1α,25-Dihydroxyvitamin D_3

A Metabolite of Vitamin D That Promotes Bone Repair

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1α,25-dihydroxyvitamin D_3, the hormonal form of vitamin D_3 that mediates calcium translocation in intestine and bone, was tested for its ability to promote fracture repair. Chicks were raised on a vitamin D-deficient diet supplemented with 1α,25-dihydroxyvitamin D_3 for 3 weeks. Following fracture of the humerus, those chicks that did not receive continued 1α,25-dihydroxyvitamin D_3 supplementation showed prolonged fracture healing, abnormal enchondral bone formation, delayed remodeling of woven bone and osseous union, but normal formation of callus. Fracture repair in chicks receiving 1α,25-dihydroxyvitamin D_3 was normal. These data indicate that 1α,25-dihydroxyvitamin D_3 promotes bone repair in the absence of vitamin D_3, 25-hydroxyvitamin D_3, and 24,25-dihydroxyvitamin D_3. (Am J Pathol 1982, 106:171–179)

1α,25-DIHYDROXYVITAMIN D_3 is the hormonal form of vitamin D that facilitates calcium and phosphate absorption from the intestine.[4,8,12,16] The mechanism of vitamin D action on bone is more controversial. 1α,25-dihydroxyvitamin D_3 acts directly on bone in organ culture to promote calcium resorption without the presence of parathyroid hormone or other vitamin D metabolites.[14] Measurement of biochemical markers *in vitro* suggests that 1α,25-dihydroxyvitamin D_3 induces bone resorption by activating osteoclasts and inhibiting osteoblasts.[17] The demonstration of a vitamin D-dependent calcium-binding protein in bone indicates that vitamin D and/or its metabolites have a direct effect on bone metabolism.[1] Further evidence supporting a direct role for 1α,25-dihydroxyvitamin D_3 is the localization of the hormone in bone by autoradiography.[5]

Vitamin D also promotes bone formation.[4] The question of whether mineralization occurs indirectly through maintenance of serum calcium and phosphate or from direct action of vitamin D and/or a specific metabolite persists. Vitamin D_2 has been shown to increase bone ash at a fracture site in the rat humerus,[15] indicating a trophic effect of vitamin D on mineralization of bone. Attempts at identification of a specific metabolite responsible for bone formation have yielded conflicting results.

Oral administration of 1α,25-dihydroxyvitamin D_3 was reported to prevent both histologic and radiographic signs of rickets in vitamin D-deficient chicks,[2,10] suggesting that this metabolite is the principle form of vitamin D involved in bone formation. However, 24,25-dihydroxyvitamin D_3 was reported to be essential for bone formation.[13] In studies of experimental fracture repair, breaking strength of femoral fractures was increased in normal rats given 1α,25-dihydroxyvitamin D_3.[9] In separate studies, however, histologic evaluation of fracture repair in vitamin-D-depleted chicks supplemented with 1α,25-dihydroxyvitamin D_3 was abnormal.[3] In the present study the effect of 1α,25-dihydroxyvitamin D_3 on bone repair was investigated temporally, in chicks withdrawn from the hormone at the time of fracture. This approach has several advantages over prior studies. First, the phases of repair, callus formation, mineralization, and remodeling[7] can be assessed independently. Second, delays in fracture repair can be assessed. Finally, since the chicks are normal at the time of fracture, fracture repair is not being evaluated in abnormal bone.

Materials and Methods

Thirty-four White Leghorn cockerels were raised from hatching on a diet deficient in vitamin D[10] but

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supplemented daily with 260 pmoles 1α,25-dihydroxyvitamin D_3 administered orally in 1,2-propanediol. The diet was composed of ground wheat, ground corn, soybean meal, and alcohol extracted casein, charcoal, water-soluble vitamins, and vitamins A, E, and K. Eighteen control animals received vitamin D_3 throughout the experiment. After 3 weeks the humerus of each animal was fractured manually under pentobarbital anesthesia, and one group of animals (16 chicks) was removed from 1α,25-dihydroxyvitamin D_3 supplementation. Four animals in each of the three groups were sacrificed following fracture. The previously fractured humerus and the tibial-talus joint were removed, sectioned longitudinally, fixed in 10% formalin, decalcified in formic acid, and embedded in paraffin. Six sections were stained with hematoxylin and eosin (H & E) and examined by light microscopy. Fracture healing was assessed by the method of Makley et al.[11] Grading was performed in blind fashion independently by two observers, as shown in Table 2. Of the 48 fractures that were assigned 144 different grades for callus, union, and compact bone, there were 3 cases of discrepancy between graders, each of which was by one grade.

High detail radiographs of the upper and lower extremities were obtained in the lateral projection utilizing duPont Lo Dose film and screen combination and a Faxitron X-ray unit. Average exposure factors were 45 kv and 18-second exposures. Faxitron Series, manufactured by Hewlett-Packard, has a stationary anode tube with a 20 degree angled tungsten target, a 0.5-mm focal spot, and a beryllium window. Calcium and phosphorus were determined by duPont Automated Clinical Analyzer.

