EXPERIMENTAL LATHYRISM

An Autoradiographic Study

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ABSTRACT

In normal and lathyritic chick embryos bone collagen was synthesized primarily in the periosteum of the femurs, and was organized as radioactive spicules in these bones. Saline extraction of the lathyritic bones removed the radioactive spicules, although they eventually seemed to become non-extractable. Normal bone seemed to be unaffected by saline extraction. Marked variation in the degree of isotope incorporation was seen in collagenous and non-collagenous tissues. All the tissues of any one embryo, however, showed a similar degree of isotope incorporation. Tritiated β-aminopropionitrile was diffusely distributed throughout bone and was completely removed by saline extraction. This autoradiographic study supports the postulate that a portion of extractable lathyritic collagen is recently synthesized and is organized in fibrous structures in bone.

The pathogenesis of experimental lathyrism may involve alterations of collagen molecular structure (1), suggested by the occurrence of two abnormal properties of lathyritic collagen. Fibrils formed from lathyritic collagen, either *in vivo* or *in vitro*, are temperature sensitive, going back into solution upon cooling (2). In addition to this manifestation of altered intermolecular bonding, lathyritic collagen contains virtually no intramolecular cross-links (3, 4).

A portion of lathyritic collagen seems to be newly synthesized (1, 5, 6), although Tanzer and Gross (1) have presented evidence indicating that lathyrism may also affect older collagen fibrils. Furthermore, their studies suggested that lathyritic collagen occurs as fibrils *in vivo*, contrary to the postulate (5, 6) of impaired fibril formation.

This paper reports autoradiographic studies concerning the origin of lathyritic collagen and its morphologic state in bone.

EXPERIMENTAL PROCEDURE

Materials

Tritiated L-proline-3,4-H^3 (5,000 mc/mM) and L-glycine-1,2-C^{14} (82 mc/mM) were obtained commercially and were found to be radiochemically pure by chromatographic methods. β-aminopropionitrile (βAPN) fumarate was generously provided by Abbott Laboratories, North Chicago, Illinois.

Labeling of Normal and Lathyritic Embryos

White leghorn hybrid chicken eggs were incubated for 14 days at 38°C and constant humidity. Twenty mg of β-aminopropionitrile fumarate in 0.1 ml of sterile water were injected through a pinhole onto the chorio-allantoic membrane. Fifty μc of tritiated proline were administered to each embryo in similar fashion; several embryos received 50 μc of glycine-C^{14} instead of tritiated proline. Control embryos received isotope alone.

In several experiments, all substances were in-

jected *intravenously* (7), to obviate any differences in diffusion across the chorio-allantoic membrane.

Preparation of Autoradiographs

After the desired period of incubation the embryos were removed, and their femurs were dissected free. The intact right femur was placed in neutral, buffered 10 per cent formalin, while the intact left femur was placed in 10 ml of ice cold 1 M NaCl, shaken for 48 hours at 4°C, and subsequently placed in neutral 10 per cent formalin. In two pilot studies, we found that cold saline removed equal amounts of collagen from intact femurs and from sliced femurs. For comparative purposes, skin, liver, kidney, and frontal bones were also removed from the embryos and were fixed in neutral 10 per cent formalin.

All bones were decalcified for 12 hours in 10 per cent formalin containing 0.2 M ethylenediaminetetraacetate (EDTA), pH 7.5, prior to embedding in paraffin. Duplicate, 5-micron tissue sections were cut; the sections were affixed directly to glass slides, deparaffined, and covered with Kodak AR-10 stripping film. Slides exposed for 4 or 6 weeks gave similar results, but only the former are reported. Following exposure, the autoradiographs were developed and were stained with nuclear fast red.

Preparation of Special Autoradiographs

100 μc of uniformly labeled, tritiated beta-aminopropionitrile (βAPN)[1] and 20 mg of non-radioactive βAPN fumarate were injected intravenously into several animals. The femurs were removed ½ hour later, quickly frozen in dry ice–acetone, and sectioned on a cryostat microtome at −20°C. Autoradiographs were made by the procedure of Fitzgerald (8).

