PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 163, 249–257 (1980)

Mineralization during Long-Term Cultivation of Chick Embryos *in Vitro* (40757)

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Introduction. Recently, Auerbach and colleagues (1) described a procedure for the long-term cultivation of shell-less chick embryos in petri dishes from the 3rd to the 20th day of incubation. The purpose of our study was to employ this procedure to investigate avian embryonic cartilage and bone development in continuous long-term culture conditions in the absence of the egg shell, partly with the idea of determining whether the egg shell was necessary for chick morphogenesis, and to also assess the suggested functions of the egg shell in the mineralization of the chick skeletal system.

Early chemical analyses of the domestic fowl's egg indicated that albumin and yolk contained 0.03 g of calcium, whereas the chick which hatched from the egg shell some 21 days later contained 0.14–0.18 g of calcium (2). The egg shell was reported to contain approximately 2 g of calcium carbonate in the form of calcite (3–5). It was suggested by Buckner *et al.* (6) that carbon dioxide produced by the forming chick embryo was transported by the chorioallantois and its blood vessels to the vicinity of the egg shell and participated in the solubilization of the shell calcium into calcium carbonate. Since Plimmer and Lowndes (2) reported that the hatching chick contained five times as much calcium as that detected in the original egg contents (i.e., yolk and albumin), numerous investigations have concluded that calcium in the hatching chick was essentially derived by translocation of calcium ions from the calcium carbonate of the egg shell (6–8).

The information regarding calcium metabolism of the chick embryo and its relationship to skeletal tissue mineralization has classically been based upon a substantial number of theories proposed to account for the transfer of calcium ions from the egg shell to the developing embryo (9–11). The present investigation attempts to reexamine the function of the egg shell in avian embryonic morphogenesis and skeletal mineralization during prolonged *in vitro* cultivation of shell-less embryos.

Materials and methods. White Leghorn fertile eggs were obtained from a commercial source (Demler Farms, Anaheim, Calif.) and stored in a cold box (15°) for no longer than 2 days before initiating the incubation experiments. Control embryonated eggs were incubated for periods up to hatching at 21 days. Control embryos were dissected free from the adjacent yolk tissues at 48, 72, and 96 hr and 6, 9, 12, 14, 16, and 21 days of incubation and processed for light and transmission electron microscopy by methods previously reported by this laboratory (12). Identification and designation of embryonic chick developmental staging used the Hamburger–Hamilton classification (13). In embryos of 7 days (ca stage 32) and older, biopsy samples from the mandibles, calvaria, femurs, and visceral organs were examined.

The techniques for the long-term continuous cultivation of shell-less chick embryos were modified from those methods published by Auerbach and colleagues (1) and from procedures related to us by Dr. Arnold Caplan (personal communication). The procedures described in this report have now been used by us for growing 700 embryos during the past year. Fertile eggs were either first incubated for 72 hr in a forced air incubator at 37.5° with optimal humidity and then candled to determine the location of the blastoderm, or the eggs were not incubated before initiating the experiment. In this case 0-day-old egg contents were immediately placed in the bottom of a 20 × 100-mm plastic petri dish by first cracking the underside of the egg shell against the edge of that dish. The egg shell surfaces were thoroughly washed with 70% alcohol prior to cracking open the shells

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0037-9727/80/020249-09$01.00/0

and releasing the egg contents into the petri dishes. Prior to cracking the egg shell, 10 ml of Dulbecco's (calcium-magnesium-free) phosphate-buffered saline, pH 7.2 (PBS), containing 200 units/ml of gentamycin, mycostatin, penicillin, and streptomycin (Grand Island Biological Co., Berkeley, Calif.) was placed into the plastic dishes. The plastic dishes, containing chick embryo with associated yolk and albumin in the isotonic solution, were then placed into larger 25 × 150-mm plastic petri dishes also containing PBS for additional humidification. The assembly was covered and continuously incubated at 37.5° in an optimally humidified atmosphere of air for periods up to 19 days. Forced air ventilation was found to be essential. Pilot studies using 5% CO_2 indicated that atmospheric carbon dioxide was not necessary. Artificially cultivated embryonated eggs were continuously monitored for survival results, and tissues from selected, developmentally staged embryos were processed for light and transmission electron microscopy for comparison with *in situ* skeletal morphogenesis.

