

## THE ROLE OF ALKALINE PHOSPHATASE IN OSTEOGENESIS

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Almost two centuries ago Belchier (1) demonstrated that bone is a living and actively metabolizing tissue. Since then, however, relatively little has been discovered about its basic physiology. The mechanism of new bone formation, and in turn the role played by the enzyme alkaline phosphatase, have been among the most confusing aspects of the problem. The present investigation, which is part of a larger inquiry into the physiology of osteogenesis and bone healing, represents a study of the role of alkaline phosphatase by histochemical techniques.

Robison in 1923 (2) demonstrated the presence in growing cartilage and bone of an enzyme that has the capacity to split off inorganic phosphates from organic phosphates. At first this discovery of alkaline phosphatase seemed to solve the problem of the mechanism of bone calcification. It was assumed that phosphatase attacks the organic phosphates present in tissue fluids and stock-piles large concentrations of inorganic phosphates. When a sufficient number of these inorganic phosphate ions accumulated, so that when combined with calcium ions present locally the solubility product of calcium phosphate was exceeded, bone salts would be chemically precipitated into osteoid. It was soon recognized, however, that this reasoning was an oversimplification. Even Robison (3) had suggested a "second mechanism" present in calcifying tissues, which was concerned with the deposition of calcium salts into the ground substance. Other tissues containing phosphatase failed to calcify because they lacked this "second mechanism." Since the phosphoric esters that normally serve as a substrate for phosphatase activity are not present in the tissues at the site of calcification, it became necessary to assume the existence of another system whereby a suitable phosphate ester substrate would be made available to the enzyme. Gutman (4) has postulated that the enzyme phosphorylase, concerned with glycogenolysis, serves this purpose. This early phase of calcium phosphate deposition is only the first stage in the eventual elaboration of complicated apatite crystals involving further complex, as yet unexplained, mechanisms.

The demonstration of alkaline phosphatase in various non-calcifying tissues such as liver, fibrosarcomata, rachitic bone, and non-ossifying osteogenic sarcomata, further undermined the concept that phosphatase alone is concerned with calcification.

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Its presence in preosseous tissues that are undergoing transformation into bone, long before calcification occurs, suggests the closer relationship of the enzyme to metabolic processes involved in the deposition of matrix than to actual calcification. Bloom and Bloom (5) conceived of calcifiability as a property conferred upon osseous tissues as they were deposited, and that the process of calcification itself was merely a matter of the available mineral being deposited into the osteoid when it had reached a chemical stage capable of accepting it. Confusion exists as to the precise role of phosphatase in these various possibilities, the question of whether it is involved in matrix formation, or bone calcification, or both, remaining obscure. One explanation of the different metabolic characteristics of different tissues, all containing alkaline phosphatase, is the possibility that there are different alkaline phosphatases with different roles. However, in spite of the demonstration of the differing specificity of apparently different phosphatases, as evidenced by the substrates on which they are capable of acting, Gomori (6) feels that there is insufficient evidence for the existence in animal tissues of phosphatases other than the common acid and alkaline types.

The present investigation was instituted in the hope that comparative studies using various histochemical techniques might cast some further light on the role of alkaline phosphatase in osteogenesis, and its relationship to inorganic phosphate and matrix metabolism.

#### *Materials and Methods*

The bones of growing rabbits and newborn human beings were employed for the epiphyseal and metaphyseal studies. For the bone grafts and studies involving radioactive materials, only growing rabbits were employed. The human epiphyses consisted of ten costochondral junctions obtained from two newborn infants within 12 hours of their death. For the phosphatase studies the bones were fixed in cold 80 per cent ethyl alcohol, and for the phosphate studies in 10 per cent formalin and 1 per cent cobalt nitrate. Over thirty epiphyses of 8 week old female rabbits were stained for phosphatase and phosphates. The upper tibial epiphysis was used as routine, although the lower radial, upper humeral, and lower femoral epiphyses were also studied to confirm the results. Twenty-three bone grafts were stained for phosphatase, and of these eight were also stained for phosphates. In addition numerous specimens of exuberant callus around the bone grafts were stained for both phosphatase and phosphates. All specimens obtained from rabbits were fixed within 20 minutes of death, which was induced by an intravenous injection of nembutal.