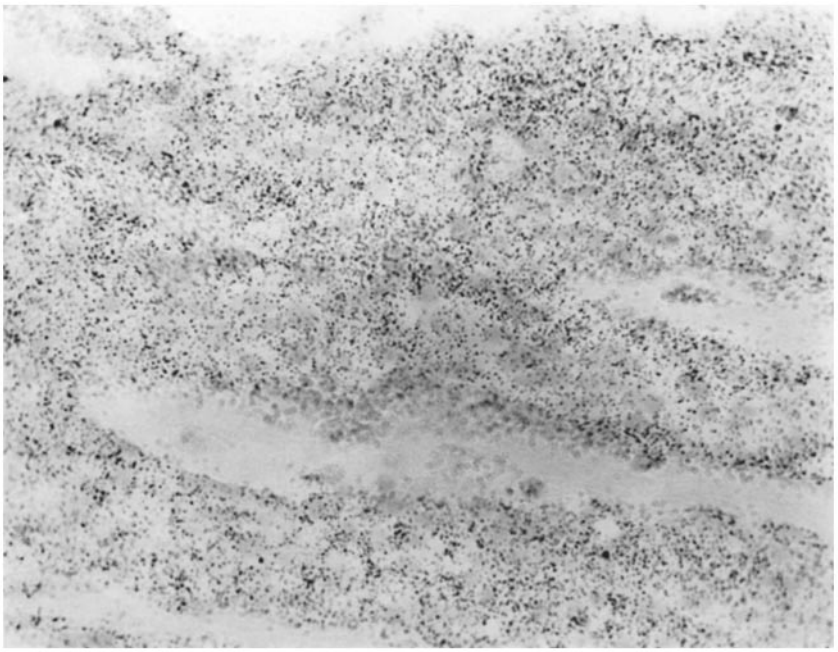

FIGURE 1 Right femur, normal chick embryo, 2 hours after tritiated proline. The periosteum is at the top. The silver grains are diffusely present over cells and are not concentrated over spicules. Identical picture in opposite femur after cold saline extraction. × 400.

Analyses

The isolation, chemical analysis, and radioactivity assay of hydroxyproline were performed as previously reported (1).

RESULTS

Amino Acid Incorporation into Chick Embryo Bone

The histologic sequence of isotopic proline or glycine appearance in chick embryo bone was as follows:

[1] Kindly supplied by Dr. J. Gross.

Initially, the silver grains were diffusely distributed over the periosteal osteoblasts (Fig. 1). Subsequently, they were intensely concentrated over the subperiosteal bone spicules (Fig. 2). Only the outermost spicules were radioactive, while the innermost spicules showed no label. No radioactive spicules were seen at the epiphyseal junction. Thus, confirming previous studies (9), these results indicate that the chick embryo forms new bone primarily by subperiosteal deposition.

With time, there was a considerable decrease in the radioactivity of the growing, outermost spicules. The highly labeled collagen became well outlined by an internal region of non-radioactive collagen and by an external region of slightly radioactive collagen (Fig. 3).

The amount of internal, non-radioactive collagen diminished with time, and eventually the labeled collagen was adjacent to the endosteum. This appeared to be a result of uniform marrow cavity expansion, its margins remaining equidistant from the labeled regions.

Localization of Lathyritic Collagen in Bone

Embryos were injected with a solution containing βAPN and tritiated proline. Subsequently, the femurs were harvested and extracted at seven graded time intervals (0.5 to 10 hours). This procedure removed the labeled spicules, as seen in Figs. 4 and 6 (before extraction) and Figs. 5 and 7 (after extraction), but did not seem to affect the amount of intracellular isotope (Figs. 6 and 7). Whereas there was no loss of labeled spicules from any of the control femurs (Fig. 2), even from those which contained label 1 hour following the injection of tritiated proline, there was removal of the isotopic spicules from every lathyritic femur. Furthermore, virtually all the labeled spicules were removed from the lathyritic femurs (Fig. 5), implying that lathyrism occurs throughout the bone. In several separate experiments of this type, this difference in appearance of extracted control and lathyritic bones was always found.