Results

The serum calcium and phosphorus determinations on each group of animals is shown in Table 1. Results at time 0 are from two animals sacrificed 3 weeks after hatching (without fracture). The serum calcium progressively declined in animals that were not supplemented with 1α,25-dihydroxyvitamin D_3 from Day 3 to Day 22 following fracture. At 16 and 22 days after fracture there was a significant difference between the serum calcium in the supplemented and nonsupplemented chicks. There was no significant change in serum phosphorus. The mean body weight increased from 162 ± 8 g to 422 ± 32 g in the supplemented chicks. The mean body weight decreased to 148 ± 5 g in the nonsupplemented chicks.

Radiographic evaluation of the growth plates included careful attention to the amount and extent of mineralization in the zones of provisional calcification and subjacent bony trabeculations, the organization and mineralization of these trabeculations, and the general configuration of the metaphysis. Normal growth plates showed compact, defined, well-mineralized trabeculas with a sharply delineated margin adjacent to the cartilaginous portions (nonradiopaque) of the growth plate (Figure 1A). Early rachitic changes were subtle and consisted of irregular margins between the mineralized and unmineralized portions of the growth plate and subjacent bony trabeculations and a general decrease in mineralization (Figure 1B). More advanced rachitic changes showed a minimal number of disorganized trabeculations beneath the growth plate, with decreased mineralization. The growth plate may be widened in the transaxial dimension with a concave cuplike structure (Figure 1C).

Histologic section of the tibia 3 days after fracture in the 1α,25-dihydroxyvitamin D_3-supplemented chick is shown in Figure 2A. The growth plate is similar to that of the normal control chick (not shown) and shows well-organized zones of maturation, hypertrophy, provisional calcification, and ossification. The growth plate in the chicks that had been withdrawn from 1α,25-dihydroxyvitamin D_3 for 3 days (Figure 2B) shows marked widening of the growth plate and resting zone with some irregularity in border between the zones of hypertrophy and provisional calcification. The parallel arrangement of the chondrocytes undergoing hypertrophy and calcification is retained.

Twenty-two days after fracture, the tibia of the supplemented chick shows mild evidence of vitamin D deficiency (Figure 2C). There is mild expansion of the growth plate and resting zone and some irregularity in the zone of hypertrophy, and calcification is retained. By 22 days the nonsupplemented chicks showed histologic evidence of severe rickets (Figure 2D). The growth plate is expanded, with severe disorganization of the zones of hypertrophy and provisional calcification. The parallel arrangement of chondrocytes is absent.

Table 2 contains histologic criteria for grading bone repair adapted from Makley et al.[11] Grading of the fracture healing is shown in Table 3 for chicks that were supplemented with 1α,25-dihydroxyvitamin D_3, those withdrawn from 1α,25-dihydroxyvitamin D_3, and controls (that received vitamin D). Both the grade range and mean grade for each group of animals at each time period are shown. Within the first 3 days there was development of profuse cellular callus with bridging of the fracture site in the control

Table 1—Serum Calcium and Phosphorus at Each Time Period

Group	Ion	Time period (days) 0	3	8	16	22
1α,25-dihydroxyvitamin D_3 withdrawn	Ca mg/dl ± SD	10.3 ± 0.3[a]	10.1 ± 0.4	8.5 ± 0.3	8.0 ± 0.5*	7.5 ± 0.4*
	P mg/dl ± SD	6.2 ± 3	5.9 ± 0.4	5.6 ± 0.4	5.3 ± 0.3	5.3 ± 0.3
1α,25-dihydroxyvitamin D_3 supplemented	Ca mg/dl ± SD	10.3 ± 0.3	10.0 ± 0.5	10.3 ± 0.6	9.8 ± 0.2	9.6 ± 0.3
	P mg/dl ± SD	6.2 ± 0.3	6.0 ± 0.1	5.3 ± 0.3	5.8 ± 0.3	6.1 ± 0.4
Control	Ca mg/dl ± SD	10.0 ± 0.4				
	P mg/dl ± SD	6.0 ± 0.1				

* Differs from *a*, $P < 0.05$.

chicks. At 8 days there was osseous union within the callus and an osteochondral union in the medullary shaft. An osseous union composed of compact bone had developed by 16 days. Twenty-two days after fracture the healing process was complete, including reorganization of the shaft and repopulation of bone marrow.