*1. Phosphatase Localization.*—The identification of alkaline phosphatase was accomplished according to the principles of Gomori (7), later applied to bone by Lorch (8), Greep (9), and McKelvie (10). In general it involves the incubation of bones in an alkaline medium in the presence of calcium ions and an appropriate organic phosphate ester. At the site of phosphatase activity in the tissues inorganic phosphate ions are liberated from the phosphate ester, and immediately precipitated by the calcium ions to form an insoluble salt. This invisible salt, calcium phosphate, is transformed into a black silver or cobalt sulfide deposit for histochemical visualization. By using carefully buffered solutions the enzyme can be preserved through the process of decalcification in weak acids, and can be fully reactivated in alkaline solutions.

*2. Phosphate Deposition.*—Free or ionic phosphates present normally in the tissues consist of those salts involved either in normal tissue metabolism or in calcification. By a study of the localization of free phosphates in the tissues, their relationship to enzyme activity and

matrix elaboration can be better clarified. Silver or cobalt impregnation of fresh tissues (11) binds soluble phosphates as insoluble silver or cobalt salts at their normal sites of metabolism. These insoluble phosphates are easily converted into sulfides which remain intact during the process of decalcification with 10 per cent formic acid. Comparative studies with phosphatase can be carried out on the same section or on different sections. It should be borne in mind that when free phosphates are identified by these methods, carbonates and soaps are also precipitated by the silver or cobalt, and therefore a quantitative estimation of the phosphates cannot be made. Although most results were confirmed by the silver method, cobalt was used primarily in this investigation since it gave better histochemical detail, and since it was also used for visualization in the phosphatase studies. In order to preclude washing out the most soluble phosphate salts, cobalt (1 per cent) was added directly to the fixing solution (10 per cent formalin). Different sections of the same specimen were studied separately for phosphates and phosphatase, because the frequent coexistence of the two makes differentiation on the same section difficult.

The validity of the cobalt technique was established by studies using radioactive phosphorus. Since  $P^{32}$  is removed along with bone salts in decalcification, the standard methods of studying bone salts entail the use of undecalcified bone. Little detailed information is obtainable, however, because the sections of undecalcified bone are too thick to give accurate autographs, and the bones of growing rabbits are too dense to be cut well. A method was devised (12) whereby the bones of young rabbits were decalcified by formic acid in the presence of lead ions. The phosphates that were liberated were immediately bound by the lead as insoluble lead phosphates, and hence the bone could be decalcified and yet retain large amounts of radioactive phosphorus. Detailed autographs were thus obtainable from thin decalcified sections. A combined study of histological section and radioautograph was possible by coating the stained section with 1 per cent celloidin or with a layer of vinyl plastic, after which a liquid photographic emulsion<sup>1</sup> sensitive to the beta radiations of  $P^{32}$  was painted on the slide in the dark, and allowed to be overexposed to reduce the length of contact of the slide with the developing solutions. Although photographic developers and fixers remove histological stains, the protection offered by the celloidin and plastic sufficed during rapid developing and fixing so that the stain was not affected. In this manner the exposed emulsion (radioautograph) overlay the stained section, and the sites of radioactive phosphorus localization in the sections could be identified as black deposits on the emulsion. These studies confirmed the fact that phosphates are physiologically present at the sites at which they can be demonstrated by the cobalt technique.

3. *Matrix Elaboration.*—Bone matrix was best demonstrated by the use of the Van Gieson stain, which identified the matrix as bright red, as opposed to the yellow-stained non-collagenous tissues. It permitted the accurate observation of the formation of collagen fibers. The non-fibrillar portions of the cartilage matrix and the young connective tissue of graft beds (presumably chondroitin sulfate and hyaluronic acid respectively) were identified by their metachromatic staining with toluidine blue.

## RESULTS

The observations on the sites of localization of phosphatase and phosphates in epiphyseal ossification were compared with the findings on the ossification of bone graft beds. The distribution of chondroitin sulfate and the elaboration of bone matrix in relation to phosphate deposition were also studied.

<sup>1</sup> Supplied by H. Hoerlin of Ansco.

*Epiphyseal Ossification*

*Region of Undifferentiated Cartilage Cells.*—The cells are small and the nuclei stain for phosphatase but not for free phosphates (Figs. 1 and 2). The cytoplasm of the cells and the cartilage matrix are clear and contain neither phosphatase nor free phosphates. There is marked metachromatic staining of the cartilage matrix.

