenolytic activity is markedly temperature-dependent.

TABLE II

DIGESTION OF COLLAGEN GEL SUBSTRATE*

	Hypro in supernate	Free hypro in supernate	Dialyzable hypro in supernate	pH at end of incubation
	per cent	*per cent*	*per cent*	
Control	1	0	0	7.9 7.95
(27°C)	15	0	49	7.65 7.85 7.9
(37°C)	59	0	78	7.9 8.2 8.1

* Tail fin tissue grown on reconstituted calf skin collagen gels in amphibian Tyrode solution for 48 hours.

Living tissues are required for production of the enzyme in culture since freezing and thawing prevent the appearance of collagenolytic action. It would thus seem that this enzymatic activity is not contained within the lysosomes or otherwise stored, to be released upon autolysis of the cells. Rather it is more likely that the enzyme is produced and accumulates in the culture medium. Killing of the tissue either stops its production or its activation.

Examination of twelve different tissues of non-metamorphosing tadpoles including kidney, liver, gonads, heart, back and tail muscle, notachord, bone, gut, tail and back skin, and gill has revealed collagenolytic activity in only four of these tissues, namely, tail and back skin, gill, and gut. The details of these experiments have been described elsewhere (Gross and Lapiere, 1962; Gross, Lapiere, and Tanzer, 1963; Lapiere and Gross, 1963).

We have now obtained the enzyme in crude form as a powder, produced by cultivating large amounts of tail fin tissue on filter paper suspended in Tyrode's solution in Petri dishes (Nagai, Lapiere, and Gross, 1963). Enzymatic activity in the culture medium rises progressively for 3 or 4 days, then levels off. At this time the tissues have undergone almost complete autolysis leaving suspensions of free epithelial and mesenchymal cells in the medium. These are removed by centrifugation, the supernant solution dialyzed free of breakdown products, then lyophilized. Enzymatic activity is measured by the release of radioactive peptides from small fixed volumes of reconstituted C^{14}-labeled collagen.

By gel filtration on sephadex G 100 columns we have demonstrated that the tadpole collagenase is of lower molecular weight than bacterial collagenase. If the two enzymes are combined, we obtain two entirely separate peaks of collagenolytic activity, the bacterial enzyme eluting from the column much earlier. We have been able to separate the tadpole collagenase from nearly all non-specific proteolytic activity, as measured by casein digestion, by lowering the pH of an aqueous solution of the crude enzyme powder from 6.0 to 5.4 by ammonium sulfate fractionation. Tadpole collagenase precipitates leaving proteolytic activity in solution. Its solubility characteristics in this regard are different from those of bacterial collagenase. The tadpole enzyme is inactivated at 60° for 10 minutes, has a pH optimum between 7 and 8, and is inactive below pH 6 and above pH 8.6. It is reversibly inhibited by EDTA and requires calcium, similar to the bacterial enzyme. The specificity of the purified tadpole enzyme is now being tested on a series of synthetic peptides and also its point of attack on the collagen molecule is under study.

The effect of thyroxine-induced metamorphosis on collagenase activity was tested by cultivating small bits of tail fin tissue from tadpoles exposed to thyroxine for 2 to 6 days. Using the measurement of released radioactivity from the collagen substrate per unit area of tissue we could show a significant increase in collagenolytic activity in the metamorphosing animals over that of the controls (Lapiere and Gross, 1963). However, it is necessary to take into account the concentration of cells as

a result of removal of water from the tissues. This should increase the activity in the absence of any other change by a factor of two. In a series of similar experiments there was a two- to fourfold increase in activity in the tissues of animals treated for 6 days over that of the control tadpoles.

In a recent series of experiments on turnover of collagen in the tail fin and back skins of non-metamorphosing and thyroxine-treated (metamorphosing) tadpoles we have observed little diminution in rate of collagen synthesis in the tail fin but rather an increase in resorption to account for the net loss in collagen (Lapiere and Gross, 1963). There is also a considerable shift in the distribution of radioactivity between the different collagen fractions suggesting a selective attack on the old insoluble fibrils leaving behind newly deposited insoluble fibers. From this data, we have suggested that the process of resorption involves a continual laying down of a new collagen scaffolding to replace the older fibrils undergoing resorption. The new fabric is in some way protected partially from enzymatic attack at least for the limited period of time necessary to maintain the functional integrity of the tissue.

The manner in which the enzyme is carried to the fibers is probably important. Weiss and Ferris (1954) and Kemp (1961) observed active invasion of the basement lamella by mesenchymal cells during metamorphosis. It is quite possible that these cells carry the enzyme at their surfaces and attack those fibers with which they come in contact. The fact that isotope data indicate continued collagen synthesis in the tail fin during its active resorption raises the question as to which cells are synthesizing and which are producing collagenase. Could different regions of the same cell be performing these two opposing jobs?