In another study, βAPN was injected into 14-day-old embryos and 24 hours later (day 15)

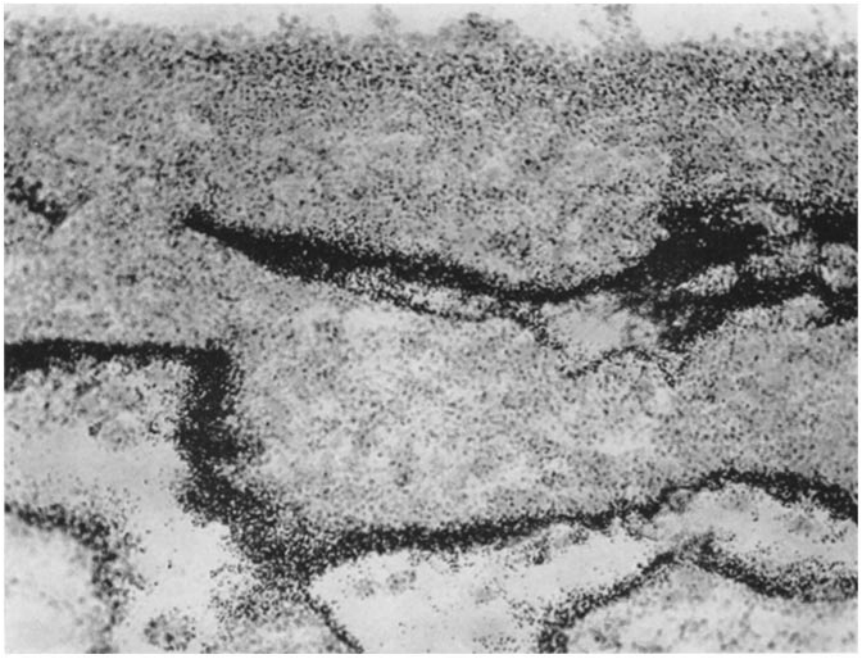

FIGURE 2 Right femur, normal embryo, 6 hours after tritiated proline. There is marked film blackening over the spicules. The cellular silver grains are slightly out of focus. Identical picture in opposite femur after cold saline extraction. × 400.

FIGURE 3 Right femur, normal embryo, 60 hours after tritiated proline. The periosteum is at the top. There is a continuous band of radioactive spicules near the endosteum. Identical picture in opposite femur after cold saline extraction. × 150.

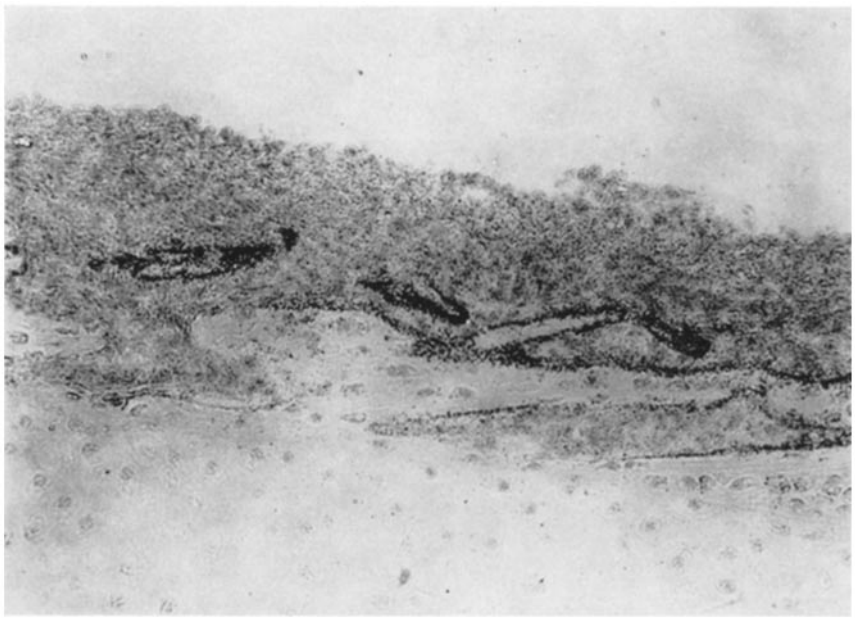

FIGURE 4 Right femur, lathyritic embryo, 6 hours after βAPN and tritiated proline. The radioactivity is concentrated in bone spicules. × 225.

they received tritiated proline. Subsequently, the femurs of these animals were dissected at 2, 6, 12, 24, and 48 hours and were extracted. The labeled, lathyritic spicules were not extractable at 24 and 48 hours but were removed at the earlier times.