*Region of Palisading Cartilage Cells.*—In addition to the localization of phosphatase in the nuclei of the cartilage cells, moderate amounts make their appearance in the cartilage matrix itself (Fig. 1). Phosphates, however, are absent from the matrix (Fig. 2). There is no change in the intensity of metachromatic staining as phosphatase appears.

*Region of Hypertrophy and Swelling of Cartilage Cells.*—The site of phosphatase deposition is unchanged in this area, but it stains more heavily. Phosphates are first found in the cartilage matrix in the region where cartilage cell hypertrophy occurs (Fig. 2), but they are absent from the nuclei of the chondrocytes. In the region of cartilage cell destruction both phosphates and phosphatase coexist in the matrix, and continue into the cartilage remnants of the metaphysis. Toluidine blue stains chondroitin sulfate metachromatically throughout the epiphyseal cartilage, and it persists along with phosphatase in the cartilage remnants.

*Cartilage Remnants of the Metaphysis.*—(Fig. 3). Phosphatase, free phosphates, and chondroitin sulfate can be demonstrated in large concentrations in the remnants of epiphyseal cartilage in the metaphysis that are being surrounded by new bone. As long as cartilage remnants persist, both chondroitin sulfate and phosphatase can be noted in the matrix. Free phosphate concentrations apparently diminish greatly in the remnants in the mid-metaphyseal region. Local phosphatase concentrations do not appear to be increased in those areas where the cartilaginous matrix of the remnants is being replaced by the newly forming bone matrix (Fig. 3). It seems, therefore, that the enzyme is not specifically associated with the process of the replacement of the cartilage remnants by bone. The only phosphatase deposited in these areas is that related to the formation of the matrix around invading bone cells. There does not appear to be a stage of actual removal of cartilage matrix before the new bone is deposited.

These observations suggest that bone matrix is deposited in the ground substance of the cartilage remnants, as though some use were being made of its components. At the point of deposition of bone matrix in the cartilage matrix, the latter loses its metachromatic staining characteristics.

*Metaphyseal Bone Trabeculae.*—(Fig. 3). Immediately with the appearance of osteoblasts on the surface of the cartilage remnants, a red-staining material, appearing homogeneous and amorphous with the Van Gieson stain, comes to surround each cell in the form of a separate island. These islands of matrix

grow and coalesce into a single homogeneous mass of material in which the osteoblasts remain imbedded (Fig. 4). As this matrix increases in density, fine, irregular, collagen fibers make their appearance. Matrix is never deposited independently of cellular activity, and presumably represents ground substance of bone which is either elaborated or secreted by the osteoblast. Phosphatase is demonstrable in the nucleus of the osteoblast, in the lacunae and canaliculi, and in the newly forming matrix itself. The enzyme appears to diminish rapidly in the bone matrix of the metaphyseal trabeculae. The only demonstrable new phosphatase in this area is in the matrix around those osteoblasts that are rapidly producing new bone. The enzyme is completely absent from the matrix of mature bone. Free phosphates coexist with phosphatase in the canaliculi of the osteoblasts and in the matrix surrounding young bone cells. Phosphates are also present on the surface of the trabeculae where early calcification is occurring. Chondroitin sulfate is absent from all stages of bone matrix elaboration during the formation of metaphyseal trabeculae.

The sites of phosphate disposition noted above were visualized with cobalt. When silver was employed, the results were similar except that black deposits were present in most of the cartilage cell nuclei, whereas with the cobalt method they were absent from the nuclei.

#### *Ossification in a Bone Graft Bed*

The incorporation of autogenous bone grafts, both iliac and tibial, was studied in rabbits by the following techniques.

A 1 cm. defect was made in the lower third of the ulna in young female rabbits weighing 2.5 kilos. Chips of either iliac or tibial bone, removed from the same animal, were placed into the defect. The tissues overlying the graft site were sutured to prevent displacement of the transplant. No form of immobilization was employed, and the animals were permitted to walk on the operated limb. The grafts were left *in situ* for 1, 2, 3, 4, and 5 weeks, and their incorporation was studied by the techniques already described.

The detailed results of the experiments will be reported in a future publication. Since the findings in fibrocallus formation were identical with those of cartilage callus formation, only the latter will be described.