Results. In situ calcium metabolism. The total incubation time for the egg of the domestic fowl is approximately 21 days. Calcium analysis performed by Plimmer and Lowndes (2) indicated that the chick which hatched from the egg contained five times as much calcium as was present in the original egg contents (albumin and yolk). A detailed analysis of calcium metabolism of the embryo during development *in situ* observed three separate rates of calcium increase in the ash content of the embryo (8). During the first 10 days of incubation, 85–95% of the calcium in the embryo was assumed to have been derived from the yolk. Subsequently, calcium ions from the egg shell were removed and translocated to the embryo, and the egg shell was assumed to function as the main source of calcium during the latter half of incubation (8).

Effects of storage treatments of embryonated eggs. Aging of the avian blastoderm during preincubation storage is known to progressively reduce the capacity for normal embryogenesis (14). The results of our experiments to assess storage conditions and survival rates of embryonated chick eggs are summarized in Table I. Comparison among various storage treatments of chick eggs, using the criteria of embryo viability after 9 days of incubation, indicated that storage in air at 15.5° for no more than 2 days is appropriate for experimental studies *in vitro*.

Long-term cultivation of shell-less chick embryos in vitro. The results from experiments designed to evaluate survival rates of chick embryos cultured *in vitro* for periods up to 19 days (stage 42) are summarized in Table II. The number of embryos listed represent successfully transferred shell-less embryos into the culture apparatus. Approximately 6/10 embryos can be transferred from embryonated eggs into petri dishes without disrupting investing membranes or injury to the egg yolk. All disrupted embryos were discarded at this point and were excluded from additional analysis. We observed that injury to the membranes or distortion of the yolk contents prevented subsequent survival and morphogenesis of the shell-less embryos *in vitro*.

In experiments in which we cultured 0-

TABLE I. STORAGE CONDITIONS AND SURVIVAL RATES OF INCUBATED EMBRYONATED CHICK EGGS

Storage treatment prior to incubation	Number of eggs incubated	Number of viable eggs after 9 days incubation
No storage; eggs still warm after laying	25	22
No storage; eggs already cooled to 15.5°	50	40
Storage in air at 15.5° for 2 days	25	21
Storage in air at 15.5° for 6 days	50	36

TABLE II. SURVIVAL RATES OF CHICK EMBRYOS CULTIVATED *IN VITRO*[a]

Age of embryos prior to culture	Number of embryos	Number of viable embryos recovered (days)				
		3	6	9	15	19
0	50	10	5	1	0	0
1	50	11	2	0	0	0
2	50	8	3	1	0	0
3	50	50	43	40	8	5
3.5	500	500	435	390	200	190

[a] All embryos cultivated were obtained from fertile White Leghorn chick eggs stored in air at 15.5° for 2 days prior to initiating *in vitro* studies.

day-old chick embryos, an 80% loss of viable embryos during the first 3 days of subsequent incubation of shell-less embryos was observed (Table II). Five out of fifty embryos reached stage 29 under these experimental conditions. In contrast, survival data indicated a 70–80% viability for cultures up to 9 days of incubation (stages 33–35) using 3.5-day-old preincubated and precandled embryonated chick eggs (Table II). From 9 to 15 days of incubation (3.5-day-old embryos *en ova* plus days of incubation) we observed a 50% reduction in viability. Although shell-less embryos survived for periods up to stage 42, the vast majority of long-term cultures of chick embryos did not survive beyond stage 38.

The method originally described by Auerbach and his colleagues (1) was confirmed to be suitable for continuously monitoring chick embryonic morphogenesis *in vitro* from Days 0 through 19 days of incubation (Figs. 1A–D and 2A–B). Transmitted light facilitated the observations of morphogenesis during shell-less embryos *in vitro*. Embryos cultivated in petri dishes developed comparable to, albeit smaller than, normal control embryos (Fig. 3). Whereas developmental stages reached were quite similar between shell-less embryos and controls, embryos cultured *in vitro* were generally smaller than controls. Major congenital malformations of the beak, limbs, or cranium were rarely detected in these long-term, shell-less embryos cultured *in vitro*.

In vitro mineralization. The cartilagenous models of long bones such as the femur are sharpely defined with well-developed perichondria. In such long bones, ossification begins at the middle of the cartilaginous model. A thin layer of osteoid is laid down between perichondrium and that portion of the model containing hypertrophied cartilage cells. The general histological characteristics of ossification in shell-less embryos cultured to stage 34 and beyond were very comparable to control specimens at equivalent stages of development (Fig. 4). The histological characteristics of chondrogenesis and osteogenesis associated with femur development in shell-less embryos cultured *in vitro* (3.5-day-old embryos *en ova* plus 12 days *in vitro*) were similar to controls except the size of the femurs was smaller in the shell-less embryos (stages 34–42).

