Mouse Bone Collagenase: Isolation, Partial Purification, and Mechanism of Action* (33747)

M. SHIMIZU, M. J. GLIMCHER, D. TRAVIS, AND P. GOLDHABER

Departments of Oral Histology and Periodontology, Harvard School of Dental Medicine and Department of Orthopedic Surgery, Harvard Medical School and Massachusetts General Hospital, Boston, Massachusetts 02114

Animal collagenase, first isolated from tadpoles (1, 2), has also been detected in a number of other tissues (3–14). To date, however, only the enzymes obtained from tadpole fin (15, 16), rat uterus (14), rheumatoid synovial tissue (12), and human skin (13) have been partially purified and characterized, and their mode of action in degrading the collagen macromolecule studied.

If tissue collagenase is physiologically important and plays a significant role in tissue collagen degradation, one would expect considerable quantities of such an enzyme to be synthesized in bone, since the turnover of collagen in bone is amongst the highest of any of the collagenous tissues (17). Indeed, collagenase activity has been observed in tissue culture media of bone cultured *in vitro* from animals which had received parathyroid hormone (3) and which had been undergoing resorption *in vivo*, and in the tissue culture media of bone actively undergoing bone resorption *in vitro* (4). In the latter study, increased collagenolytic activity was shown to parallel the increased rate of bone resorption when parathyroid hormone, and parathyroid hormone and heparin were added to the tissue culture medium (4). The present communication deals with the isolation, partial purification, and mode of action of mouse bone collagenase, obtained from the tissue culture media of living bone cultured *in vitro* in the presence of heparin (18).

Methods. Tissue culture of bone. Tibiae of 5-day old Swiss albino mice of the Webster strain were removed aseptically and muscle and connective tissue carefully removed. Immediately thereafter, four bones were placed in a roller tube containing 2 ml of tissue culture medium to which was added 0.1 ml of heparin (100 units) dissolved in Gey's solution, and the tubes were gassed with 95% O_2 and 5% CO_2 for approximately 1 min. The tissue culture medium was composed of 95 ml of mammalian Tyrode solution, 1 ml each of 100 times concentrated amino acid mixture, 100 times concentrated vitamin mixture, and 200 m*M* L-glutamine (all from Microbiological Associates, Inc., Bethesda, Maryland), and 2 ml containing 5000 units each of penicillin and streptomycin.

The roller tube cultures were incubated for 4 days at 37°; the bones were removed and the pooled media, clarified by centrifugation, was dialyzed extensively against water at 2°. This crude extract was freeze–dried and stored at −20°.

Purification of the collagenase. Approximately 100 mg of the freeze–dried crude extract were dissolved in 20 ml of 0.05 *M* Tris buffer (pH 7.6), 5×10^{-3} *M* $CaCl_2$ at 2°, and the solution was clarified by centrifugation. Solid $(NH_4)_2SO_4$ was added slowly to 20% saturation and the solution was allowed to stand at 2° for 1–2 hr. The precipitate was collected after centrifugation, solid $(NH_4)_2SO_4$ was added slowly to the supernatant to 50% saturation, and the mixture was allowed to remain at 2° for 12–16 hr. The precipitate was again collected after centrifugation. Collagenolytic activity, measured either by the release of [^{3}H]-hydroxyproline and [^{3}H]-proline from reconstituted collagen gels (2), or by changes in the viscosity of purified collagen solutions at 20° was found only in the second precipitate [20–50% saturation of $(NH_4)_2SO_4$]. This precipitate was redissolved in 0.05 *M* Tris buffer (pH 7.6), 5×10^{-3} *M* $CaCl_2$, and after extensive dialy-

Supported by research grants from the U. S. Public Health Services, National Institute of Health (DE-01298), AM-06375, 5-T1-De-112) and the John A. Hartford Foundation, Inc.

sis against this buffer was frozen and stored in solution at —20°. The partially purified enzyme showed negligible proteolytic activity using casein as a substrate (19). Acrylamide disc gel electrophoresis of the partially purified enzyme, run at pH 7.5, 0.01 *M* Tris buffer, 1×10^{-3} $CaCl_2$ at 2° showed seven bands when stained with amido black. Preparative acrylamide gel electrophoresis was carried out as follows: A 0.5 in. test tube approximately 2 in. long was filled with acrylamide gel equilibrated in 0.05 *M* Tris buffer (pH 7.8), 5×10^{-3} *M* $CaCl_2$ at 2°. The enzyme, dissolved in the same Tris buffer was added to 4 *M* sucrose solution which was layered over the starting gel. After electrophoresis, and based on the position of the fractions as revealed by amido black staining on previously run samples, the gel was cut into eight pieces, each of which was eluted with Tris buffer. Aliquots of the eight eluates were assayed for collagenolytic activity (2).

