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ALKALINE PHOSPHATASE AND CALCIFICATION

HISTOCHEMICAL INVESTIGATIONS ON THE RELATIONSHIP
BETWEEN ALKALINE PHOSPHATASE AND CALCIFICATION

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PREFACE

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The Danish text has been translated by *Harry Cowan, B.Sc.*

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Chapter I.

INTRODUCTION

Previous hypotheses on the role of phosphatase in the mechanism of calcification.

The aim of this study is to review, from an essentially histological standpoint, the association between alkaline phosphatase activity, calcification and bone formation. It is based in part on the author's previously published investigations.

Robison (1923) was the first to suggest an association between the enzyme alkaline phosphatase and calcification in cartilage and bone. He showed that the enzyme was found in cartilage where calcification occurred, but not in cartilage which did not calcify (tracheal cartilage, uncalcified costal cartilage). Later, the enzyme was also demonstrated in bone at the stage of calcification (*Robison & Soames* 1924, *Martland & Robison* 1924). Originally, *Robison* explained calcification as a hydrolysis of a phosphoric acid ester, the calcium salt of which is easily soluble in water, releasing phosphate ions. He assumed that the blood is normally in equilibrium with the inorganic constituents of bone — the bone salt — and that the condition of supersaturation necessary for the precipitation of this salt occurs locally in hypertrophic cartilage and in osteoid tissue, as a result of the splitting up of the phosphoric esters present in the blood by the alkaline phosphatase present at these sites. However, further investigations showed that this simple theory was inadequate to explain why some tissues calcify and others, under normal physiological conditions, do not. For example, alkaline phosphatase has been demonstrated in kidney tissue (*Robison* 1923), and although embryonic avian cartilage has a high content of alkaline phosphatase (*Fell & Robi-*

son 1934), it does not calcify in the embryonic stage prior to the formation of the primary medullary cavity, as does mammalian embryonic cartilage (*Fell* 1925). It was also shown that rachitic cartilage could calcify *in vitro* in solutions which contained only inorganic compounds of calcium and phosphorus (*Shipley et al.* 1925 and 1926, *Robison & Soames* 1930).

In his further formulation of the phosphatase theory of the calcification mechanism, *Robison* conceived two factors as operating. 1) The phosphatase mechanism, causing a supersaturation of phosphate ions in the matrix of bone and cartilage, and 2) the inorganic mechanism, or local factor, favouring the deposition of calcium phosphate from supersaturated solutions, whether this state of supersaturation is caused by an enzymatic hydrolysis of phosphoric acid esters, or by a supersaturated solution diffusing into the cartilage from outside (*Robison, MacLeod & Rosenheim* 1930, *Robison & Rosenheim* 1934). *Roche* and co-workers, in particular, carried out further investigations on the relation between alkaline phosphatase and calcification (see review of these studies by *Roche* 1946). The result of these studies, which will be discussed later together with the work of *Robison* & co-workers, was a changed conception of the significance of phosphatase for calcification and bone formation. Calcification is conceived by *Roche* as being due to a local factor, which causes calcium phosphate to be precipitated from the dissolved salts of the blood and bound to the ground substance of the tissue involved. The phosphatase is supposed to be significant both for the ground substance acquiring this local factor or property, and also for hastening the calcification by bringing about a local surplus of phosphate ions. The function of the phosphatase should thus be to speed up the calcification, but not to trigger off this process.

The demonstration of phosphorylating enzymes in hypertrophic cartilage, whereby the break-down of glycogen could explain the appearance of ester-linked phosphate as a substrate for the alkaline phosphatase, has not clarified the significance of the latter for calcification (*Gutman & Gutman* 1941).

As a result of recent developments, primarily a major research

program by several American groups, there has been a clear tendency to attribute less significance — or none at all — to the function of alkaline phosphatase in the calcification process and at most grant it significance for the formation of the ground substance. The calcification process is conceived as being due to a local factor bringing about calcification, provided the necessary humoral conditions are present. This local factor also includes Robison's second factor, but it is not considered proven that the phosphatase — even though found present at the site of calcification — has anything to do with the calcification (*McLean et al.* 1946). Further investigation on this local factor has provided much information on the relation between ground substance and the precipitated crystals, and on the histochemistry of the ground substance. A more physicochemical orientation towards the calcification process has developed as a result of physicochemical investigations on the crystal growth and the composition of the minerals of the bones. The direction of the work has been away from the phosphatase theory, but as yet nothing has been put in its place. A local characteristic or property is conceived, associated with proteins, and causing the formation of the first crystals: these, by their further growth, would provide the association between organic structure and mineral which is characteristic of bone tissue. Instead of further work on the phosphatase mechanism, it is considered "more profitable to speculate on ways and means by which crystallization might be initiated" (*Neumann & Neumann* 1953). The following unchallenged remark by *McLean* (1955) clearly shows that in the opinion of this and other American investigators phosphatase lacks significance in calcification: "With reference to phosphatase, I am happy to note that, for the first time in years, it has been possible to sit through a conference on mineralized tissues without having to discuss the role of alkaline phosphatase in calcification. I have long subscribed to the belief that this enzyme is not required to provide phosphate ions for the bone salt, but that it probably assists in making the matrix of bone and of cartilage calcifiable. I hope that the absence of arguments to the contrary at this time means that this ghost is laid".

Histochemical methods of phosphatase staining.

A number of circumstances have led to renewed histochemical investigation on the role of alkaline phosphatase in the calcification process. One of these is the introduction of new principles in the histochemical demonstration of phosphatase activity. This activity was first demonstrated histochemically by *Robison*, who described the precipitation of calcium phosphate in rachitic rat cartilage in this first study on the significance of phosphatase for calcification (1923). The cartilage was incubated in a solution of calcium hexose monophosphate, and the precipitated calcium phosphate became visible after silver staining by the *von Kossa* technique. Practically the same histochemical staining procedure was subsequently described by *Takamatsu* (1939) and *Gomori* (1939). The latter used the *von Kossa* technique, but was also able to demonstrate the precipitated calcium phosphate by treatment with cobalt nitrate and ammonium sulphide, whereby the calcium ions were exchanged with cobalt ions, which were then precipitated as sulphide. The advantages and disadvantages of the method will not be gone into here. Attention will merely be drawn to the difficulty of investigating the relationship between the alkaline phosphatase activity and calcification, as the calcium phosphate present in the preparation is stained in the same way as that formed by the enzymatic activity. This difficulty has undoubtedly been reduced by prior decalcification in a weakly acid solution, so as not to inactivate the phosphatase completely (*Lorch* 1946, *Greep Fischer & Morse* 1947), but as will be shown later, decalcification, in spite of all precautions, reduces the phosphatase activity, and this has introduced errors in the estimation of the alkaline phosphatase content of osteoid tissue.

The new histochemical methods for determining phosphatase activity use naphthyl phosphate as a substrate for the phosphatase. The naphthol released in the enzymatic decomposition is coupled to a diazo-compound, forming a waterfast dye (*Menten, Junge & Green* 1944). The method was much improved by the introduction of stable diazo-compounds for the coupling (*Manheimer & Seligman* 1948), and diazo-compounds have subsequently been

found which not only give more sparingly soluble dyes on coupling to naphthol but have less inhibitory effect on the enzymatic process (*Grogg & Pearse* 1952). The diazo methods have several advantages: above all, the adsorption artefacts are avoided which were frequent with the *Takamatsu-Gomori* method (*Novikoff* 1951, *Gomori* 1951). Further there is the most important advantage that when investigating the relation between phosphatase and calcification, the calcium phosphate already present does not stain in the histological preparation.

Author's investigations.

Another circumstance which led the way to further investigation of the phosphatase-calcification relationship was the demonstration of the fact, not previously recognized, that alkaline phosphatase activity can arise during the cellular degeneration of previously phosphatase-free cells (*Henrichsen* 1956 a), and that certain calcifications in tissue cultures had to be considered as the result of alkaline phosphatase activity (*Astrup & Henrichsen* 1954). These circumstances led to two conclusions. First, the possibility had to be considered of alkaline phosphatase activity during cellular degeneration in tissue which otherwise contained no alkaline phosphatase, and where it had thus been impossible previously to associate calcification with phosphatase activity. Secondly, there appeared to be grounds for investigating the histochemical relation between alkaline phosphatase activity and calcification in tissue cultures, which investigation had not previously been carried out. The results of these investigations have already been published (*Henrichsen* 1956 b, c).

The present thesis discusses two topics, the occurrence of alkaline phosphatase activity in cellular degeneration, and the connection between phosphatase and calcification. The work comprises both a summary of the results of the author's own histochemical investigations, which are discussed on the basis of the results of previous investigators, and a hypothesis as to the mechanism of calcification, based on the histochemical results. The hypothesis is discussed on the basis of earlier investigations on the mechanism of calcification.

Chapter II.

ALKALINE PHOSPHATASE ACTIVITY IN CELL DEGENERATION

Previous investigations on alkaline phosphatase activity in tissue cultures.

Histochemical investigations on the occurrence of alkaline phosphatase in cells cultivated *in vitro* have been carried out by Wilmer (1942), Rodova (1948), and Chèvremont & Firket (1949), all using the Takamatsu-Gomori method. These investigations showed that the cytoplasm of heart fibroblasts and fibroblasts from the subcutaneous tissue contained only extremely slight amounts of phosphatase, or none at all, whereas osteoblasts contained considerable amounts of the enzyme, as can also be seen in histological preparations of bone or periosteum. In all cases, the nuclei showed the staining reaction for alkaline phosphatase.

The expression "fibroblast culture" in the present study denotes a tissue culture from pieces of the heart or the musculature of the extremities of 9-day-old chick embryos. The expression "osteoblast culture" denotes a tissue culture from pieces of the frontal bones of 11-day-old chick embryos. The osteoblast cultures consist of two cell types, 1) phosphatase-containing spindle-shaped cells, osteoblasts growing out from the innermost layer of the periosteum, and 2) non-phosphatase-containing spindle-shaped cells, growing out from the peripheral layer of the periosteum, these cells being regarded as fibroblasts. However, an examination of the periosteum showed a gradual transition between these two cell types. This will be discussed later.

The author's initial investigations on the phosphatase activity in tissue cultures from chick embryos showed that a powerful phosphatase activity was present in the osteoblasts of the first pas-

sages. Thereafter, the activity diminished somewhat, but could still be demonstrated even after 7 weeks of cultivation (21 passages). Alkaline phosphatase could not be demonstrated in heart fibroblast cultures and in cultures of fibroblasts from the musculature of the extremities. The results of the investigations thus agreed overall with those of the investigations cited above. However, phosphatase could not be demonstrated in the nuclei, which was in agreement with more recent investigations using the diazomethods for demonstrating phosphatase (*Henrichsen* 1956 a).

After this confirmation of earlier investigations, demonstrating an absolute difference in the alkaline phosphatase content of fibroblasts and osteoblasts, experiments were started on bone induction in tissue cultures with the aid of bone marrow extract, as previously attempted by *Bertelsen* (1944). Here, the intention was to use phosphatase activity to indicate whether fibroblasts had been converted to osteoblasts in the tissue cultures. From these investigations, and by examining tissue cultures where circular zones of calcification had appeared, as described by *Astrup & Henrichsen* (1954), it was found that phosphatase activity *could* arise in tissue cultures of fibroblasts cultivated in normal media without the addition of bone marrow extract. As a consequence of this observation, further investigation of the phosphatase activity in osteoblast cultures and fibroblast cultures became necessary.

The formulation of the problem.

The problems presented at the start of the work can be formulated as follows:

- 1) Under which circumstances does alkaline phosphatase activity appear in tissue cultures of fibroblasts cultivated in normal media?
- 2) With which region of the cell is the phosphatase activity associated?
- 3) Is the phenomenon associated with cell cultivation *in vitro*, or are similar conditions also found *in vivo*?

4) Is the phenomenon particular to fibroblasts, or do similar effects appear in other cell types?

Alkaline phosphatase activity on cell degeneration in tissue cultures of fibroblasts.

Cover slip cultures of fibroblasts from the heart and from the musculature of the extremities in 9-day-old chicken embryos and of osteoblasts from 11-day-old chicken embryos were used for the investigations. The cultures were transferred to new media at intervals of three, two and two days (Monday - Wednesday - Friday). At every third transfer, only the outer two migration zones were used for further cultivation. This was the usual method of cultivation used in our laboratory, which throughout many years of use had never given unsatisfactory results. However, it was later learned that in a few other tissue culture laboratories the cultures are always transferred every second day, as a three day interval can give unsatisfactory results (*Lettré*, personal communication).

With the cultivation technique used, very pronounced phosphatase staining was found in the osteoblast culture during the first passage; the phosphatase activity was reduced on further cultivation, but could nevertheless still be demonstrated after cultivation for 7 weeks *in vitro* (21 passages).