The data in Table 3 were analyzed by a Kruskal-Wallis one-way ranked-order analysis of variance of the grades of each group at each time period after fracture. A matrix of *P* values for each group at each time period showed significant differences at 8, 16, and 22 days in the grades of fracture union and at 16 and 22 days in the grades of compact bone at the fracture site. Further, there were no significant differences in the grades of callus formation at any time period, and there were no significant differences in fracture repair at 3 days after fracture. Those groups that showed differences in grades were further analyzed by Kruskal-Wallis one-way analysis of variance with regard to comparison of normal versus 1α, 25-dihydroxyvitamin-D_3-withdrawn, normal versus 1α,25-dihydroxyvitamin-D_3-supplemented, and 1α, 25-dihydroxyvitamin-D_3-supplemented versus 1α, 25-dihydroxyvitamin-D_3-withdrawn. The analysis indicates that the grades of fracture repair at 8, 16, and 22 days for union and 16 and 22 days for compact bone at the fracture site in the 1α,25-dihydroxyvitamin-D_3-withdrawn chicks were significantly different from the 1α,25-dihydroxyvitamin-D_3-supple-

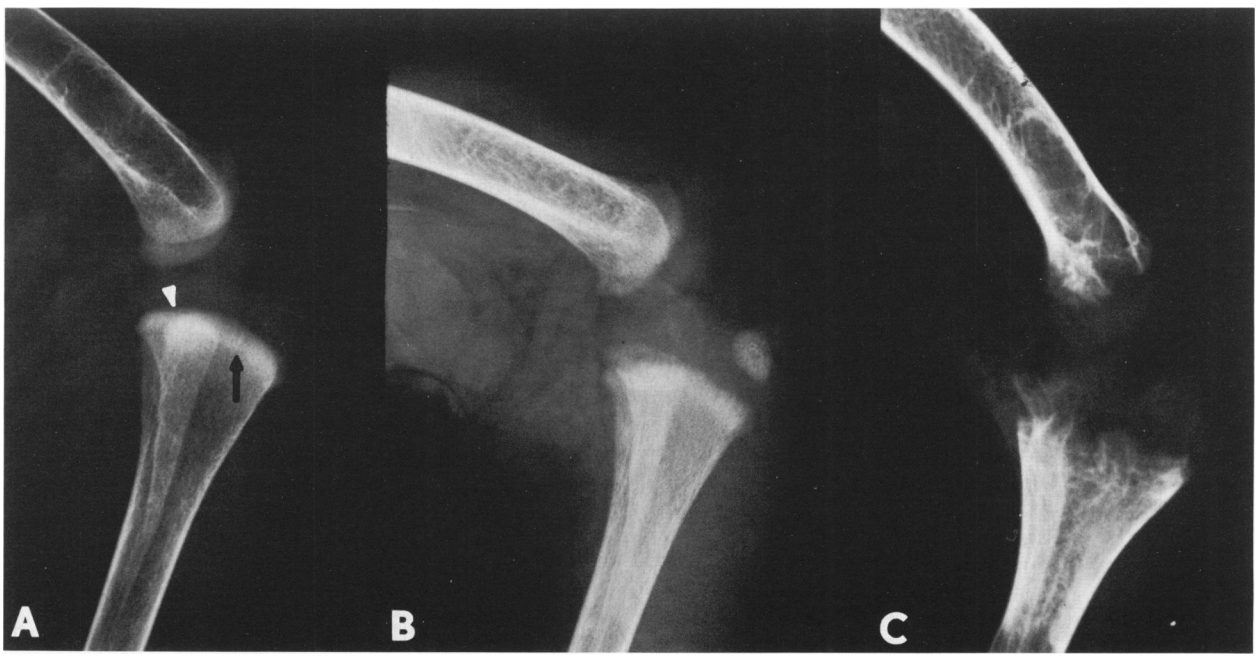

Figure 1—Lateral radiograph of knee in chicks given supplements of 1α,25-dihydroxyvitamin D_3 (**A**, 3 days after fracture; **B**, 22 days after fracture) and chicks withdrawn from 1α,25-dihydroxyvitamin D_3 (**C**, 22 days after fracture). **A**—No rachitic changes are identified. Note the excellent mineralization of the bony trabeculations subjacent to the cartilaginous growth plate (*arrows*). The delineation of these trabeculations from the growth plate is sharp (*arrowhead*). **B**—Mild rickets is demonstrated by a decrease in the mineralization and number of bony trabeculations beneath the growth plate. The interface between the cartilaginous growth plate and the trabeculations is irregular. **C**—Advanced rachitic changes. Note widening of growth plate in transaxial dimension and slight concave configuration.

