From: The Division of Oral Pathology, University of Illinois College of Dentistry, Chicago, Ill. U. S. A.
Head: J. P. Weinmann, M. D.

and

The Norwegian Institute of Dental Research, Oslo, Norway.
Head: B. Nygaard Östby, Ph. D.

BONE TISSUE FORMATION

A Morphological and Histochemical Study

by

HARALD LÖE

This investigation was supported by: Research Grants D-625 and D-658 from the Division of Research Grants, National Institutes, U. S. Public Health Service; U. S. Government under Public Law 584, Fulbright Act, and the Norwegian Research Council for Science and the Humanities.

Published as supplementum 27, 1959
of
ACTA ODONTOLOGICA SCANDINAVICA
vol. 17, no. 3 (311—427), 1959

Color matrices from Bernh. Middelboe Reproduktionsanstalt, Copenhagen
Color plates printed by Bianco Luno's Bogtrykkeri A/S, Copenhagen

Printed in Sweden
STOCKHOLM 1960
AB FAHLCRANTZ' BOKTRYCKERI

TABLE OF CONTENTS

PREFACE

The major portion of the present work was carried out at the Dental College, University of Illinois, U.S.A. During the last months of 1957 and through the year of 1958 I held a position as a Research Associate at the Department of Oral Pathology, Head: Joseph P. Weinmann, M.D. I am sincerely grateful for the financial support, for the excellent working conditions and for the prudent guidance he gave me. I am also very much obliged to Dr. Julia Meyer, Ph.D. Her constructive assistance and collaboration have greatly influenced the character of this publication.

During the initial period of experimentation Dr. Robert D. Ray, M.D., Ph.D., opened his well-equipped laboratories for me. This generosity is deeply appreciated. I want to thank Dr. James A. Yaeger, D.D.S., Ph.D., for inumerable discussions on the subject and for the personal friendship which developed from mutual professional interests.

The completion of the investigation has been performed at the Norwegian Institute of Dental Research, Oslo, Norway, where I presently hold a position as a Research Associate. I wish to express my gratitude to the Director of the Institute, Dr. Birger Nygaard Östby, D.D.S., Ph.D., for the confidence he has shown me, as well as for the economic support and technical facilities provided. Furthermore, I feel indebted to my colleague at the Institute, Dr. Einar Hals, D.D.S., Ph.D., for pertinent suggestions.

All laboratory work was done by myself, except for the microradiograms which were produced in the Department for Medical Physics, Karolinska Institutet, Stockholm, Sweden. I owe thanks to Dr. Arne Engström, M.D., for this kindness and to Miss Gudrun Bergendahl for her skilled assistance.

Oslo in May 1959.

H. L.

GENERAL INTRODUCTION

The presence of fundamentally different constituents in bone and the arrangement of the organic and inorganic portions, make it clear that bone represents a difficult tissue as far as histologic technique is concerned.

Up to recent days, mainly two methods have been available for microscopical study of hard tissues: Sectioning after decalcification or the making of ground sections and ground surfaces.

The first technique involves the removal of the inorganic salts; in the second no soft tissues will be recognizable, or at least no detailed study at the cellular level is feasible.

The shortcomings of these procedures are obvious and generally recognized. There has always been a desire to investigate the soft and hard tissues in their proper relationship. Some studies have been carried out with modified paraffin and celloidin techniques by which it was possible to obtain histologic sections of undecalcified bone. It appeared, however, that this method had to be limited to fetal or very young spongious bone.

The methacrylate embedding technique (*Woodruff & Norris*, 1955; *Yaeger*, 1958) seemed to provide a more effective and reliable method for the preparation of undecalcified sections for microscopical investigation. One of the main purposes of the present study was, therefore, to elaborate this method for the making of sections of mature cortical bone which would allow morphologic and histochemical study of cells and mineralized intercellular substance of the same specimen.

Most of the knowledge on the formation of mature bone stems from studies on decalcified material from different parts of the skeleton. The relatively few reports on osteogenesis, based on studies of undecalcified preparations, have been restricted to observations on embryonic bone tissue or primary and secondary spongiosa of the epiphyseal region of the extremities.

A hundred years ago histologists using partly or completely decalcified long bones, observed that when bone was formed, it was invariably preceded by an organic matrix, which subsequently became mineralized (*Virchow*, 1851; 1853).

