ing from this point of view, one could say that we have built and tested a crude membrane model in which such a phenomenon might be observed, and that thus far the binding energies agree satisfactorily with those calculated from *in vivo* experiments only when the model contains lipid. In so far as we understand the behavior of the model, it does not permit us to state whether the interactions occur primarily between lipid film and narcotic or between water and narcotic, facilitated by the presence of lipid. In either interpretation, our data suggest that lipid plays an essential role and show that even substances which are not intrinsically surface active can have profound effects when the composition of the interfacial phase is suitably chosen.[17] The sensitivity of the model to inert gases and the quantitative similarity of its reactions to their biological effect suggest that further studies of its changes of physical state under narcotics may be very informative.

We wish to thank Sheldon Gottlieb and other officials of the Linde Company of Tonawanda, New York, for helpful discussions and for their generosity in supplying purified rare gases for these studies.

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*COLLAGENOLYTIC ACTIVITY IN AMPHIBIAN TISSUES: A TISSUE CULTURE ASSAY**

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Precisely timed and localized removal of structural tissue elements in delicate balance with synthesis is essential to normal growth and development. To date, there is little evidence in animal tissues for specific enzyme systems functioning in the removal of particular structural components. Collagen, representing a major fraction of animal protein, is removed rapidly during remodelling processes;

yet a true animal collagenase has not been found.[1] Fibrous collagen, as a substrate for detecting and measuring such specific enzymatic activity, has the unique advantage of being relatively immune to attack, under physiologic conditions, by any of the commonly known proteolytic enzymes or cathepsins.

Proceeding on the assumption that appreciable amounts of unbound active collagenase may rarely, if ever, accumulate *in vivo*, a method was devised to allow the diffusion of a collagenolytic principle away from the tissue and to detect and measure its activity. Tissue fragments were cultivated on fibrous gels formed from purified, solubilized collagen. Collagenolytic activity was detected and measured by an increasing area of lysis around the explant and by the hydroxyproline or C^{14} content of degraded radioactive collagen.

Precipitated collagen has been used as a culture substrate in recent years by several investigators,[2-4] primarily because it resists the lytic activity of growing cells, in sharp contrast with the fibrin clot.

The Anuran tadpole was selected for our first study because of the rapid dissolution of large tissue masses such as tail, gill, and gut during natural or hormone-induced metamorphosis. This report describes the collagenolytic activity of the tissues of tadpoles not undergoing active metamorphosis, except where so stated.