Ultrastructural characteristics of osteoblasts and osteocytes and the osteoid extracellular matrix were comparable between embryos cultured *in vitro* and controls (Fig. 5). Matrix vesicles containing electron-dense, crystal-like structures were first detected in the progenitor osteoid extracellular matrix beginning with stage 34. Matrix vesicles have been repeatedly found to be nucleation sites for mineralization and calcium hydroxyapatite crystal formation in embryonic chick long bone formation (15–17).

Discussion. A key finding in this study is that the developmental pattern of skeletal mineralization in shell-less chick embryonic femurs *in vitro* is comparable, albeit smaller in size, to that in controls. There was no detectable difference between cultured and

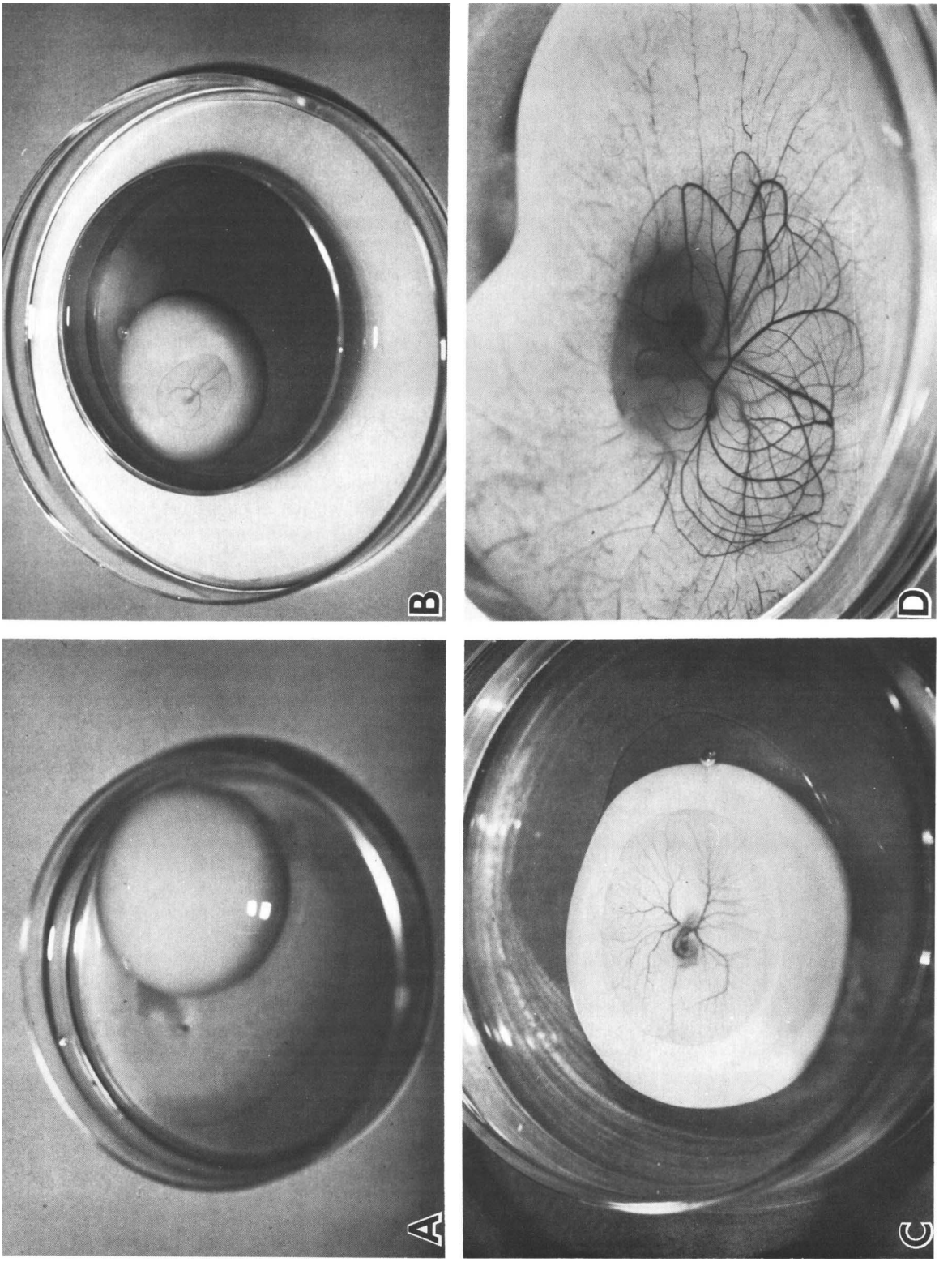

FIG. 1. Cultivation of 0-day-old chick embryos shell-less *in vitro*. (A) Initial culture, 0 days of incubation. ×0.504 (B) Culture of 3.5 days of incubation. ×0.378. (C) Culture of 4.5 days of incubation. ×0.693. (D) Culture of 7.5 days of incubation (Hamburger–Hamilton stage 32). ×0.9.

