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CALCIFICATION AND PHOSPHATASE *

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In 1923, Robison made the observation that if calcium or barium salts of hexosephosphate are incubated with an extract of bone containing phosphatase, a precipitate of calcium or barium phosphate, respectively, will form. This phenomenon led him to the idea that phosphatase may play a rôle in calcification of bone as it occurs *in vivo*. One year later he found that phosphatase is also present in developing teeth of young animals. Further studies by Martland and Robison showed that phosphatase is not present in cartilage before the appearance of the center of ossification but can be demonstrated in large amounts after the appearance of the center. Fell and Robison cultured embryonic fowl femora *in vitro* and found that the appearance of phosphatase coincides in time with the development of hypertrophied cartilage. Huggins showed that considerable amounts of phosphatase are present in and around bone-producing transplants of bladder mucosa. This finding was confirmed by Regen and Wilkins. The findings mentioned, together with the well known changes in plasma phosphatase level in diseases of the bones (Kay), support the assumption of a close causal relationship between calcification and the presence of phosphatase. Although it has been known since the experiments of Shipley that *in vitro* calcification of calcifiable tissues will occur even in purely inorganic solutions, apparently without phosphatase action, the results of recent studies by Gutman and Gutman indicate that even in the case of inorganic solutions phosphatase plays an important rôle by breaking down hexosephosphoric esters formed locally from glycogen and inorganic phosphate. In conclusion, it seems that phosphatase action is at least one of the mechanisms utilized by the organism to increase the $[Ca^{++}] \times [PO_4^{\equiv}]$ product locally beyond the critical value of 3.3×10^{-6}, which is the solubility product of secondary calcium phosphate at the pH of the blood.

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In 1939 a microtechnical method for the visualization of phosphatase action in tissue sections (Gomori, Takamatsu) became available. Shortly afterward, Ross, and Freeman and McLean published morphological observations on the presence of phosphatase in calcifying trichina cysts and in bone. So far no other histological observations on the relationship between calcification and phosphatase have been published.

In the present paper the relationship between phosphatase and calcification under normal and under a variety of pathological conditions, as shown by a new microtechnical method for the simultaneous visualization of preformed calcium salt deposits and of phosphatase activity, is presented.

Material and Methods

Since calcium salts are removed and phosphatase destroyed by decalcification, all material was embedded and cut without decalcification. This imposed a certain limitation on the material since tissues too hard to be sectioned without decalcification had to be excluded.

Slices of tissues were fixed in 80 per cent alcohol not later than 4 hours after death or removal at operation. Small embryos were fixed *in toto*. The tissues were subsequently dehydrated in 95 per cent and absolute alcohol and embedded in paraffin. Some of the tissues were fixed in ice-cold acetone for studies on acid phosphatase. Paraffin sections were cut from 4 to 6 μ in thickness.

The sections were stained with a modification of my method for the demonstration of phosphatase, devised for the purpose of simultaneous visualization of preformed deposits of calcium salts and of sites of phosphatase activity. The principle of the modification is this: insoluble calcium salts are first demonstrated by transforming them into black cobalt sulfide. The section is subsequently incubated with a solution of calcium glycerophosphate. The calcium phosphate precipitate formed by enzymatic action is stained in a different shade.

The method is as follows:

1. Fix fresh tissues in 80% alcohol, embed in paraffin.
2. Run paraffin sections through xylol and alcohols to distilled water.
3. Treat sections for from 6 to 12 hours with a 2% solution of cobalt acetate. Calcium phosphate and carbonate are transformed into the corresponding cobalt salts. Rinse sections thoroughly in distilled water.
4. Immerse sections for 10 minutes in dilute buffered solution of yellow ammonium sulfide (5 to 6 drops of yellow ammonium sulfide to a Coplin jarful of phosphate or maleate buffer of about pH 7). Unbuffered, strongly alkaline solutions of ammonium sulfide may destroy the enzyme. Cobalt phosphate and carbonate are transformed into black cobalt sulfide. Rinse in water.