Preparation of collagen substrate. Five times reconstituted, acid soluble, rat skin and rat tail tendon collagen, as well as five times reconstituted guinea pig skin collagen, the latter labeled with [^{3}H]-hydroxyproline and [^{3}H]-proline, were prepared as previously described (4, 20). The specific activity of the radioactively-labeled guinea pig skin collagen was 1×10^5 dpm/mg of collagen. Aliquot samples of the freeze-dried collagens were dissolved as 0.1–0.2% solutions in sodium phosphate buffer (pH 7.6) $\mu = 0.45$ at 2°, and clarified by ultracentrifugation in a Beckman Spinco model L preparative ultracentrifuge at 30,000 rpm for 4 hr. The concentration of the collagen solutions was calculated from the hydroxyproline concentration (21) of acid hydrolysates.

Assay for collagenase activity. (2) Aliquots of 100 μl of the guinea pig collagen solutions were gelled by warming to 37° in small test tubes for 20 hr. To solution containing 50 μl of the enzyme solution (0.7 mg of protein/ml), 200 μl of 0.1 *M* Tris maleate or HCl buffer, and 50 μl of 5×10^{-3} *M* $CaCl_2$ were then added. The pH of the solutions added to the reconstituted collagen fibrils (gel) was varied from 5.0 to 8.6. The mixtures were incubated for 30 min at 37° and the reaction was inhibited with 10 μl of 0.4 *M* EDTA solution, pH 7.0 and the tubes were centrifuged at room temperature. Aliquots of the supernatants were dissolved in Bray's solution (22) and counted in a liquid scintillation counter.

The effect of cysteine and EDTA on collagenase activity was tested in the same fashion, except that 200 μl of 0.06 *M* Tris–HCl buffer at pH 7.6 containing 5×10^{-3} *M* $CaCl_2$ and 50 μl of EDTA or cysteine were used as the solution added to the collagen gels.

Viscosity and optical rotation. The specific viscosity of control solutions of rat tail tendon and rat skin collagen and collagen solutions reacted with enzymes was measured in Ostwald viscometers with water flow times varying from 90–120 sec at 20°. The usual reaction mixture contained 1 ml of 0.1% collagen solution, 0.5 ml of enzyme solution in a Tris–HCl buffer solution at pH 7.6, 0.4 *M* NaCl. The concentration of the enxyme solutions varied from 1 to 3 mg of protein/ml. Viscosity measurements were made at regular intervals at 20° for a period of approximately 24 hr. The optical rotation of untreated solutions of rat tail tendon and rat skin collagen and of solutions reacted with the enzyme was measured at 20° in a Perkin-Elmer model 141 polarimeter. At the end of 24 hr of incubation of both the control and enzyme-reacted samples, the denaturation temperatures were measured after dialysis against 0.15 *M* NaCl, 0.05 *M* acetic acetic acid.

Examination of the products of reaction. (i) After 24 hr of incubation at 20°, the collagen solutions, both control and enzyme reacted samples were brought to 30% saturation with $(NH_4)_2SO_4$ at 2°, and allowed to stand for 1 hr. The precipitates were collected after centrifugation, resuspended in 0.05 *M* acetic acid and dialyzed for 48 hr in the cold against this solution. Aliquots of the solutions were denatured at 50° for 10 min and examined by acrylamide disc gel electrophoresis (16).