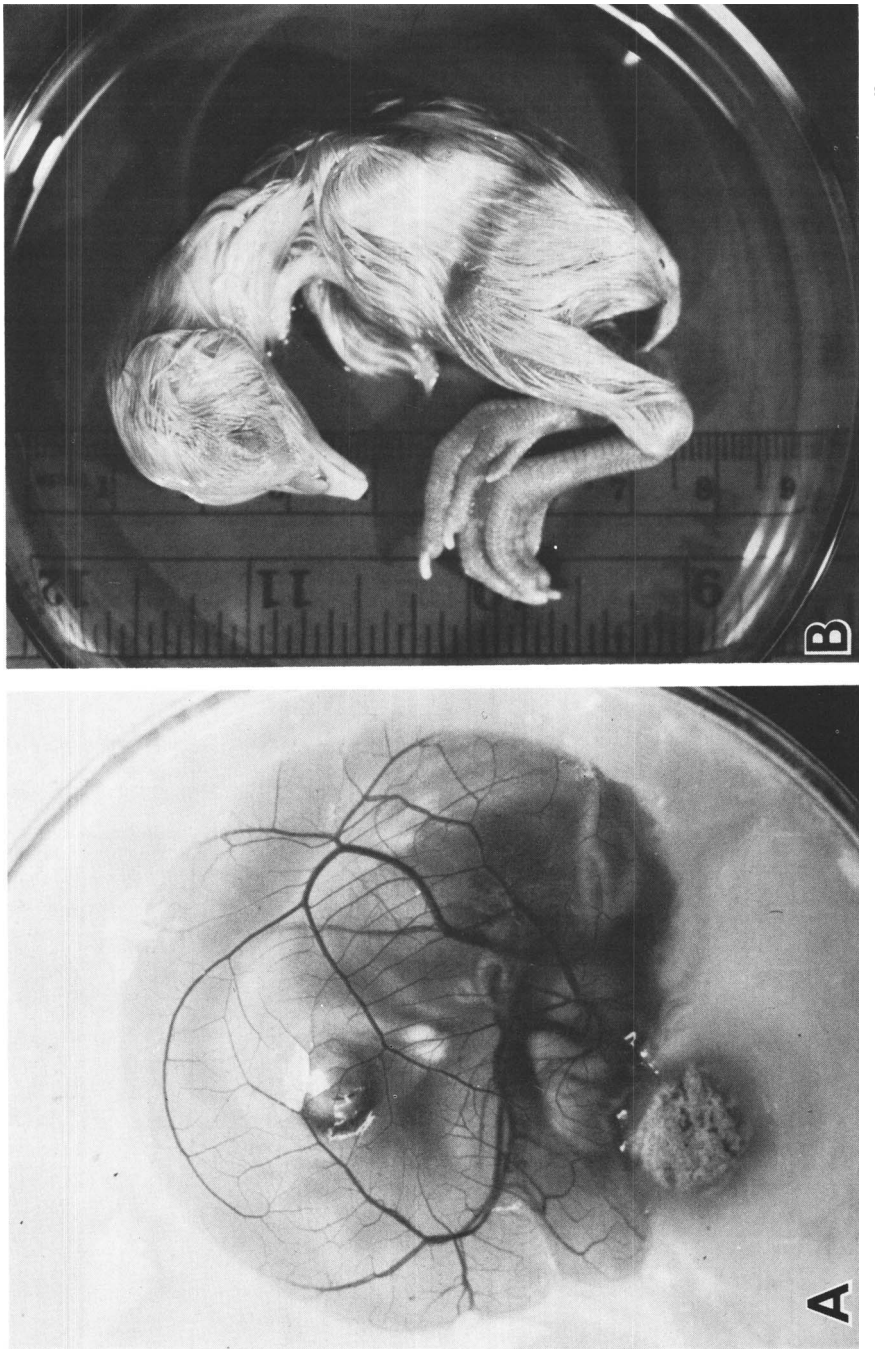

FIG. 2. Long-term cultivation of 3.5-day-old chick embryos shell-less *in vitro*. (A) Culture of 9 days of incubation (Hamburger–Hamilton stage 38). Chorio-allantoic membrane now covers most of the surface. ×0.96. (B) A typical example of a viable shell-less embryo after 19 days of incubation (3.5-days-old *en ova* plus 16 days in culture). The embryo has been dissected free from investing membranes and placed in a clean, sterile dish for photographic purposes (stage-42 embryo). ×1.48.