Effect of Decalcification on Extractable Collagen

Since the labeled, lathyritic spicules were not extractable 24 hours after their formation, it seemed important to determine whether bone mineral (10) was interfering with collagen extraction. As shown in Table I, following decalcification more collagen (extract 2) was removed from normal and lathyritic bones, and higher amounts of collagen were obtained from the older, decalcified lathyritic bones. The amount of collagen removed from undecalcified, normal bones (extract 1) is slightly greater than that previously reported (1), perhaps due to more extensive extraction in the present experiments. An insignificant amount of collagen was detected in a fourth extract made prior to decalcification.

Effect of βAPN on Prelabeled Collagen

In order to determine the effect of βAPN on prelabeled spicules, isotopic proline was injected 24 hours prior to βAPN administration. The femurs were obtained and extracted at seven sequential time points (26 to 72 hours following tritiated proline). There was no loss of radioactive spicules from any of the lathyritic or control femurs. Fig. 8 demonstrates that extraction did not even remove the spicules which were adjacent to the endosteum.

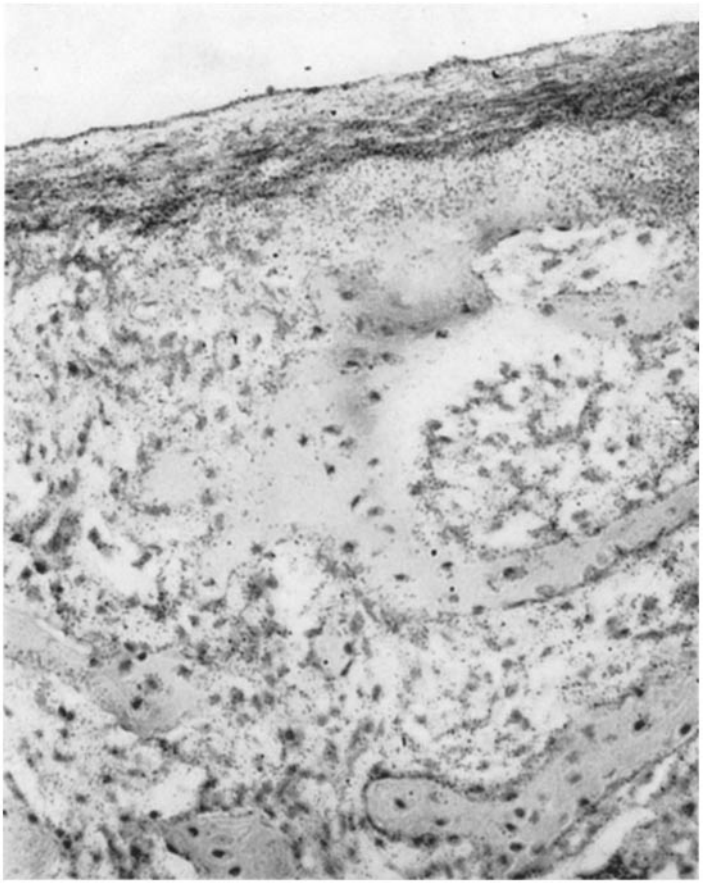

FIGURE 5 Left femur, same embryo as that in Fig. 4, extracted in cold saline. There is no concentration of radioactivity in the spicules. The silver grains are slightly out of focus. × 225.

FIGURE 6 Same as Fig. 4, higher magnification. The silver grains are concentrated over spicules and are diffusely present over cells. × 400.

