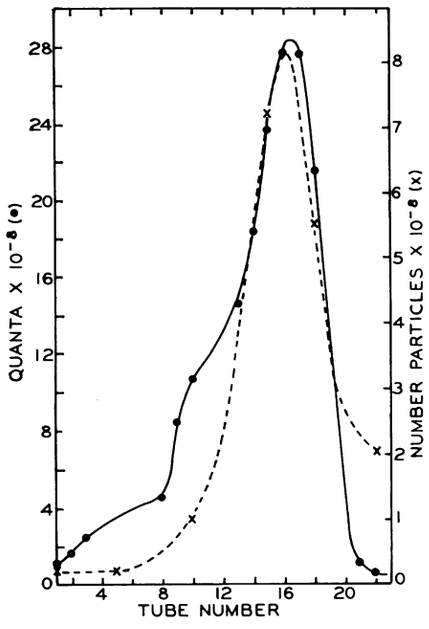

Fig. 2. Sedimentation pattern of scintillons by centrifugation in a sucrose density gradient prepared as described in the text. Bioluminescence of the fractions is correlated with the presence of scintillons. Ordinate, *left*: bioluminescence after rapid mixing with acetic acid, at 20°C, expressed in terms of the total number of quanta emitted (photon yield); *right*: number of the particles in the assay mixture. Although the number of quanta emitted per scintillon appears to be quite low, a considerable loss in activity occurred during the purification procedure; the value for the intact cell is believed to be several orders of magnitude higher.

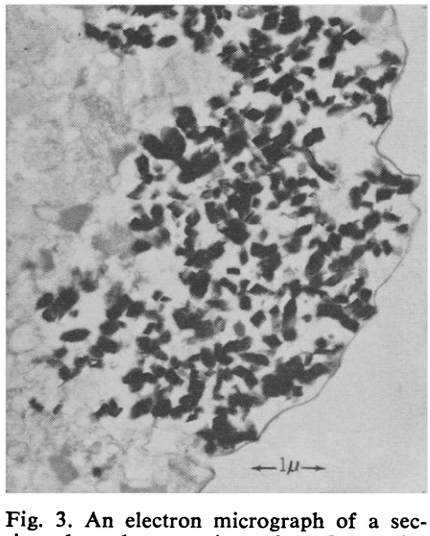

Fig. 3. An electron micrograph of a section through a portion of a *Gonyaulax polyedra* cell. The opaque structures are scintillons. Their distribution may be scattered or, as illustrated here, in clumps located primarily near the periphery of the cell. Some fine structure is evident in the sectioned crystals. A chloroplast is located at the lower left.

long axis of the particle is about 0.3 μ; however, the size ranges from 0.1 to 0.6 μ. The width and thickness are approximately equal and range from 0.1 to 0.3 μ.

For additional purification of the particles, 10 ml of the particle fraction was layered on a linear (5 to 20 percent) sucrose gradient in a 150-ml plastic centrifuge tube and centrifuged (for 2 hours at 3200 rev/min) in the swinging-bucket rotor of the model PR-1 International centrifuge. The gradient was then fractionated by puncturing the bottom of the tube with a needle and collecting drops. Samples of each fraction were subsequently assayed for light emission. The number of particles in each fraction was determined with the electron microscope by the spray droplet method of estimating particle numbers (*6*).

The quantitative relationship between luminescence in vitro and the number of particles (Fig. 2) constitutes evidence supporting the presumed light-emitting role of the scintillon. Although evidence of a more direct nature concerning this point is difficult to obtain, the high purity of the preparation strongly supports the conclusion. No alternative candidate for the light-emitting particle has been found in the electron-micrograph studies.

Figure 3 shows that scintillons occur abundantly in the cell. Although the particles are most numerous towards the periphery of the cell, they are also found in other regions. Their unusually high electron opacity is particularly striking.

Under a polarizing microscope the scintillons can be shown to possess an extremely high degree of birefringence. The coefficient of birefringence, 10^{-1}, was determined from retardation measurements in a rectified polarizing microscope with a rotating mica compensator (*7*). Because of their small size and asymetric shape, the scintillons exhibit a striking scintillation in polarized light. This is undoubtedly due to their rotatory Brownian motion. A similar scintillation attributable to the oscillatory rotation of the particles is readily visible in the living cell.