(ii) Aliquots of solutions of rat skin collagen and collagen solutions reacted with the

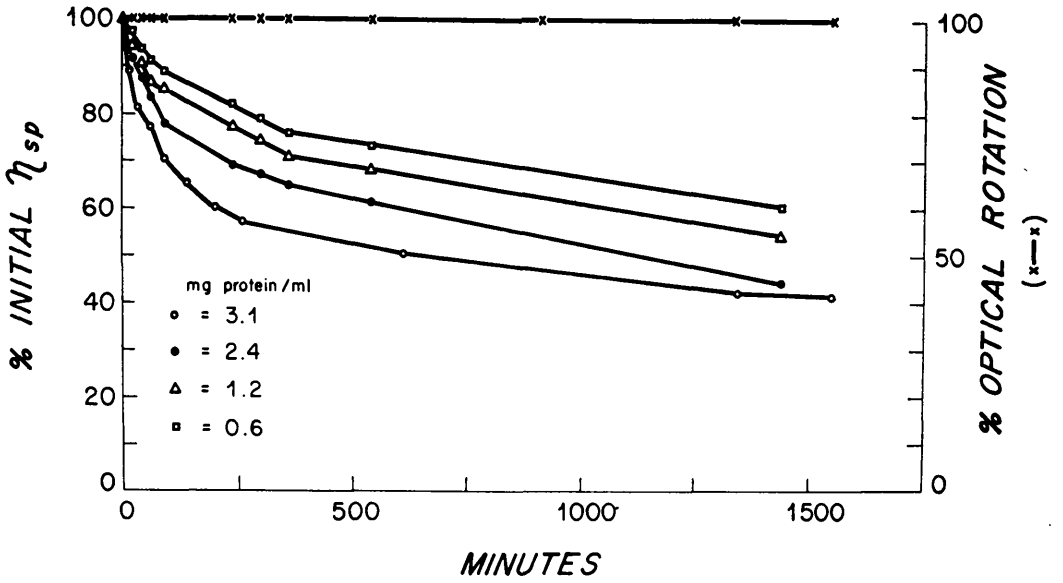

FIG. 1. The effect of mouse bone collagenase on the viscosity and optical rotation of rat skin collagen solutions at 20° (see text for details): the percentage change in the specific viscosity and in the optical rotation is shown on the ordinate and the time after the addition of the enzyme on the abscissa; the viscosity of the control solutions of rat skin collagen remained constant during the period of the experiment.

bone enzyme at pH 7.6 at 20° for 12 and 24 hr, were dialyzed exhaustively against 0.05% acetic acid, and the collagen precipitated by the addition of ATP as the free acid at a final concentration of 0.2–0.3%. The suspensions so formed were examined in an Elmiskop I electron microscope. A usual reaction mixture contained 1 ml of collagen solution (0.45 *M* NaCl at pH 7.5), 0.5 ml of 0.05 *M* Tris buffer (pH 7.5) containing 1×10^{-3} *M* $CaCl_2$; 2 ml of 0.45 *M* NACl, 0.05 *M* Tris buffer (pH 7.5), and 0.4 ml of the enzyme (2.8 mg of protein/ml) dissolved in 0.05 *M* Tris buffer, 1×10^{-3} *M* $CaCl_2$ (pH 7.5).

Results. Collagenase activity. The bone enzyme, partially purified by $(NH_4)_2SO_4$ fractionation, reduced the specific viscosity of collagen solutions at pH 7.6 at 20° (Fig. 1). The rate at which the viscosity was reduced was dependent on the concentration of the enzyme (Fig. 1). There was no further significant reduction in the viscosity after approximately 1500 min of incubation. There was no change in the specific optical rotation after 1500-min incubation with the enzyme (Fig. 1).

Purification of the enzyme by $(NH_4)_2SO_4$ fractionation increased its activity by about 10- to 15-fold as compared with the crude extract, but there was insufficient material obtained by preparative acrylamide gel electrophoresis to accurately assess its activity relative to the $(NH_4)_2SO_4$ fraction from which it was derived. Moreover, as in the case of highly purified tadpole collagenase (15), the most highly purified mouse bone collagenase obtained by preparative acrylamide gel electrophoresis was unstable when stored frozen or freeze–dried.

The mouse bone collagenase also degraded collagen in the solid state, and the release of soluble [^{3}H]-proline and [^{3}H]-hydroxyproline-labeled peptides was linear with enzyme concentration (Fig. 2).