*Preosseous Tissue.*—In the cartilage callus, bone formation occurs in a manner similar to that noted at the epiphyseal line, with the exception that the orderly stages of palisading and hypertrophy of cartilage cells are absent. The nuclei of the chondrocytes, their capsules, and the matrix stain heavily for phosphatase. Unlike the findings in the epiphyseal cartilage, there are no clear cut areas of young cartilage cells around which the matrix is devoid of phosphatase. However the site of metaplasia of immature mesenchyme into cartilage or more dense fibrocallus can be identified by the appearance of phosphatase in the matrix. Phosphatase exists also in the nuclei of the precallus mesenchyme

cells. There are no free phosphates in the matrix of either the cartilaginous or the fibrous callus, both of which are rich in phosphatase. Phosphates make their appearance just as bone matrix forms from the callus (Fig. 5). Chondroitin sulfate can be identified at the time of appearance of phosphatase in the cartilage matrix, and coexists with the enzyme throughout the cartilage callus. Similarly, metachromatic staining material coexists with phosphatase in the fibrocallus. There is no metachromatic material in the mature bone trabeculae.

*Osseous Tissue.*—The sites of phosphatase deposition in the newly formed trabeculae are comparable to those in the metaphyseal trabeculae. The enzyme can be demonstrated on the trabecular surface, in the canaliculi and nuclei of the osteoblasts, and to a less degree homogeneously deposited throughout the bone matrix of the immature trabeculae. Free phosphates are deposited either on the surface of the new trabeculae or beneath the surface. In the latter case a clear zone of osteoid tissue, devoid of demonstrable salts, often exists between the site of phosphate deposition and the surface, where the enzyme is found (Figs. 7 and 8). In the bone grafts, new bone cells invade the necrotic transplants. Only small amounts of phosphatase are noted in the vicinity of the new cells. This sharp differentiation between the large amount of phosphatase in the graft bed and invading new bone, and its absence in the necrotic transplants serves as a valuable aid in mapping the progress of bone graft incorporation (Fig. 6).

#### DISCUSSION

The observation that, in tissues incubated with a suitable substrate, alkaline phosphatase can split off inorganic phosphates from organic phosphate esters at sites where inorganic phosphates frequently are not normally encountered in the tissues, suggests various possibilities as to the physiological function of the enzyme. Among these is the suggestion of Gutman that an adequate substrate upon which the enzyme is capable of acting does not exist. Gutman demonstrated (13) that phosphorylase is concerned with glycogen degradation and suggested that one of the esters of the glycolytic series might act as the substrate on which phosphatase would be capable of acting. In view of the widespread distribution of phosphatase in various non-ossifying tissues, in rachitic bone, and in uncalcified proliferating cartilage and fibrocallus, it seems unlikely that phosphatase and phosphorylase are mutually dependent universally. A relationship may exist only in the calcifying areas at the epiphyseal line. The need for phosphorylase as a substrate former is apparently not present in rachitic trabeculae (14) and in newly forming trabeculae in a graft bed (Figs. 7 and 8), where an uncalcified osteoid seam exists between the sites of phosphatase and phosphate deposition.

The close association of phosphatase with preosseous matrix formation in

the cartilaginous callus and fibrocallus in the absence of demonstrable phosphates strongly suggests that the enzyme is basically concerned with the cellular functions related to the elaboration of both cartilage and bone matrix. In this regard it might be related non-specifically to phosphate transfer and phosphorylation mechanisms. There are many cellular metabolic processes of more vital concern than bone calcification that involve phosphate transfer, and where phosphatase but no phosphates are demonstrable histochemically (liver, intestine, granulation tissue). Recent observations (15) using radioactive phosphorus to study phosphorus transfer by means of phosphatase activity *in vitro*, have demonstrated phosphorylation to be a common phenomenon in many basic metabolic systems other than calcification. In rickets, cartilage and osteoid proliferation occur in the presence of phosphatase and in the absence of demonstrable salt deposition. This rachitic osteoid is calcifiable, and will calcify when a normal diet is instituted or *in vitro* when immersed in a suitable calcium phosphate solution (16). It appears, therefore, that calcification is not in any way necessary for matrix formation, and that in the formation of matrix without calcification, the distribution of phosphatase does not materially differ from its distribution when calcification occurs. Under these circumstances it becomes easier to relate phosphatase to matrix formation than to calcification. The enzyme may be involved in some as yet unexplained way in the task of making inorganic salts available to this calcifiable matrix, but it appears to be more closely related to the more basic metabolic functions of young bone and cartilage. Since rachitic bone calcifies when incubated with normal serum (17) it is apparent that suitable salts are normally available in tissue fluids and serum. The immediate unsolved problems in this regard are whether a separate calcifying system is necessary, or whether the chemical nature of the osteoid matrix is responsible for the state of calcifiability; that is, does complex bone salt formation from simple calcium phosphate occur under the influence of enzymes, or does it occur passively as a chemical function of increasing complexity of the organic bone matrix?