By means of simultaneous studies on morphology, biosynthesis, and degradation of a single structurally important component in a biological system which can be easily manipulated with a hormone, we hope to gain access to some of the mechanisms regulating morphogenesis.

This is Publication No. 343 of the Robert W. Lovett Memorial for the Study of Diseases Causing Deformities, Harvard Medical School at the Massachusetts General Hospital. Some of the work discussed herein was accomplished with the aid of Grant AM-05142-02 from the Institute of Arthritis and Metabolic Diseases, National Institutes of Health, United States Public Health Service.

REFERENCES

ALTGELT, K., HODGE, A. J., and SCHMITT, F. O., 1961, Gamma tropocollagen: A reversibly denaturable collagen macromolecule, *Proc. Nat. Acad. Sc.*, **47**, 1914.

BEAR, R. S., 1952, The structure of collagen fibrils, *Adv. Protein Chem.*, **7**, 69.

BENSUSAN, H. B., and HOYT, B. L., 1958, The effect of various parameters on the rate of formation of fibers from collagen solutions, *J. Am. Chem. Soc.*, **80**, 719.

CASSEL, J. M., MANDELKERN, L., and ROBERTS, D. E., 1962, The kinetics of the heat precipitation of collagen, *J. Am. Leather Chemists Assn.*, **57**, 556.

COWAN, P. M., and MCGAVIN, S., 1955, Structure of poly-L-proline, *Nature*, **176**, 501.

CRICK, F. H. C., and RICH, A., 1955, Structure of polyglycine II, *Nature*, **176**, 780.

DOTY, P., and NISHIHARA, T., 1958, The molecular properties and thermal stability of soluble collagens, *in* Recent Advances in Gelatin and Glue Research, (G. Stainsby, editor), New York, Pergamon Press, 92.

FESSLER, J. H., 1960, Some properties of neutral–salt-soluble collagen. I and II, *Biochem. J.*, **76,** 452, 463.

GALLOP, P. M., SEIFTER, S., and MEILMAN, E., 1957, Studies on collagen. I. The partial purification, assay and mode of activation of bacterial collagenase, *J. Biol. Chem.*, **277,** 891.

GRASSMANN, W., HANNIG, K., and ENGEL, J., 1961, Das quantitative Verhältnis zwischen α- und-β-komponente des denaturierten löslichen Kollagens in der Ultrazentrifuge sowie Beschreibung einer schneller sedimentierenden γ-Komponerte, *Z. physiol. Chem.*, **324,** 284.

GRASSMANN, W., HANNIG, K., and SCHLEYER, M., 1960, Zur Aminosäure sequenz des Kollagens. II, *Z. physiol. Chem.*, **322,** 71.

GROSS, J., 1958, Studies on the formation of collagen. III. Time-dependent solubility changes of collagen *in vitro, J. Exp. Med.*, **108,** 215.

GROSS, J., 1959, On the significance of the soluble collagens, *in* Connective Tissue, Thrombosis and Atherosclerosis, (I. H. Page, editor), New York, Academic Press Inc., 77.

GROSS, J., 1961, Collagen, *Scient. Am.*, **204,** 120.

GROSS, J., 1961, Ageing of connective tissue; The extracellular components, *in* Structural Aspects of Ageing, (G. Bourne, editor), London, Pittman, 177.

GROSS, J., 1963*a*, Comparative biochemistry of collagen, *in* Comparative Biochemistry, A Comprehensive Treatise, (M. Florkin and H. S. Mason, editors), New York, Academic Press Inc., 307.

GROSS, J., 1963*b*, An intermolecular defect of collagen in experimental lathyrism, *Biochim. et Biophysica Acta*, **71,** 250.

GROSS, J., and KIRK, D., 1958, The heat precipitation of collagen from neutral salt solutions. Some rate-regulating factors, *J. Biol. Chem.*, **233,** 355.

GROSS, J., and LAPIERE, C. M., 1962, Collagenolytic activity in amphibian tissues: A tissue culture assay, *Proc. Nat. Acad. Sc.*, **48,** 1014.

GUSTAVSON, K. H., 1956, The Chemistry and Reactivity of Collagen, New York, Academic Press Inc.

HARKNESS, R. D., 1961, Biological function of collagen, *Biol. Rev.*, **36,** 399.

HARRINGTON, W. F., and VON HIPPEL, P. H., 1961, The structure of collagen and gelatin, *Adv. Protein Chem.*, **16,** 1.