The effective pH range of the enzyme was from 7 to 8.6, with an optimum at about 8.0 (Fig. 3). There was no collagenase activity at pH 5.0. The activity of the enzyme was completely inhibited by EDTA at a concen-

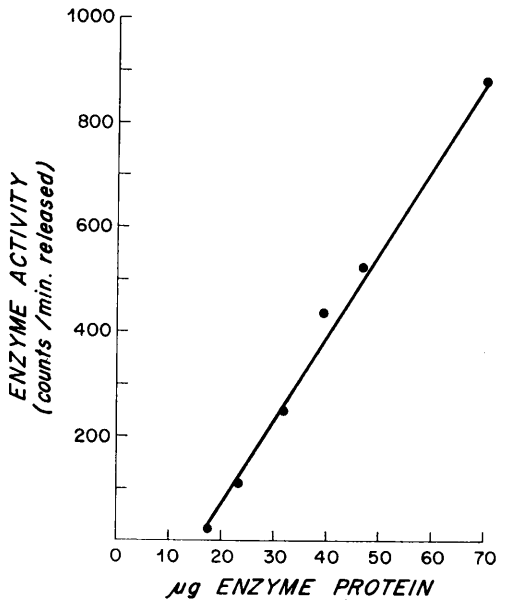

FIG. 2. The release of (3H)-proline and (3H)-hydroxyproline containing peptides from reconstituted guinea pig skin collagen gels by mouse bone collagenase as a function of enzyme concentration: collagen gels and enzyme reacted at 37° for 30 min. Negligible radioactivity was noted in control samples incubated without the addition of enzymes. Trypsin released less than 5% of the total radioactivity under similar conditions (see text for details).

tration of 5×10^{-3} *M* and by cysteine at a concentration of 1×10^{-3} *M*.

Only the third, fourth, and fifth fractions (no. 1 being the slowest moving fraction) obtained from the 20–50% $(NH_4)_2SO_4$ fraction eluted from the preparative acrylamide gel electrophoresis showed collagenase activity as measured by the release of radioactive labeled peptides from collagen gels at 37°.

Characterization of reaction products. (i) Acrylamide gel electrophoresis of the degradation products of collagen after incubation with partially purified bone collagenase showed patterns (Fig. 4) similar to those reported after collagen treatment with collagenase of tadpole fin (15, 16), rat uterus (14), human skin (13), and human rheumatoid synovium (12). The same was true for the reaction products produced by the third, fourth, and fifth fractions eluted from preparative acrylamide gel electrophoresis (Fig. 5).

(ii) Electron microscopy of the aggregates produced by the addition of ATP revealed small fragments cleaved from the "B" end of the macromolecule similar to the TC^B aggregates and larger aggregates similar to the TC^A fragments described by Gross and Nagai (23). However, there were larger variations in the lengths of the smaller and larger fragments than that reported by Gross and Nagai (23) for tadpole collagenase, in which case, there was a marked preponderance of large fragments whose lengths were approximately three-quarters of the length of the collagen macromolecule, and short fragments approximately one-quarter of the molecular length.

Discussion. The incubation of living mouse bone in a synthetic medium enriched with heparin, markedly increases the lysis of purified reconstituted collagen gels, as com-

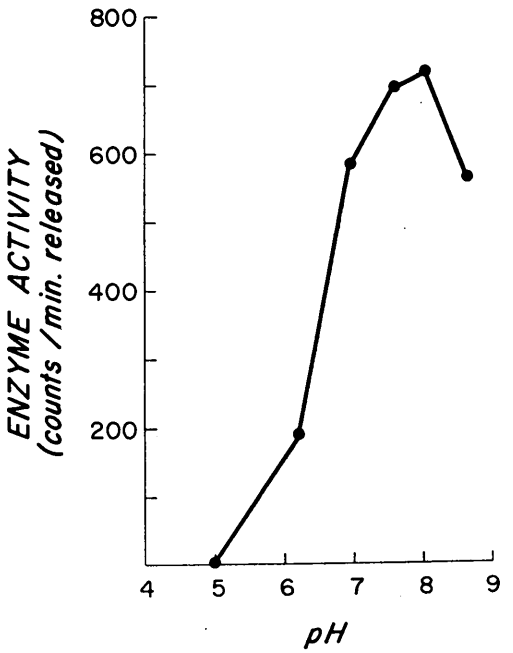

FIG. 3. The effect of pH on the enzyme activity of mouse bone collagenase: reconstituted, guinea pig skin collagen fibrils labeled with (3H)-proline and (3H)-hydroxyproline were incubated with the bone enzyme for 30 min and the radioactivity released from the collagen gels into the media was measured in a scintillation counter (see text for details). Negligible radioactivity was noted in control samples incubated without the addition of enzyme.