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References and Notes

1. J. M. Bassot, *Arch. Anat. Microscop. Morphol. Exptl.* **49**, 23 (1960); J. B. Buck, *Ann. N.Y. Acad. Sci.* **49**, 397 (1948); F. H. Johnson, *J. Cell. Comp. Physiol.* **53**, 259 (1959); E. N. Harvey and I. M. Korr, *ibid.* **12**, 319 (1938).
2. E. N. Harvey, *Bioluminescence* (Academic Press, New York, 1952). Additional discussion of, and references to, the older literature related to this question may be found in this volume.
3. Supported in part by grants from the National Science Foundation and the National Institutes of Health, and by a contract from the Office of Naval Research. One of us (R.D.S.) is a predoctoral fellow of the National Institutes of Health. We thank Dr. Shinya Inoué for his assistance with these observations.
4. J. W. Hastings and B. M. Sweeney, *J. Cell. Comp. Physiol.* **49**, 209 (1957); J. W. Hastings and V. C. Bode, in *Light and Life*, W. D. McElroy and B. Glass, Eds. (Johns Hopkins Press, Baltimore, 1961), p. 294.
5. Q. H. Gibson and J. W. Hastings, *Biochem. J.* **83**, 368 (1962).
6. R. C. Backus and R. C. Williams, *J. Appl. Phys.* **21**, 11 (1950).
7. S. Inoué, in *Encyclopedia of Microscopy*, G. L. Clark, Ed. (Reinhold, New York, 1961), pp. 480–485.

1 July 1963

Osteoclasts: Organization in Chick Embryo Bone

Abstract. *A multinucleated endosteum was observed in the long bones of chick embryos aged 14 to 17 days. The endosteum appears to be responsible for extensive bone resorption; the bulk of the diaphyseal bone is almost completely replaced during this particular 3-day interval. Eventually, the endosteum appears to be transformed into numerous individual osteoclasts. Both the endosteum and the osteoclasts are found almost entirely on the medial surfaces of the innermost bone spicules and they seem to destroy collagen by extracellular proteolysis.*

During the course of investigations into the pathogenesis of experimental lathyrism in the chick embryo, we examined the histology of the long bones from normal embryos. Although a detailed histological analysis of bone development in the chick embryo has been reported (*1*), we observed the presence of a multinucleated embryonic endosteum which does not seem to have been described previously.

The femurs and tibias of chick embryos aged 14 to 19 days were cleaned of muscle, fixed in neutral 10-percent formalin, and decalcified in 0.2*M* ethylenediaminetetraacetic acid (EDTA) at *p*H 7.5; they were then embedded in paraffin, sectioned longitudinally at 5 μ, deparaffined, and stained with hematoxylin and eosin.

The major histological features of these bones were as described (*1*), but we also observed a multinucleated endosteum, covering the innermost bony and cartilagenous surfaces (Fig. 1). Occasionally, this structure was found displaced from the bone, but maintaining its continuity (Fig. 2). Infrequently, shallow lacunae were seen on the surface of the underlying bone (Figs. 1 and 2). Distinct intercellular boundaries were not found upon scanning the endosteum, and the nuclei were often two or three deep (Fig. 3). The endosteum was almost exclusively located on the medial surfaces of the innermost spicules which were usually considerably thinner than the more lateral spicules. Only occasionally would the endosteum be found bridging adjacent spicules or terminating at the spicule end in continuity with a typical osteoclast; a distinct osteoclast was rarely seen within the endosteum, but when it was found, it appeared as an integral component (Fig. 4). Typical osteoclasts were not common in the bones of 14- to 17-day embryos, but seemed to be more numerous in those aged 18 to 19 days. In these older embryos, the endosteum appeared to have changed from a multinucleated layer to single, uninucleated flat cells interspersed with many osteoclasts. The osteoclasts did not seem to be present in areas other than the medullary cavity, although they have been observed previously on periosteal bone in younger embryos (*1*).

The histological characteristics of the endosteum indicated that its function might be that of actively removing bone. It was also possible that, as in adult bone, it was simply a supporting structure, or one could even conceive of the endosteum contributing to embryonic hematopoesis, since it is adjacent to the marrow. Additional information concerning the functional role of the multinucleated endosteum has therefore been obtained by autoradiographic and histochemical studies (*2*).

In a series of autoradiographic experiments, tritiated proline was injected intravenously and found to be incorporated into bone collagen, primarily in the periosteal and perichondral regions. In the longitudinal tissue sections, the radioactivity appeared as an uninterrupted pair of subperiosteal lamellae, one member of the pair extending along each side of the entire length of the diaphysis. Within 48 to 72 hours after deposition, these two longitudinal bands of labeled collagen were found at the marrow surface. In time-sequence experiments, the well-demarcated, radioactive laminae could be traced as they changed location relative to the periosteum and endosteum. The formation of these laminae and their changing location seemed to be a consequence of two factors: (i) progressive deposition of more collagen (slightly radioactive) external to the intensely radioactive collagen; and (ii) progressive resorption of the internal nonradioactive collagen, as shown by the diminishing distance between the endosteum and the labeled collagen. Upon reaching the marrow cavity, the symmetrical bands of radioactive collagen disappeared completely. It is important to note that these lamellae maintained both their linearity and their continuity, even when adjacent to the endosteum. The absence of discontinuities suggests that previous, localized, bone removal had been minimal. Therefore, it appears that bone destruction occurs primarily in the area of the marrow cavity in the 14- to 17-day chick embryo, and that there is virtually complete replacement of diaphyseal bone collagen during this particular 3-day interval.