Employing the same technique of cultivation, phosphatase activity was only rarely found in fibroblast cultures, and then only as a faint staining reaction, clearly different from the strong red stain characterizing the phosphatase activity in osteoblast cultures. It was found on analyzing a large number of cultures that phosphatase activity could be demonstrated only in those culture portions originating from migration zones where three days had elapsed between transfers. When a change was made to transferring the cultures every third or fourth day, and older parts of the cultures were retained instead of cultivating only from the two outer migration zones, a vigorous staining for alkaline phosphatase activity was found with great regularity along the cut edges produced by cutting out the cultures when transferring them to new

growth medium. The phosphatase activity could also be demonstrated in the form of a circular staining reaction in the periphery of the cultures when 4—5 days had elapsed since the last transfer.

Phase contrast microscopy of the phosphatase-stained fibroblasts, which was possible after alcohol extraction of the precipitated dye, showed clear morphological changes in these cells compared to the "normal" phosphatase-free fibroblasts. The cell form had changed from slender spindle-shaped cells to larger, more irregular cells with several short processes. In the non-phosphatase-stained "normal" fibroblasts, oval nuclei were found with two distinct nucleoli, placed each at opposite ends of the nucleus. In the fibroblasts containing phosphatase, the nuclei were swollen, somewhat rounded, and the nucleoli had fused together into one large mass, or were very indistinct. In several cases it was difficult if not impossible to recognize the nucleus by phase contrast microscopy. Finally, a coarse granulation of the cytoplasm was usually found, not present in the "normal" fibroblasts. *Zollinger* (1948 a and b), using phase contrast microscopy, had found similar changes in cells undergoing "hydropic degeneration". As the phosphatase activity occurred in the cells as a result of cultivation beyond the optimal period for normal growth, and at those points in the cultures where experience has shown that cell degeneration arises (*Fischer, Fogh and Bohus Jensen* 1945), the conclusion was unavoidable that alkaline phosphatase activity arose during cell degeneration in fibroblasts cultivated *in vitro*. In further confirmation, it might be mentioned that particularly pronounced phosphatase activity was found in cultures of heart fibroblasts where the plasma used as growth medium came from cocks in their moulting period, i. e., spring and autumn (*Henrichsen*, unpublished observation). The use of such plasma causes poor growth in the cultures and pronounced cellular degeneration. This fact has been known for several years in tissue culture laboratories, and has given trouble for short periods in spring and autumn.

It has been possible to reproduce the phenomenon of alkaline phosphatase occurrence in fibroblasts, using other tissue culture methods. For example, it occurs in Carrel flask cultures, where

the cells are cultivated in a coagulum with a supernatant fluid phase consisting of serum and Tyrode's solution, and also in cultures grown on perforated cellophane in fluid medium, as described by *Evans & Earle* (1947).

In those cells where the stain precipitate was still slight enough to reveal the cell structure and appeared only as individual granules of stain, phase microscopy always showed these granules, and therefore the phosphatase, lying in the cytoplasm, clearly distinct from the cell nucleus.

It is possible that *Chèvrement* and *Firket* had already observed alkaline phosphatase activity *in vitro* as a result of fibroblast degeneration. They described how they occasionally found cells in the cultures, somewhat enlarged and with intensely stained cytoplasm and nucleus, clearly different from the very faint staining of normal cells; however, they assumed that the condition was an artefact. *Gomori* (1943) described alkaline phosphatase activity in the degenerated cells found in the margin of tuberculous necrotic areas. *Gomori* assumed that the phosphatase originated from the blood plasma and was adsorbed on the degenerated cells, being once more liberated by them at the onset of necrosis, as the central, necrotic region showed no sign of phosphatase staining. An adsorption phenomenon cannot be involved, however, as the phosphatase staining also appears in fibroblasts cultivated in a fluid medium which has been heated to 60° for half an hour, irreversibly inactivating any possible phosphatase in the growth medium (*Henrichsen* 1956 a).

Alkaline phosphatase activity on cell degeneration in tuberculous lymph nodes.

Further investigations were carried out with mesenterial lymph nodes from pigs destroyed on account of infection with avian tuberculosis (*Henrichsen* 1956 c). In this material, as also described by *Gomori*, positive phosphatase staining was found in those cells forming the most peripheral part of the tuberculous inflammatory process. In contrast to the cells in the tissue cultures, hydropic

degeneration was not found in this case, but pyknosis and karyorrhexis. As *Gomori* showed, the phosphatase activity was only present as long as the cells were still in the stage of degeneration; by the time actual necrosis and karyorrhexis developed, the phosphatase activity had disappeared. This fact will be discussed later in the section on calcification and phosphatase.

Peripheral to the region with phosphatase-containing, degenerated cells, cells were found in which glycogen had become stored, and peripheral again to these, collagenous connective tissue was found. As the degeneration develops and the phosphatase activity appears, the glycogen gradually disappears; hypertrophic hyaline cartilage shows a similar state of affairs (see below). It seems probable that the occurrence of alkaline phosphatase activity in the degenerating cells is a stage in a complete change of cell metabolism, but we do not know as yet what changes really take place. So far, the first histochemically demonstrable expression of this change is storage of glycogen. The change could be conceived as a transition from aerobic to anaerobic metabolism, in which the break-down of glycogen is the essential source of energy. The transition to anaerobic metabolism must in this case be considered as due to changes in the enzyme mechanism of the cell, and not to reduced oxygen tension, as it has been shown that the cell degeneration which occurs in the peripheral cells of tissue cultures, and which in the experiments mentioned here produced a circular phosphatase staining, is not due to reduced oxygen tension (*Fischer, Fogh & Bohus Jensen* 1945).

Alkaline phosphatase activity in cartilage.

Robison (1923) was the first to demonstrate alkaline phosphatase activity in hypertrophic cartilage. He showed that an aqueous extract of hypertrophic cartilage contained an enzyme which could split the phosphate ion from hexose monophosphate. It has been mentioned already that *Robison* was also the first to demonstrate the phosphatase in hypertrophic cartilage by a histochemical method, without actually describing his procedure as such.

The presence of phosphatase activity in hypertrophic hyaline cartilage was subsequently confirmed repeatedly by histochemical investigations, using both the *Takamatsu-Gomori* method with or without decalcification, and the diazo methods (*Moog* (1944), *Lorch* (1947), *Greep, Fischer & Morse* (1948), *Zorzoli* (1948), *Follis & Berthong* (1949), *Morse & Greep* (1951), *Siffert* (1951), *Schajowicz & Cabrini* (1954), *Manheimer & Seligman* (1948)). The present author's investigations have added nothing new to the above (*Henrichsen* 1958). The hypertrophy of the cartilage cells was generally considered by the earlier observers as an expression of cellular degeneration (*Häggqvist* 1929), and several authors still share this impression (*Bloom* 1943). The demonstration of alkaline phosphatase activity in hypertrophic cartilage resulted in *Fell & Robison* (1933) putting forward the view that the hypertrophy was not an expression of a cellular degeneration but of a specific differentiation. When *Gomori*, by a histochemical technique, subsequently found phosphatase activity associated with undoubtedly degenerated cells, he assumed that this was due to adsorption, as he did not believe that a cell in the process of degeneration could synthesize a new enzyme. Once it was demonstrated that phosphatase activity could in fact arise in cells in the course of degeneration, there was no reason for not continuing to regard cartilage cell hypertrophy as a phenomenon of degeneration. Actually, the question of nomenclature is purely semantic; it is also possible to argue against using the term cellular degeneration for a condition which is a normal stage in the life history of every cartilage cell, from its formation by mitosis until its death and calcification. By using the word degeneration, however, agreement is established between the changes taking place in the cell (vacuolization of the cytoplasm, chromatolysis) and general verbal usage. On the other hand, the phosphatase content of osteoblasts must be considered as expressing a process of differentiation; the enzyme is present in completely normal cells without the changes characteristic of degeneration. The amount of phosphatase increases with increasing degree of differentiation (*Henrichsen* 1958) and decreases with the onset of ossification, as osteocytes do not con-

tain alkaline phosphatase, although it appears again in the degeneration of osteocytes, the so-called "onkose" (*Manjo & Rouiller* 1951).

If it is assumed that the phosphatase activity in hypertrophic cartilage is an expression of a degenerative phenomenon, and the phosphatase activity in osteoblasts an expression of a process of differentiation, the difference between the phosphatase activity of hypertrophic cartilage cells and osteoblasts in severe scurvy can be explained. As *Follis jr.* (1952) showed, phosphatase activity in cartilage develops quite normally during severe scurvy in young guinea-pigs, whereas osteoblasts do not become differentiated and do not contain phosphatase. This state of affairs has been difficult to explain on the assumption that the phosphatase in hypertrophic cartilage expressed a differentiation analogous to that of the osteoblast. If, on the other hand, the phosphatase in cartilage cells is considered as expressing a degenerative process, there is nothing to hinder it appearing in a different manner to that in which phosphatase appears when osteoblasts differentiate, and without Vitamin C having any significance for its appearance.

Chapter III.

THE HISTOLOGICAL RELATIONSHIP BETWEEN ALKALINE PHOSPHATASE AND CALCIFICATION

The previous chapter described how alkaline phosphatase activity in mesenchymal cells either expressed a process of differentiation or a process of degeneration. Calcification appears in connection with both forms of phosphatase activity, and just as there is a difference in principle in the occurrence of phosphatase in these two instances, there is a difference in principle between the corresponding calcified tissues, for example between bone tissue and calcified cartilage. Structurally, bone tissue is a highly organized, living tissue, continually being broken down and rebuilt; the phosphatase-containing osteoblasts continue living in the tissue as phosphatase-free osteocytes. In contrast to this, calcified cartilage is a necrotic tissue, which may be broken down, but where no rebuilding of the tissue takes place; the phosphatase-containing chondrocytes become completely necrosed.

In view of these differences, the two forms of calcified tissue must be sharply distinguished; this has not always been appreciated.

In the present chapter the relation between alkaline phosphatase and calcification in necrotic tissue and bone tissue is considered, as seen in the light of histological experiments. The investigations on which the discussion is based have been published previously (*Henrichsen* 1956 b, c and 1958).

Calcification in tissue cultures.

Carrel flask cultures of heart fibroblasts to which organic phosphate has been added show a circular calcification in the peripheral

part of the culture after 4-5 days' incubation (*Astrup & Henrichsen* 1954). Phosphatase staining of control cultures to which no organic phosphate has been added shows a circular phosphatase-stained ring with the same localization as the calcification. This phosphatase staining is usually present about one day earlier than the calcification. In the cultures in question, pronounced calcifications are also seen along the cut edges of the middle piece of the test culture. These calcifications correspond to the places where one would expect to find alkaline phosphatase activity, but as a rule such activity was not found at the places in question.

Calcification in tissue cultures can occur, however, without organic phosphate being added to the medium, which consists of plasma or embryo extract, diluted if necessary with Tyrode's solution. Such spontaneous calcifications are occasionally seen in ordinary coverslip cultures. The calcifications, consisting of precipitated calcium phosphate as *Hueper & Russel* (1933) previously showed, are found partly along the cut edges produced on cutting out the cultures for passage to fresh medium, partly in the original fragment of embryonic tissue if this is retained in the culture. The localization of the calcification in the tissue cultures corresponds exactly to the regions where alkaline phosphatase activity is found, as described in chapter 1.

If various cultures are compared, as well as serial sections of the same culture, stained in various ways, a close association between the phosphatase activity and the calcification is found. It is possible to distinguish 4 stages in the calcification:

- 1: Alkaline phosphatase is found in degenerated but not necrotic cells; there is no calcification. This stage corresponds to the phosphatase activity in degenerated cells, as described in chapter 1.
- 2: Alkaline phosphatase and calcification are present simultaneously. The calcification is only slightly pronounced, and is always limited to the individual cells. This stage seems to be brief, as it is more usual to find cells with phosphatase but without calcification (stage 1), or cells with calcification but without phosphatase (stage 3 or 4).
- 3: Alkaline phosphatase activity cannot be demonstrated. Cal-

cification is found localized to the individual cells, usually more pronounced than in stage 2.

4: Calcification is more widespread than in stage 3, spreading beyond the limits of the individual cells to produce calcification also in the medium between the cells. The outlines of the individual cells are no longer visible. No phosphatase staining can be found, either corresponding to the cells or the medium.

All four stages are often found together in the same tissue culture. Stage 3 or 4 found in the peripheral part of the region within a cut edge, stage 1 in the innermost part of the region representing the passage in question, and stage 2 in between. The same four stages in the development of the calcification could be observed in cultures on perforated cellophane in fluid medium, according to the method described by *Evans & Earle* (1947).

The relationship between alkaline phosphatase activity, calcification and nuclear staining is of particular interest. Methyl-green pyronin staining was used for these investigations, as the calcified tissue is strongly stained by haematoxylin. In the cells containing phosphatase (stage 1) nuclear staining is usually distinct, although frequently somewhat weaker than in normal phosphatase-free fibroblasts. A weak nuclear staining was occasionally found in the slightly calcified cells (stage 2), but usually this was not the case. On the other hand, in regions representing stage 3 and 4, no nuclear staining was observed.