Methods.—Acid-soluble calf skin collagen was prepared as described previously[5] and stored in the lyophilized state at −20°C. Solutions were prepared by dissolving 10 mg of collagen in 10 ml of cold sterile phosphate buffer, pH 7.6, $\Gamma/2 = 0.4$, by shaking overnight at 5°C. The viscous solution was then dialyzed 24 hr against 0.4 *M* NaCl and clarified by centrifugation at 100,000 *g* for one hr. Radioactive guinea pig skin collagen was prepared from actively growing 250 gm animals injected intraperitoneally with 100 μc of C^{14} glycine 6 hr prior to sacrifice. Collagen was extracted from the dermis with 0.14 *M* NaCl or 0.45 *M* NaCl.[6] The solubilized collagen was purified by repeated salt precipitation.[7] The specific activity of the batch used in these experiments was 20,000 cpm/mg of protein. Simple culture cells were constructed by sealing (with polyvinyl acetate) two pairs of concentric plastic rings onto standard microscope slide. Dimensions of the inner rings are 13 mm O.D., 9 mm. I.D., and 1.7 mm deep. The outer rings, provided to facilitate bathing the gels with medium are 16 mm I.D., 19 mm O.D., and 3 mm deep. Cold collagen solution (125 μl of approximately 0.1% collagen in 0.4 *M* NaCl) was added to the central chamber to a level flat with the top of the ring and incubated in a moist atmosphere at 37°C for at least three hr. At this temperature, collagen in neutral solution will precipitate as a mass of typical cross-striated fibrils in an opalescent gel.[5] These relatively rigid gels were bathed with warm sterile medium containing 150 units each of penicillin and streptomycin per ml, then drained of free fluid. Thus, the saline of the gel was replaced by a physiologic medium. Larger cultures were prepared in 30 mm culture dishes on 2 ml of collagen gel. *Rana catesbiana* tadpoles, stage IX to XXI (Kollros scale for *R. Pipiens*)[8] were sterilized prior to use by keeping them in dishes containing 500,000 units of penicillin and 750 mg streptomycin per liter for 12 to 18 hr. Small pieces of tissue 0.5 to 4 mm across were washed in Tyrode solution and applied to the surface of the collagen gels without adding additional medium. The chambers covered with square glass slips were incubated at 27° or 37°C in a moist atmosphere containing 5 per cent CO_2. Cultures were observed on a side-illuminated black platform stage of a dissecting microscope. The lysed area showed as a black hole in a white opalescent disk. Areas of explant and lysis were measured with an ocular micrometer. Whole stained mounts of cultures were prepared by fixing in 10 per cent formalin, removing the rings, staining in aniline blue, and sealing in balsam with a cover slip. Radioactivity in solution from degraded collagen was determined after 50,000 *g* centrifugation at room temperature. The supernatant fluids were dried and counted in a low-background gas flow counter. Throughout the experiments, the pH of the culture media was checked after incubation and found in all cases to be between 7.2 and 8.2. Sterility of the cultures was frequently confirmed by inoculating the medium in broth and agar plates. Bacterial contamination, when it infrequently occurred, was clearly visible as discrete colonies which in no case produced lysis of

the collagen gel. Contaminated cultures were discarded. Hydroxyproline was measured by the method of Neuman and Logan[9] scaled down by a factor of ten for increased sensitivity.

Evidence for Collagenolytic Activity.—The criteria for collagen degradation were the lysis of the fibrous gel at neutral pH at either 27 or 37°C and the appearance of large amounts of peptide-bound, dialyzable hydroxyproline or radioactivity in the supernatant fluid.

1. *Lysis of collagen gels:* Figure 1 illustrates lysis of the collagen substrate sur-

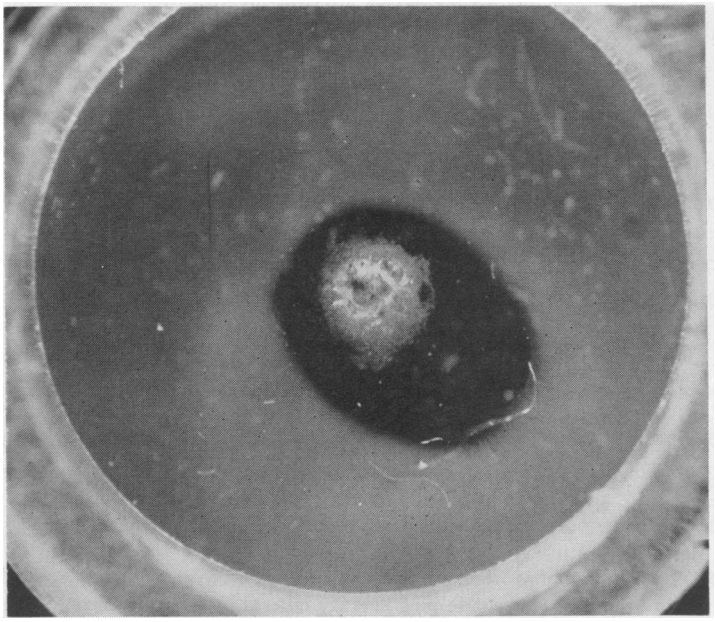

FIG. 1.—Photograph of culture of tadpole fin skin illustrating area of lysis around explant. Remaining collagen gel substrate is seen as surrounding opalescent region within ring (magnification 9 ×).

rounding an explant of tail fin tissue after 48 hr incubation at 27°C. Cellular outgrowth was variable. Any correlation between proliferation and extent of substrate lysis is not presently clear.