The thesis that phosphatase is related to cellular metabolism and matrix elaboration suggests that when the enzyme is demonstrable at sites where there are no cells, it is physiologically inactive. A study of newly forming bone trabeculae supports this assumption. There are no cells in the central cartilage remnants of the metaphyseal trabeculae. Phosphatase is abundant in these remnants and appears to remain undiminished in concentration until the cartilage remnants are actually replaced by bone. There is never a demonstrable change in the character of the matrix or in the apparent concentration of chondroitin sulfate in the remnants to indicate that phosphatase is exerting any physiological role. It is suggested, therefore, that in the remnants where there are no cells the enzyme is inactive and is merely left over from the huge

amounts present in the matrix of the epiphyseal cartilage. Since there are no cells in the phosphatase-laden remnants, it is unlikely that the presence of the enzyme might be due to its diffusion into the matrix of the remnants during histological preparation of the specimens. Phosphatase can be demonstrated in the central cartilage remnants of metaphyseal bone transplants from the ilium as long as 3 weeks after grafting, even though the bone fragments themselves have become necrotic. When these phosphatase-laden remnants come into contact with invading bone, or into contact with the surrounding graft bed, there is no evidence that the presence of the enzyme affects the invading bone in any way, nor does it appear to stimulate calcification, ossification, or any other changes in the graft bed. The persistence of the enzyme for several weeks in cartilage remnants of necrotic transplants, and its ability to be reactivated in alkaline solution are evidence of its sturdiness in resisting long periods of inactivity. Bone preserved for 2 years in alcohol or acetone in the refrigerator still reveals large amounts of the enzyme. There are also no demonstrable changes to indicate that any physiological role is played by the small amounts of phosphatase that are scattered throughout the immature trabeculae in a graft bed (18). In evaluating phosphatase stains, therefore, it becomes important to realize that the histochemical demonstration of the enzyme does not necessarily indicate its physiological activity. The demonstration of physiological activity only in the presence of living cells adds further weight to the impression that the enzyme is a function of basic cellular metabolism as related to matrix elaboration.

Observations of enchondral ossification in a graft bed indicate that phosphatase is heavily deposited in the cartilage callus matrix, but no phosphates are demonstrable. The salts make their appearance just before the bone matrix itself is formed. Roughly at this point phosphatase concentrations begin to diminish. It seems likely therefore that the deposition of phosphates at this site is related to the changes occurring in the matrix and not to where phosphatase is deposited.

Histochemical evidence appears to support the concept that the most immature bone matrix is elaborated under the influence of the osteoblast, perhaps in a way somehow related to the omnipresent phosphatase, rather than secreted by the bone cell itself. Van Gieson stain colors the cytoplasm of the osteoblast a distinctive yellow, whereas the surrounding bone matrix is red. There are no traces of red material within the confines of the bone cell.

Sylvén (19) postulated that cartilage depended for its integrity upon mucopolysaccharide chondroitin sulfate. When chondroitin sulfate was destroyed, he stated, cartilage matrix became vulnerable and was immediately destroyed as well. On the basis of quantitative determinations by other authors of phosphatase at various levels of enchondral osteogenesis in epiphyses, he suggested



that chondroitin sulfate disappeared as phosphatase made its appearance, and that, therefore, the removal of chondroitin sulfate was a link in the chain of events producing an alkaline medium which is a prerequisite for the action of alkaline phosphatase. The present histochemical studies show that phosphatase and chondroitin sulfate can both be demonstrated together in epiphyseal cartilage, cartilage callus, and cartilage remnants, and disappear together in the callus and remnants as bone replaces the cartilage matrix. The coexistence of phosphatase with metachromatic material in fibrocallus can also be shown. In the technique used to demonstrate phosphatase in bone it is necessary to decalcify the bone in an acid solution. As long as the acidity is carefully buffered to a pH above 4.8 the enzyme is only temporarily inactivated and can be readily reactivated in an alkaline solution. It seems reasonable therefore to assume that phosphatase is similarly physiologically inactivated but not destroyed in the weak acid medium of the cartilage remnants, and that it is histochemically demonstrable only after reactivation in an alkaline medium. The enzyme would then be physiologically inactive *in vivo* in these regions, even though both chondroitin sulfate and phosphatase are demonstrable at these sites *in vitro*.