5. Incubate sections for from 5 to 6 hours at 37° C. in the following solution:

2% sodium glycerophosphate	25 cc.
2% sodium barbital	25 cc.
Distilled water	50 cc.
2% calcium chloride	5 cc.
2% magnesium sulfate	2 cc.
Chloroform	a few drops

This solution will keep in the ice box for months. Before use add to this mixture a few drops of a 1% solution of some soluble sulfide, such as ammonium or sodium sulfide, in order to depress the solubility of the cobalt sulfide precipitate.

After incubation rinse sections thoroughly in distilled water.

6. Transfer sections for 15 minutes to a 1% solution of lead nitrate. The calcium phosphate precipitate formed at the sites of phosphatase activity is transformed into lead phosphate. Rinse with distilled water.

7. Stain sections in the following mixture for 15 minutes:

0.5% solution of methyl green	2 to 3 parts
0.5% solution of acridine red (Grübler)	1 part

Other brands of acridine red gave results which were much inferior. The staining solution should be not more than 4 weeks old. It should be kept in the ice box when not in use.

8. Differentiate in 95% alcohol, dehydrate in absolute alcohol, clear in xylol, mount in balsam.

Results: preformed deposits of calcium salts, black; sites of phosphatase activity, purplish red; nuclei, green-blue.

Observations

The normal material consisted of numerous embryos of the mouse, rat, guinea-pig, rabbit, dog, pig, chicken and man. The findings were essentially the same in all species.

The earliest sites of phosphatase-positive staining in the skeletal system were the perichondrium of the vertebrae and of the ribs, but in a somewhat later stage the perichondrium of practically all cartilage that in later life develops into bone became positive. The perichondrium contained phosphatase either all around the circumference of the cartilage or in certain patches only. Somewhat later the cartilage itself, especially the portion adjoining the positively-staining perichondrium, showed an intense reaction both in the cell nuclei and in the matrix. It was in these sites of positive phosphatase reaction within the cartilage that the first traces of granular calcification appeared. The deposits of calcium salts were always found in the centers of phosphatase-positive areas. Such areas were about 20 to 50 μ wide. At practically the same stage or somewhat later, cellular strands of connective tissue, representing the anlage of later membranous bones, became intensely positive. In their centers deposits of calcium salts, at first granular but later coalescing to solid strands, made their appearance. Not a single instance of deposition of calcium salts in phosphatase-negative areas could be observed in more than 50 embryos

of various species and ages. Phosphatase-positive cartilage or connective tissue without any signs of calcification was seen quite often, but since no serial sections were made it is impossible to tell whether these positive areas did or did not contain centers of calcification at some other level.

In the bone tissue the osteoblasts remained positive for some time but soon lost their positivity in the more central, older portions of the bone, whereas in all areas where apposition was active, *i.e.*, under the periosteum and in the epiphyses, the osteoblasts were always strongly positive.

Teeth were examined only in a few rat embryos. Intense phosphatase reaction was obtained in the stratum intermedium of the enamel organ and also in the connective tissue of the pulp, especially in a layer immediately subjacent to the odontoblasts in regions where calcified dentin was present. The ameloblasts and the odontoblasts were negative or, in some cases, showed traces of positive staining.

It seems to be appropriate to include here the observations on one case of experimental rickets in a rat, although rickets is not a normal condition. The proximal tibial epiphysis was examined. Phosphatase was found to be present only in the hypertrophied zone of cartilage, while the remainder of the epiphyseal plate was negative. No calcification was present in the cartilage matrix. The osteoid borders of the bone trabeculae were negative for phosphatase but the osteoblasts lining them were positive. These observations are in full agreement with those of Freeman and McLean.

Bone formation under the influence of transplants of bladder epithelium was studied in 8 dogs, 4 guinea-pigs and 4 rabbits. Six pieces of the epithelium of the urinary bladder were transplanted in each animal to both rectus sheaths (three on each side) according to the technic of Huggins. They were spread as flatly as possible, the surface of the epithelium facing the skin. In addition, in 4 dogs small pieces of bladder epithelium were buried in the parenchyma of the liver and spleen and between the muscular layers of the stomach. The transplants were removed at regular intervals over a period of 10 weeks.