Living explants are required for substrate lysis. Repeatedly frozen and thawed skin failed to induce visible lysis while living tissue from the same animal caused extensive dissolution (Fig. 2).

The collagenolytic principle passes through a filter separating the tissue from the substrate. In these experiments, tail fin fragments were placed on a moistened 25-micron-thick Millipore filter, pore size 0.4 microns, covering the gel in the culture chamber. Figure 3 illustrates the clear areas of lysis which appeared beneath the explants.

The extent of lysis of the substrate was compared with the size of tissue explant. For tissues ranging from 1 to 5 square centimeters it is clear from Table 1 that the area of lysis is directly proportional to that of the explant.

Furthermore, the amount of collagen solubilized was correlated with the area of lysis (Fig. 4). Cultures of tail fin skin of approximately equal size were incubated for periods ranging from 24 to 96 hr. Lysed areas and the supernatant radio-

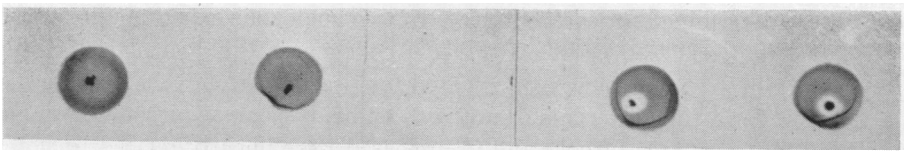

FIG. 2.—A typical pair of cultures. Fixed and stained preparations of cultures of living tadpole fin (right) and nonliving tissue frozen and thawed repeatedly (left).

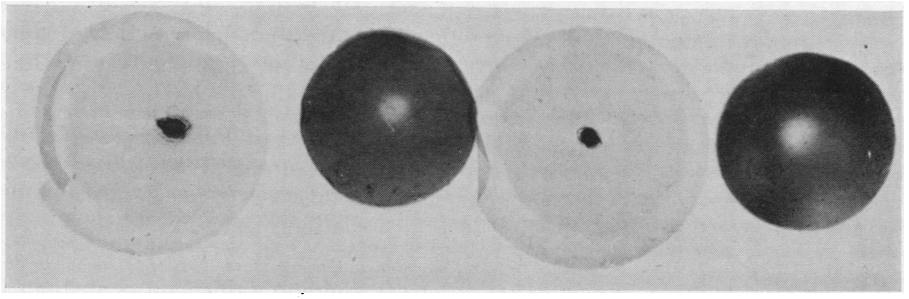

FIG. 3.—Explants of tadpole fin skin placed on Millipore filters separating them from the collagen gels. The filters carrying the explants are seen to the left of the stained substrate. After incubation for 72 hr, the hole produced in the collagen substrate by the lytic factor is clearly visible (magnification 2.5 ×, formol fixation, aniline blue stain).

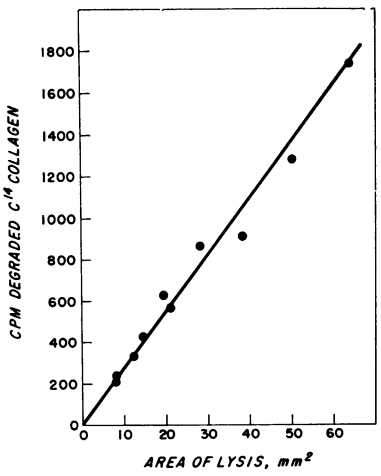

FIG. 4.—Relationship between released radioactivity and area of lysis in tail fin skin cultures.