More recent works on undecalcified material, however, could not confirm this detail. This led to the belief that the osteoid borders found in the diaphyseal region and elsewhere, were either artefacts (*Weidenreich*, 1923) or signs of pathologic conditions (*McLean & Bloom*, 1940; *Bloom, Bloom & McLean*, 1941). This, in turn, laid the foundation for the statement that bone matrix normally was calcifiable and became calcified simultaneous with its laying down (*McLean & Urist*, 1955). There is, however, evidence for the presence of osteoid at sites of bone tissue formation even if it is not seen in the light microscope (*Robinson & Cameron*, 1956). The same phenomenon occurs when dentine is formed. Dentine is always preceded by an organic matrix, the predentine, which later becomes mineralized. The width of the predentine has permitted the study of, and given valuable information about the processes which result in calcified dentine (*Irving & Weinmann*, 1948, *Hals* 1953). Similar processes of tissue differentiation have been postulated to take place in the osteoid prior to mineralization (*Weinmann & Sicher*, 1955).

The amount of osteoid varies considerably in different areas of one and the same skeleton (*Erdheim*, 1914) as well as in various species and ages (*Lehnert*, 1910; *Pommer*, 1885, 1925).

Knowing that the diaphyses of dog bones normally exhibit a considerable width of the osteoid (*Lehnert*, 1910), it was thought that this tissue would be favorable for the study of the differentiating processes leading to calcified bone tissue.

Studies on the mineralization mechanism itself have almost without exceptions been performed on calcifying cartilage. Although there exist most likely some common characteristics in bone and cartilage, and although there may be some common conditions required for mineralization to take place in any tissue, it also seems true that there are basic differences as to the various constituents involved, as well as to the process itself (*Gutman & Yu*, 1950) and to the end result (*Robinson & Cameron*, 1957).

For these reasons, it was decided to take advantage of the material and the technique already mentioned, and to study the processes which lead to the formation of mature bone tissue by the application of conventional methods of investigation to undecalcified sections of bone known to have wide osteoid borders. In order to permit a comparison with the findings recorded in the literature, the present investigation included sections of bone treated with the classical fixatives and decalcifying agents.

As will be seen, the present investigation has shown that the osteoid is a highly differentiating tissue. Three major zones of the osteoid are defined. On the basis of the structural appearance of these zones, and their histochemical behavior, the characteristics of the tissue components within the different parts of the osteoid are described.

In this way it has been possible to disclose some of the dynamic processes taking place in this tissue.

The characteristic structural arrangement of bone tissue, together with its specific chemical composition and important physiological activity have been a challenge to researchers for a long time. The complexity of this tissue, however, calls for profound knowledge in a multitude of sciences.

Against this background it may seem courageous of a morphologist to forward a concept on the formation of bone tissue. On the other hand, the new morphologic and histochemical findings, made possible by the choice of material and techniques, claim to be worked into already existing hypotheses on the processes involved in bone tissue formation.

EARLIER INVESTIGATIONS

CHAPTER I · BONE TISSUE

Bone is a specialized type of connective tissue which owes its characteristics to its structural arrangement and chemical composition. As any other living tissue, bone consists of cells and intercellular substance. The cells, known as the osteocytes, in mature lamellated bone extend numerous branching processes in all directions and planes, whereby processes from one cell fuse with those of the cells in the neighborhood. The most superficial osteocytes, in addition, communicate with cells on the surface of bone by these means. A syncytium thereby exists between the cells themselves, and a communication is established between the cells and the tissues surrounding the bone. The osteocytes, or better the osteocytium is enclosed in the calcified intercellular substance. Whether the processes actually fill the entire length and lumen of the canaliculi in mature bone appears not to be clear (*Ham*, 1953). There seems to be no doubt, however, that the cellular arrangement constitutes part of the nutritional pathways in bone tissue, and as such is of importance in the maintenance of the vitality of the tissue.

Except for the narrow portion bordering upon the osteocytes (the osteocytic capsule), and structures like cementing lines (*Weinmann & Sicher*, 1955), the composition of all of the calcified intercellular substance has been considered basically identical.

Varying somewhat with the type and maturation (*Amprino & Engström*, 1952; *Weinmann & Sicher*, 1955) bone tissue contains by weight about 70 per cent inorganic matter, 20 per cent organic structures and 10 per cent water (*Eastoe & Eastoe*, 1954).

More than 90 per cent of the organic component are collagen fibers (*Rogers, Weidmann & Parkinson*, 1952); also varying in

amount, thickness and arrangement according to age and function of the bone tissue in question (*Weinmann & Sicher*, 1955).