Epithelial cyst formation was observed in from 6 to 10 days at the sites of all transplants in dogs and in guinea-pigs and in 1 rabbit. In 3 rabbits the transplants disappeared.

Up to the point of completed cyst formation there was no difference between the histological pictures of the fascial and the intraparenchymatous transplants. In all transplants the epithelium retained its originally phosphatase-positive staining in the transplanted as well as in the regenerated portions. However, after the stage of cyst formation

the events took an entirely different course in transplants to the liver, the spleen and the stomach, on the one hand, and in transplants to the fascia, on the other hand.

In the liver, the spleen and in the wall of the stomach the cysts grew to a certain size, became rather distended, the epithelium became flattened and the entire structure was surrounded by a thick capsule of connective tissue. The epithelial lining remained positive throughout. No phosphatase was seen in the capsule.

In the fascial transplants on the 6th day the connective tissue subjacent to the newly formed epithelium showed a few scattered, strongly phosphatase-positive fibroblasts. In the next few days the number of these phosphatase-positive cells rapidly increased, and at the same time the fibrillar ground substance between them also became diffusely positive. By the 9th and 10th day a thick plaque of coarsely fibrillar, osteoidlike, intensely phosphatase-positive connective tissue was found under the newly formed epithelium. The thickness of the plaque was from 20 to 200 μ. It was not in immediate contact with the epithelium but was separated from it by an intermediate phosphatase-negative layer of connective tissue, about 20 μ in thickness. No reaction of the type described was seen under the old surviving epithelium of the transplant. By the 13th to the 20th day some of the densest strands in the centers of phosphatase-positive areas showed a granular deposit of calcium salts. The calcified areas rapidly extended and ramified. By the end of the 1st month well developed bone with hemopoietic marrow was seen in all transplants. The bone was surrounded by a wide zone of a rather cellular, intensely phosphatase-positive connective tissue, simulating the picture seen in embryonic osteogenesis. The entire process was essentially the same in dogs, guinea-pigs and in the rabbit.

As mentioned, bone formed only in the connective tissue underlying the newly formed epithelium. This is in complete agreement with the observations of Huggins.

The pathological material will be divided in three groups: (1) bone tumors; (2) tuberculosis; (3) calcification in hyaline connective tissue.

1. *Bone Tumors*

Four cases of bone-forming osteogenic sarcomas were observed. The pictures they presented were closely similar. All of the tumors were very strongly positive for phosphatase, especially in their most cellular, peripheral portions. Both the cells themselves and the fibrillar intercellular substance partook in the reaction. Granular deposits of

calcium salts were seen in strands of connective tissue within the positive areas. These deposits gradually coalesced until the entire stroma was outlined in black, with clear cells encased in the meshes. Two of the tumors had a marked tendency to sclerosis. In the sclerosing areas the reaction was far less strongly positive than in the cellular ones, except for groups of strongly positive cells growing within the vessels. Highly sclerotic, almost acellular areas were found to be entirely negative.

In addition to the cases mentioned, one case of osteoplastic metastasis of a cancer of the breast was observed. The tumor cells themselves were entirely negative for phosphatase, whereas the stroma was strongly positive, with extensive calcification and bone formation in the centers of positive areas.

Two giant cell tumors and two fibrosarcomas of bone, without new bone formation, were entirely negative for phosphatase, except for a few phosphatase-positive capillaries.

2. *Tuberculosis*

The material consisted of 42 rabbits, 58 guinea-pigs and 3 human cases. The rabbits were inoculated with 0.2 to 2 mg. of the culture of a bovine strain subcutaneously or intratracheally, the guinea-pigs with 2 mg. of a human strain subcutaneously. The animals were killed at regular intervals or allowed to die over a period of 55 weeks.

Observations on the three species will be given separately.

Rabbit. In the normal rabbit's lung phosphatase occurs in variable amounts in three different sites: first, the lining of the alveoli; second, the endothelium of blood vessels; third, in leukocytes and lymphocytes. The rabbit's neutrophils ("specials") are by no means as uniformly positive for phosphatase as those of the guinea-pig. Only a certain percentage of them are positive, and the ratio of positive to negative is usually much lower within the blood vessels than it is within the tissues. There is no morphological difference between the positive and negative cells; in fact, the two kinds are entirely indistinguishable from each other without the use of the phosphatase reaction. For the time being it is impossible to tell what factors control the number of positive and negative leukocytes. The above statements apply also to the lymphocytes. Within the blood stream most of them are negative, whereas in lymphocytic infiltrates the positive type prevails.