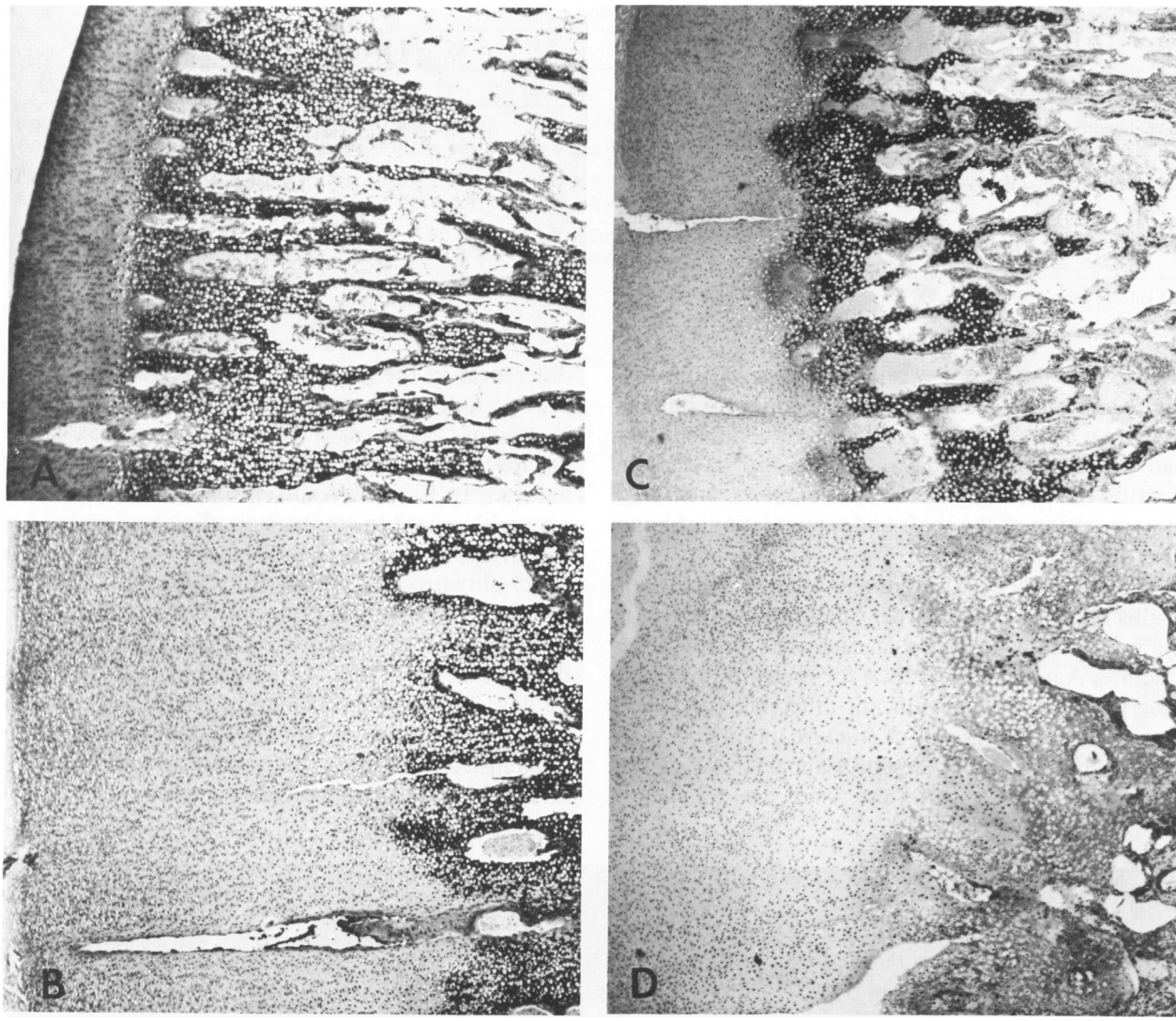

Figure 2—Photomicrographs of histologic sections of epiphyseal growth plate of chick tibias. **A**—Three days after fracture in a 1α, 25-dihydroxyvitamin-D_3-supplemented chick. **B**—Three days after fracture in a nonsupplemented chick. **C**—Twenty-two days after fracture in a 1α,25-dihydroxyvitamin-D_3-supplemented chick. **D**—Twenty-two days after fracture in a nonsupplemented chick. Widening of the growth plate is noted after removal of 1α,25-dihydroxyvitamin D_3 supplementation, although organization of the zone of provisional calcification is maintained (**B**). There is marked widening and disorganization of the growth plate 22 days following removal of 1α,25-dihydroxyvitamin D_3. (H&E, × 100)

mented and from the normal chicks. There was no significant difference between grades of the normal and 1α,25-dihydroxyvitamin-D_3-supplemented chicks. Thus, statistical evaluation of grades of fracture repair suggests that there is significantly delayed healing in the chicks withdrawn from 1α,25-dihydroxyvitamin D_3 and no difference between the healing in the normal and 1α,25-dihydroxyvitamin-D_3-supplemented chicks. However, the initial phase of healing, the development of cellular callus, was similar in all three groups of animals. Osseous bridging within the callus was delayed, as well as the development of osseous union by compact bone. Radiographs showed varying delays of fracture healing in rachitic chicks. Callus formation was identified but showed decreased mineralization. Endosteal healing was also delayed (Figure 3A).