In mature bone, however, be it lamellated or bundle bone, the fibers always display a definite pattern of organization. This arrangement may sometimes, e.g., in the *Haver*sian system, be very subtile and specific (*Schmidt*, 1938, *Lacroix*, 1951).

Most of the studies on collagen have been performed on collagen from other tissues (*Wasserman*, 1951; *Gustavson*, 1956; *Bear*, 1956) but enough information seemed to be at hand to assume that bone collagen has essentially the same characteristics and properties as collagen of other connective tissues (*Neuman*, 1949, *Eastoe*, 1955).

Structurally, collagen of bone has been characterized on a microscopic level by the organization in fibers or fiber bundles. These fibers were found by electron microscopy to be composed of fibrils with an average diameter between 50 to 100 Å (*Robinson & Watson*, 1953; *Jackson & Randall*, 1956). *Robinson & Watson* (1953) noticed a considerable increase of the diameter of the fibrils from childhood to old age.

Electron micrographs of the fibers of bone revealed cross-banding with axial repeating periods of approximately 640 Å, which corresponded to what was found in other collagen. It has been suggested that there exists a relationship between these cross-bands and the internal arrangement of the collagen molecule (*Bear*, 1952).

Chemically, collagen is a protein or a group of proteins, built up from polypeptide chains arranged in parallel and characterized by its content of certain amino acids (*Neuman*, 1949).

Between the fibrous structures of bone is found a variable amount of interfibrillar substance and fluid, probably fundamentally very much like fluids of other tissues (*Gersh*, 1952). From isotope studies (*Bélanger*, 1954; *Amprino*, 1956) it appeared evident that the extracellular fluid must be in close relationship to the so-called ground substance. In fact, the statement has been put forward that the tissue fluid and the ground substance are "co-extensive and homogeneous, interconnected throughout" (*McLean & Urist*, 1955).

Based on results obtained by chemical assays, this interfibrillary phase of bone has been found to consist of a mucopoly-

saccharide-protein complex (*Masamune et al.*, 1950). *Rogers* (1951) estimated the amount of polysaccharides to be about 0.5 % of the organic material in cortial bone.

Meyer, Davidson, Linker & Hoffman (1956) reported the carbohydrate component of mature ox bone powder after decalcification to consist mainly of chondroitin sulphates, and a much smaller amount of hyaluronic acid and kerato-sulphate. *Dische, Danilczenko & Zelmenis* (1958) decalcified femur shafts from calves and steers in EDTA. Besides an acid mucopolysaccharide fraction they found a complex of neutral polysaccharides. Although the same components might be found in various other body tissues, the amount and the ratio of the components may be specific for bone (*K. Meyer*, 1956).

The presence of sulphated carbohydrates has been confirmed by isotope studies revealing incorporation of radiosulphur in the organic matrix (*Dzietwiatkowski*, 1949, 1951; *Bélanger*, 1954; *Engfeldt & Hjertquist*, 1954; *Amprino*, 1956).

Rogers, Weidmann & Parkinson (1951) and *Eastoe & Eastoe* (1954) reported of a "resistant protein" in bone which they could not dissolve by decalcification and autoclaving. The significance of this protein in bone tissue is not quite clear.

Highly polymerized carbohydrates have been demonstrated in the intercellular substance of normal and pathologic bone by histochemical means (*Cobb*, 1949; *Heller-Steinberg*, 1951; *Engel*, 1952; *Yaeger*, 1958). Using periodic acid-*Schiff* technique, they all reported results which substantiated the statements that the degree of polymerization of the mucopolysaccharides was proportionate to the degree of calcification.

Sylvén (1947) observed that in undecalcified sections, the calcified portions of bone did not show metachromasia. *Levine, Rubin, Follis & Howard* (1949), however, demonstrated the presence of metachromatic material evenly distributed in bone, provided the sections were decalcified before staining. Incubation of calcified tissues with collagenase, trypsin or hyaluronidase prior to staining with periodic acid-*Schiff* (*Burstone*, 1952) or toluidine blue (*Wislocki & Sognnaes*, 1950), did not appreciably change their staining qualities.

The inorganic bone salts appear to consist of a crystalline calciumphosphate complex and a non-crystalline component in

the form of calcium carbonate. In addition, smaller amounts of various ions may be present (*McLean & Urist*, 1955).

X-ray diffraction studies have revealed that the main arrangement of the crystalline structure may be close to that of a hydroxyapatite, and that this most likely is surrounded by a non-crystalline shell of varying composition (*Carlström*, 1955).