Early tuberculous granulation tissue without signs of necrosis was found to be entirely negative for phosphatase except for that contained in leukocytes and lymphocytes. As soon as necrosis set in phosphatase

appeared in the centers of the necrotic areas, first in the shape of an irregular, coarse network, later coalescing to a uniform, more or less rounded area of very intense phosphatase reaction. The enzyme was not carried in leukocytes since it appeared in all necrotic tubercles, regardless of whether they did or did not contain leukocytes. The positivity of the areas of necrosis did not last long as phosphatase soon disappeared from the centers, only to appear in the area of fresh necrosis in the peripheral zone of spread. In this way ever receding and enlarging rings of phosphatase-positive staining occupied the inner, necrotic area of the zone of spread. If the process came to a standstill, phosphatase disappeared altogether.

The intensity of phosphatase-positive staining in the early necroses was so high that it is impossible to account for the amount of enzyme present in them from purely local sources. As mentioned, phosphatase is not carried in by leukocytes. The synthesis of such a highly specific enzyme by a necrotic mass is improbable. The most probable explanation seems to be the adsorption of the enzyme from the blood and from the tissue fluids by the fresh necrotic material, owing to some physicochemical property of the latter at a certain stage of development. At a later stage this property is lost and consequently the enzyme is eluted from the lesion. It should be mentioned here that in two rabbits necrosis of the lung and liver were produced by the intraparenchymatous injection of alcohol. No phosphatase was found in areas of necrosis produced by this method.

The changes mentioned were remarkably uniform in the pulmonary lesions of all animals except two. In the latter cases phosphatase failed to appear in the necrotic areas. The phosphatase picture of lesions in other organs was far less regular. Most areas of necrosis were found to be free of phosphatase, regardless of their age; and even when phosphatase was present, the reaction was much fainter than in the lesions of the lungs.

Calcification was observed in 15 cases out of 42. It involved pulmonary lesions only, although extensive tuberculosis of the spleen, liver and kidneys was present in many of the cases. The earliest date at which calcification could be demonstrated was 38 days after inoculation (average: 101 days).

If calcification took place, its earliest traces invariably occurred in the center of some phosphatase-positive area. Calcification did not alter the peripheral shift of phosphatase as described. Phosphatase receded in the shape of a ring from the central calcification in the typical way. In the peripheral positive area new centers of calcification often developed which, if they coalesced, led to the formation of im-

perfect concentric calcareous shells. This pattern of concentric rings is well known from x-ray studies on human tuberculosis.

Guinea-Pig. The normal lung of the guinea-pig does not contain any phosphatase at all, except in the scattered leukocytes which are constantly and strongly positive in this species and in the epithelial lining of some of the bronchi.

Tuberculous granulation tissue as well as areas of necrosis were entirely free of phosphatase, except for the leukocytes. In my series of guinea-pigs the lesions were of a markedly exudative character and the tubercles in practically all cases were densely infiltrated by leukocytes. The latter often formed strongly phosphatase-positive abscesses in the centers of the lesions. After the breakdown of the leukocytes the enzyme diffused in all directions. All the phosphatase in guinea-pig tubercles comes from leukocytes. These data are in complete agreement with those presented by Takeuchi and Takamatsu.

Calcification was observed in only 3 cases out of 58. It involved both pulmonary and splenic lesions. The earliest date at which calcification could be found was 55 days after inoculation. It always occurred in the centers of necrotic areas. Phosphatase could be seen around some of the calcified centers, but owing to the small number of cases no further conclusions can be drawn.

Man. The normal human lung contains extremely variable amounts of phosphatase in three sites: first, in the lining of the alveoli; second, in the endothelial lining of capillaries; third, in the epithelium of the bronchi. Human leukocytes are free from phosphatase.