In the replacement of the metaphyseal cartilage remnants the new bone matrix appears to be elaborated into the cartilage matrix. This supports the concept that the components of the cartilage matrix may become part of the new bone matrix. Phosphatase is present in amounts normally seen around young bone cells, and does not seem to be involved directly in the removal of cartilage matrix. The cartilage matrix of the remnants itself is saturated with phosphatase, and remains unchanged until new bone matrix is deposited. As this occurs, the cartilage matrix loses its metachromasia. Similarly phosphatase does not seem to be implicated in the removal of the bone of necrotic transplanted fragments. In support of the belief that the enzyme is not involved in bone or cartilage removal, the clinical findings can be cited of a low serum phosphatase in conditions, such as multiple myeloma, that involve bone destruction only with no new bone formation.

#### SUMMARY

The role of alkaline phosphatase in osteogenesis has been investigated by histochemical techniques with particular attention to its relationship to phosphate metabolism and matrix elaboration. The upper tibial epiphysis mainly, and other epiphyses as well of growing rabbits, and the costochondral junctions of newborn human beings were studied, as were bone grafts in growing rabbits. The findings in the newborn human beings were identical with those in the rabbits.

Phosphatase activity and free phosphate localization do not universally coincide. The enzyme appears to be intimately related to preosseous cellular metabolism and to the elaboration of a bone matrix that is chemically calcifiable. It remains possible, however, that phosphatase may be in some way involved in making inorganic salts available to the calcifiable matrix. If this function does exist it is a secondary one, since the elaboration of bone matrix, which is always associated with phosphatase activity, can and does occur in the absence of calcification. Calcification may occur later, in the absence of the enzyme.

There is evidence to suggest that cartilage matrix is utilized in the formation of bone matrix.

Phosphatase is physiologically active only in the presence of living cells. Where it is demonstrable in the absence of living cells, as in the cartilage remnants of the metaphysis, it appears to be physiologically inactive. Since phosphatase is temporarily inactivated in weakly acid media, and readily reactivated by alkaline solutions it is possible that the enzyme might survive in a physiologically inactive state in weakly acid tissues, and yet remain capable of histochemical demonstration *in vitro* in an alkaline medium.

Phosphatase is not related to the disappearance of chondroitin sulfate.