Whether the shape of the crystals is that of platelets (*Robinson & Cameron*, 1956), rods (*Carlström & Engström*, 1956) or needles (*Sognnaes*, 1955) does not seem to be fully clarified. Possibly all these forms are found, and the apparent discrepancies are dependent on the area of the tissue from which the specimens are taken (*Sognnaes*, 1958).

Polarization optical analysis of bone tissue (*Schmidt*, 1938) has shown that the bone salts exist as negative uniaxial crystallites which are elongated along their optical axes, and arranged parallel to the lime-embedded fibers. In untreated condition the positive birefringence of bone is largely due to the collagen fibres which overcompensate the much weaker negative birefringence of bone salts.

Also x-ray diffraction (*Carlström & Finean*, 1954; *Wallgren*, 1957) and electron microscopy (*Robinson & Watson*, 1953; *Jackson*, 1957) revealed an intimate relationship and a definite orientation between the organic fibrillar structure and the mineral crystals. *Robinson & Watson* (1953) observed the crystals to be situated within as well as on or very near the surface of the collagen fibrils. This finding was later confirmed by *Sheldon & Robinson* (1957) and *Jackson* (1958). *In vitro* studies by *Glimcher, Hodge & Schmitt* (1957) seemed to corroborate the statements about the interconnection between collagen and bone mineral.

Most workers, however, seem to believe in the mucopolysaccharide-protein complexes as a cementing substance of some sort. Whether these substances, however, are solely cementing the fibrils and fibers together (*Partridge*, 1948), or the mucoproteins act as a calcium binding matrix (*Levine & Schubert*, 1952; *Yaeger*, 1958), or a collagen-polysaccharide-protein complex is necessary for molecular interconnection between bone matrix and bone salts (*Neuman et al.*, 1952), does not seem to be clearly understood.

CHAPTER II - BONE TISSUE FORMATION

Introduction

As seen from the preceding chapter, bone tissue consists of cells, organic matrix and minerals. The problems, therefore, involved in the understanding of osteogenesis are the origin of the bone cells, the production and organization of the matrix, and finally the mechanism of deposition of mineral salts in this matrix.

The process of bone formation seems to be essentially similar whenever and wherever it occurs *(Weinmann & Sicher,* 1955), in fetal or post-fetal life *(Gardner,* 1956). Any distinction between endochondral and intra-membraneous formation of bone will consequently refer to the tissues which are to be replaced, rather than to the actual process of bone formation. Bone tissue is always laid down in loose connective tissue, in which the organic elements required in osteogenesis are qualitatively present. Minerals are normally available at adequate rates in the environmental tissue fluid through the blood supply.

Although ectopic calcifications have been known to take place in the absence of any specific cellular activity (*McLean & Bloom,* 1940) it seems generally accepted that the cells related to the formation of bone are the osteoblasts (*Pritchard,* 1956). The location of these cells indicates their relationship to the process in general, and their morphology and cytochemistry have given evidence for specific functions (*Heller-Steinberg,* 1951; *Pritchard,* 1952; *Jackson & Randall,* 1956). These studies revealed that the main function of the osteoblasts would be the production of fibers and interfibrillar substance.

Since these functions are characteristic of many cells of mesenchymal origin (*Wassermann,* 1951), the most important rôle

played by the osteoblasts — which also would justify their name — must be to organize the organic elements and confer the calcifiability upon this tissue. The details concerning these abilities of the cells seem, however, still to be a matter of conjecture.

Most osteoblasts, eventually, are embedded in the bone matrix and become osteocytes. The cells lining the bone surface where no bone apposition occurs, are called resting osteoblasts (*Pritchard*, 1956).

Presence of Osteoid as a Stage in Osteogenesis

Virchow (1851, 1853) was probably the first to describe the organic precursor of bone. From his studies on shell fish and human bone, he was able to show the existence of "an osteoid connective tissue" which, he felt, became ossified by simple deposition of calcium salts. He also applied the name "osteoid" to this tissue.

Pommer (1885, 1925) using partly decalcified sections confirmed the observations of *Virchow*, and furthermore observed that osteoid did occur not only in growing individuals, but that this tissue also was encountered in adults.

From his study of the formation of the mandible, *Schaffer* (1888) described "eine weiche, zellenlose und anscheinend homogene Masse", that he thought would change into a fibrillar structure, in which calcification finally would occur. *Stoltzner* (1902) also believed that osteoid was a representative stage in osteogenesis, that would last "as long as the bone forming cells kept their osteoblastic character".