Calcification in tissue cultures is thus a case of calcification of necrotic tissue. This was previously described by *von Möllendorff* (1932) in the case of the central piece in fibroblast cultures. With respect to calcification in the peripheral parts of the culture, *von Möllendorff* did not observe that this to begin with was localized to the individual cells, and that inter-cellular calcification is a secondary phenomenon. As it is quite possible to find uncalcified cells among the calcified ones, it is understandable that a certain similarity to the histological picture presented by bone tissue was found, and the assumption made that differentiation into osteoid tissue had taken place. *Von Möllendorff* had not studied alkaline phosphatase activity, and was therefore unable to discuss the

association between phosphatase and calcification. As it is now known that alkaline phosphatase activity can arise by cell degeneration, and that the phosphatase-containing cells calcify and do not continue as living cells, as do osteoblasts, there is no reason to consider that calcification in fibroblast cultures is an expression of differentiation into bone tissue.

Hanks (1949) found that calcium phosphate could precipitate in a mixture of serum and embryo juice. This was due to the action of the alkaline phosphatase in the embryo juice. The precipitation took place even though there were no cells in the medium in question, but if cells were being cultivated in the medium, the calcification took place in close association with them. No histochemical demonstration of alkaline phosphatase activity was made in these investigations, but it may be assumed that the localized precipitation was caused by the phosphatase activity associated with the cells.

Calcification in tuberculous lymph nodes.

In the same way as in the other tissues where alkaline phosphatase activity has been demonstrated in regions of calcification, this activity has been correlated with the calcification taking place in the degenerated cells of tuberculous lymph nodes (*Gomori* 1943). However, apart from a reference to Robison's theory, investigations on a so-called local factor in this tissue have not been made.

The present author investigated alkaline phosphatase activity and calcification in mesenteric lymph nodes from pigs with avian type tuberculosis (*Henrichsen* 1956 c). It was demonstrated that the same relation existed between alkaline phosphatase, necrosis and calcification as was found when investigating calcification in tissue cultures.

There is no sign of calcification in the most peripheral part of the tuberculous lesion, where phosphatase-containing cells without any pronounced nuclear degeneration are found. Somewhat more centrally, in the transitional zone between the cells containing phosphatase and the actual necrotic area which comprises the

largest, central region of the process, phosphatase-containing cells showing pyknosis or karyorrhexis are found, and here a few calcified cells are visible. Still more centrally, the calcification is more pronounced. At first it is still limited to individual cells, which no longer stain for phosphatase activity, but more centrally still the calcification has spread beyond the individual cells to produce a large solid calcification. No alkaline phosphatase can be demonstrated in this region either.

Thus, in the calcification of necrotic tissue, degenerating cells do not calcify as long as there is no histological sign of cell death. It is only when these histological signs of cell death are found (chromatolysis, karyorrhexis) that the individual cell can be shown to calcify. At this stage phosphatase can still be demonstrated in the cells in question. The calcification can then continue and spread beyond the cell boundaries, and at this point phosphatase activity can no longer be demonstrated.

Calcification in hyaline cartilage.

As previously mentioned, the presence of alkaline phosphatase in hypertrophied cartilage has been known since Robison's first studies on phosphatase (1923). Since then, numerous histochemical investigations have demonstrated its presence. Numerous studies have also been made on the calcification *in vitro* of hypertrophied cartilage, particularly the rachitic form. It has long been known that both embryonic mammalian cartilage and embryonic avian cartilage contain alkaline phosphatase in the hypertrophied cartilage cells, but that only the mammalian cartilage calcified in the embryonic stage. Still, no comparative investigations have hitherto been made on the two forms of cartilage with respect to the relation between phosphatase activity and calcification.

However, investigations on the embryonic hyaline cartilage of the long bones of mouse embryos (Henrichsen 1958) showed the same relation between phosphatase activity, histological signs of necrosis and calcification as in tissue cultures and tuberculous lymph nodes. While the calcification is proceeding, three zones

can be distinguished in the cartilage; the small-celled cartilage which does not contain alkaline phosphatase, the hypertrophic cartilage, where alkaline phosphatase is found in the hypertrophic cartilage cells and somewhat later also in the cartilage matrix, and finally the calcified cartilage, where the calcium salt is precipitated in the cartilage matrix. Normally, no phosphatase activity is found in the calcified cartilage, but the transition to uncalcified, phosphatase-containing cartilage is not completely abrupt, so that phosphatase-containing cells surrounded by slightly calcified matrix can be seen. Methyl-green or Feulgen staining shows normal cell nuclei in the small-celled cartilage, more faintly but still visibly stained nuclei in the hypertrophic, phosphatase-containing cartilage, but no stained nuclei in the calcified cartilage.

In embryonic avian cartilage, one finds both small-celled phosphatase-free cartilage and hypertrophic cartilage with phosphatase staining of the hypertrophic cells and, to a lesser degree, of the cartilage matrix. Nuclear staining is normal in the small-celled cartilage and somewhat weaker in the hypertrophic cartilage, but it must be stressed as characteristic that there is distinct nuclear staining of the hypertrophic cartilage cells right up to the medullary cavity. Those cartilage cells which do not calcify in spite of their content of alkaline phosphatase, show signs of cell degeneration, but do not reach the stage of becoming necrotic before being released from the ground-substance by the encroaching bone marrow. When preparations stained by the McManus method are examined, the hypertrophic glycogen-containing cartilage cells released from the ground-substance can even be recognized once more as round chondrocyte-like cells in the bone marrow close to the cartilage tissue. However, the glycogen content disappears rapidly, while at the same time the cell shape changes from round to spindle form. During this conversion the cell nucleus once more assumes a fully normal appearance. *Brachet* (1893) has described how the cartilage cells, after release from the ground-substance, can be converted to reticular cells. It is difficult to decide with certainty whether it is actually reticular cells which are formed by the conversion mentioned; as far as the association between

phosphatase and calcification is concerned, however, the cell type to which the previous cartilage cell is ascribed is indifferent. The point of interest is that the cartilage cells in the avian embryo do not necrotize but continue as living cells in the bone marrow.

In contrast to other necrotizing tissue, calcification in cartilage does not take place in the individual cells but in the cartilage matrix. It has been impossible so far to provide any satisfactory explanation for this difference. The low rate of diffusion of the phosphatase in the cartilage matrix may be the reason for its slow disappearance from the latter, so that it becomes bound to collagen fibrils; this again may determine the localization of the calcification. In spite of this variation in the localization of the calcification, calcification in cartilage must be grouped with other calcifications in necrotic tissue. The phosphatase appears during cell degeneration, and the calcification first develops with the necrosis of the phosphatase-containing cartilage cells; if necrosis is absent, so is the calcification.

Leblond, Belanger & Greulich (1955) investigated the uptake in bone and cartilage of the radioactive isotopes C^{14} , S^{35} , Ca^{45} and P^{32} . They found that Ca^{45} and P^{32} were taken up by the part of the cartilage which calcified during the period of the experiment, but not C^{14} and S^{35} . On the other hand, C^{14} and S^{35} were taken up by the remaining part of the cartilage. As these latter two elements enter into the formation of chondroitin sulphuric acid, produced by the cartilage cells, it could thus be demonstrated that the vital process, the synthesis of chondroitin sulphuric acid, did not take place in that part of the cartilage which calcified. The authors remark that it appears as if the calcification of the cartilage matrix is made possible by cell death.

Phosphatase and calcification in cell degeneration and necrosis of other tissue.

Gomori (1943) was unable to demonstrate alkaline phosphatase in 6 cases of early arteriosclerosis of the aorta, in 2 cases of sclerosed and calcified pyelonephritis, in 2 cases of calcified struma and in

one case of calcified tumor of the islets of Langerhans in the pancreas. He mentions the possibility that phosphatase may have been present earlier in these cases, but nevertheless considers the negative phosphatase staining in all cases as an argument against assuming any significance of phosphatase for the occurrence of these calcifications. This argument is hardly valid: as stated above, the alkaline phosphatase disappears quickly when the degenerated cell becomes necrotic and calcifies. This was found to be the case in tissue cultures, in tuberculous lymph nodes and in cartilage. In the material of calcified, tuberculous lymph nodes mentioned, older, calcified necroses are found, without signs of fresh processes. The calcified tissue is encapsuled in collagenous connective tissue, growing between the irregular, calcified parts. Alkaline phosphatase cannot be demonstrated at any point. *Gomori* in fact describes the calcium phosphate grains as found in an almost non-cellular connective tissue in all cases; this could suggest that completed calcification processes have been investigated, and according to our present knowledge alkaline phosphatase activity would not be expected in such cases, even though present when the calcification started or was developing. *Henrichsen* (unpublished observation) found alkaline phosphatase activity and calcification in the stroma of a rectal adenocarcinoma and in a uterine fibromyoma with necrosis. In both cases, the same relation as described above was found between cell degeneration, phosphatase activity, necrosis and calcification.

In calcification of the kidney tubules, the same relation is found between phosphatase activity, necrosis and calcification. Here, however, the phosphatase has not arisen from cell degeneration, but is found normally in the kidney tubules, as first shown by *Takamatsu* 1939 and *Gomori* 1939, and confirmed by numerous investigations. When calcification develops in these cells, as is seen for example in mercuric chloride poisoning, the calcification is preceded by necrosis (*Ogilvie* 1932). In experiments with mercuric chloride poisoning in rats, *Henrichsen* (unpublished observation) found that the calcification takes place in kidney tubule cells containing phosphatase, the cell nuclei of which would not

stain either by the Feulgen or Methyl-green staining. Thus, in this case also the phosphatase does not cause calcification as long as it is found in a living cell, but the calcification starts as soon as the phosphatase-containing cell becomes necrotic.

Bone tissue.

There has been no doubt as to the presence of alkaline phosphatase in hypertrophic cartilage cells, and its occurrence in cartilage matrix has only been denied by a few investigators (*Freeman & McLean* 1941), who, however, did not examine normal tissue. On the other hand, there is no agreement as to the localization of alkaline phosphatase in bone tissue. There is general agreement that phosphatase is found in osteoblasts and not in osteocytes or in the ground substance in the calcified bone, but there is some disagreement as to whether the enzyme is found in the uncalcified osteoid tissue. Some investigators do not mention the osteoid tissue specifically, but it is unlikely that they have observed phosphatase activity there, as they merely remark that the ground substance of the bone does not contain phosphatase (*Greep, Fischer & Morse* 1948, *Follis & Berthong* 1949). Others, using decalcified material (by the method of *Lorch* (1947) or *Greep, Fischer & Morse* (1948)) state that phosphatase is not found in osteoid tissue (*Zorzoli* 1948, *Schajowicz & Cabrini* 1954), while others have investigated rachitic bone, and have been unable to demonstrate phosphatase in the widespread osteoid regions in this tissue (*Freeman & McLean* 1941, *Gomori* 1943).

Contrary to this, *Takamatsu* (1939) describes phosphatase activity in the bone ground-substance, and *Moog* (1944), who did not use decalcification, states that the osteoid tissue contains alkaline phosphatase which disappears with the onset of calcification. *Bevelander & Johnson* (1950), who were particularly interested in the question of phosphatase in osteoid tissue, found distinct phosphatase staining in osteoid tissue in non-decalcified embryonic intramembranous bone. The enzyme activity disappeared on calcification. *Lorch* (1947), using decalcified material, states that

weak phosphatase activity is found in osteoid tissue, and *Siffert* (1951), using *Lorch's* method for decalcification, describes phosphatase activity in osteoid tissue, both in normal bones and in callus formation. In both cases the phosphatase disappeared quickly on calcification. *Manjo & Roullier* (1951), also using *Lorch's* method for decalcification, find phosphatase in osteoid tissue under normal circumstances. If the bone formation took place with particular rapidity, as in cases of bone regeneration, osteoid tissue which was first phosphatase-negative, later phosphatase-positive, was found in association with the calcification. *Manjo & Roullier* point out that *Gomori's* method is not very well suited to investigating the phosphatase content of osteoid tissue (cf. chapter 1). However, the fact that calcified bone tissue is always found phosphatase negative and osteoid tissue always phosphatase positive is nevertheless remarkable. Finally, *Morse & Greep* (1951) found particularly strong phosphatase activity in osteoid tissue in rachitic rats. All the investigations referred to were carried out with the *Takamitsu-Gomori* phosphatase method. *Manheimer & Seligman* (1948) do not mention osteoid tissue, but their fig. 1 C appears to show that the enzyme is present here. *Henrichsen* (1958), using *Manheimer & Seligman's* method on non-decalcified bones from mouse and chick embryos, found a very pronounced phosphatase staining in osteoid tissue, contrasting strongly with the phosphatase-free, calcified bone.

As mentioned previously, when using *Gomori's* phosphatase staining method without decalcification, the phosphatase-stained regions must be determined by comparison with control sections showing the localization of the calcification. It is difficult to decide whether the staining of a bone lamella in the phosphatase-stained preparation extends a little further than in the control section. The illustrations in *Bevelander & Johnson's* work, however, show that it is possible to decide this point. The clear pictures they obtained of the localization of the phosphatase are undoubtedly due to the use of freeze drying in the fixation. The loss of the phosphatase during fixation is thereby reduced to a minimum. In spite of all precautions, the use of decalcified tissue undoubtedly involves a loss

of phosphatase activity. As suggestive of this, *Lorch* found only a weak phosphatase staining in osteoid tissue, while *Manheimer & Seligman's* technique, which cannot be considered as particularly sensitive, shows a very powerful phosphatase staining in osteoid tissue. Another factor making it difficult to demonstrate phosphatase in osteoid tissue may have been the presence of only a slight amount of the osteoid tissue in the preparation. *Urist & McLean* (1941) showed that the bone matrix calcifies immediately on formation, provided the humoral conditions for calcification are optimal. If this were the case for all types of bone formation, it should strictly speaking be impossible to demonstrate osteoid tissue under normal (optimal) physiological conditions. If this is so, it makes the interpretation of a Gomori-stained preparation even more difficult, as in the immediate vicinity of a phosphatase-stained, possibly quite thin layer of osteoid tissue, there is a row of strongly phosphatase-stained osteoblasts on the one side and calcified bone on the other side.