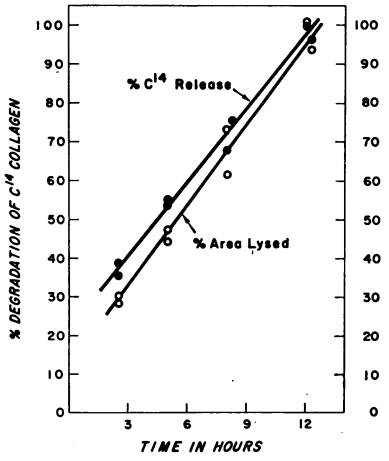

FIG. 5.—Action of bacterial collagenase on collagen gel substrate. Release of degraded collagen (measured as dissolved radioactivity) and area of lysis as a function of incubation time.

TABLE 1

RELATIONSHIP BETWEEN SIZE OF EXPLANT AND AREA OF LYSIS, TADPOLE FIN

Three categories of explant size selected arbitrarily from the data

Number of cultures	Explant area	Mean explant area in mm²	Mean area of lysis in mm²
14	0–1.5	0.8 ± 0.3	4.1 ± 1.6
10	1.6–3.0	2.3 ± 0.4	9.0 ± 5.0
10	3.1–5.0	3.9 ± 0.5	16.8 ± 3.5

activity were determined for each culture. In two analogous experiments, approximately 0.004 unit of *Clostridium histolyticum* collagenase[10] in 1 μl placed on filter paper disk 2 mm in diameter was substituted for tissue. Triplicate sets were incubated at 37°C for 4 time intervals from 2.5 to 12 hr, lysis areas measured, and dissolved C^{14} counted. Area of lysis and C^{14} released were linearly related to time of incubation (Fig. 5). In Figure 6, the lytic activity of bacterial collagenase and tail fin tissue are compared. The action of collagenase is immediate and linear with time, whereas that of the tissue is logarithmic and slower.

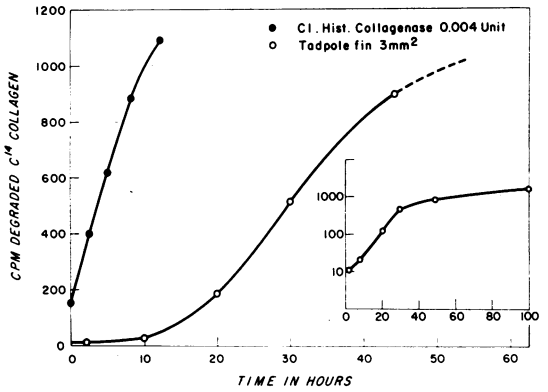

FIG. 6.—Comparison of lytic activity of bacterial collagenase and tadpole tissue as a function of time. Insert is logarithmic plot of radioactivity release in the tissue culture.

2. *Measurement of dialyzable collagen fragments:* After incubation of tail fin skin cultures at 37° and 27°C, the supernatant fluids obtained on centrifugation were dialyzed against water. Free and bound dialyzable hydroxyproline was measured in the dialyzate with results shown in Table 2. Negligible amounts of

TABLE 2

DIGESTION OF COLLAGEN GEL SUBSTRATE IN DISH CULTURES OF TADPOLE TAIL FIN TISSUE

Two ml of 0.1% calf skin collagen, pH. 7.6. Nine 2-mm² tissue fragments per dish, three dishes per experiment. Cultivation time, 48 hr at 27° and 37°. Contents of each dish centrifuged at 27°C and hypro measured in precipitate and supernate. The latter were then dialyzed and dialyzable and free hypro determined. Controls were collagen gels plus tissue centrifuged without incubation.

	μg total hypro	μg hypro in residue	μg hypro in supernate	Per cent hypro in supernate	Per cent free hypro in supernate	Per cent dialyzable hypro in supernate	pH at end of incubation
Control	175	174	1				7.9
	163	160	3	1	0	0	7.9
	169	166	3				7.95
(27°C)	175	150	25				7.65
	160	132	28	15	0	49	7.85
	169	148	21				7.9
(37°C)	163	70	93				7.9
	157	54	103	59	0	78	8.2
	168	76	92				8.1

hydroxyproline were present in the supernatant fluid of the control as compared to the test preparations. There was a fourfold increase in degradation of collagen at 37°C as compared to that at 27°C. There was also nearly twice as much dialyzable hydroxyproline in the 37°C cultures (based on percentage of the supernatant fluid). It should be noted that no free hydroxyproline was present; thus we were dealing with peptide fragments.