The disorganization of the endochondral bone formation in the 8-day-old fractures of the nonsupplemented chicks is shown in Figure 4C. Compared with the control (Figure 4A) and supplemented chicks (Figure 4E), the ossification of the callus in the nonsupplemented animal is similar to the rachitic lesion at the epiphyseal plate (Figure 2D). Photomicrographs from the medullary cavities at 16 days after fracture (Figure 4B, D, and F) demonstrate compact bone formation and early repopulation of the bone marrow in the control and 1α,25-dihydroxyvitamin-

Table 2—Criteria for Grading Fracture Repair

Callus	
No callus	0
Small amount of callus	1
Moderate amount of callus	2
Profuse callus	3
Bridging callus	4
Osseous bridging	5
Union	
No sign of union, fibrous or other	0
Fibrous union	1
Osteochondral union	2
Osseous union	3
Complete reorganization of shaft	4
Compact bone at fracture site	
None	0
Beginning to appear	1
Formation well under way	2
Intact but incomplete	3
Complete reorganization	4

D_3-supplemented animals and the persistence of fibrous tissue at the fracture site in the rachitic chick.

Figure 5 illustrates the persistence of fibrous tissue and cartilaginous callus at the fracture site in the rachitic chick (Figure 5C and D). There is little progress in repair at Days 16 and 22, as compared with Day 8 in the nonsupplemented chick. Some compact bone is noted at the fracture site; however, the cellular callus remains. The bone ends are joined by primarily fibrous tissue, and the marrow cavity is filled by fibrous tissue, osteoid, cartilage, and primary bone. Healing in the control (Figure 5A and B) and supplemented (Figure 5E and F) is nearly complete with reorganization of compact bone, medullary cavity, and repopulation of the marrow.

Discussion

1α,25-dihydroxyvitamin D_3 apparently promotes normal healing of fractures in the chick. Both the D-deficient and 1α-25-dihydroxyvitamin-D_3-supplemented chicks formed cellular callus normally 3 days following fracture of the humerus. However, at 16 days there were clear differences in fracture repair. At that time there was an unossified fracture union and less compact bone at the fracture site in the D-deficient chicks. In the supplemented and normal chicks there was already evidence of reorganization of the shaft. By 22 days the fracture was almost completely healed in the 1α,25-dihydroxyvitamin-D_3-treated and in the normal chicks. At that time the D-deficient chicks still had a large amount of cellular callus, an osteochondral union, and thin trabeculas of compact bone at the fracture site (Figure 5, Table 3).

Our conclusions concerning the effect of 1α,25-dihydroxyvitamin D_3 differ from those of Dekel et al,[3] who studied the effect of 1α-hydroxyvitamin D_3 on fracture repair in chicks. However, they studied fracture repair 9 days after fracture, a time at which we were not able to detect a statistical difference among the groups that received vitamin D or metabolites and the normal group (Table 3). In addition, the differences in fracture repair that they described

Table 3—Fracture Repair in Normal, 1α,25-dihydroxyvitamin-D_3-Supplemented, and 1α,25-dihydroxyvitamin-D_3-withdrawn chicks

		Fracture repair (grade)*					
		Callus		Union		Compact bone at fracture site	
Vitamin D status	Days post-fracture	Mean	Range	Mean	Range	Mean	Range
Normal	3	3.25	(2-4)	1.5	(1-2)	0	0
	8	4.5	(4-5)	2.5	(2-3)	1	(0-3)
	16	5	5	3	3	3	3
	22	5	5	3.75	(3-4)	3.75	(3-4)
1α,25-dihydroxyvitamin D_3 withdrawn	3	3	(2-4)	0.5	(0-1)	0	0
	8	4	4	1.25	(1-2)†	0	0
	16	4.5	(4-5)	2.25	(2-3)‡	0.5	(0-2)‖
	22	4.75	(4-5)	2	2§	1	(0-3)¶
1α,25-dihydroxyvitamin D_3 supplemented	3	3	(2-4)	0.75	(0-2)	0	0
	8	4.75	(4-5)	2.25	(2-3)†	1.25	(0-3)
	16	5	5	3.5	(3-4)‡	2.75	(2-3)‖
	22	5	5	4	4§	4	4¶

* Values represent grade range (see Table 2) for each group of animals.
† Significantly different, $P < 0.05$.
‡ Significantly different, $P < 0.05$.
§ Significantly different, $P < 0.01$.
‖ Significantly different, $P < 0.05$.
¶ Significantly different, $P < 0.05$.