From the investigations mentioned, it cannot be decided whether phosphatase is found in the widespread osteoid regions in rachitic bones or not, as they have given both positive and negative results. If, in view of the above, phosphatase is assumed to be present in osteoid tissue under normal circumstances, it must also be assumed that it is found at a certain stage in rachitic osteoid tissue. It is possible that the disappearance of phosphatase from the osteoid tissue simultaneously with the appearance of calcification merely expresses the fact that phosphatase does not continue to be supplied to this extra-cellular region by the osteoblasts. If this is the case, a difference should be expected between the phosphatase content of newly formed osteoid tissue, even in rachitis, and the phosphatase content of older osteoid tissue which is uncalcified due to rachitis. This would explain the conflicting observations. Further investigation on the phosphatase activity in osteoid tissue from rachitic animals is necessary to decide whether there is any dependence in particular on the duration of the rachitic lesions. On the basis of the available investigations, it may be concluded that alkaline phosphatase activity is found localized to osteoid tissue under nor-

mal physiological conditions, but that its occurrence in osteoid tissue from rachitic animals is still unknown.

Thus, from the histochemical investigations carried out, there is no reason to assume that alkaline phosphatase is without significance for the calcification of osteoid tissue in spite of the claim to the contrary by *McLean, Lipton, Bloom & Barron* (1946) on the basis of *Freeman & McLean's* investigations of phosphatase in osteoid tissue from rachitic dogs.

Histochemical investigation has thus shown the presence of alkaline phosphatase in those regions of bone tissue where calcification is taking place. The enzyme is present prior to the commencement of calcification, and rapidly disappears after the calcification has taken place. This signifies that at least the first 3 of the 4 steps, relating calcification to alkaline phosphatase activity as described above, are found present in bone tissue. On the basis of silver staining with von Kossa's method it is impossible to decide whether calcification takes place in bone tissue after the alkaline phosphatase has disappeared from the ground-substance, corresponding to stage 4 in the calcification of necrotic tissue. The calcification of the osteoid tissue gives rise very early to such an intense and homogeneous blackening, that it is difficult to decide whether this blackening is even more pronounced at a later stage. However, other methods of investigation, to be discussed later, suggest that such a secondary calcification also takes place in bone tissue.

The histological investigations on the association between alkaline phosphatase and calcification mentioned in this chapter may be summed up as follows:

- I. Calcification occurs both in bone tissue (extracellular, osteoid tissue) and in connection with tissue necrosis. (Examples: tissue cultures, tuberculous lymph nodes, cartilage, kidney tubules).
- II. Alkaline phosphatase has been demonstrated, or can presumably be demonstrated, where calcification takes place.
- III. Calcification does not occur in necrotizing phosphate-containing cells until the nuclear staining shows signs of cell

death. Further, it is possible histologically to distinguish between 4 stages in the development of calcification:

1st stage: The phosphatase activity is found localized in noncalcified cells.

2nd stage: The phosphatase activity and calcification are found localized in the same cell.

3rd stage: The calcification is found localized in cells which do not show phosphatase staining.

4th stage: The calcification has spread beyond the cell boundaries at a time when phosphatase activity can no longer be demonstrated.

IV. Alkaline phosphatase is found in the osteoblasts during the formation of bone tissue. In osteoid tissue, in addition, a strong staining for phosphatase activity is found. In the completely formed, calcified bone, phosphatase activity can be demonstrated neither in the ground substance nor in the osteocytes.

Alkaline phosphatase and the formation of collagen fibrils.

When discussing phosphatase activity in osteoid tissue it would seem natural to mention the possible significance of phosphatase for the formation of collagen fibrils. *Fell & Danielli* (1943), investigating the healing processes in wounds, found pronounced phosphatase staining of newly formed collagen fibrils by the use of *Gomori's* technique.

It was subsequently found that vitamin C was necessary for the occurrence of phosphatase in the above experiments (*Danielli, Fell & Kodicek* 1954). These authors believe that there is a connection between the phosphatase activity and the formation of collagen fibrils, but point out the possibility of the enzyme being adsorbed on the fibrils, and maintain that the blackening by *Gomori's* method is not certain to provide an exact indication of the localization of alkaline phosphatase. *Danielli* (1946) showed subsequently that collagen fibrils also have a pronounced affinity for phosphate

ions, as the same blackening of the fibrils which appeared with *Gomori's* phosphatase staining method could be produced by treating the tissue section with a solution of calcium nitrate and sodium phosphate. The same blackening of the fibrils could also occur in the tissue section after the phosphatase had been destroyed by heating. These sections were treated as by the *Gomori* method, but instead of enzymatic splitting of the organic phosphate, hydrogen peroxide was used, splitting the organic portion of the molecule and releasing the phosphate.

Marchant (1950), repeating *Fell & Danielli's* experiments by investigating nerve regeneration after division, could not demonstrate by *Gomori's* technique any phosphatase activity in connection with the formation of collagen fibrils in the scar tissue around the site of division. *Robertson, Dunihue and Novikoff* (1950) investigated the connective tissue reaction to cellophane placed perirennally or subcutaneously. Using *Gomori's* technique, faint staining was found in the fibrils nearest the kidney, while the remaining fibrils were unstained. Using *Manheimer & Seligman's* method, there was no staining whatever of the collagen fibrils. These authors conclude that the fibrils do not contain alkaline phosphatase, and that the weak staining found with the *Gomori* method is due to diffusion and adsorption of phosphate ions from the renal cortex.

Gold & Gould (1951) find that newly formed collagen has the power to adsorb alkaline phosphatase. There appears to be a direct adhesion between the collagen and the enzyme. The interesting observation was made that neither protein, hyaluronic acid nor nucleic acid acted as a link between the collagen and the enzyme.

Henrichsen (1956 a) found that phosphatase-stained fibrils in tissue cultures were formed from phosphatase-containing cells (degenerating fibroblasts). However, if the fibrils were formed from phosphatase-free fibroblasts, they did not stain for phosphatase. The collagen fibrils found around tuberculous inflammatory processes in lymph nodes did not contain alkaline phosphatase (*Henrichsen* 1956 c). The phosphatase staining in osteoid tissue

was so pronounced that the individual fibrils could not be evaluated, but in view of the preceding observations it must be assumed that they contain phosphatase. Numerous collagen fibrils without any sign of phosphatase activity were found in the phosphatase-free outer layer of the periosteum, the fibroblast layer.

It must be concluded, therefore, that collagen fibrils can be formed without alkaline phosphatase being involved. However, they can contain the enzyme, if the cell or cells from which they have been formed contain phosphatase. Further, it must be assumed that a fibril which is originally free of phosphatase can adsorb phosphatase if the enzyme is present in the free state around the fibril.

Chapter IV.

THE MECHANISM OF CALCIFICATION

Robison's theory.

As mentioned in the introduction, Robison found it necessary to modify his original theory of the significance of phosphatase for calcification. In addition to the alkaline phosphatase, a local factor (second factor or second mechanism) had to be introduced. Robison assumed that wherever alkaline phosphatase was found, it would cause a rise in the concentration of free phosphate ions (*Robison & Rosenheim* 1934). In order to explain why calcium phosphate is precipitated in those sites where calcification takes place, but not, for example, in the kidney tubules or in embryonic avian cartilage, both containing alkaline phosphatase, he ascribed a special property to his "second factor", namely, that its presence was necessary for the precipitation of calcium phosphate to take place from a solution supersaturated with calcium and phosphate ions. The precipitated calcium phosphate is then bound to the tissue, whereby the latter calcifies.

If the difference between phosphatase-containing tissues which calcify and those which do not were to be characterized in the light of our present knowledge of the histological relationship between alkaline phosphatase activity, cell death and calcification, it would be natural to regard the necrosis of the phosphatase-containing cells as being the essential factor.

In accordance with the definition of *Robison's* local factor, the absence of calcification in sections of kidney tissue *in vitro* in solutions containing organic phosphate and calcium ions may be interpreted as a lack of the local factor (*Rosenheim & Robison*

1934). Experiments of this kind are really a phosphatase staining with *Gomori's* method, carried out as vital staining. Provided there has been an enzymatic splitting of the organic phosphate, and provided the lack of calcification is only due to the absence of the local factor, then it should be possible in such experiments to demonstrate the organic part of the enzymatically split molecule, for example the β -naphthole in *Manheimer & Seligman's* phosphatase staining method. *Henrichsen* (1956 b) showed that this could not be done. On the other hand, phosphatase staining could be demonstrated, without prior fixation, in those regions of a chick embryo bone where calcification was taking place, i. e., corresponding to the perichondrially formed bone, but not in the phosphatase-containing hypertrophic cartilage, which does not calcify.

It is difficult to interpret this experiment with certainty, but it would be reasonable to conclude that an enzymatic splitting of organic phosphate has probably not taken place in *Rosenheim & Robison's* experiment. After the lapse of two days, *Rosenheim & Robison* could demonstrate a faint calcification in the kidney tubules, possibly indicating that the cells were by then dead, or that the phosphatase had been released in some other way (extraction). The experiment also suggests that there is a difference between the action of the phosphatase arising as the result of the function of the enzyme in the living cell and the effect demonstrated from experiments with the isolated enzyme. This latter effect, which can explain the role of the phosphatase in the calcification process as well as the phosphatase staining, is found only where calcification is taking place: in phosphatase-containing, necrotic cells and extracellularly, for example in osteoid tissue.

Author's theory.

Histochemical investigations suggest that in certain cases phosphatase contributes to the synthesis of mucopolysaccharides (*Kroon* 1952). The cases in question are phosphatase in osteoblasts and in the earliest stage of cartilage development, where the presence of the phosphatase is unrelated to cell degeneration. *Kroon* has

also demonstrated a similar relation between acid phosphatase and the formation of mucopolysaccharides. Certain experiments suggest that phosphatase contributes to the synthesis of glycogen. In such cases the phosphatase is localized between the blood vessel and the glycogen depot. This is the case, for example, in the placenta (*Wislokski & Dempsey 1945*) and in hair follicles (*Johnson & Bevelander 1946*). In other cases it must be assumed considering the localization that the phosphatase plays a part in the breaking-down of glycogen: this is so for example in hypertrophic cartilage and in tuberculous lymph nodes, where phosphatase is found localized near the glycogen, but not in regions where the cellular degeneration is more pronounced (*Henrichsen 1956 c*).

As far as the enzymatic synthesis and breaking down of glycogen is understood, this is a process in which various enzymes take part, whereby the phosphate ion is split off from one link to become attached to another. A varying placing of the phosphate ion may be imagined, whereby energy is either accumulated (anabolism) or released (catabolism).

It may thus be conceived as a property of the living cell that the interplay between the various enzymes does not allow free phosphate ions to accumulate at any one stage in the process. With the onset of cell necrosis, the interplay between the various enzymes may be imagined to cease, the phosphatase is released from its dependence on other enzymes and the phosphatase effect appears with which we are familiar from extracted phosphatase and the phosphatase staining of fixed tissue.

The assumption of a difference of this nature between the action of the phosphatase in a living cell and in a necrotic cell, and the further assumption that the effect of the phosphatase when it appears extra-cellularly in osteoid tissue is of the same nature as its effect in necrotic tissue, provides an explanation for the relationship between phosphatase and calcification, in agreement with the histological findings (Stages 1—2).

As mentioned in chapter 3, it could be demonstrated that phosphatase was only related to the initial calcification in necrotic tissue; thereafter, the calcification could develop at a stage where

the alkaline phosphatase activity could no longer be demonstrated (Stages 3—4). The *von Kossa* staining method used was unable to show whether a corresponding diphasic calcification also takes place in osteoid tissue, but other investigations suggest this is possible. *Leblond, Belanger & Greulich* (1955) have demonstrated by radioautography of dentine that calcification in this tissue, which is closely related to bone tissue, takes place in two stages. 1) The calcification commences on the transition from predentine to dentine, and 2), the calcification continues as an increasing calcification of the already calcified dentine. This process could further be demonstrated by an increasing degree of hardness of the dentine during the first few days after its formation. In this connection it is interesting to note that *Wislocki & Sognnaes* (1950) have demonstrated alkaline phosphatase in predentine, while on the contrary phosphatase could not be demonstrated in dentine, apart from nuclear staining (*Gomori's* method) *Sognnaes* 1955, p. 554, fig. 21.

It may therefore be assumed that all calcification takes place in two stages: 1) the formation of calcium phosphate crystals, apatite, when the phosphate ions become concentrated locally as a result of the alkaline phosphatase activity, and 2), a secondary crystal growth, for which purpose the necessary phosphate ions need not arise from a local enzymatic splitting of ester-bound phosphate.