The number of human cases observed being only three, no definite conclusions can be drawn as to the phosphatase picture in human tuberculosis. Tuberculous granulation tissue was found to contain no phosphatase. There was a marked perifocal intensification of the reaction in the alveolar lining. In one case the outer fibrous wall of a cavity was found to be positive. Takeuchi and Takamatsu made practically the same observations. Calcification was observed in one old, encapsulated lesion, with no trace of phosphatase around it.

3. *Calcification of Hyaline Connective Tissue*

This group includes 6 examples of early arteriosclerosis of the aorta; 2 of sclerosing and calcifying pyelonephritis in human beings and 1 in a rabbit; 2 calcifying goiters and 1 calcifying islet cell tumor of the pancreas. In all of these, granular calcium salt deposits were observed in very dense, almost acellular, connective tissue. No trace of phosphatase was found around any of the granules of calcification.

As mentioned, sections of practically all specimens were also stained

by my technic for the demonstration of acid phosphatase. In no instance could the presence of this enzyme in or around calcareous deposits be demonstrated.

Comment

Calcification of living or recently necrosed tissue seems to start invariably in the centers of intensely phosphatase-positive areas. On the other hand, calcification of sclerosed, hyaline connective tissue apparently can and does occur without phosphatase action. Although it is possible that the latter type of calcification may be initiated by phosphatase action followed by a rapid disappearance of the enzyme, the fact that phosphatase was not found in a single instance around deposits of calcium in hyaline connective tissue is against such an assumption. Obviously there are other mechanisms besides phosphatase action capable of raising the $[Ca^{++}] \times [PO_4^{\equiv}]$ product beyond the critical level. The results of Ross clearly show that under certain conditions tissues may calcify far below the $[Ca^{++}] \times [PO_4^{\equiv}]$ value of 3.3×10^{-6}, required for the calcification of bone. The nature of the mechanism involved in calcification at low $[Ca^{++}] \times [PO_4^{\equiv}]$ products is not understood at present. One possibility, unexamined so far, would be the presence of some protein with a higher dissociation constant than that of plasma proteins, resulting in a higher local concentration of calcium ions at the same level of total calcium.

An interesting problem is the source of phosphatase in the connective tissue around transplants of bladder epithelium. The two possibilities considered are: (1) simple diffusion of the enzyme (and, according to Huggins, of calcium and phosphate) through the thin layer of newly formed epithelium into the surrounding tissue, and (2) specific inductive power of the bladder epithelium on certain kinds of connective tissue, resulting in the proliferation of phosphatase-producing fibrocytes. The theory of simple diffusion does not explain the fact that calcification and bone formation is regularly produced at certain sites (fascia), while consistently negative results are obtained at other sites (liver, spleen, stomach), unless the presence of an antiphosphatase in these latter organs is hypothecated. On the other hand, the theory of specific induction would fit well into proved facts of embryology. Gruenwald published striking instances of strictly region-bound inducibility of the mesenchyme by the growing wolffian duct.

An attempt was made to solve this problem by transplanting other, normally phosphatase-positive, kinds of epithelium to the fascia and by observing whether or not phosphatase and bone formation would

appear around them. In 3 dogs pieces of duodenal mucosa and in 5 more dogs and in 3 guinea-pigs pieces of the epididymis (finely minced to provide for a large surface of contact) were transplanted to the rectus sheath. The transplants of duodenal mucosa were complete failures. They all disappeared within 3 weeks. The transplants of epididymis took well and grew considerably in 10 weeks. Many epithelium-lined cysts were observed. Although the epithelium in many places retained fully its phosphatase-positive character, no phosphatase and no bone formation were observed in any of the transplants. The results of these experiments seem to support the theory of specific induction rather than that of diffusion. Induction of bone formation by bladder epithelium seems to be a favorable case in which it is possible to trace the induction mechanism one step farther back than in most other instances.

SUMMARY

A new microtechnical method for the simultaneous demonstration of preformed calcium salt deposits and of sites of phosphatase activity is presented, together with observations made with this method on normal and pathological calcification.

Calcification of living or recently necrosed tissues seems invariably to involve phosphatase activity. On the other hand, calcification of hyaline connective tissue occurs without any phosphatase action. An attempt is made to explain the difference between the mechanisms of these two types of calcification.