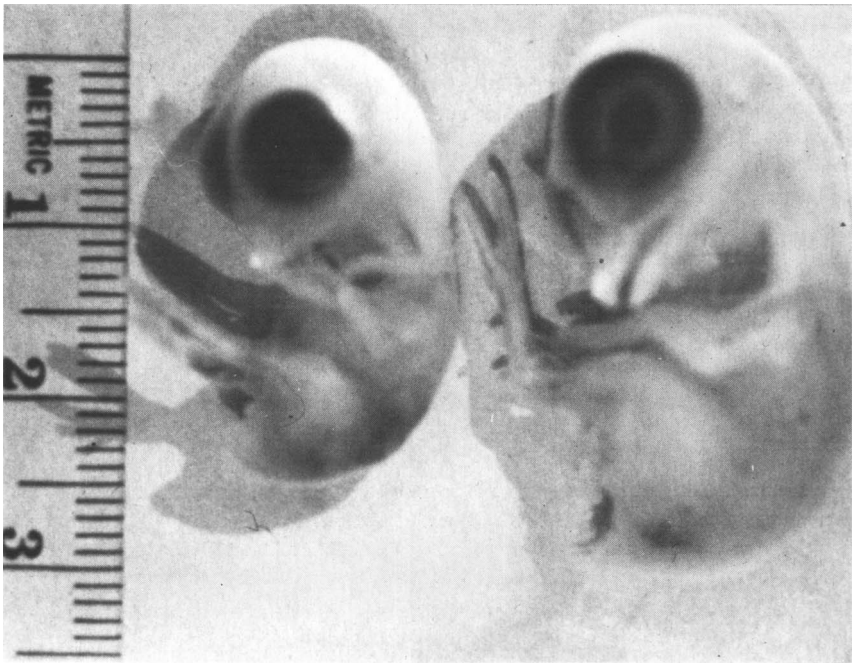

FIG. 3. Comparison of 0-day-old chick embryo cultured shell-less for 7.5 days *in vitro* (left) with a normal, age and developmental stage-matched control (stage 34). Morphogenesis in shell-less culture is comparable to controls although the cultured embryos are generally reduced in size. ×2.23.

control embryos with respect to the number and developmental pattern of trabeculae during femur morphogenesis. Light microscopic and ultrastructural characteristics of chondrogenesis and initial osteogenesis were remarkably equivalent within both shell-less embryos (starting with stages 34 through 42) cultured *in vitro* and controls. Our data show that matrix vesicles appear to mediate the nucleation of calcium hydroxyapatite during *in vitro* and *en ova* chick embryonic long bone mineralization as early as Hamburger–Hamilton stage 34. Previous reports have indicated that normal chick embryonic femurs begin to mineralize after 7 days incubation *in situ* (stage 34) (15–18). Anderson demonstrated that matrix vesicles were diagnostic for initial mineralization during embryonic chick femur osteogenesis (15). Matrix vesicles have also been observed to mediate mineralization in various mammalian developing systems including long bones (16, 19) and dentin (12, 16, 20, 21).

Recently Narbaitz and Jande showed that 3-day-old chick embryos could be cultured *in vitro* until they reached stages 37 or 39 (22). They reported that shell-less embryos showed a marked reduction in the amount of mineralization as compared with controls, yet they also reported that histological and ultrastructural characteristics of tibia osteogenesis was comparable in both *in vitro* and in controls (22). Our data supports their evidence (22) and that provided by Johnston and Comar (8) indicating that prior to reaching Hamburger–Hamilton stage 36 most if not all of the calcium for initial bone mineralization in the chick embryo is derived from the yolk; thereafter, *in vivo,* the egg shell provides a significant amount of calcium ions for additional skeletal mineralization. Our results agree with those of Narbaitz and Jande (22) showing that even in the complete absence of the egg shell most of the cultured embryos demonstrated mineralizing bone formation from stages 34 through 39–42. This experimental model provides a unique opportunity to explore the dual origins of cal-