Whereas the authors mentioned invariably used decalcified material, *Wieland* (1909) examined undecalcified celloidin sections of embryonic and very young bone. He found, as other researchers had done, that osteoid was regularly present and easily distinguished from the calcified bone as well as from the osteoblasts. *Wieland* applied the term "physiological osteoid", thereby stressing the normality of its occurrence.

In a thorough study of growing and adult normal and rachitic rats, *Erdheim* (1914) showed that osteoid was present both in growing and adult normal animals, as well as in rachitic ones,

but that the width of the osteoid might differ in the various parts of the skeleton. He was generally unable to find osteoid on primary spongiosa. In the secondary spongiosa the amount of osteoid was scarce. The diaphyseal bone always showed osteoid of considerable width.

Weidenreich (1923), on the other hand, denied the presence of any osteoid in the normal process of bone formation. He characterized the findings of the above mentioned authors as artefacts produced by faulty techniques.

From his point of view, there was no definite difference between calcification and true bone formation, as both processes merely involved the deposition of calcium in a ground substance. If bone were formed in the presence of osteoblasts, he believed, these cells formed the fibrous matrix and secreted the calcium salts simultaneously. *Watt* (1928) studying the development of bone in embryos, corroborated the statements of *Weidenreich* on the absence of osteoid and the rôle of the osteoblasts as "bone-secreting" cells.

McLean & Bloom (1940) and *Bloom, Bloom & McLean* (1941) supported the view that the matrix was normally calcified simultaneously with its deposition, or at least so soon thereafter that no intermediate stage in the form of osteoid could be recognized. The study was made on undecalcified sections of bones of rats, puppies and kittens. In agreement with observations made by *Erdheim* (1914) they were unable to find osteoid borders on primary spongiosa. Neither did the secondary spongiosa usually have osteoid. In the shaft, however, they commonly observed osteoid lining in the growing Haversian systems. On the basis of these observations they made the statement that osteoid was not a necessary stage in the process of bone formation, and that when osteoid occurred, this might have been due to deficient local supply of bone minerals. Work along the same line on embryonic rats by *Bloom & Bloom* (1940) revealed that although osteoid was present at the very beginning of the formation of bone trabeculae in the connective tissue, further growth occurred without the presence of osteoid.

In examining bone from pigeons during the egg-laying cycle, *Bloom, Bloom & McLean* (1941) noticed that during the preovulatory formation of new bone — when the bone formation is

rapid — the bone newly laid down just opposite the osteoblasts was not densely calcified as revealed by the *von Kossa* technique. On the other hand, true osteoid was not seen.

Follis & Jackson (1943) also stated from studies on human bone that the presence of osteoid "in any quantity" indicated disturbances in the deposition of bone salts.

Microradiographs compared with decalcified histologic sections, however, confirmed the observations of earlier investigators that osteoid was present as a stage in osteogenesis and, moreover, that the osteoid was uniformly uncalcified (*P. G. Meyer,* 1956). The translucence of this tissue to "soft" x-rays was also evident from pictures published by *Vincent* (1955) and *Lacroix* (1956). The latter authors demonstrated a zone surrounding the osteoid that definitely showed a lesser degree of calcification than older bone.

Examination in the fluorescence microscope showed that calcified bone has a primary fluorescence, whereas osteoid has not (*Hals,* 1953).

Robinson & Cameron (1956) investigated the formation of bone at the epiphyseal line in the electron microscope. These authors found a zone of uncalcified tissue between the osteoblasts and the calcified bone. The completely uncalcified zone usually exhibited a width of less than 1.5 microns, whereas the region of mineralization seemed to be an additional 2 to 3 microns wide. In a more recent paper (1958) the same authors report the width of osteoid in the epiphysis to vary between 500 Å and 2 microns.

Without giving any measurements of the width of the osteoid, *Jackson & Randall* (1956) concluded from similar work that "considerable amounts of osseous organic matrix are formed before the process of mineralization occurred". It is clear, however, that the measurements given by *Robinson & Cameron* (1956, 1958) corresponded to the figures given by *Erdheim* (1914) for the epiphyseal region (below 1 micron).