3. *Comparison of the collagenolytic activity of living tadpole fin tissue with that of repeatedly frozen and thawed tissue:* Dish cultures of living and frozen-thawed tissues prepared as described above were incubated at 37°C for 48 hr, then transferred to tubes (to obtain better mixing) for five more days at 37°C. Complete vis-

ible lysis of collagen occurred in the living cultures, none in the dead. Microscopic examination of some of the tissue remaining showed numbers of large, slowly moving cells. Only amorphous cell fragments remained in the nonliving explants.

By analysis as shown in Table 3, the living cultures solubilized all of the collagen,

TABLE 3

DIGESTION OF COLLAGEN GEL SUBSTRATE IN DISH CULTURES OF TAIL FIN SKIN OF METAMORPHOSING TADPOLE

Cultures were incubated at 37°C in dishes for 48 hr. The whole contents were fragmented to coarse suspensions in tubes and incubated at 37°C for 5 days. Controls were prepared with frozen-thawed tissue (killed tissues).

	μg total hypro in culture	μg hypro in residue	μg hypro in supernate	Per cent hypro in supernate	Per cent free hypro in supernate	Per cent dialyzable hypro in supernate
Killed tissue	149	126	23			
	161	140	21			
	161	146	15	12	0	7
	156	144	12			
	155	140	15			
Living tissue	132	2	130			
	100	0	100			
	149	1	148	100	4	80
	160	0	160			
	170	0	170			

most of which became dialyzable; little solubilization occurred in the nonliving cultures. Again, little free hydroxyproline was detected.

4. *Collagenolytic activity of medium in the absence of living tissue:* After freezing and thawing of tail fin skin cultures at 50 per cent lysis, continued incubation resulted in complete lysis. This experiment with controls is described in Table 4.

TABLE 4

DIGESTION OF COLLAGEN GEL SUBSTRATE IN PETRI DISH CULTURES OF TAIL FIN TISSUE

To determine whether or not living tissue is required for continuing collagenolytic activity, three sets of cultures were carried in dishes for 42 hr, at which time one set was analyzed for activity (A); a second group was fragmented and suspended in tubes for continued culture for four days (B); and a third group was frozen and thawed three times prior to continued tube culture for four days (C).

	μg total hypro	μg hypro in residue	μg hypro in supernate	Per cent hypro supernate
A	161	71	90	53
	157	80	77	
B	177	1.7	175	99
	101	0.6	100	
C	170	2.0	168	99
	184	1.2	182	

That the collagenolytic principle was free in the medium was further demonstrated in the cell free fluid obtained from gill cultures after complete lysis of the substrate. The viscosity-reducing and fiber-lysing activity[11] was equivalent to that of bacterial collagenase in concentrations of 0.01 to 0.02 unit per ml.

Collagenolytic Activity of Different Tadpole Tissues.—Dish cultures bearing clusters of different tissues were incubated at 37°C in triplicate. Figure 7 illustrates a typical culture. Only three tissues displayed collagenolytic activity—skin, intestine, and gill; muscle, heart, notocord, liver, kidney, and gonads were inactive. Judging from the degree of lysis, it would appear that the gill produced the greatest concentration of activity. A quantitative comparison of collagenolytic activity of the different tissues will be reported elsewhere.