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## EXPLANATION OF PLATES

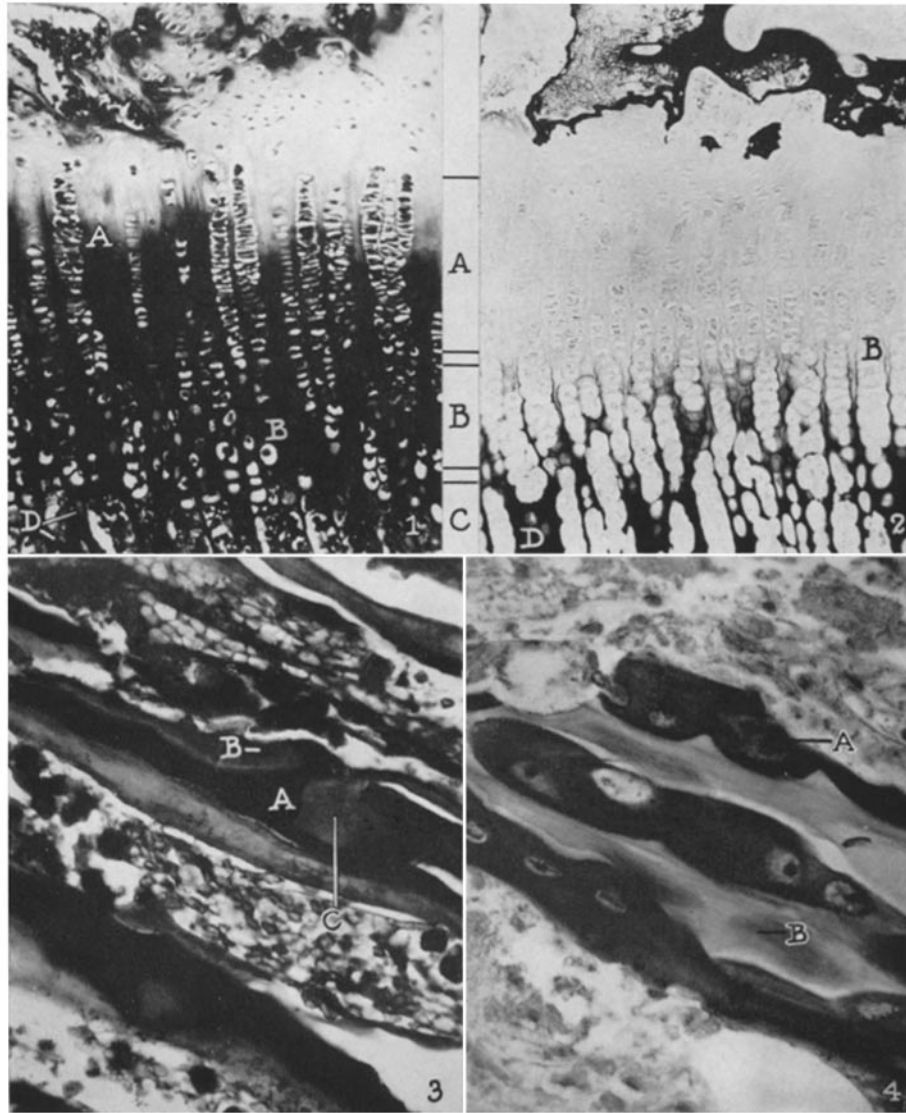
## PLATE 29

FIG. 1. Epiphyseal line of the upper tibia of a rabbit. The nuclei of all the cartilage cells stain for phosphatase, but the enzyme does not make its appearance in the matrix until the region of palisading cartilage cells is reached (*A*). Phosphatase exists in the region of hypertrophy of cartilage cells (*B*), and persists in the cartilage remnants (*D*), in the region of cartilage cell destruction (*C*). Gomori technique for alkaline phosphatase.  $\times 65$ .

FIG. 2. Another section of the specimen furnishing Fig. 1, stained for phosphates and photographed by indirect light. The cartilage cells are devoid of demonstrable free salts, and the phosphates in the matrix do not make their appearance until the region of hypertrophy of the cartilage cells is reached (*B*). Phosphates, along with phosphatase (Fig. 1), persist in the cartilage remnants (*D*), in the region of cartilage cell destruction (*C*). Cobalt technique for phosphates.  $\times 65$ . When the silver technique is employed, black deposits are noted in the cartilage cell nuclei.

FIG. 3. Trabeculae in the metaphysis of the upper tibia in a growing rabbit; stained for phosphatase. The enzyme is present in the central cartilage remnants (*A*) and surrounding osteoblasts that are engaged in bone formation (*B*). There is no increase in phosphatase concentration at the site of replacement of cartilage matrix by bone (*C*). Gomori technique for alkaline phosphatase.  $\times 65$ .

FIG. 4. Trabeculae in the metaphysis of a long bone of a rabbit, stained for bone matrix with the Van Gieson stain. The matrix is deposited around each osteoblast (*A*). As the islands grow they coalesce into a single homogeneous mass, which gradually replaces the cartilage remnants (*B*). In the remnants both chondroitin sulfate and phosphatase are demonstrable.  $\times 286$ .



(Siffert: Alkaline phosphatase in osteogenesis)