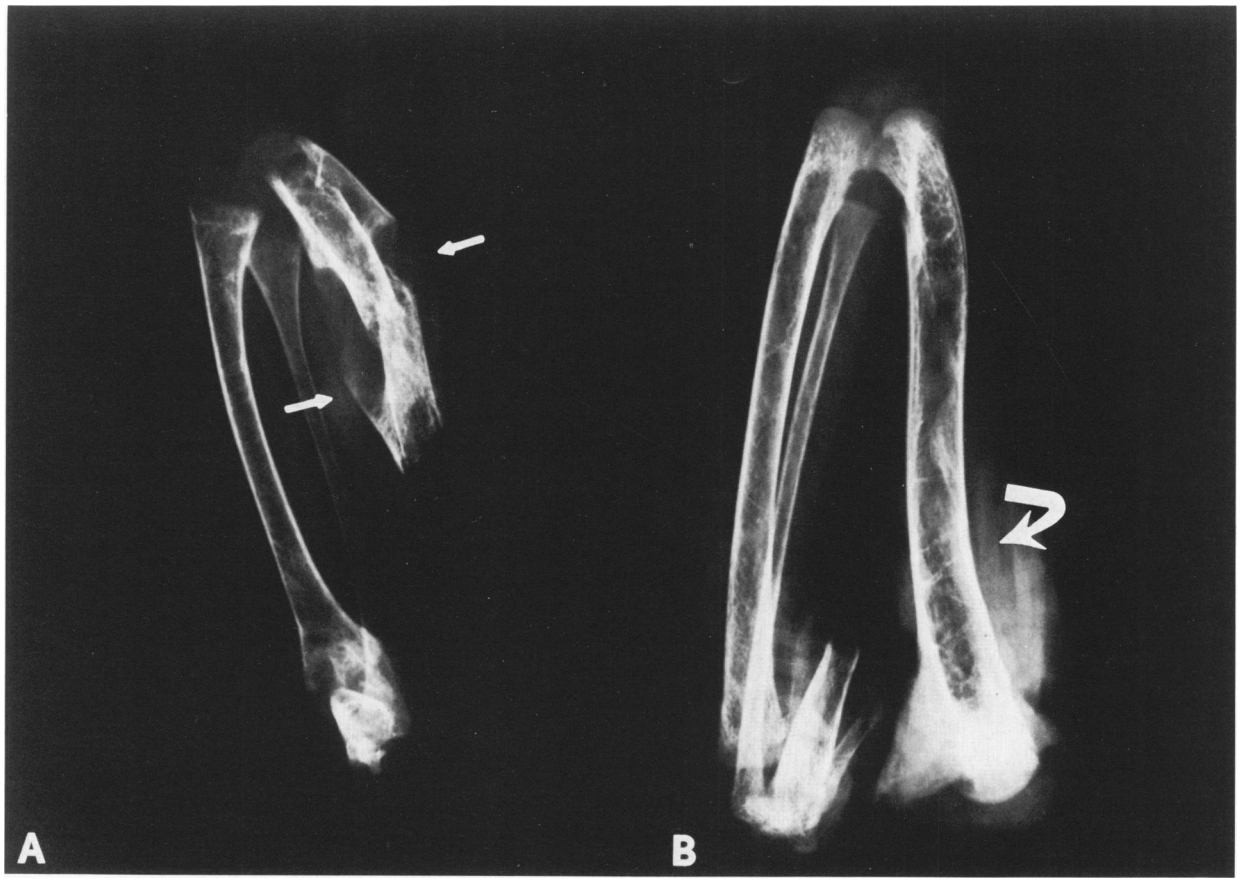

Figure 3—Fractured humeri from 43-day-old chicks (22 days after fracture). **A**—Withdrawn from 1α,25-dihydroxyvitamin D_3. Callus bridges the midshaft fracture but is grossly undermineralized (*arrows*). **B**—Supplemented chick shows some complete healing of humeral fracture with remodeling (*arrows*).

were primarily in callus formation, and not in the development of compact bone and an osseous union, which in our model occurs later (Days 16–22).

The chick humerus model for evaluation of fracture repair has several advantages. The chicks were noted to splint their wings with little apparent motion or abnormal positioning, and there was very little overriding of bone ends at the fracture site. Thus, the additional variables introduced by internal fixation, infection, and a foreign object in the marrow cavity were eliminated.

The evidence of mild rickets in the animals treated by 1α,25-dihydroxyvitamin D_3 (Figure 2) may be explained by an insufficient dose of the hormone, too great a time interval (24 hours) between doses, and the requirement of a metabolite of vitamin D other than 1α,25-dihydroxyvitamin D_3 for the prevention of rickets. Daily oral administration of 195 pmoles 1α,25-dihydroxyvitamin D_3 has previously been shown to prevent rickets in 3-week-old chicks.[2,10] However, in the present study, the daily dose of 260 pmoles may have been insufficient to meet the needs of a 6-week-old chick healing a fracture of the humerus. The dosing schedule is also of critical importance in assessing the effectiveness of 1α,25-dihydroxyvitamin D_3 on bone mineralization. After intravenous administration of tritiated 1α,25-dihydroxyvitamin D_3 to normal humans, only 10–17% of the tritium remained in the plasma after 4 hours.[6] Presumably, the half-life of the hormone is also rapid in the chick. Although oral administration should prolong absorption and help maintain a serum level of the hormone, it is possible that a more frequent dosing schedule would have completely prevented disorganization at the growth plate. Finally, another metabolite of vitamin D such as 25-hydroxyvitamin D_3 or 24,25-dihydroxyvitamin D_3 may be involved in bone formation. However, fracture repair in the control and 1α,25-dihydroxyvitamin-D_3-supplemented chicks was similar, indicating that this metabolite is the major, if not the only, vitamin D principle that promotes fracture repair.