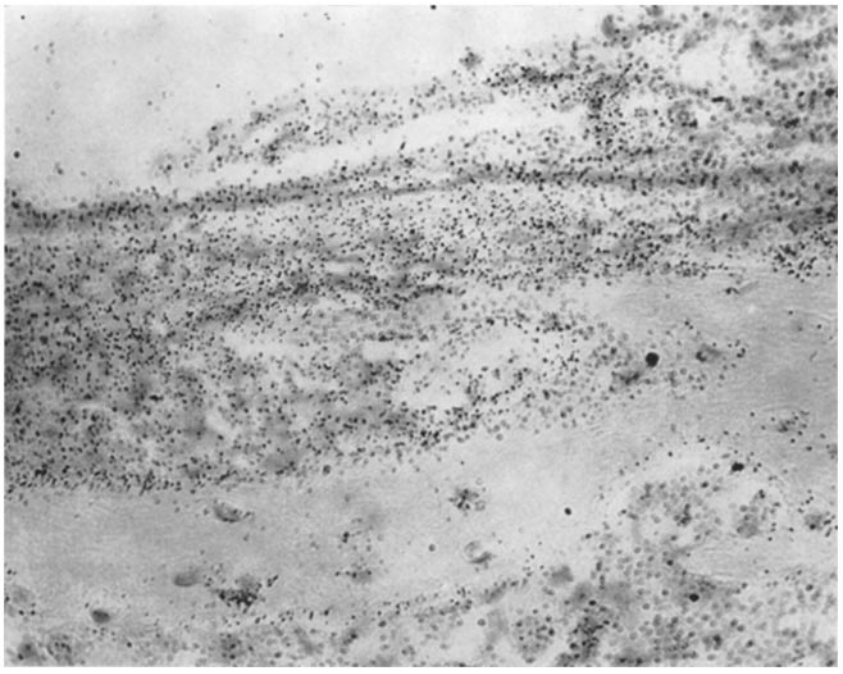

FIGURE 7 Same as Fig. 5, higher magnification. Concentrated silver grains are absent. The cellular radioactivity is similar to that of Fig. 6. × 400.

TABLE I

Effect of Decalcification on Extractable Bone Collagen

Sample	Time	Hydroxyproline, μg/G*			
		Extract 1	Extract 2	Residue	Total
	Hrs.				
Control	6	328	59	3890	4277
	12	199	100	4741	5040
	24	189	120	6108	6417
	48	143	60	6900	7103
	72	237	105	4126	4468
Lathyritic	6	634	126	3893	4653
	12	731	120	3348	4199
	24	2100	215	4096	6411
	48	1820	260	3952	6032
	72	1580	190	3428	5198

* G = wet weight, bone.

Five embryos per time point, all bones pooled, weighed, and extracted three times (24 hrs. each) in 6 volumes (V/W) 1 M NaCl, at 4°C. Extracts combined, dialyzed against distilled water, and resultant precipitate hydrolyzed (extract 1). Bones then decalcified by shaking twice, overnight, in 50 ml of 0.2 M EDTA, pH 7.5, followed by extraction twice in 6 volumes 1 M NaCl. EDTA and saline solutions combined, dialyzed against distilled water, and precipitate hydrolyzed (extract 2).

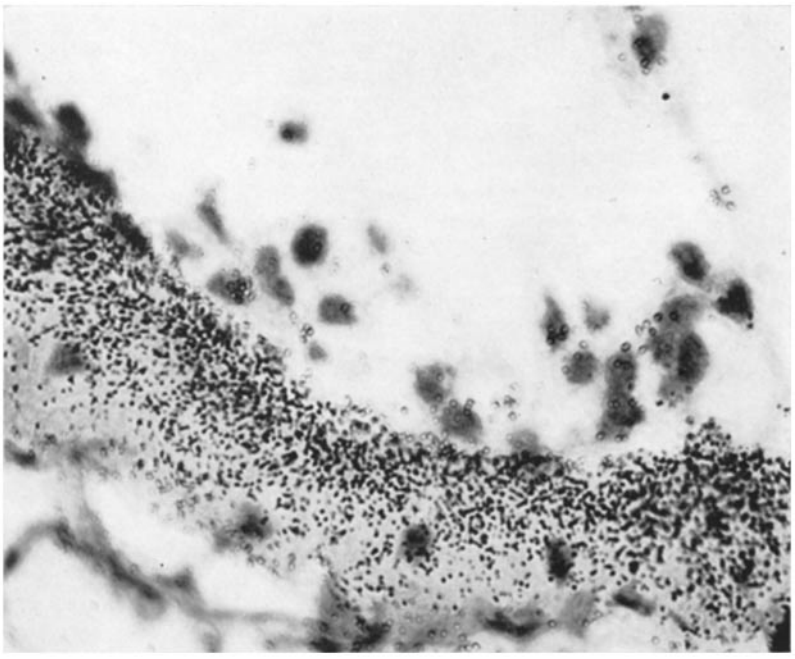

FIGURE 8 Right femur, lathyritic embryo, 72 hours after tritiated proline and 48 hours after βAPN. A labeled spicule is adjacent to the endosteal cells. At this magnification, the cells are out of focus because of shallow depth of field. Identical picture in opposite femur after cold saline extraction. × 900.