Susceptibility of the Collagen Gel Substrate to Known Hydrolytic Enzymes.—The

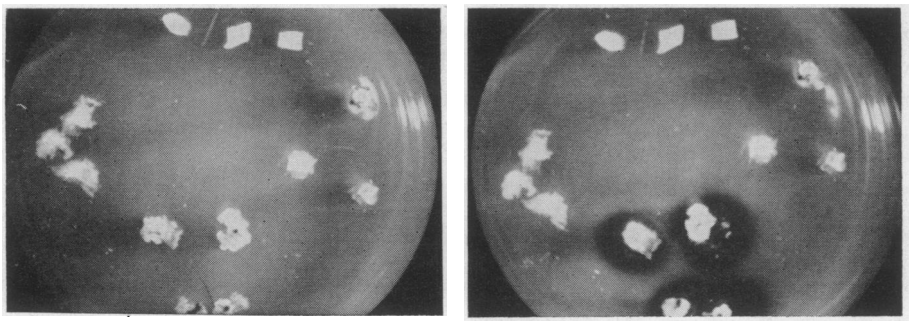

FIG. 7.—Collagenolytic activity of four different tadpole tissues illustrated in photographs of dish cultures before (left) and after (right) incubation at 37°C. Areas of lysis surrounding explants are black on a light background of opalescent collagen gel. Left, body muscle. Right, bone. Top, gonads. Bottom, gills.

collagenolytic activity of a variety of enzymes and tissue extracts was compared with that of bacterial collagenase and of tadpole tissues in culture. One μl of 5 per cent solutions of the enzymes listed in Table 5 were placed on 2 mm diameter

TABLE 5

ACTION OF VARIOUS ENZYMES ON COLLAGEN GELS

Release of C^{14} at 37°C after 40 hours, 2,400 cpm per gel (pH 7.6)

Enzyme		Per cent release of C^{14}	Enzyme		Per cent release of C^{14}
NaCl 0.12 *M*		2.7	Chymotrypsin	50 μg	6.7
Cathepsin C (+SH)	0.6μ	2.7	Collagenase	0.002 units	30.5
Papain (+SH)	50 μg	3.7	N. Tadpole extract	1 μl	3.1
Hyaluronidase	50 μg	2.7	Met. " "	1 μl	4.3
Tyrosinase	50 μg	2.7	Killed tail skin	3 mm^2	4.0
Pepsin	50 μg	2.7	Tadpole tail skin	3 mm^2	43.4
Trypsin	50 μg	9.0	Tadpole gut	3 mm^2	18.6

filter paper disks on the surface of radioactive collagen gels. Aliquots of concentrated aqueous tissue extracts were similarly applied. All preparations were run in triplicate. After 40 hr of incubation at 37°, pH 7.6, the release of radioactivity revealed (Table 5) that only living tadpole tissues and bacterial collagenase appreciably digested collagen.

Discussion.—Although collagen has a relatively low turnover rate in most tissues of mature animals, its rapid removal during physiologic remodelling is well recognized. The best known examples are the remodelling of bone, resorption of the post-partum myometrium, the regression phase of carrageenan granulomas, and the early healing stage of experimental cirrhosis. (See the recent review by Harkness[12] for detailed discussion and bibliography.) When radical changes in body shape and associated disappearance of organs occur during amphibian metamorphosis, the removal of collagen must proceed at a rapid pace.

The existence of collagenase in certain bacteria, development of methods for its purification and assay, and increasing knowledge of its mode of action have led to an intensive search for such enzymes in animal tissues,[1] chiefly by assaying aqueous extracts by methods commonly used, i.e., digestion of native collagen fibrils or lowering of the viscosity of collagen solutions at neutral pH.[11] These efforts have, to date, been uniformly unsuccessful.[1] This lack of success may be ascribed to several factors. Gallop *et al.*[11] observed that bacterial collagenase binds to the

solid substrate and is released only after complete digestion; thus, soluble enzyme in a connective tissue homogenate might be hard to find. It seems unlikely that appreciable amounts of an enzyme capable of dissolving a major structural element would be widely distributed in active form. The sharp limitation of areas of bone resorption to the immediate vicinity of osteoclasts suggests a local release of activity in limited amounts and only where required. The enzyme may be stored within the cell to be released upon demand, or its *de novo* synthesis may be induced. The possible presence of collagenase inhibitors in tissue fluids may also serve to limit and even completely mask collagenolytic activity. Failure to detect a collagenolytic enzyme system has led to the speculation that collagen is removed in stages; a structural alteration of the protein is produced by low pH, elevated temperatures, or denaturing substances which would predispose the altered protein to degradation by nonspecific cathepsins (for discussion see refs. 1 and 12).