Acid phosphatase plays no rôle in calcification.

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[*Illustrations follow*]

DESCRIPTION OF PLATE

PLATE 24

Calcifications, black; sites of phosphatase activity, purplish red; nuclei and ground substance of cartilage, green-blue.

FIG. 1. Parasagittal section of a rat embryo, 18 mm. long, showing processes of vertebrae. Positive reaction in the perichondrium. × 36.

FIG. 2. Lower incisor of a newborn rat. × 30.

FIG. 3. Bone-producing transplant of bladder mucosa. Positive reaction in the basal layer of the epithelium and in a thick strand of connective tissue in the upper right part of the field. The bone is surrounded by a wide zone of positive reaction. × 36.

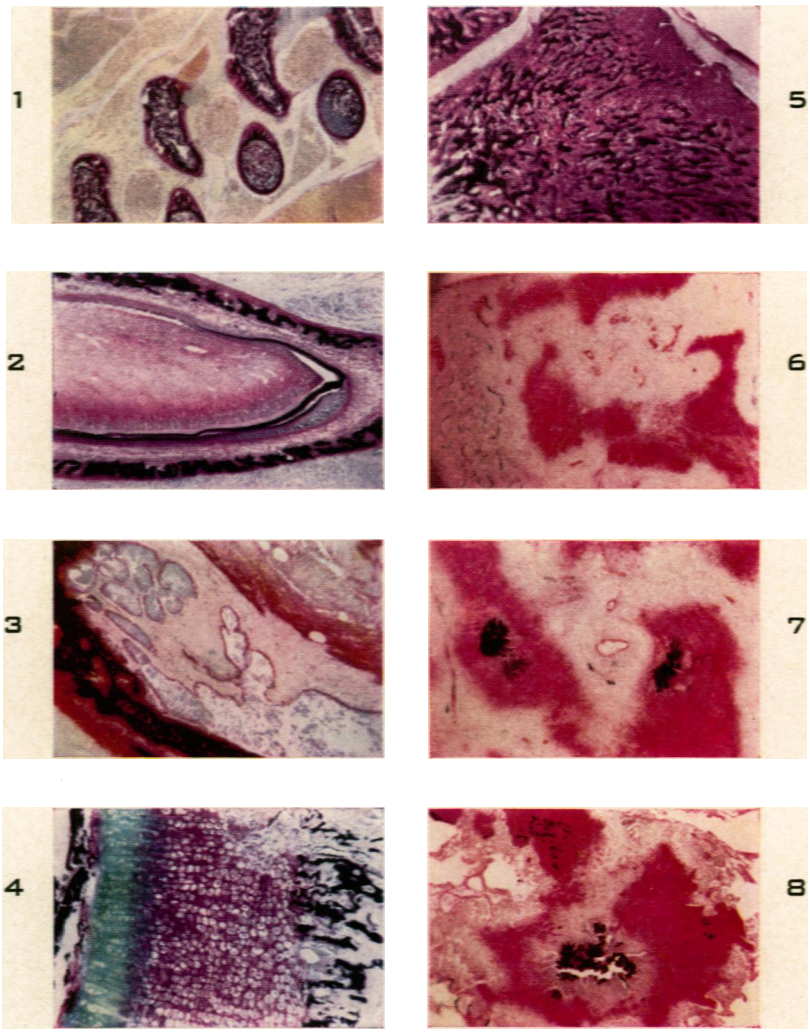
FIG. 4. Upper tibial epiphysis of a rachitic rat. The zone of hypertrophied cartilage is strongly positive for phosphatase. × 48.

FIG. 5. Osteogenic sarcoma of the tibia. The tumor is strongly positive, the surrounding connective tissue is negative. × 36.

FIG. 6. Phosphatase reaction in the center of an early necrotic tubercle in the rabbit's lung. × 36.

FIG. 7. Calcification in centers of phosphatase-positive areas (rabbit's lung). × 36.

FIG. 8. Peripheral recession of phosphatase reaction with secondary calcification at the periphery (rabbit's lung). × 30.



Gomori Calcification and Phosphatase