Fluctuation of Isotope Incorporation

We found a considerable variation in degree and localization of isotope incorporation among individual embryos which had received label for the same time interval. Infrequently, barely detectable amounts of isotope were found in some bones as late as 10 hours following injection. Fig. 2 demonstrates the maximum isotope incorporation, while Fig. 6 illustrates average labeling.

In sequence studies, the label location did not correlate well with its duration in the embryo. Thus, between ½ and 8 hours following isotope injection there was diffuse grain distribution in some bones, as seen in Fig. 1. In other bones, the label was already concentrated in the spicules (Figs. 2 and 6). Furthermore, Figs. 2 and 6 show that there was no association between isotope location and its degree of incorporation, since, in both figures, the spicules are labeled but there is a considerable difference in the extent of film blackening.

The degree of proline or glycine incorporation was also variable in non-collagenous tissues such as liver, muscle, and kidney. It is of special interest that all tissues of any one animal were found to have very similar concentrations of silver grains.

Intravenous injection of isotope, while generally increasing activity, did not eliminate the variations in degree and location of the label. Furthermore, in all experiments, animal weights were very similar, obviating a dose artifact.

Film sensitivity and accuracy were checked by hydrolyzing portions of the skin and bones and determining the hydroxyproline specific activity. The specific activities agreed qualitatively with the degree of film blackening.

The variations in bone labeling could not be ascribed to losses of collagen in the formalin or EDTA solutions. Very small amounts of hydroxyproline (0.1 to 0.25 μg per bone) and insignificant radioactivity were detected in these solutions.

Distribution of Tritiated βAPN

Tritiated βAPN was found uniformly and diffusely distributed throughout the bones and seemed to be completely removed by extraction.

DISCUSSION

The two major findings of interest in this autoradiographic study are the localization and morphology of lathyritic bone collagen, and the variability of isotope incorporation among embryos.

Biochemical experiments (1) indicate that a portion of extractable lathyritic collagen is recently synthesized and is in fibrillar aggregates *in vivo*. The present autoradiographic results support this interpretation by demonstrating extractable, lathyritic, bone collagen to be localized in newly formed spicules. The inability to extract labeled lathyritic collagen 24 hours following isotope administration suggests that the lathyritic alteration may not be persistent. It does not appear as though mineralization prevents a substantial amount of lathyritic collagen from being extracted by saline (Table I). The persistence of labeled spicules in extracted, mineralized, normal bone probably reflects the fact that only a small amount of this collagen can be removed by saline.

The apparent lack of βAPN effect on prelabeled fibres favors the view that lathyrism may preferentially alter dispersed collagen and recently formed fibrils. However, biochemical studies indicate that extractable lathyritic collagen is heterogeneous (1), deriving from two or more sources of collagen, in various aggregation states. Probably the contribution from older fibres is relatively small and not detectable by our autoradiographic methods, which are less sensitive than the biochemical techniques.

Although collagen is rapidly resorbed in chick embryo bone (11), our findings indicate that the resorbing spicules, next to the endosteum, are not removed by saline. Perhaps, in bone, collagen may be resorbed without prior conversion to an extractable form (12). The symmetrical deposition of collagen and the symmetrical expansion of the marrow cavity may account for the continued alignment of the labeled collagen layer. Furthermore, the preservation of the layer's alignment and continuity is probably due to insignificant resorption of its radioactive spicules, prior to endosteal removal (11).

The complete extraction of labeled βAPN from the tissue sections by saline is consistent with the observation (13) that βAPN is not bound to collagen.

A prominent feature of these autoradiographic studies is the wide temporal range and wide degree of intensity of label appearance in the collagenous and non-collagenous tissues of individual embryos. This may be due to variable rates of protein synthesis (1, 14), alternations in the turn-

over of the precursor pools, or, in the case of collagen, variations in cellular excretion. While fluctuations in isotope incorporation in the chick embryo appear in the data of some observers (1–3, 15), they are minimal in the results of others (16, 17). However, the number of embryos used, their age, and the mixing of tissues are factors which limit comparison.

Although we expect to find a population distribution for any biologic function, it is the wide range which is so striking in this study. Possibly, we may be observing a specialized example of rhythmic activity and, at any one time, individual embryos are in different phases of the cycle (18, 19).

Dr. Tanzer was formerly Captain, MC, United States Army. Dr. Hunt was formerly Captain, VC, United States Army.

The principles of laboratory animal care as stated by the National Society for Medical Research were observed during this study.

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