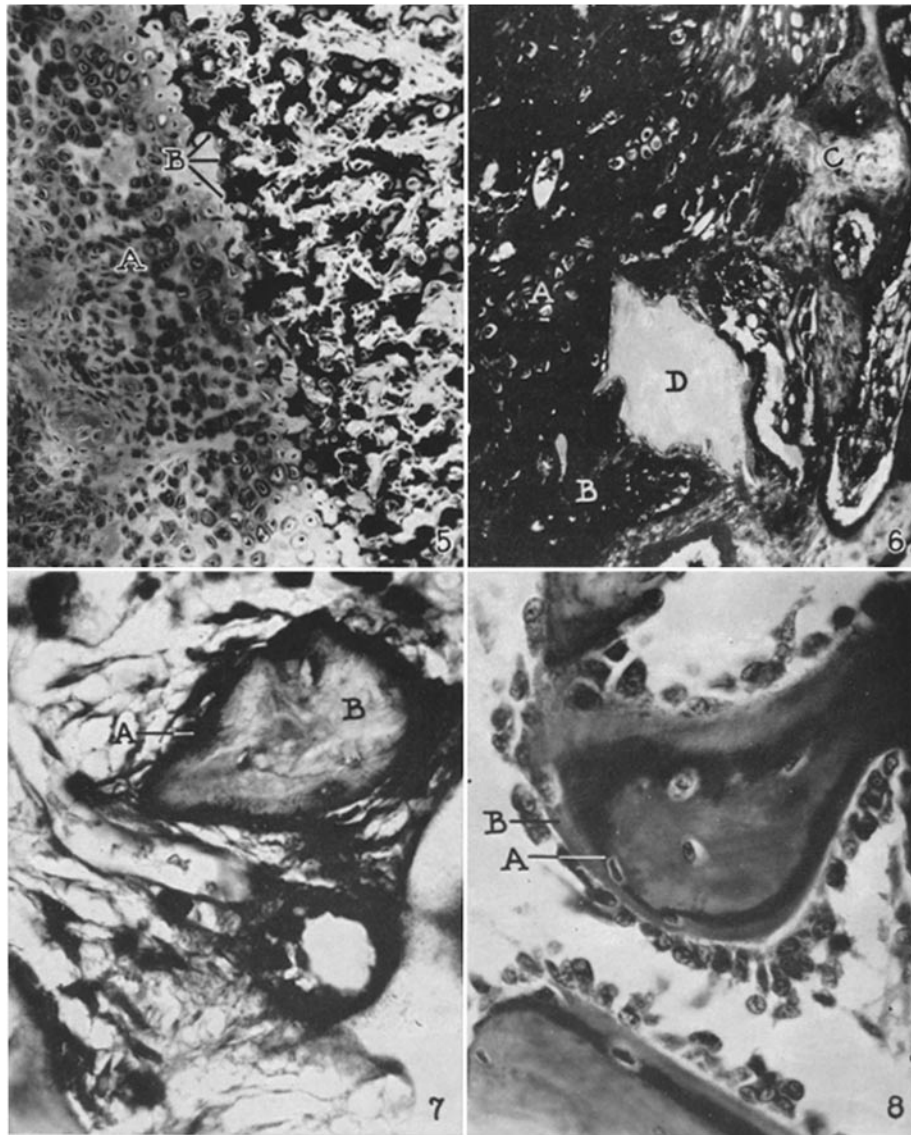
PLATE 30

FIG. 5. Enchondral bone formation in a graft bed in a rabbit; stained for phosphates (counterstained with hematoxylin and eosin to show the cartilage nuclei). The cartilage matrix (*A*) which stains heavily for phosphatase, contains no demonstrable phosphates. The phosphates appear at the site of new bone formation (*B*). Cobalt technique for phosphates.  $\times 66$ .

FIG. 6. Combined mesenchymal and enchondral bone formation in a graft bed in a growing rabbit; stained for phosphatase. The enzyme is deposited in the cells and matrix of the cartilage callus (*A*) and fibrocallus (*B*). It diminishes in concentration in the newly formed trabeculae (*C*). The necrotic bone transplant (*D*) containing no enzyme is easily identified in the osteogenic bed. Cobalt technique for phosphates.  $\times 66$

FIG. 7. Newly forming trabecula in a bone graft bed of a growing rabbit; stained for phosphatase. The enzyme is deposited around the osteoblasts on the surface of the trabeculum (*A*). The underlying bone matrix is relatively free of phosphatase (*B*). Gomori technique for alkaline phosphatase.  $\times 290$ .

FIG. 8. Newly forming trabecula in a bone graft bed in a growing rabbit; stained for phosphates. Although the enzyme and salt deposition frequently coincide, they often do not, as in this specimen. The phosphates are deposited beneath the surface of the trabecula (*A*), leaving clear matrix (*B*) between the surface and the site of phosphate deposition (see FIG. 7). Simultaneous demonstration of phosphatase and phosphate on the same section also showed the uncalcified osteoid seam beneath the surface phosphatase. Cobalt technique for phosphates.  $\times 290$ .



(Siffert: Alkaline phosphatase in osteogenesis)