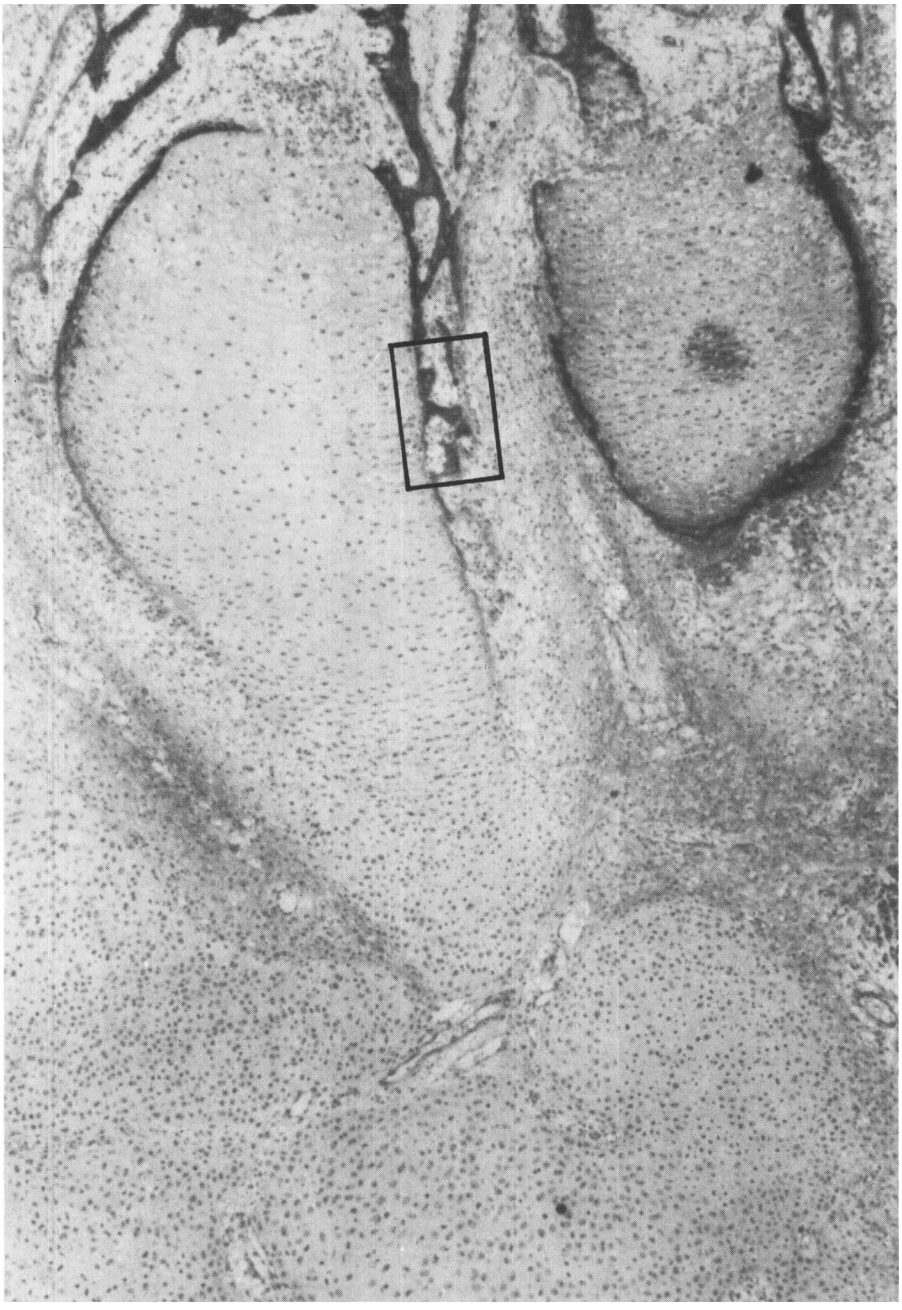

FIG. 4. Region of ossification in femur development within 3.5-day-old chick embryo cultured for 12 days shell-less *in vitro*. The histological characteristics of chondrogenesis and osteogenesis were comparable in both shell-less embryos and controls. Rectangle indicates region observed in Fig. 5. ×100.