The question of whether 1α,25-dihydroxyvitamin D_3 acts directly on bone to promote fracture repair

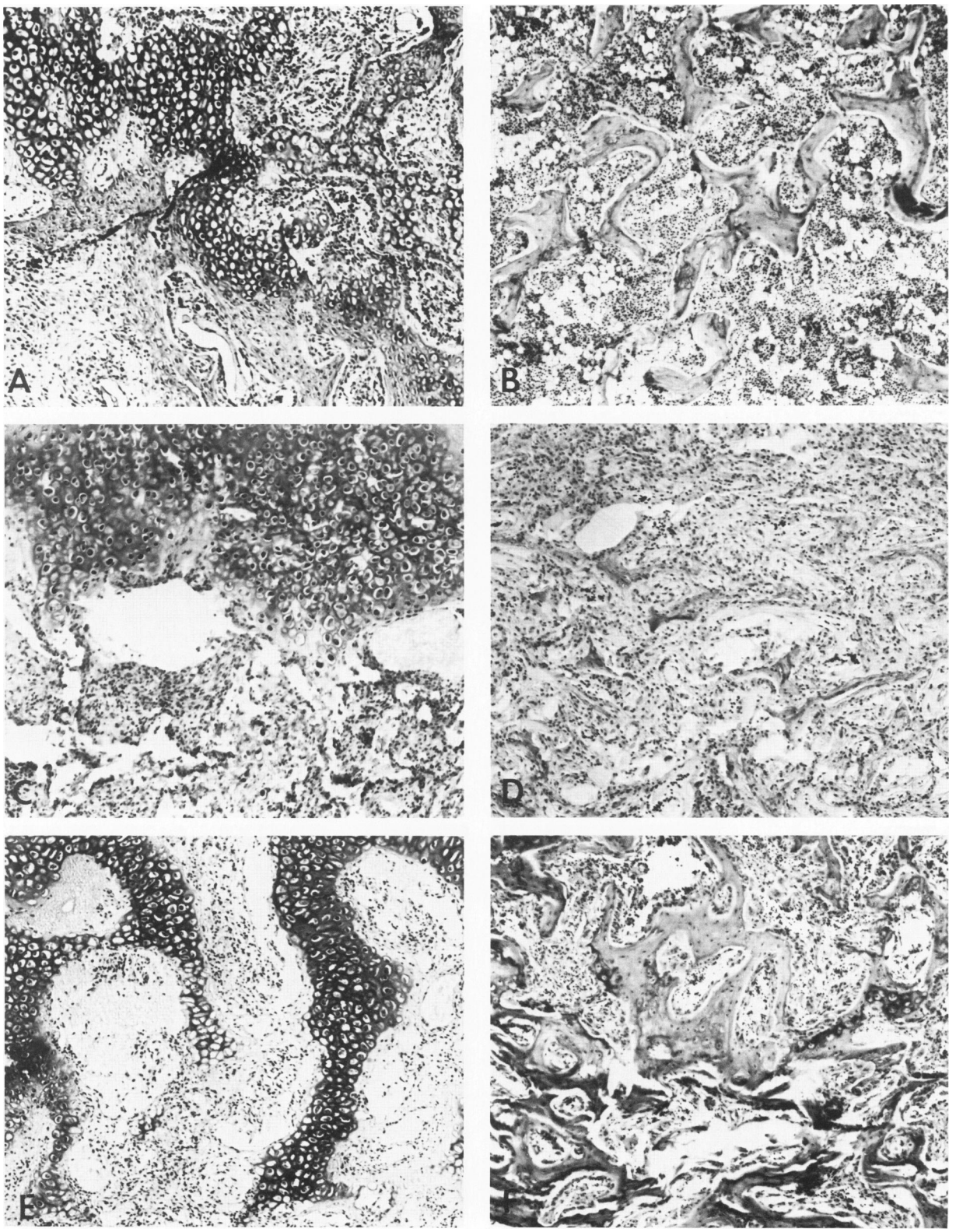

Figure 4—Photomicrographs of histologic sections of humerus fractures 8 days after fracture (**A**, **C**, and **E**) and 16 days after fracture (**B**, **D**, and **F**). Photomicrographs are from the site of union in the medullary area. **A** and **B**—Normal (received vitamin D) chicks. **C** and **D**—Chicks that did not receive 1α,25-dihydroxyvitamin D_3. **E** and **F**—Chicks that received 1α,25-dihydroxyvitamin D_3. There is an osseous union with marrow repopulation in the control (**B**) and 1α,25-dihydroxyvitamin-D_3-treated (**F**) chicks at 16 days. (H&E, × 200)