Substrates for the phosphatase.

Robison imagined that all the phosphate which was deposited first passed through an intermediate organic stage, from which it was split by the action of the phosphatase. Such a mechanism must require the supply of large quantities of ester-bound phosphate, and one of the arguments raised against *Robison's* theory was that such large amounts of ester-bound phosphate are not available, as only very small amounts are reported present in the blood. If, on the other hand, it is supposed that only the initial stage of calcification takes place via ester-bound phosphate, large

amounts would not be required, and not by supply from the blood or tissue fluids. With such a calcification mechanism, however, any local availability of ester-bound phosphate is of interest. *Gutman & Gutman* (1941) demonstrated phosphorylating enzyme in hypertrophic cartilage, and later it could be demonstrated that glycolysis of glycogen could play a part in the processes leading to calcification (*Gutman* 1946, *Gutman & Yu* 1949). Organic phosphoric acid esters are formed by the glycolysis, whereby a concentration of organic phosphate takes place as a result of the supply of inorganic phosphate from intercellular fluid and blood. When these phosphoric acid esters are later split by means of the alkaline phosphatase, the phosphate ions can be imagined as occurring locally in higher concentrations than in the blood. Several of the stages in the glycolysis of glycogen from glucose-1-phosphate to glycerophosphoric acid can be dephosphorylated by alkaline phosphatase. Even though the glycolysis can be shown to take part in several cases of calcification *in vitro*, it is not known with certainty to what extent this takes place *in vivo*, or which steps in the process function as substrate for the phosphatase under normal physiological conditions. If, however, the glycolysis should stop on the death of the cell, it must be assumed that alkaline phosphatase will split the phosphoric acid esters present at that stage. In addition to functioning in cartilage, glycolysis can be imagined to play a part in calcification in tuberculous lymph nodes and perhaps in other tissues too, if it is the general rule that glycogen occurs before the alkaline phosphatase at the commencement of cell degeneration.

Lecithin and lecithinase have also been demonstrated in cartilage, the latter splitting phosphocholine from lecithin (*Levine & Follis jr.* 1949). Phosphocholine can be broken down further by alkaline phosphatase. Large amounts of adenylyphosphopyruvic acid or adenosinetriphosphoric acid (ATP) have been demonstrated in hypertrophic epiphyseal cartilage by *Cartier* (1950) and by *Albaum, Hirschfeld & Sobel* (1952). As ATP can be dephosphorylated by alkaline phosphatase and this presumably will take place with very great speed on account of the great lability of ATP,

it is possible that ATP can function as a substrate for the alkaline phosphatase in the initial calcification of cartilage, even though such a function of ATP in cartilage has not yet been demonstrated.

De Stefano, Neuman & Rouser (1953) demonstrated by paper electrophoresis that hypertrophic cartilage contains an organic phosphate which can be split by alkaline phosphatase, presumably phosphorylated hexosamine. *De Stefano et al.* consider this substance to be associated with the calcification process, believing that it initiates the formation of the first crystal without the intervention of phosphatase (cf. *Neuman* p. 5). With equally good reason, however, the substance can be assumed to function as a substrate for alkaline phosphatase.

In necrotic tissue, the further possibility must be allowed for that depolymerized ribonucleic acid (RNA), which is found in the cells that become necrotic, can be split by the phosphatase, and thus function as a substrate (*Follis jr.* 1950). As it may be assumed that dephosphorylated ribonucleic acid will stain with the McManus-Hotchkiss staining method (PAS) (*Everson Pearse* 1953), there is the possibility of accounting for the presence of PAS positive material in calcified, necrotic tissue, without having to explain this by the presence of mucopolysaccharides, as assumed by *Levine, Rubin, Follis & Howard* (1949). It is possible that ribonucleic acid also plays a role in the calcification of osteoid tissue. If this is the case, the difference between the large content of RNA in osteoblasts and the slight content in osteocytes can be explained (*Follis jr.* 1950, 1952; *Schajowicz & Cabrini* 1954).

As to the significance of the substances mentioned for the calcification process, our knowledge is still incomplete, but it is hardly unjustified to assume that locally, where the calcification takes place, there is enough ester-bound phosphate present to explain the initial calcification by means of alkaline phosphatase activity.

Crystals in bone.

It has been maintained (*Neumann & Neumann* 1953) that the precipitation of calcium phosphate as a stage in an enzymatic

process could not lead to the formation of the crystals characteristic of bone tissue. ("Furthermore how precipitation can result in a highly orientated pattern of minerals has never been explained.").

Even though the conditions of calcification *in vivo* and in the histochemical demonstration of phosphatase by means of *Gomori's* method are not identical, it is nevertheless interesting to note that the precipitation of calcium phosphate as it takes place in *Gomori's* method is actually in the form of crystals with apatite structure, i. e., the crystal form typical of bone tissue (*Carlsen, Jensen & Johansen* 1953). There is thus nothing to suggest that in the form in which it is split from the ester-bound phosphate by the alkaline phosphatase, the phosphate ion cannot precipitate as calcium salt in the complex crystal form characteristic of both bone tissue and other calcified tissue in the organism. The detailed mechanism of the formation of the apatite crystal is still unknown. It has been claimed that the solubility product for CaHPO_4 is decisive for the commencement of precipitation (*Shear & Kramer* 1928, *McLean et al.* 1946, *Neumann & Neumann* 1953). Against this is the fact that CaHPO_4 cannot be demonstrated by X-ray diffraction investigations, but only apatite crystals (*Hodge* 1950, 1955). Further, using inorganic solutions in which the calcium content is kept constant but phosphate content and pH are variable, the calcification *in vitro* follows the solubility curve for $\text{Ca}_3(\text{PO}_4)_2$ and not the solubility curve for CaHPO_4 , provided the pH is under 7.4 (*McLean et al.* 1946). As mentioned previously, research has not yet solved the problem of the detailed mechanism of the formation of the apatite crystal. One of the difficulties appears to be that a solubility curve cannot be obtained for the crystal form in question (*Hodge* 1955). It has therefore been impossible to obtain agreement between the biological processes occurring naturally and the known chemical processes, the precipitation of CaHPO_4 and $\text{Ca}_3(\text{PO}_4)_2$ respectively.

Electron microscope investigations have revealed a close structural association between the apatite crystals in bone tissue and collagen fibrils (*Robinson & Watson* 1955). This observation might perhaps be interpreted as supporting *Sobel's* theory that a complex

of collagen and chondroitin sulphuric acid could be responsible for the formation of the first crystal (*Sobel* 1955), and *Neumann & Neumann's* (1953) concept of a structure associated with the organic matrix, triggering off the calcification by causing the formation of the first crystal. The relation between collagen and crystals can also be explained in another way, however, if an enzymatic release of phosphate ions is assumed as the triggering mechanism in calcification. As previously mentioned, collagen fibres have a very great affinity for phosphate ions (*Danielli* 1946) and for alkaline phosphatase (*Gold & Gould* 1951); it is therefore easy to imagine that phosphate ions released in a ground substance with numerous collagen fibrils will become adsorbed on these fibrils, and thus determine the localization in the ground substance of the apatite formed as a result of the accumulation of phosphate ions and the calcium in solution.

Robison's "local factor".

Shipley, Kramer & Howland (1925, 1926) showed that rachitic hypertrophic cartilage could calcify *in vitro* in solutions containing calcium and phosphate ions as inorganic compounds alone. These investigations were carried further by *Robison* and his colleagues among others, who furthermore investigated calcification *in vitro* in solutions which also contained ester-linked phosphate.

As mentioned in chapter 1, *Robison* defined the second or local mechanism as a tissue property favouring the deposition of calcium phosphate from supersaturated solutions, whether the supersaturation was brought about by enzymatic hydrolysis of phosphoric acid esters or arose by diffusion of a supersaturated solution from outside into the tissue (*Robison, McLeod & Rosenheim* 1930, *Robison & Rosenheim* 1934). In addition to distinguishing between phosphatase-containing tissues which calcified and those which did not calcify, the hypothetical second factor could in this way be invoked to explain the *in vitro* precipitation of calcium phosphate in rachitic cartilage in supersaturated solutions. Furthermore, the definition implied that it should be possible to obtain information on the

second factor by continuing the study of such calcification experiments *in vitro*.

However, *Robison*'s own experiments show that the definition of the local mechanism as given cannot be used. If the effect of the local mechanism were to encourage calcium phosphate deposition independently of how the supersaturation takes place, either enzymatically or by diffusion from supersaturated solution, it might be expected that a treatment which hindered calcification in supersaturated solutions would also hinder the precipitation of calcium phosphate when the supersaturation was due to phosphate ion accumulation from the enzymatic splitting of hexosephosphate, for example. However, experiments show that while NaF in a concentration of 10^{-5} M. has a pronounced inhibitory effect on the calcification from supersaturated solutions, there is no inhibition of calcification in solutions containing ester-bound phosphate. Other experiments show that treatment of rachitic cartilage with distilled water or solutions with a low calcium and phosphate ion content, to a high degree inhibits subsequent calcification in supersaturated solutions, but has no effect on subsequent calcification in solutions also containing hexosephosphate (*Robison & Rosenheim* 1934). These experiments thus suggest that calcium phosphate can be precipitated and bound to tissue, even though the local mechanism as defined by *Robison* is absent. Nor, as previously mentioned, is there any good reason to assume that the difference between phosphatase-containing tissues which calcify and those which do not calcify is due to the presence of a special local factor. There are thus hardly any grounds for assuming that a local factor, such as defined by *Robison*, could explain calcification in supersaturated solutions, and it may be accepted that the process investigated when experiments are made with supersaturated solutions is different to that which *Robison* imagined.

McLean's theory.

McLean et al. (1946) do not believe that phosphatase takes part in the actual calcification, but assume that it may have to do with establishing what these investigators call the local me-

chanism for the calcification, "the local mechanism which we define as whatever it is that operates locally and brings about calcification in the presence of favourable humoral conditions". (*Mc Lean et al.* 1946, p. 17). *McLean* thus assumes that there is only one mechanism of calcification. As previously mentioned, however, it must be assumed that there are two mechanisms for the calcification, the phosphatase mechanism, which starts off the calcification, and a secondary calcification, taking place at a time when no local phosphatase activity is found.

McLean assumes that the ability to bring about calcification, represented by his local factor, is the result of the activity of living cells. Pointing out that rachitic cartilage does not lose its power to cause calcification after treatment with acetone, alcohol or chloroform, a treatment which undoubtedly destroys living cells, *McLean et al.* write (*loc.cit.* p. 18) "One may safely assume, it seems to us, that living cells were necessary in the process of conferring calcifiability on the cartilage matrix — that is, of creating the conditions for calcification — but it is clear that once created, these conditions may outlive the cells that created them. Our interpretation of these findings is that the conditions for calcification were produced in these tissues during the life of the animals, and that they were not lost with the death of the tissues."

It should be recalled in this connection that calcification did not take place in necrotic tissue until there were histological signs of cell death. If, for example, the tubule cells in kidney are considered, calcification does not normally occur there, and as a consequence no local factor is present, neither according to *Robison's* nor *McLean's* definition. On the other hand, such a factor must be considered as present when the tubule cells calcify at the onset of necrosis. In this case it is difficult to imagine a local factor which has arisen as a result of the activity of living cells. Nor can a local factor associated with collagen and chondroitin sulphuric acid be imagined in tubule cells, as assumed by *Sobel* (1955), who has continued the investigations on the local factor defined by *McLean*.

Both *McLean et al.* and *Sobel* assume, with *Robison*, that information on "the local factor" can be obtained by following up cal-

cification experiments *in vitro*. However, just as with *Robison's* local mechanism, the local factor defined by *McLean* is based on a hypothesis which is not in agreement with the histological facts. It is therefore not surprising that the experiments in question have not provided a decisive answer concerning the nature of "the local factor".

Roche's theory.

Roche (1946), on the basis of his own earlier investigations and those of his colleagues (see review of these studies by *Roche* 1946) has established a theory for the calcification process which lies almost half way between the theories of *Robison* and *McLean*.

On the basis of investigations on bone tissue and callus in particular, he divides the calcification process into 4 stages.

The first stage is the formation of the protein of the bone tissue, a process requiring the presence of Vitamin C.

The second stage is defined as the production of a preparatory mechanism for the calcification ("la g n se du m canisme pr paratoire   la calcification"). The phosphatase activity is as yet slight and does not produce calcification, as the proteins are still unable to bind precipitated calcium phosphate. On analyzing the callus from experimental fractures in doves, a rise in the phosphorus and calcium content of the callus is found at this period. This rise cannot be explained as indicating precipitated calcium salts, as the ratio Ca/P lies between 0.9 and 1.67, while the ratio Ca/P corresponding to $\text{Ca}_3(\text{PO}_4)_2$ is 1.93, and CaHPO_4 , 1.29. It is therefore believed that the rise is due to calcium ions and phosphate ions being bound to protein. However, the results of analysis show that the rises in the Ca and P content are very slight in relation to the total increase in the content of mineral substances. On the basis of the results quoted it is impossible to state the form in which the Ca and P have been present in the callus. It may be assumed that at least a part of the phosphorus content is present in nucleic acids and incorporated in proteins in other ways, and part is possibly present as phosphoric acid esters. At any rate, the assumption that phosphorus is found bound to proteins, in the same

manner as calcium might be imagined bound as calcium proteinate, is a hypothetical one. There is no proof that a conversion of proteins, such as Roche imagines taking place in the 2nd stage, is a necessary condition for later calcification (cf. the considerations advanced above in the discussion on *McLean's* local factor).