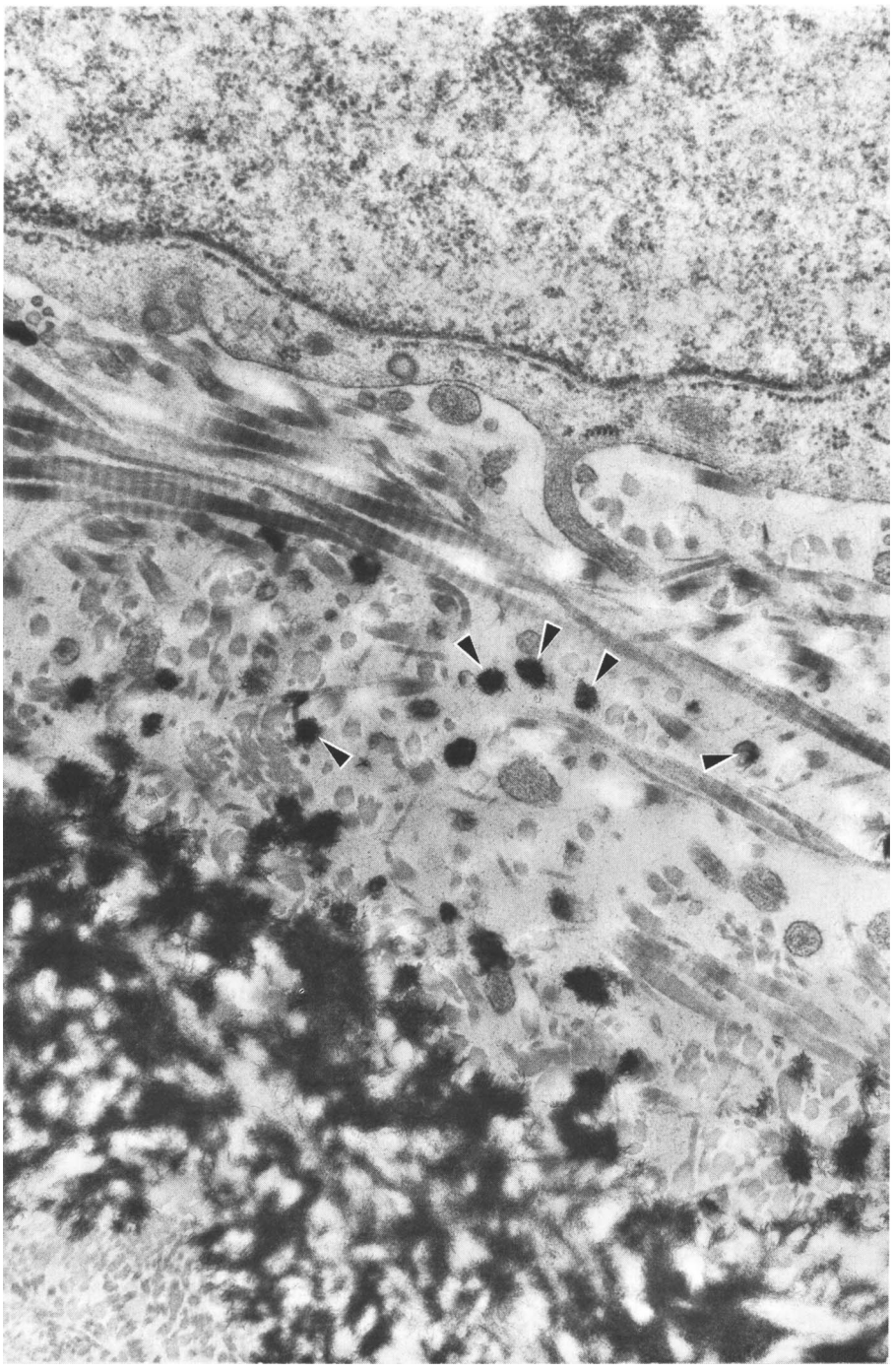

FIG. 5. Ultrastructural characteristics of osteoblasts and osteocytes, the extracellular osteoid matrix including matrix vesicles, were equivalent between cultured embryos and controls. Matrix vesicles (arrows) associated with calcium hydroxyapatite crystal-like structures were routinely observed in association with newly forming osteoid extracellular matrix in femurs from embryos (Fig. 4) cultured shell-less *in vitro*. The cultured embryos were generally smaller in size and showed a reduction in the amount of mineralization in developing bone. ×27,000.

cium ions during embryonic osteogenesis and also overcomes several of the technical issues related to calcium metabolism (9–11, 18, 23). This model should also be useful in studies of induced congenital malformations.