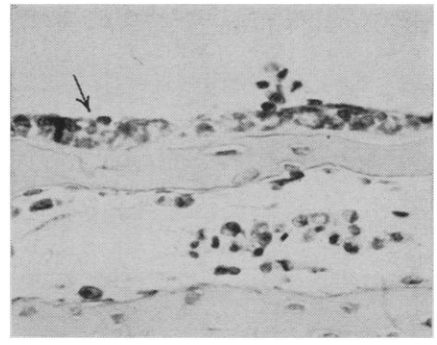

Fig. 1. Longitudinal section of a femur from a chick embryo aged 14 days. The periosteal direction is toward the lower portion of the figure. The multinucleated endosteal cell layer (arrow) is apparently eroding the spicule surface. (× 370)

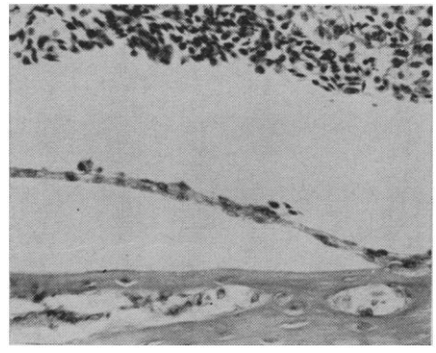

Fig. 2. Section of a region similar to that in Fig. 1, showing the detached, but continuous, intact endosteum. It has remained attached to the spicule at a shallow lacuna. The detachment is probably artifactual. (× 250)

In another series of autoradiographic experiments, the relationship of the endosteum to the labeled bone spicules was studied under circumstances where these structures were adjacent (48 to 72 hours after the injection of tritiated proline). The labeled spicules, which were in direct contact with the cytoplasmic border of the endosteum, were usually thinner than those which were not yet in contact. Furthermore, we did not observe any radioactive bone collagen within the endosteal cytoplasm, suggesting that collagen digestion in the bone may be an extracellular process. (Any small radioactive products of collagen digestion would probably not have been seen as they were presumably washed out of the tissues by the aqueous solutions used during fixation, film application, and staining.)

The contrast between such rapid and uniform bone removal, and the presence of only limited numbers of scattered osteoclasts seem to provide strong, but admittedly indirect, evidence that the endosteum serves in an osteoclastic capacity. Additional information favoring this concept was obtained by histochemical studies. We based these studies upon earlier reports (*3*, *4*) which show that certain enzymes are histochemically associated with typical osteoclasts; hence, their presence within the endosteum would imply osteoclastic activity. Accordingly, the distribution of several enzymes was studied in fresh frozen, undecalcified tissue sections of the femurs and tibias from 14-day chick embryos. Acid phosphatase and leucine amino-peptidase were found to be localized at virtually all the medial surfaces of the innermost spicules, and did not seem to be present at any other sites. In as much as these two enzymes are commonly associated with osteoclasts (*3*), the present findings provide additional support for the concept of an osteoclastic endosteum. Two other enzymes, alkaline phosphatase and succinic dehydrogenase, were histochemically de-

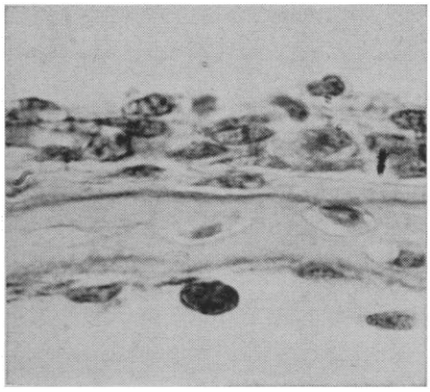

Fig. 3. High magnification of the endosteum, demonstrating the lack of distinct intercellular margins within the cytoplasm. (× 900)

tected, but these assays did not provide definitive results concerning the endosteum. A very slight reaction for alkaline phosphatase was seen in the endosteal area, while a much greater reaction was present in the osteoblastic cells of the periosteum. The outermost, fibrous periosteum, on the other hand, was unreactive. Succinic dehydrogenase was present in all the embryonic bone cells and did not appear concentrated in any particular area. (The succinic dehydrogenase reaction was monitored by noting its activity in a piece of muscle adjacent to the bone.) The distribution of all these enzymes in the bone of the chick embryo appears to be qualitatively similar to that seen in other animals, although there may be quantitative differences (*3*, *4*).