HAUSMANN, E., 1963, Gelling and time-dependent solubility changes of lathyritic collagen *in vitro, Fed. Proc.*, **22,** 191.

JACKSON, D. S., and BENTLEY, J. P., 1960, on the significance of the extractable collagens, *J. Biophysic. und Biochem. Cytol.*, **7,** 37.

KEMP, N. E., 1961, Replacement of the larval basement membrane lamella by adult-type basement membrane in anuran skin during metamorphosis, *Develop. Biol.*, **3,** 391.

LAPIERE, C. M., and GROSS, J., 1963, Animal collagenase and collagen metabolism, *in* Mechanisms of Hard Tissue Destruction, (R. Sognnaes, editor), Washington, American Association for the Advancement of Science, 1963, 663.

LEVENE, C. I., and GROSS, J., 1959, Alterations in state of molecular aggregation of collagen induced in chick embryos by β-aminopropionitrile (lathyrus factor), *J. Exp. Med.*, **110,** 771.

MARTIN, G. R., GROSS, J., PIEZ, K. A., and LEWIS, M. S., 1961, On the intramolecular cross-linking of collagen in lathyritic rats, *Biochim. et Biophysica Acta*, **53,** 599.

MARTIN, G. R., PIEZ, K. A., and LEWIS, M. S., 1963, The incorporation of [^{14}C] glycine into the subunits of collagens from normal and lathyritic animals, *Biochim. et Biophysica Acta*, **69,** 472.

NAGAI, Y., LAPIERE, C. M., and GROSS, J., 1963, Isolation and partial characterization of an animal collagenolytic enzyme, *Fed. Proc.*, **22,** 648.

OREKHOVICH, V. N., and SHPIKITER, V. O., 1958, Procollagens, *Science*, **127,** 3311.

OREKHOVICH, V. N., SHPIKITER, V. O., MAZUROV, V. I., and KUNINA, O. V., 1960, The procollagen: Classification, metabolism, and action of proteinases, *Bull. soc. chim. biol.*, **42,** 505.

ORLOFF, S. D., and GROSS, J., 1963, Experimental lathyrism in the chick embryo, The distribution of β-aminopropionitrile, *J. Exp. Med.*, **117,** 1009.

PIEZ, K. A., EIGNER, E. A., and LEWIS, M. S., 1963, The chromatographic separation and amino acid composition of the subunits of several collagens, *Biochemistry*, **2,** 58.

PIEZ, K. A., LEWIS, M. S., MARTIN, G. R., and GROSS, J., 1961, Subunits of the collagen molecule, *Biochim. et Biophysica Acta*, **53,** 596.

PORTER, K. R., and PAPPAS, G. D., 1959, Collagen formation by fibroblasts of chick embryo dermis, *J. Biophysic. and Biochem. Cytol.*, **5,** 143.

RANDALL, J. T., (editor), 1953, Nature and Structure of Collagen, London, Butterworth and Co., Ltd.

RICE, R. V., 1960, Reappearance of certain structural features of native collagen after thermal transformation, *Proc. Nat. Acad. Sc.*, **46,** 1186.

RICH, A., and CRICK, F. H. C., 1961, The molecular structure of collagen, *J. Molecular Biol.*, **3,** 483.

RUBIN, A. L., PFAHL, D., SPEAKMAN, P. T., and SCHMITT, F. O., 1963, Tropocollagen: Significance of protease-induced alterations, *Science*, **139,** 37.

SCHMITT, F. O., 1959, Interaction properties of elongate protein macromolecules with particular reference to collagen (tropocollagen), *Rev. Mod. Physics*, **31,** 349.

STAINSBY, B. Y. G., (editor), 1958, Recent Advances in Gelatin and Glue Research, New York, Pergamon Press.

TUNBRIDGE, R. E., (editor), 1957, Connective Tissue, A Symposium, Oxford, B. H. Blackwell Ltd.

VEIS, A., and COHEN, J., 1960, Reversible transformation of gelation to the collagen structure, *Nature*, **186,** 720.

WARNER, J. R., RICH, A., and HALL, C. E., 1962, Electron microscope studies of ribosomal structures synthesizing hemoglobin, *Science*, **138,** 1399.

WEISS, P., and FERRIS, W., 1954, Electron microscopic study of the texture of the basement membrane of larval amphibian skin, *Proc. Nat. Acad. Sc.*, **40,** 528.

WOOD, G. C., 1960, The formation of fibrils from collagen solutions. 2. A mechanism of collagen-fibril formation, *Biochem. J.*, **75,** 598.