Summary. An experimental model is described which permitted the long-term and continuous cultivation of shell-less chick embryos in petri dishes from 0 to 19 days *in vitro*. This procedure permits the study of development, skeletal morphogenesis and mineralization during embryogenesis in the absence of the egg shell. Embryos cultivated in Dulbecco's (calcium-magnesium-free) phosphate-buffered saline developed in petri dishes were similar to normal chick embryos incubated *in situ,* albeit somewhat smaller in overall size. Congenital malformations of the beak, limbs, or cranium were rarely detected in embryos cultured *in vitro*. Light microscopic and ultrastructural characteristics of chondrogenesis and osteogenesis among developmentally staged, shell-less embryos and controls were remarkably similar. *In situ* and *in vitro* mineralization of femur bone development was initially detected in stage-34 embryos and examined until embryos reached stage 42 (19 days of incubation). The calcium in the chick embryo has a dual origin: Experiments by Johnston and Comar show that prior to stage 36 most of the calcium originates in the yolk (8), whereas after stage 38 calcium ions are rapidly translocated from the egg shell to the chick skeletal system (8, 11, 18, 22). In the absence of the egg shell during *in vitro* chick embryogenesis, mineralization is regulated and sustained using calcium present in the yolk.

The authors wish to thank Mrs. Peggy Meek for her assistance in typing the manuscript. This research was supported by NIH Grants DE-02848-09, DE-03569-07, and DE-07006-04, U.S.P.H.S.

1. Auerbach, R., Kubai, L., Knight, D., and Folkman, J., Dev. Biol. **41**, 391 (1974).
2. Plimmer, R. H. A., and Lowndes, J., Biochem. J. **18**, 1163 (1924).
3. Mayneard, M. V., and Brit, F., Radiology **23**, 19 (1927).
4. Warren, D. C., and Scott, H. M., J. Agr. Res. **51**, 565 (1953b).
5. Warren, D. C., and Scott, H. M., Poult. Sci. **14**, 195 (1935a).
6. Buckner, G. D., Martin, J. H., and Peter, A. M., Amer. J. Physiol. **71**, 253 (1925).
7. Nozaki, H., Horri, S., and Takei, Y., Bull. Nat. Inst. Agr. Sci. (Japan) **9**, 85 (1954).
8. Johnston, P. M., and Comar, C. L., Amer. J. Physiol. **183**, 365 (1955).
9. Simkiss, K., Biol. Rev. **36**, 321 (1961).
10. Gilbert, A. B., *in* "Advances in Reproductive Physiology" (A. McLaren, ed.), pp. 111–180. Logos Press, London (1967).
11. Tuan, R. S., and Zrike, J., Biochem. J. **176**, 67 (1978).
12. Slavkin, H. C., Bringas, P., Croissant, R. D., and Bavetta, L. A., Mech. Age. Dev. **1**, 139 (1972).
13. Hamburger, V., and Hamilton, H. L., J. Morphol. **88**, 49 (1951).
14. Konishi, T., and Kosin, I. L., J. Embryol. Exp. Morphol. **32**, 557 (1974).
15. Anderson, H. C., *in* "The Comparative Molecular Biology of Extracellular Matrices" (H. C. Slavkin, ed.), pp. 201–205. Academic Press, New York (1972).
16. Anderson, H. C., *in* "Hard Tissue Growth, Repair and Remineralization" (R. F. Sognnaes, ed.), pp. 213–225. Elsevier Excerpta Medica North–Holland, Amsterdam (1973).
17. Ali, S. Y., Fed. Proc. **35**, 32 (1976).
18. Schraer, R., and Schraer, H., *in* "Biological Calcification: Cellular and Molecular Aspects" (H. Schraer, ed.), pp. 347–373. Appleton–Century–Crofts, New York (1970).
19. Bernard, G. W., and Pease, D. C., Amer. J. Anat. **125**, 271 (1969).
20. Slavkin, H. C., *in* "International Colloquium on Physical Chemistry and Crystallography of Apatites of Biological Interest" (G. Montel, ed.), pp. 161–177. Centre Nacional Recherche Scientifique, Paris (1975).
21. Slavkin, H. C., Croissant, R. D., Bringas, P., Matosian, P., Wilson, P., Mino, W., and Guenther, H. G., Fed. Proc. **35**, 127 (1976).
22. Narbaitz, R., and Jande, S. S., J. Embryol. Exp. Morphol. **45**, 1 (1978).
23. Slavkin, H. C., Croissant, R. D., Guenther, H. G., and Sorgente, N., *in* "Formation and Calcification of Hard Tissues" (R. V. Talmage and H. Ozawa, ed.), pp. 59–82. S. Hoken, Tokyo (1978).

Received June 7, 1979. P.S.E.B.M. 1980, Vol. 163.