Additional evidence concerning the nature of the medullary lining was provided by autoradiography after the administration of tritiated thymidine.

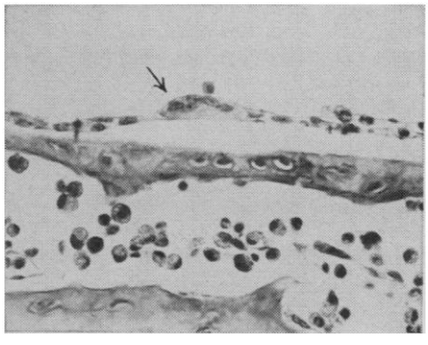

Fig. 4. Longitudinal section of a femur from a chick embryo aged 17 days. Orientation as in Fig. 1. A small osteoclast (arrow) is in continuity with the endosteum. (× 370)

Very few of the nuclei within the multinucleated endosteum were labeled 1 hour after the injection of isotope. By contrast, numerous nuclei of the marrow, periosteum, and cartilage cells were labeled at this time. This difference is in agreement with previous studies (*5*) which indicate that osteoclasts incorporate tritiated thymidine considerably later than other bone cells.

The results of our experiments are consistent, and indicate that rapid bone removal takes place in the 14- to 17-day chick embryo and that such removal is probably due to osteoclastic activity of the endosteum. Contrary to these conclusions, expansion of the marrow cavity due to erosion of the inner bone spicules has not been thought to occur during osteogenesis in the avian embryo (*6*), although continuous removal of the preceding cartilage by erosion from within has been recognized for many years. Perhaps the sparseness of typical osteoclasts has fostered the concept of insignificant bone resorption in these embryos. In addition to the other studies, we also examined fresh tibias from chick embryos aged 14 to 17 days, and observed a considerable widening of the marrow cavity with increasing age (*2*).

Although our results differ from previous concepts concerning the occurrence of bone resorption during osteogenesis in the chick embryo, they are in complete agreement concerning the process of bone deposition. As previously described (*1*, *6*), we did not see any histological evidence of endochondral ossification until very late in embryogenesis (about day 19). Our autoradiographic results confirm the fact that subperiosteal and subperichondral bone formation are the major sources of new bone in these embryos.

The apparent lack of distinct intercellular membranes within the multinucleated endosteum, the occasional presence of typical osteoclasts in continuity with it, and its apparent ultimate transformation into numerous, small osteoclasts suggest that the endosteum may represent a network of fused primordial osteoclasts acting in coordination. The relative lack of typical deep Howship's lacunae can probably be attributed to uniform erosion of the spicule surface by the covering cell layer. The symmetrical deposition, continued linear uniformity, and symmetrical removal of the labeled collagen laminae imply a well-coordinated structural and functional organization of bone in the chick embryo. It is conceivable that there is some regulating mechanism which closely synchronizes the activities of the periosteal and endosteal regions (*7*).

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References and Notes

1. H. B. Fell, *J. Morphol.* **40**, 417 (1925).
2. A description of the complete details is in preparation.
3. M. S. Burstone, *J. Histochem. Cytochem.* **7**, 39 (1959); W. Lipp, *ibid.*, p. 205.
4. F. Schajowicz and R. L. Cabrini, *Science* **131**, 1043 (1960).
5. E. A. Tonna, *Anat. Record* **137**, 25 (1960); A. M. Hunt and K. J. Paynter, *J. Dental Res.* **40**, 652 (1961); B. Messier and C. P. Leblond, *Am. J. Anat.* **106**, 247 (1960); L. F. Belanger and B. B. Migicovsky, *Anat. Record* **145**, 385 (1963).
6. A. L. Romanoff, *The Avian Embryo* (Macmillan, New York, 1960), pp. 917–924.
7. The principles of laboratory animal care as stated by the National Society for Medical Research were observed.

18 July 1963

Circulating Small Lymphocytes: Immunologically Competent Cells with Limited Reactivities

Abstract. *Highly purified small lymphocytes from peripheral blood are capable of producing acute transplantation disease. However, such lymphocytes do not transfer from sensitized to normal mice the capacity to produce antibody to bovine serum albumin under conditions where mixed populations of lymphoid cells from the sensitized donors are effective.*

There is now compelling evidence that purified, small circulating lymphocytes are capable of producing acute transplantation (that is, graft-versus-host) disease in mammals (*1*, *2*). After injection, such adult lymphocytes or their immediate descendants promptly attack antigenically disparate newborn hosts and mediate graft-versus-host reactions that constitute a model of immunologic disease (*3*). Moreover, small lymphocytes from adult mice may protect isogenic newborn mice against transplantation disease (*4*). The mechanisms by which these lymphocytes bring about immune reactions—whether cellular, humoral or both—are not clear.