Our demonstration of a diffusible collagenolytic factor in living amphibian tissue, capable of degrading undenatured calf skin collagen to dialyzable fragments at neutral pH and temperature as low as 27°C, suggests that true collagenolytic enzyme systems do indeed exist in certain animal tissues. We have also observed collagenolysis in cultures of post-partum myometrium, chick embryo skin, and newborn mouse and rat bone. These experiments will be reported elsewhere.

Detectable activity was not found in tadpole tissue extracts using the assay of Gallop[11] or our culture technique. Failure of dead tissue fragments to release appappreciable amounts of collagenolytic factor in culture suggests that the enzyme is not stored in large amount. A fixed amount of bacterial collagenase, allowed to diffuse freely through the substrate from a filter paper "explant," digests collagen at a relatively rapid, linear rate. This is in contrast with the logarithmic increase in activity of the living cultures, suggesting continual production by the cells with accumulation in the substrate. Diffusion away from the tissue may allow survival of enzyme activity which might otherwise be inhibited or masked.

Characterization of the collagenolytic factor and its mode of action awaits preparation and purification of the enzyme. Peptide analysis at this point is subject to indeterminacies due to the possible presence of other peptidases. That collagen peptides are formed in our system is clearly evidenced by the presence of bound, dialyzable hydroxyproline in the lysate.

The observation that only skin, gut, and gill have relatively strong collagenolytic potency is of particular interest since these tissues undergo most radical remodeling during metamorphosis. Other structures such as tail muscle and notochord are also removed in the absence of detectable collagenolytic activity; no doubt other lytic enzymes are functioning here, although the collagen framework is also resorbed.

Preliminary studies indicate that the collagenolytic activity of metamorphosing and nonmetamorphosing tail skin are of comparable degree, in contrast to Weber's observation[13] of a thirtyfold increase in "catheptic" activity of whole tail tissue during metamorphosis. In organ cultures of whole tadpole tail, thyroxine is required to initiate resorption,[14] whereas in contrast, collagenolytic activity is evident in small tissue fragments in culture without thyroid hormone. Perhaps tissue culture reveals basic potentialities more overtly than organ culture because of the absence of regulating factors.

Summary.—Production of a diffusible collagenolytic factor operating at neutral pH and physiologic temperature on undenatured collagen was demonstrated in living animal tissues in culture. Cultivation of bullfrog tadpole tissues (skin, gut, and gills) on thermally reconstituted neutral calf and guinea pig skin collagen gels resulted in degradation of the substrate to dialyzable collagen peptides. This substrate was not attacked by the common proteolytic enzymes, by Cathepsin C, by tadpole tissue extracts, or by killed tadpole tissue. It is proposed that the experimental system used here may be developed into a quantitative assay for collagenolytic activity in small tissue fragments.

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EPR IN CHROMATOPHORES FROM RHODOSPIRILLUM RUBRUM AND IN QUANTASOMES FROM SPINACH CHLOROPLASTS

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I. *Introduction.*—The photo-induced electron paramagnetic resonance (EPR) observed in photosynthetic materials has implicated electron transfer reactions in the primary quantum conversion act.[1] Aside from this, it has provided little new information of biological significance. The reason for this dearth of new information stems mainly from the difficulty encountered in relating the characteristics of the resonance to biological parameters. Such approaches to the problem as the use of mutant species,[2, 3] special growth conditions,[4, 5] special metabolic inhibitors,[6] extreme physical conditions,[7] etc., have been tried, but progress toward