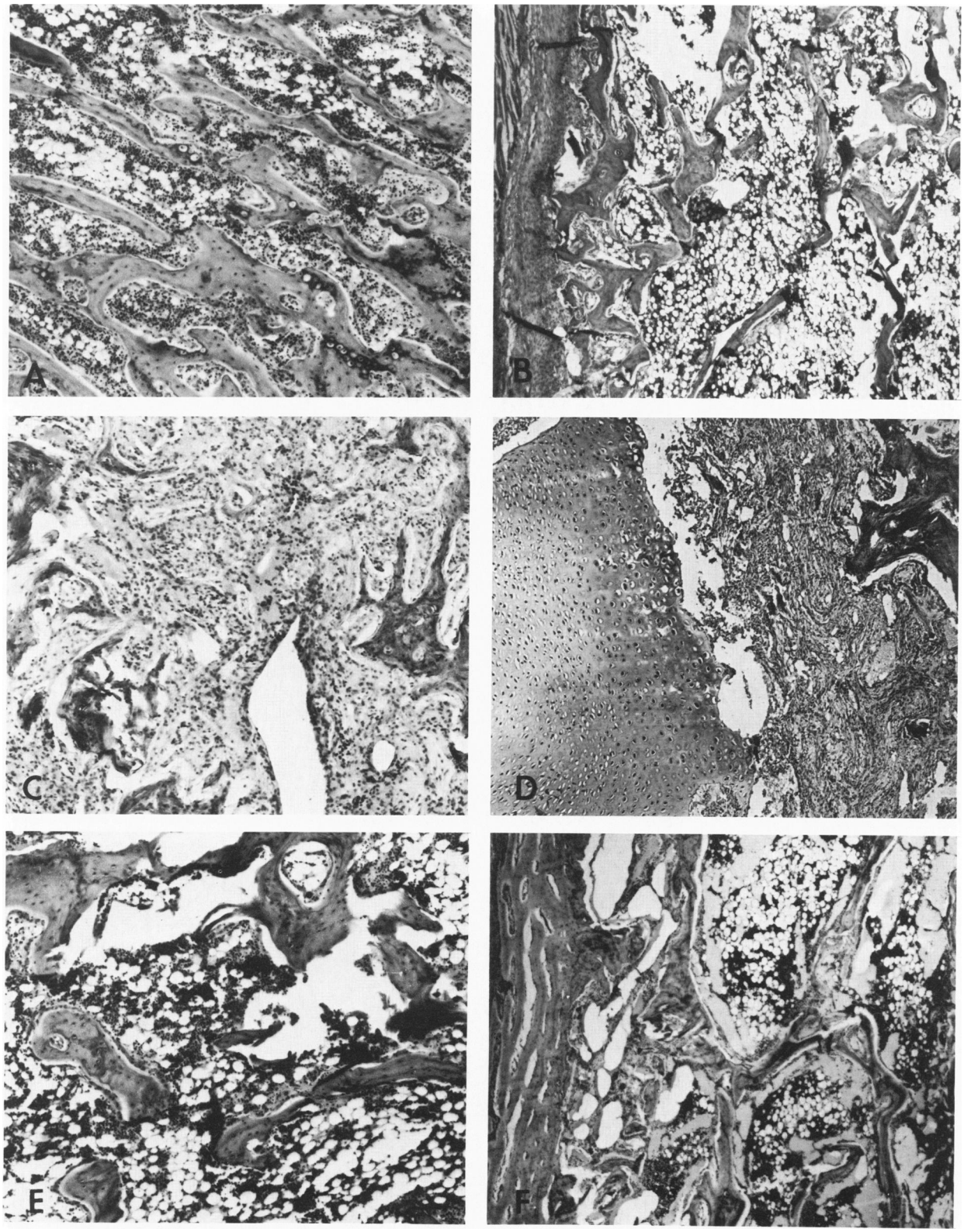

Figure 5—Photomicrographs of histologic sections of humerus fractures 22 days after fracture. **A** and **B**—Normal (received vitamin D) chicks. **C** and **D**—Chicks that not receive 1α,25-dihydroxyvitamin D_3. **E** and **F**—Chicks that received 1α,25-dihydroxyvitamin D_3. Photomicrographs **A**, **C**, and **E** are from the medullary area. Photomicrographs **B**, **D**, and **F** are the cortex and at the fracture site. There is complete healing with reorganization and repopulation of the marrow in the normal (**A** and **B**) and 1α,25-dihydroxyvitamin-D_3-supplemented chicks (**E** and **F**). There is persistence of an osteochondral union in the rachitic animals.

remains. Although there were clear histologic differences at the epiphyseal plate and at the fracture sites, chicks 3 days after fracture showed similar fracture healing (as graded in Table 3) whether or not they had received 1α,25-dihydroxyvitamin D_3. At that time the vitamin-D–deficient animals also had normal serum calcium and phosphorus levels, perhaps implying that 1α,25-dihydroxyvitamin D_3 exerts its effect on bone through maintenance of serum calcium and phosphorus and not through direct action on bone. However, at 3 days the primary response to the fractures was the formation of cellular callus, which may not be affected by the hormone. By the time ossification was well under way at the fracture (8 days), the serum calcium in the D-deficient chicks was abnormally low (Table 1). Thus, 1α,25-dihydroxyvitamin D_3 may act directly or indirectly on bone to promote fracture repair.

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