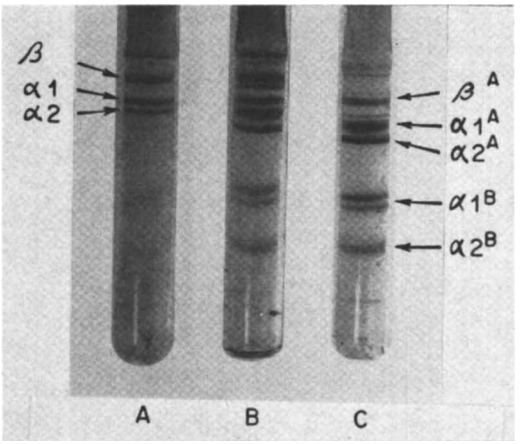

FIG. 4. Acrylamide gel disc electrophoresis of thermally denatured rat skin collagen after reaction with mouse bone collagenase [20–50% $(NH_4)_2SO_4$ fraction] at 20° for: (A), zero time; (B), 1 hr; and (C) 24 hr; enzyme concentration, 3.1 mg/ml.

pared to controls incubated without heparin (18). In the present study, an enzyme was isolated from the tissue culture medium of similar experiments carried out in the absence of the collagen gels, which is capable of degrading purified collagen fibrils *in vitro* at 37°, at a pH of 7.4. Since no enzymatic activity could be extracted from bone tissue homogenates or was recovered from the tissue culture medium when freeze–thawed, dead bone was cultured *in vitro* with heparin (18), it appears that there is little if any storage of the active enzyme in the tissue. These findings are consistent with those reported for tadpole tail (15), human skin (13), and synovium (12). The range of pH and the optimum pH (8.0) are also similar to those of the other tissue collagenases (12, 13, 15). These data combined with the inability to extract the enzyme from tissue homogenates even after procedures designed to disrupt membranes (18), are consistent with the suggestion that tissue collagenases are not of lysosomal origin (24). The finding that mouse bone collagenase is inhibited by EDTA and cysteine is similar to the results reported for human skin collagenase (13), tadpole collagenase (23), and synovium collagenase (12), but differs in this respect from the enzyme obtained from rat uterus (14).

Mouse bone collagenase was partially purified by $(NH_4)_2SO_4$ fractionation and by preparative acrylamide gel electrophoresis and shown to have negligible noncollagenolytic protease activity. At 20°, the purified enzyme cleaves the collagen macromolecule across the three chains at a site closer to the "B" end, producing a smaller TC^B fragment and a larger TC^A fragment. Both fragments retain the triple stranded helical configuration of collagen as evidenced by the maintenance of the optical rotation during the fall in viscosity when collagen is incubated with the enzyme, and the ability to reconstitute the individual fragments into segment long spacing (SLS) aggregates. The mean denaturation temperature (the temperature at which one-half the total change in optical rotation is reached) of the fragments is slightly less than the intact macromolecule (36 vs 32°). The mode of action of mouse bone collagenase is therefore similar in these respects to that of the other tissue collagenases (12–14, 23).

It was previously demonstrated that the degradation of bone collagen in tissue culture (25) and of reconstituted collagen fibrils incubated with bone during active bone resorption (4), results in the release into the media of small peptides and of free amino

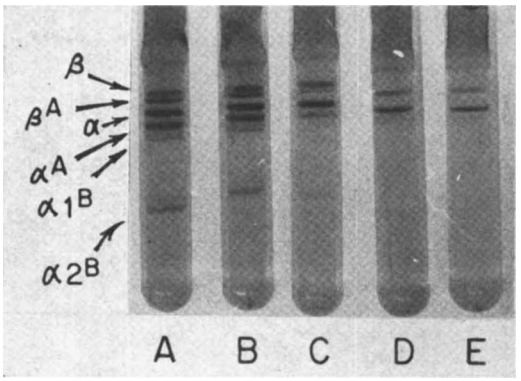

FIG. 5. Acrylamide gel disc electrophoresis of thermally denatured rat skin collagen incubated with mouse bone collagenase eluted from preparative acrylamide gel electrophoresis: (A), fraction 3; (B), fraction 4; (C), fraction 5; (D), fraction 6; (E), fraction 7. Collagenase activity was noted only in fractions 3, 4, and 5. Reaction carried out at 20° for 24 hr.