According to *Roche*, the third stage consists of the conversion of the proteins in such a manner that the tissue can now calcify, but the nature of these changes is unknown. "Elle traduit un phénomène ne se prêtant pas à une étude chimique, car notre connaissance des protéines osseuses est très réduite et l'on ne peut pas espérer définir leur remaniments structureaux en l'état actuel de nos méthodes. Seules les conséquences physiologiques de ses remaniments nous apparaissent." It is known from histological investigations that changes do take place as an increased basophilia is observed in the osteoid tissue immediately prior to the calcification. But neither with regard to this stage is there any proof that the ability to calcify is due to a conversion of protein.

The fourth stage is characterized by a powerful and rapid rise in the phosphatase activity, and simultaneously with this the precipitation of calcium phosphate takes place. The maximal phosphatase activity is reached in the course of a few days, and thereafter an equally strong and rapid fall in the phosphatase activity is seen, while at the same time the calcification proceeds more slowly. On the basis of *Roche et al.*'s investigations it is impossible to decide whether the calcification takes place later than the termination of the phosphatase activity, corresponding to stages 3 and 4 in the present author's investigations.

It is impossible to be certain whether the stages 1, 2 and 3 described by *Roche* play any role in the calcification of bone tissue or whether they merely represent the physiological tissue changes during the formation of callus. As stated above, such a process at any rate cannot be considered in all cases of calcification. On the other hand, the investigations of *Roche et al.* clearly show the association between phosphatase activity and calcification *in vivo*, and the results are not compatible with *McLean's* concept that phosphatase has no significance for the actual calcification, but only for a conversion of the tissue, so that it becomes able to calcify.

Chapter V.

CALCIFICATION EXPERIMENTS IN VITRO

Shipley (1924) showed that cartilage from rachitic rats could calcify *in vitro* in serum from normal rats or from rats whose rachitis was in the process of healing as a result of treatment. *Shipley, Kramer & Howland* (1925, 1926) showed that a corresponding calcification of rachitic cartilage could take place in aqueous solutions containing calcium ions and phosphate ions in the form of inorganic compounds. Several investigators carried these experiments further, as it was felt that information might be obtained by this means on the variously defined local factors which might have significance for the calcification process. Even though these investigations have not confirmed the different theories on local factors, the experiments have provided much information on the calcification process. The investigations on the breaking down of glycogen by phosphorylation and the influence of this mechanism on the calcification have been particularly valuable.

Before examining further the various investigations available, it must be pointed out that due to various circumstances, the results from these *in vitro* experiments cannot be directly transferred to the conditions obtaining in the normal living organism. Most of the experiments have been made with rachitic cartilage, some few with embryonic bone and cartilage. It cannot be taken for granted in advance that the calcification process occurring *in vitro* during the course of a few hours is identical either with the *in vivo* process which produces calcification during the healing of rachitis, or with the calcification process under normal physiological conditions.

If the conditions of the *in vitro* and *in vivo* experiments as known from histological investigations are compared, it can be expected that the limited experimental period will result in the phosphatase mechanism (stage 2) operating throughout that whole period, and that the calcification corresponding to histological stages 3 and 4 will either occur to a lesser extent or occur simultaneously with stage 2, for which reason it may be difficult to draw reliable conclusions. Another circumstance which has made it difficult to interpret *in vitro* experiments is the fact that at least two different mechanisms are active in the calcification: the phosphatase mechanism and the crystal growth, and that the calcification can further be varied by changes in the glycolysis, whereas an attempt has been made to interpret the experiments as if only one or two mechanisms were active in the calcification. Finally, the experiments have been interpreted on the assumption that there is no calcification in the rachitic cartilage at the commencement of the experiments, because it has been impossible to demonstrate such calcification by silver staining according to *von Kossas's* method. Electron microscopy has shown that the earliest demonstrable calcification in osteoid tissue consists of crystals associated with the collagen fibrils, the size of the crystals in the regions where calcification is commencing being 20—50 Å (*Robinson & Watson* 1955). As the light microscope will only show particles larger than 0.2 μ , i. e., about 100 times larger than the particles demonstrated in the commencing calcification of osteoid tissue, the negative findings with light microscopy after silver staining by *von Kossas's* method need not signify that there are no small crystals even in rachitic cartilage. Electron microscopic investigations of the rachitic cartilage used for calcification research *in vitro* is therefore necessary, to decide whether and to what extent growth of pre-formed crystals can explain the calcification found. Such investigations have not been made so far. In what follows, a review will be given of the most important results from *in vitro* calcification experiments, with a discussion of the extent to which these results agree with the hypotheses advanced for the course of the calcification.

Experiments demonstrating the phosphatase mechanism (stage 2).

The calcification of hypertrophic, rachitic cartilage *in vitro* can take place with ester-bound phosphate as the sole source of phosphate (Robison 1923). Further experiments showed that the calcification did not take place when there was a low content of calcium and inorganic phosphate. However, in such cases calcification could be achieved by adding small amounts of phosphoric acid ester (Robison & Soames 1930). In solutions containing phosphoric acid esters as the sole source of phosphate, the calcification could be inhibited by treatment with various phosphatase inhibitors. This could be observed, for example, by treatment with AgNO_3 1/10,000, or HgCl_2 in concentrations of 10^{-2} M. In both cases the phosphatase was completely inhibited, and no calcification of the rachitic cartilage took place (Waldman 1948, 1950). The alkaline phosphatase was inactivated by heating to temperatures above 60° , and calcification using phosphoric acid esters as substrate did not take place after heating to 60° , 80° or 100° (Waldman 1948, 1950). Aldridge (1950) showed that beryllium is a powerful phosphatase inhibitor, 2×10^{-7} M. Be^{++} inhibiting the non-activated enzyme by 50 per cent, and 6.6×10^{-7} M. Be^{++} inhibiting the magnesium-activated enzyme by 50 per cent. Gutman & Yu found that with a concentration of 1×10^{-6} M. Be^{++} the inhibition was 12 per cent, with a concentration of 1×10^{-5} M. Be^{++} it was 64 per cent and with a concentration of 1×10^{-4} M. Be^{++} and 1×10^{-3} M. Be^{++} it was 72 per cent. All results were determined in experiments on phosphatase from cartilage in 1 per cent sodiumglycerophosphate. In the calcification experiments with rachitic cartilage, inhibition of the calcification was found when phosphoric acid esters were the only source of phosphate. However, the inhibition was dependent on the concentration of the medium: glucose-1-phosphate in amounts corresponding to 10 mg per cent phosphorus was completely inhibited by 10^{-6} M. Be^{++} . In amounts corresponding to 20 mg per cent phosphorus the calcification is first completely inhibited at 10^{-4} M. Be^{++} . Using β -glycerophosphate (10 mg per cent P) inhibition of the

calcification takes place at 10^{-5} M. Be^{++} , while 20 mg per cent phosphorus requires 10^{-3} M. Be^{++} in order to achieve complete inhibition of the calcification. *Hiatt & Shorr* (1951) find results which correspond to the above.

The same authors have investigated the action of l-histidine which inhibits alkaline phosphatase from cartilage, 50 per cent inhibition being obtained with 3×10^{-3} M. l-histidine (*Bodansky* 1948). *Hiatt & Shorr*, in calcification experiments *in vitro*, find that 10^{-2} M. l-histidine inhibits calcification completely when glycerophosphate in a concentration of 10 mg per cent is the sole source of phosphate, while the inhibition is slightly less when phenyl-phosphate or hexose-diphosphate is the only source of phosphate, and much less with glucose-1-phosphate.

These experiments show that an apparently normal calcification of cartilage can occur *in vitro* with the aid of phosphoric acid esters and alkaline phosphatase, and that phosphatase inhibitors inhibit the calcification. How far such a process opposes calcification *in vivo* cannot be stated on the basis of such experiments; on the other hand, there is nothing in these experiments which contradicts the hypothesis advanced that calcification is initiated by a process in which the phosphatase acts by the enzymatic splitting-off of phosphate ions.

Calcification *in vitro* with inorganic phosphate as the sole source of phosphate can be imagined taking place via ester-bound phosphate, split by alkaline phosphatase. *Shipley, Kramer & Howland* (1925, 1926) had already shown that if the solutions containing only inorganic phosphate, calcification was inhibited by potassium cyanide, formalin, chloroform, ether, alcohol and by boiling. *Robison, McLeod & Rosenheim* (1930) confirmed these findings, but found that while calcification in inorganic solutions was completely inhibited by treatment with chloroform, acetone, alcohol and vacuum drying over sulphuric acid, the calcification could still take place when phosphoric acid esters were present. The explanation of these facts may be that the treatments mentioned (with the exception of boiling) hinder the glycolytic breaking-down of glycogen, but do not inactivate the alkaline phosphatase. As previous-

ly mentioned, the glycogen content of cartilage decreases simultaneously with the calcification. *Harris* (1932) put forward the hypothesis that the glycogen is broken down by phosphorylation: in this way hexose phosphate would be formed, serving as substrate for the alkaline phosphatase. *Glack* (1940) believes that the inhibitory action of NaF on calcification *in vitro* can be explained by the inhibition of glycolysis between 2-phosphoglyceric acid and phosphopyruvic acid. *Gutman & Gutman* (1941) demonstrated a phosphorylating glycolytic enzyme system in hypertrophic cartilage, and were thus able to confirm *Glack's* hypothesis experimentally. If, as previously suggested, the close correlation between the various enzymes which together effect the glycolytic break-down of glycogen is considered as a vital function, this can explain why fixation of the tissue with formalin, chloroform, ether, alcohol and acetone, as well as vacuum drying, inhibits the glycolysis, and thus the calcification in inorganic solutions. However, as the processes mentioned do not inactivate phosphatase, they have no influence on the calcification in solutions containing phosphoric acid esters. Treatment by 10 per cent formalin, however, which does not inhibit alkaline phosphatase by brief action but does so by prolonged action, also produced a powerful but incomplete inhibition of the calcification in solutions of phosphoric acid esters (*Robison, McLeod & Rosenheim* 1930). Several investigators, particularly *Gutman & Yu* (1949, 1950, 1951) and *Marks & Shorr* (1950 a), have investigated calcification *in vitro* in inorganic solutions, and the inhibition of this process by substances acting as enzyme inhibitors. Phlorizine, which hinders the conversion of glycogen to glucose-1-phosphate by inhibiting phosphorylase, inhibits the calcification in concentration of 10^{-2} M. when phosphate is only present in inorganic form, whereas the calcification proceeds normally if the phosphate is present as glucose-1-phosphate or as a later stage in the glycolysis (*Gutman, Warrich & Gutman* 1942, *Gutman* 1946, *Gutman & Yu* 1949). Iodoacetic acid in a concentration of 10^{-4} M. inhibits the calcification when the phosphate is present in the inorganic form (*Robison & Rosenheim*, 1934) and when phosphoric acid esters were used from those steps in glycolysis

prior to diphosphoglyceric acid, but not when phosphate was present in the form of later steps in the glycolysis (*Gutman & Yu* 1950). NaF in a concentration of 10^{-5} M. inhibits calcification *in vitro* in inorganic solutions (*Robison & Rosenheim* 1934) and with the use of all those phosphoric acid esters which occur as stages in the glycolysis, but not those which do not take part in the glycolysis, such as d-glycerophosphate, α -glycerophosphate and phenyl phosphate (*Gutman & Yu* 1950). It is still not clear which phosphoric acid esters finally function as substrate for the alkaline phosphatase. It is possible that a dephosphorylation of phosphopyruvic acid takes place, but a transphosphorylation to adenosine-5-phosphate or to adenosine-diphosphate may also take place, whereby respectively pyruvic acid, adenosine-diphosphate and adenosine-triphosphate (ATP) are formed, of which ATP can function as a substrate for alkaline phosphatase (*Gutman & Yu*).

The experiments described, showing the inhibition of calcification in inorganic solutions by NaF, which inhibits the glycolysis, are very difficult to evaluate with certainty, as the addition of fluorine ions alters the experimental conditions decisively. The solubility product of CaF is 3.4×10^{-11} ; this implies that the highest possible calcium ion concentration in *Robison & Rosenheim's* experiments must have been 3.4×10^{-6} , as the concentration of fluorine ion in these experiments was 10^{-5} M. This calcium concentration, however, is only about 1/1000 of the amount of calcium ion added (2×10^{-3} M. Ca^{++}). When calcification nevertheless does take place in solutions containing glycerophosphate, for example, it must signify that locally, where the phosphatase is active, a very high concentration of phosphate ions has been achieved.