acids. Since the purified mouse bone collagenase acts primarily to cleave the collagen macromolecule into only two fragments, it is not clear whether there are other proteolytic enzymes produced which further degrade the collagen into smaller peptides and the free amino acids, or whether the same enzyme is responsible for the continued degradation. The latter could occur after the initial cleavage, since, as it has been pointed out (23), the denaturation temperature of the TC^A and TC^B fragments is less than physiological temperature, and therefore the same enzyme could degrade the denatured TC^A and TC^B fragments after the initial scission of the macromolecule. Preliminary experiments in which dialyzable [^{3}H]-hydroxyproline and [^{3}H]-proline labeled peptides have been detected after incubation of collagen gels with mouse bone collagenase at 37°, are consistent with this latter proposal.

The isolation of an enzyme from bone which can degrade collagen fibrils under physiological conditions of temperature and pH, and the previous findings that the collagenolytic activity which is produced in bone undergoing active resorption is increased with increasing rates of bone resportion (4) strongly suggest an important biological role for this enzyme during bone remodeling.

We wish to thank Mr. Lawrence Bonar, Mrs. Josephina Gonzalez, Mrs. Ivan Jennings, and Miss Diane Brickley for their assistance.

1. Gross, J. and Lapiere, C. M., Proc. Natl. Acad. Sci. U. S. **48**, 1014 (1962).

2. Lapiere, C. M. and Gross, J., *in* "Mechanisms of Hard Tissue Destruction" (R. Sognnaes, ed.), Publ. No. 75, p. 663. Am. Assoc. Advance. Sci., Washington, D. C. (1963).

3. Walker, D. G., Lapiere, C. M., and Gross, J., Biochem. Biophys. Res. Commun. **15**, 397 (1964).

4. Kaufman, E. J., Glimcher, M. J., Mechanic, G. L., and Goldhaber, P., Proc. Soc. Exptl. Biol. Med. **120**, 632 (1965).

5. Woods, J. F. and Nichols, G., Jr., J. Cell Biol. **26**, 747 (1966).

6. Fullmer, H. M. and Lazarus, G., Nature **209**, 728 (1966).

7. Buetner, E. H., Trifthuasser, C., and Hazen, S. P., Proc. Soc. Exptl. Biol. Med. **121**, 1082 (1966).

8. Fullmer, H. M., Lazarus, G., Gibson, W. A., Stem, A., and Link, C., Lancet **1**, 1007 (1966).

9. Riley, W. R. and Peacock, E. E., Proc. Soc. Exptl. Biol. Med. **124**, 207 (1967).

10. Grillo, H. C. and Gross, J., Develop. Biol. **15**, 300 (1967).

11. Fullmer, H. M. and Lazarus, G., Israel J. Med. Sci. **3**, 758 (1967).

12. Evanson, J. M., Jeffrey, J. J., and Krane, S. M., Science **158**, 499 (1967).

13. Eisen, A. J., Jeffrey, J. J., and Gross, J., Biochim. Biophys. Acta **151**, 637 (1968).

14. Jeffrey, J. J. and Gross, J., Federation Proc. **26**, 670 (1967).

15. Nagai, Y., Lapiere, C. M., and Gross, J., Biochemistry **5**, 3123 (1966).

16. Sakai, T. and Gross, J., Biochemistry **6**, 518 (1967).

17. Laitinen, O., Acta Endocrinol., Suppl. **120**, (1967).

18. Shimizu, M., Glimcher, M. J., and Goldhaber, P., Proc. Soc. Exptl. Biol. Med., in press.

19. Kunitz, M., J. Gen. Physiol. **30**, 291 (1947).

20. Glimcher, M. J., Francois, C. J., Richards, L., and Krane, S. M., Biochim. Biophys. Acta **93**, 585 (1964).

21. Stegemann, H., Z. Physiol. Chem. **311**, 41 (1958).

22. Bray, G. A., Anal. Biochem. **1**, 297 (1960).

23. Gross, J. and Nagai, Y., Proc. Natl. Acad. Sci. U. S. **54**, 1197 (1965).

24. Eisen, A. Z. and Gross, J., Develop. Biol. **12**, 408 (1965).

25. Stern, B., Glimcher, M. J., Mechanic, G. L., and Goldhaber, P., Proc. Soc. Exptl. Biol. Med. **119**, 577 (1965).

Received Oct. 24, 1968. P.S.E.B.M., 1969, Vol. 130.