Marks & Shorr (1950 a and b) have shown that in inorganic solutions *in vitro*, calcification does not take place after the glycogen has been digested by ptyalin, or has disappeared, presumably by glycolysis, as a result of incubation for 18 hours in solutions not containing Ca^{++} and PO_4^{---} .

The experiments described show that inorganic phosphate can appear as phosphoric acid esters via glycolysis, and these can then be split by alkaline phosphatase. Thus, calcification experiments

in vitro, which can be explained as described here, cannot be in contradiction to the hypothesis advanced.

As mentioned above, beryllium inhibits alkaline phosphatase. The inhibition was partly dependent on the substrate used and on its concentration. Beryllium also inhibits the calcification of rachitic cartilage *in vitro* in inorganic solutions of Ca and P. It has been difficult to explain the action of beryllium on the process as Be^{++} does not produce inhibition until 10^{-3}M , while normal calcification proceeds in the presence of 10^{-4}M . Be^{++} (Gutman & Yu 1951). The same state of affairs is found when using β -glycerophosphate and fructose-1, 6-diphosphate in 20 per cent solution (calcification only slightly reduced at 10^{-4}M . Be). It could possibly be that inorganic phosphate, having taken part in the glycolysis, occurs as a phosphoric acid ester, which behaves like β -glycerophosphate, and which is present where the phosphatase is active, in the same concentration as is found in cartilage incubated in solution with 20 per cent β -glycerophosphate.

Experiments without the phosphatase mechanism.

Waldman (1948, 1950) has shown that calcification in inorganic solutions can take place after the rachitic cartilage is boiled. However, it was necessary for the phosphate concentration to be high. In experiments with Ca in a concentration of 1 mM./l, calcification took place with a phosphate concentration corresponding to 10 mM.P/l when the cartilage fragment had been heated to 60° , whereas 20 mM./l was necessary after boiling. Also McLean *et al.* (1946) have shown that calcification can occur after the enzymatic processes have been inhibited, provided the phosphate ion concentration is raised. On the basis of the available investigations it is not possible to decide whether in such a case it is a question of crystal growth or of the formation of new crystals. It could be imagined that the adsorption of phosphate ions on collagen fibrils could produce such a local rise in phosphate ion concentration that apatite crystals can form.

Sobel *et al.*, in numerous experiments, have investigated the

calcification *in vitro* of rachitic cartilage and strontium-rachitic cartilage (for a review of the investigations, see *Sobel* 1952 and 1955). These investigators have shown that the "calcification mechanism" can be reversibly inactivated. If rachitic cartilage is shaken for two hours in a solution containing 150 mE Ca^{++} and one of the following metals in the concentrations given: Be^{++} , 0.1 mE./l, Ca^{++} , 0.5 mE./l, Mg^{++} , 10 mE./l, Na^+ , 50 mE./l, Sr^{++} , 100 mE./l, K^+ , 400 mE./l, all in the form of chlorides, then washed in distilled water and finally placed in a solution containing Ca^{++} and PO_4^{---} in such concentrations that untreated cartilage would calcify, no calcification is produced. However, calcification will take place if, after the treatment with distilled water, the pieces of cartilage are then shaken for one hour in solution of CaCl_2 containing 150 mE. Ca^{++} /l. The calcification is hereafter more pronounced than in the control sections.

It is difficult to judge the changes which might be produced in the rachitic cartilage by the above treatment. A part of the glycogen may have disappeared (cf. *Marks & Shorr's* experiments). Like all heavy metals, Be^{++} , Ca^{++} and Sr^{++} can act as enzyme inhibitors. Finally, it is possible that by treatment with calcium ions in concentrations which are 30 times greater than the total calcium content in the blood, where only a small part of the calcium is ionized, modifications to the cartilage are produced which are the cause of later calcification, but it is doubtful whether this calcification can be compared with the normal calcification process. *Freudenberg & György* (1923) showed that cartilage matrix in articular cartilage and costal cartilage, which cannot calcify *in vivo*, can calcify *in vitro*. Cartilage matrix can bind calcium by being shaken with a solution of CaCl_2 . On subsequent treatment with a solution containing phosphate ion, calcification took place. As it is not possible with Ca^{45} to demonstrate *in vivo* that cartilage matrix binds calcium outside the regions which calcify (*Leblond et al.* 1955), it is unlikely that the calcification taking place in *Freudenberg & György's* experiment can be regarded as identical with the process producing calcification *in vivo*. It is possible that something similar is the case for the calcification

after treatment with 150 mE.Ca⁺⁺ in *Sobel's* experiment. This experiment must thus be regarded in its first part as an inhibition of the normal calcification mechanism, and in the second part of the experiment as calcification by means of another mechanism, in analogy with the investigations of *Freudenberg & György*. From the results of crystallographic investigations, *Hendricks* (1952) believed that the experiments of *Sobel et al.* could be explained by crystal growth. As previously mentioned, the present stage of our knowledge does not allow any definite conclusions on this.

The inhibition of calcification *in vitro* in inorganic solutions by means of toluidine blue can perhaps be explained as an inhibition of crystal growth. *Miller, Waldmann & McLean* (1952) showed that basic dyes, including toluidine blue, inhibited the calcification of rachitic cartilage in inorganic solutions, but not in solutions containing phosphoric acid esters. *Sobel & Burger* (1954) (see also *Sobel* 1955), carrying out similar investigations with toluidine blue, showed that the "calcification mechanism" could be reactivated by means of Ca⁺⁺ in a concentration of 150 mE./l, as in the experiments described above. They imagined the "calcification mechanism" associated with a collagen-chondroitin sulphuric acid complex, which binds calcium. When toluidine blue is bound to this complex, the power to bind calcium, and thereby to produce calcification, is lost. *Baker, Reaven & Sawyer* (1953) showed that toluidine blue did not hinder kidney calcification produced by large doses of parathyroid hormones. *Miller et al.*, by analysis, found that calcium and phosphate could be taken up in cartilage after staining with toluidine blue, but that in spite of this, calcification could not be demonstrated by silver staining. It is mentioned as a possibility that staining with toluidine blue can inhibit crystal growth, as is the case in crystal formation in supersaturated solutions, where the formation does not take place if the mother crystal is stained with certain dyes. Should this be the case, calcification *in vitro* in solutions containing phosphoric acid esters can be explained by the fact that new crystals are formed which are not stained, so that the calcification process can continue

normally. The absence of inhibition of calcification *in vivo*, as found by *Baker et al.*, can be explained in the same way.

To sum up, the *in vitro* experiments appear to show that the phosphatase mechanism can also be effective *in vitro* and that calcification of rachitic cartilage *in vitro*, in solutions containing Ca^{++} and Po_4^{---} as inorganic compounds, can take place by the breaking-down of glycogen; hereby phosphoric acid esters appear which can function as a substrate for the alkaline phosphatase. Finally, certain experiments may possibly be interpreted as secondary crystal growth. Further research on this point, however, appears to be necessary.

SUMMARY

Chapter 1.

Introduction.

The aim of this study has been to review, from an essentially histological standpoint, the association between alkaline phosphatase activity, calcification and bone formation. It is based in part on the author's previously published investigations.

As introduction, previous conceptions of the association between alkaline phosphatase activity and calcification are briefly reviewed. Robison demonstrated phosphatase in calcifying cartilage and believed originally that it was possible to explain calcification as a result solely of the phosphatase activity, which splits phosphate ion from ester-linked phosphate. The phosphate ions accumulate, the solubility product of calcium phosphate is exceeded and calcium phosphate is precipitated. In order to explain why calcification does not occur in all tissues containing phosphatase, and further to explain that calcification can occur *in vitro* in solutions containing phosphate ion only in the form of inorganic compounds, Robison modified his theory to comprise two factors: the phosphatase and a local factor, the result of which should be that calcium phosphate is precipitated from a supersaturated solution and bound to the tissue.

Subsequently, Roche assumed that phosphatase plays a part in bringing about such modifications in the ground substance that the latter becomes calcifiable, and also that the phosphatase has an accelerating effect on the process of calcification as a result of hydrolysis of phosphoric acid esters, but that the calcification is not triggered off by this process.

McLean, Sobel and several other authors do not believe that any significance can be ascribed to phosphatase in the actual calcification, but at the most for the formation of the ground substance. They believe that it should be possible to explain the calcification solely on the basis of physico-chemical laws of crystal formation and crystal growth, without any enzymatic process being involved.

The following circumstances have made it possible to re-investigate the problem: 1) the introduction of diazo methods for the histochemical determination of alkaline phosphatase activity, as it is hereby possible to examine the phosphatase activity without simultaneously staining the calcium phosphate already present; 2) the demonstration that alkaline phosphatase activity can arise in the course of cell degeneration, and the demonstration of the association between phosphatase activity arising in this way and calcification in tissue cultures.

Chapter II.

Review of author's investigations on the relation between cell degeneration and phosphatase activity.

A strong phosphatase activity was found in osteoblasts from tissue cultures by phosphatase staining, while no such activity could be demonstrated in normal cultures of fibroblasts from embryonic heart or extremity musculature. On sub-culturing the fibroblasts, however, phosphatase activity could occasionally occur. It was shown that this took place simultaneously with clear signs of cell degeneration, particularly visible by phase contrast microscopy. It has been possible to reproduce the phenomenon using various methods of growing cells *in vitro*.

It was possible to show that adsorption of phosphatase from the medium by degenerating cells could not be involved.

The occurrence of phosphatase activity in association with cell degeneration was also demonstrated in tuberculous lymph nodes, where it was furthermore possible to show that glycogen was

formed in the cells prior to the occurrence of phosphatase activity and signs of degeneration.

Based on investigations on the phosphatase activity in cartilage, the concept was formed that the occurrence of alkaline phosphatase during the hypertrophy of cartilage cells is an expression of cell degeneration, whereas the presence of phosphatase in osteoblasts is conceived as expressing a process of differentiation.

Chapter III.

The histological relationship between alkaline phosphatase and calcification.

On investigating the relation between phosphatase and calcification in tissue cultures, it was found that calcification takes place precisely at those sites where phosphatase could be demonstrated, but that the calcification occurred about 1—2 days later than the appearance of the phosphatase activity. It could furthermore be shown that calcification did not take place until the cells containing phosphatase showed signs of necrosis (absence of nuclear staining with methyl green). Furthermore, the following relationships between phosphatase and calcification were found: 1) Phosphatase containing cells, no calcification (nuclear staining retained), 2) phosphatase and calcification present in the same cells, 3) calcification of cells without demonstrable phosphatase activity, the calcification being limited to the cell itself, 4) the calcification more extensive than in 3), the intercellular spaces (the medium) also being calcified, no phosphatase activity. The same state of affairs was found on investigating calcification in tuberculous lymph nodes (mesenterial lymph nodes from pigs with avian tuberculosis).

On examining cartilage in mouse embryos it was found that calcification of the cartilage of the long bones developed when the hypertrophic cartilage cells became necrotic, and nuclear staining with methyl green or the Feulgen reaction was no longer present. In chick embryos, where the cartilage of the long bones

does not calcify in the embryonic stage until the primary medullary cavity is formed, phosphatase activity of the hypertrophied cartilage cells was found, but the cell nuclei could be stained with methyl green and by the Feulgen reaction. It could be shown that the hypertrophied cartilage cells could be recognized once more as living cells in the bone marrow.

The same relationship between cell degeneration and phosphatase activity, necrosis and calcification could be demonstrated in a case of calcification in the stroma of an adenocarcinoma of the rectum and in a calcified uterine fibromyoma. Calcification of the tubule cells of the kidneys is found in cases of mercuric chloride poisoning. Normally, these cells contain phosphatase. In a single experiment it was possible to show that the onset of the calcification was related to necrosis of the tubule cells, which agrees with previous histological investigations.

Previous investigations on the phosphatase activity in bone tissue are discussed. It is generally agreed that alkaline phosphatase is found in osteoblasts. The enzyme is released from these cells into osteoid tissue which thereupon calcifies, while the osteoblasts continue to live as phosphatase-free osteocytes in the calcified ground substance. The author's investigations are in agreement with this. Calcification in bone tissue thus does not occur in relation to cell degeneration and necrosis, but takes place when the phosphatase is found extracellularly in the osteoid tissue. Investigations on phosphatase activity in osteoid tissue from rachitic animals are discussed. In some cases the results show the presence of phosphatase activity, in other cases the results do not show its presence. The question is still open, but it should be stressed that it is not justifiable, on the basis of investigations on rachitic osteoid tissue, to conclude, as some authors do, that phosphatase does not occur in osteoid tissue. McLean, for example, bases his hypothesis of the lacking significance of phosphatase in calcification on such a conclusion.

Previous investigations on the role of phosphatase in the formation of collagen fibrils are discussed. Results are available which suggest an association between phosphatase activity and fibril

formation, while other investigations have been unable to show such an association. Investigations by the present author show that collagen fibrils can be formed without the participation of phosphatase-containing cells, but when a phosphatase-containing cell forms collagen fibrils, these also contain phosphatase. Some investigations have shown that collagen fibrils can adsorb alkaline phosphatase.

Chapter IV.

The mechanism of calcification.

A hypothesis for the process of calcification is established on the basis of the histologically demonstrable association between necrosis of phosphatase-containing cells and calcification. It is assumed that phosphatase, when it occurs in living cells, does not bring about any accumulation of free phosphate ions. The author's experiments on phosphatase staining of unfixed kidney sections might suggest that the effect of phosphatase in tubule cells is different from the effect of phosphatase as it is known from experiments with the isolated enzyme, which effect can explain the calcification process. The initiation of the calcification process may be imagined as due to the accumulation of phosphate ions, as the result of the action of the released phosphatase on phosphoric acid esters. The following substances could be imagined as substrates for the phosphatase: phosphoric acid esters formed during the breaking down of glycogen, adenosine triphosphoric acid, depolymerized ribonucleic acid, phosphocholine formed in cartilage by the splitting of lecithin by means of cartilage lecithinase. Further calcification could be demonstrated as taking place at a time when phosphatase was no longer present. This part of the calcification process must be conceived as taking place by crystal growth.

The hypothesis submitted is discussed in relation to previous investigations and previous hypotheses.

Chapter V.

Calcification experiments in vitro.

Previous investigations on calcification *in vitro* are surveyed. It is stressed that investigations on the calcification of cartilage *in vitro* are unsuited to a study of the mechanism of calcification in general, as two or possibly three different factors are found which can all vary according to the experimental conditions, namely, phosphatase, glycogen break-down by phosphorylation and the possibility of crystal growth from bone salt crystals which have not been demonstrated. It has not been possible to demonstrate bone salt in rachitic cartilage by ordinary microscopy after silver staining, but this does not preclude its presence, as electron microscopy has shown that the first crystals formed are about 1/100 the size of the smallest particles demonstrable by light microscopy.

The investigations published show that the phosphatase mechanism can also operate *in vitro*, and that glycogen break-down can convert inorganic phosphate to phosphoric acid esters, which in turn can be split by the phosphatase. Other investigations show that calcification can take place in highly supersaturated solutions after inactivation of the enzymes. In these cases it must be assumed that crystal growth has taken place, but the experiments should be repeated in such a way that there is a possibility of investigating each of the three different mechanisms independently. Electron microscopic investigations on the rachitic cartilages used in the experiments must thus be considered an essential requirement.

RESUME

Kapitel I.

Indledning.

Formålet med arbejdet har været at give en hovedsagelig på histologiske undersøgelser begrundet oversigt over sammenhængen mellem alkalisk fosfataseaktivitet og forkalkning samt knogledannelse, delvist baseret på egne, tidligere publicerede undersøgelser.

I indledningen gives en kortfattet oversigt over tidligere opfattelser af sammenhængen mellem alkalisk fosfataseaktivitet og forkalkning. Robison påviste fosfatase i forkalkende brusk, mente oprindelig at kunne forklare forkalkningen alene som følge af fosfataseaktiviteten, idet der som følge af denne fraspaltes fosfation fra esterbundet fosfat. Fosfationerne ophobes, opløselighedsproduktet for calciumfosfat overskrides, hvorefter calciumfosfat udfældes. For at forklare, hvorfor forkalkning ikke finder sted i alle fosfataseholdige væv og for at forklare, at forkalkning kan foregå *in vitro* i opløsninger, der alene indeholder fosfation i uorganiske forbindelser, ændrede Robison sin teori til at omfatte to faktorer, fosfatasen og en lokal faktor, der skulle bevirke, at calciumfosfat udfældes fra en overmættet opløsning og bindes til vævet.

Roche antager senere, at fosfatasen har betydning for at tilvejebringe sådanne ændringer i grundsubstansen, at denne bliver i stand til at forkalke, og at fosfatasen desuden virker fremskynende på forkalkningsprocessen ved hydrolyse af fosforsyreestre, men at forkalkningen ikke udløses af denne proces.

McLean, Sobel og flere forskere mener ikke at kunne tillægge fosfatasen nogen betydning for selve forkalkningen, højst for dannelsen af grundsubstansen. Forkalkningen menes at kunne for-

klares alene på grundlag af fysisk-kemiske love for krystaldannelse og krystalvækst, uden indvirken af nogen enzymatisk proces.

Når problemet kan tages op til ny undersøgelse skyldes det 1) indførelsen af diazo metoderne til histokemisk bestemmelse af alkalisk fosfataseaktivitet, idet det herved er muligt at undersøge fosfataseaktiviteten uden samtidig farvning af det allerede tilstedeværende calciumfosfat; 2) påvisningen af, at alkalisk fosfataseaktivitet kan opstå under celledegeneration, og påvisningen af sammenhæng mellem sådan opstået fosfataseaktivitet og forkalkning i vævskulturer.

Kapitel II.

Overview over egne undersøgelser over sammenhængen mellem celledegeneration og fosfataseaktivitet.

Ved fosfatasefarvning af osteoblaster fra vævskulturer fandtes en kraftig fosfataseaktivitet, medens sådan ikke kunne påvises i normale kulturer af fibroblaster fra embryonal hjerte- eller extremitetsmuskulatur. Ved videre dyrkning af fibroblastkulturer kunne fosfataseaktivitet dog af og til opstå. Det påvistes, at dette skete samtidig med, at der var tydelige tegn til celledegeneration, især synlige ved fasekontrastmikroskopi. Fænomenet har kunnet reproducere ved forskellige metoder til dyrkning af celler *in vitro*.

Det kunne vises, at der ikke er tale om et adsorptionsfænomen mellem degenereret celle og fosfatase fra dyrkningsmediet.

Fremkomst af fosfataseaktivitet i tilslutning til celledegeneration blev videre påvist i tuberkuløse lymfeknuder, hvor man yderligere kunne vise, at der dannes glykogen i cellerne på et tidspunkt, før der optræder fosfataseaktivitet og tegn på degeneration.

Undersøgelser over fosfataseaktiviteten i brusk fører til, at man opfatter fremkomsten af alkalisk fosfatase under bruskcellehyperτροφien som et udtryk for celledegeneration i modsætning til fosfataseindholdet i osteoblaster, der opfattes som udtryk for differentiering.

Kapitel III.

Den histologiske relation mellem alkalisk fosfatase og forkalkning.

Ved undersøgelse af relationen mellem fosfatase og forkalkning i vævskulturer fandtes, at forkalkning finder sted nøjagtig de samme steder, hvor fosfasen kunne påvises, men som regel skete forkalkningen ca. 1—2 døgn senere end fosfataseaktivitetens fremkomst. Yderligere kunne det vises, at forkalkning først fandt sted, når de fosfataseholdige celler viste tegn på nekrose (manglende kernefarvning med methylgrønt). Iøvrigt fandtes følgende relation mellem fosfatase og forkalkning. 1) Fosfataseholdige celler, ingen forkalkning. (bevaret kernefarvning). 2) Fosfatase og forkalkning til stede i samme celle. 3) Forkalkning af celler uden påviselig fosfataseaktivitet, forkalkningen begrænset til selve cellen. 4) Forkalkningen mere udbredt end 3), mellemrummene mellem cellerne (dyrkningsmediet) også forkalket, ingen fosfataseaktivitet. Samme forhold fandtes ved undersøgelser af forkalkning i tuberkuløse lymfeknuder. (Lymfeknuder fra mesenteriet af svin med aviær tuberkulose).

Ved undersøgelse af brusk fandtes hos musefostre, at forkalkningen af de lange rørnoglers brusk indtrådte, når de hypertrofiske bruskceller blev nekrotiske, og kernefarvningen med methylgrønt eller Feulgenfarvning ikke længere var til stede. Hos hønseembryoner, hvor de lange rørnoglers brusk ikke forkalker i embryonalstadiet forud for dannelsen af den primære marvhule, fandtes fosfataseaktivitet af de hypertrofiske bruskceller, men cellekernerne kunne farves med methylgrønt og med Feulgenfarvning. Det kunne vises, at de hypertrofiske bruskceller kunne genfindes som levende celler i knoglemarven.

Samme relation mellem celledeneration og fosfataseaktivitet, nekrose og forkalkning kunne påvises i et tilfælde af forkalkning i stromaet i et adenocarcinoma recti og i et forkalket fibromyoma uteri. Ved sublimatforgiftning findes forkalkning af nyrenes tubulisceller. Disse er normalt fosfataseholdige. Det kunne i et enkelt forsøg vises, at forkalkningen indtrådte i tilslutning til nekrose af

tubuluscellerne, hvilket er i overensstemmelse med tidligere histologiske undersøgelser.

Tidligere undersøgelser over fosfataseaktiviteten i knoglevæv gennemgås. Det anses for fastslået, at alkalisk fosfatase findes i osteoblaster, enzymet afgives fra disse til det osteoide væv, der herefter forkalker, medens osteoblasterne lever videre som fosfatasefrie osteocytter i den forkalkede grundsubstans. Egne undersøgelser er i overensstemmelse hermed. Forkalkning i knoglevæv indtræder således ikke i relation til celledegeneration og nekrose, men finder sted, når fosfatasen forekommer extracellulært i det osteoide væv. Undersøgelser af fosfataseaktivitet i osteoidt væv fra rachitiske dyr gennemgås, der foreligger resultater, der viser, at fosfatase er til stede og andre, der ikke har vist fosfataseaktivitet. Spørgsmålet er endnu uafklaret, men man må fastslå, at det ikke er berettiget, på grundlag af undersøgelser af rachitisk osteoidt væv, at konkludere, at fosfatase ikke forekommer i osteoidt væv, således, som det har været tilfældet, idet McLean blandt andet baserer sin hypotese om fosfatasens manglende betydning for forkalkning på en sådan konklusion.

Tidligere undersøgelser angående fosfatasens betydning for dannelsen af kollagene fibriller gennemgås, der foreligger resultater, der tydes som om der er sammenhæng mellem fosfataseaktivitet og fibrildannelse, medens andre undersøgelser ikke har kunnet vise sådan sammenhæng. Egne undersøgelser viser, at kollagene fibriller kan dannes uden medvirken af fosfataseholdige celler, men hvis en fosfataseholdig celle danner kollagene fibriller, er disse også fosfataseholdige. Nogle undersøgelser har vist, at kollagene fibriller kan adsorbere alkalisk fosfatase.

Kapitel IV.

Forkalkningens mekanisme.

På grundlag af den histologisk påviselige sammenhæng mellem nekrose af fosfataseholdige celler og forkalkning opstilles en hypotese for forkalkningsprocessen. Man antager, at fosfatasen, hvor

den forekommer i levende celler, ikke medfører nogen ophobning af frie fosfationer. Egne forsøg på fosfatasefarvning på ufixerede nyresnit kunne tyde på, at virkningen af fosfatase i tubulusceller er en anden, end den virkning af fosfatase, der kendes fra forsøg med det islorede enzym, og som kan forklare forkalkningsprocessen. Man må forestille sig, at forkalkningsprocessen indledes med, at fosfationer ophobes som følge af frigjort fosfatases virkning på fosforsyreestre. Som substrat for fosfatase kan følgende stoffer tænkes at fungere: fosforsyreestre dannede under glykogenets nedbrydning, adenosintrifosforsyre, depolymeriseret ribonukleinsyre, fosforylcholin dannet i brusk ved spaltning af lechitin ved hjælp af brusklechitinase. Videre forkalkning kunne påvises at foregå på et tidspunkt, hvor fosfatase ikke længere var til stede, denne del af forkalkningsprocessen må tænkes at foregå ved krystalvækst.

Den fremsatte hypotese diskuteres i forhold til tidligere undersøgelser og tidligere hypoteser.

Kapitel V.

Forkalkningsforsøg in vitro.

Gennemgang af tidligere undersøgelser af forkalkning in vitro. Man fremhæver, at dette materiale er uegnet til undersøgelser over forkalkningens mekanisme, idet der findes to og muligvis tre forskellige faktorer, der alle kan varieres ved forsøgsbetingelserne. Fosfatase, glykogennedbrydningen ved fosforylering og muligheden fra krystalvækst udfra ikke påviste krystaller af knoglemineral. Det forhold, at man ikke ved almindelig mikroskopi efter sølvfarvning har kunnet påvise tilstedeværelsen af knoglemineral i rachitiske bruske, er ingen garanti for at sådanne ikke findes, idet man ved elektronmikroskopi har kunnet vise, at de først dannede krystaller er ca. 1/100 af de mindste partikler, der kan påvises ved optisk mikroskopi.

De foreliggende undersøgelser viser, at fosfatasemekanismen kan være virksom også in vitro, og at glykogennedbrydningen kan omdanne uorganisk fosfat til fosforsyreestre, der atter kan spaltes

af fosfatasen. Andre forsøg viser, at forkalkning kan foregå i stærkt overmættede opløsninger efter, at enzymer er inaktiverede. Man må antage, at der i disse tilfælde kan have været tale om krystalvækst, men undersøgelserne må gentages på en sådan måde, at man har mulighed for at undersøge virkningen af de tre forskellige mekanismer hver for sig. Elektronmikroskopiske undersøgelser af de til forsøgene anvendte rachitiske bruske må derfor anses for en absolut nødvendighed.

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 - II: Alkaline phosphatase and calcification in tissue cultures.
Exp. Cell Research. 1956, 11: 403—416.
 - III: Alkaline phosphatase and calcification in tuberculous lymph nodes.
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 - IV: Bone formation and calcification in cartilage.
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