Measurements from various regions of bones (*Pommer,* 1885; *Wieland,* 1909; *Erdheim,* 1914) have disclosed that the thickness of normal osteoid varies greatly with the locations in one and the same individual, as well as with the developmental stages and species. Thus, *Lehnert* (1910) found that osteoid in the diaphyses of the long bones of normal dogs, would make one think of rachitis if it occurred in human beings.

Morphology and Histochemistry of the Osteoid

Generally, osteoid has been described as a zone of uncalcified tissue (*Vincent*, 1955; *Lacroix*, 1956; *P. C. Meyer*, 1956), whereas the organic matter was either *a homogeneous* (*Pommer*, 1925) or *hyaline* (*Weinmann & Sicher*, 1955) or *edematous* material (*Pritchard*, 1952) that would change into a fibrillar mass before calcification.

The demarcation line between the osteoid and the bone matrix was found to be sharp, but irregular. In hematoxylin-eosin stained sections this boundary was recognized because of a fine, granular, basophilic deposit, in contrast to the bulk of the osteoid which normally was eosinophilic or unstained (*P. C. Meyer*, 1956).

Collagen

From his demineralized sections *P. C. Meyer* (1956) showed that the osteoid stained more intensely with *Mallory*'s connective tissue stain than the rest of the bone. However, when viewed in polarized light, it displayed the same appearance as the calcified lamellae.

Observations in the electron microscope from embryonic bone (*Jackson & Randall*, 1956) or from the epiphyseal region (*Robinson & Cameron*, 1956) did not seem to disclose any characteristic features of the organic precursor of bone that would distinguish the latter from connective tissue fibers and interfibrillary substance. It should be mentioned, though, that *P. C. Meyer* (1956) found fine radial striations to be present in the osteoid when examining ground sections from dog bone. This phenomenon he could not observe in human material.

Mucopolysaccharides

Sylvén (1947) studied sites of mineralization in cartilage of young rats. He was able to show that the cartilage lost its metachromatic material prior to calcification. This observation led him to the conclusion that calcified bone was free of ester sul-

phates. The same phenomenon was noticed by *Wislocki, Singer & Waldo* (1948) in their study of dentinogenesis.

Levine, Rubin & Howard (1950), on the other hand, observed that when sections of rat costal cartilage and bone were subjected to toluidine blue and periodic acid-*Schiff* reagents, a marked increase in intensity of these two staining reactions occurred in the zone of transition from the noncalcified to the calcified part of the matrix. This, they believed, was indicative of a change either in the state or in the concentration of the acid mucopolysaccharides, coincident with the onset of calcification.

Bevelander & Johnson (1950), studying the development of the head in pig embryos, found that the periodic acid-*Schiff* positive material was confined to osteogenic fibers from the beginning, and later on became generally distributed throughout the osteoid.

Vincent (1955) and *Lacroix* (1956) reported that osteoid was periodic acid-*Schiff* positive, but orthochromatic, while the newly calcified bone surrounding the osteoid was metachromatic.

Pritchard (1952) in his work on developing bone in rat fetuses found that newly deposited osteoid next to the osteoblasts stained metachromatically and reacted positively to periodic acid-*Schiff*.

Heller-Steinberg (1951) observed the periodic acid-*Schiff* reaction in osteoid to be weaker than in the rest of the bone. She also noticed metachromatic material in the osteoid, but it appeared that the area of metachromasia did not always coincide with the osteoid, as measured in sections stained with silver nitrate.

Leblond et al. (1950) stated that the osteoid sometimes did not contain any polysaccharides. Similar findings were made by *Fawns & Laundells* (1953) in a comprehensive study of human knee joints. They stated that osteoid consisted of collagen only. *P. C. Meyer* (1956) was likewise unable to discover either periodic acid-*Schiff* positive material, or metachromasia in the osteoid.

In summary, although the results from the studies mentioned seemed to be somewhat controversial, it is apparent from the majority of works on the subject, that acid mucopolysaccharides, presumably in the form of chondroitin sulphates are present in osteoid tissue.

The Isoelectric Point

Stained with buffer solutions of acridine orange prior to examination in fluorescent light, the osteoid showed no "concentration effect". A certain secondary fluorescence, a fresh-green color occurred, however, above pH 4.5 *(Hals,* 1953). Using acidometric methylene blue solutions, *Hals* also observed that at and above pH 4.5 the osteoid displayed metachromasia. The blue remained fairly unchanged at the neutral point and in the alkaline range. By means of these two basically different methods *Hals* was able to show that the isoelectric point of osteoid of alveolar bone was approximately pH 4.5.

Alkaline Phosphatase

Much attention has been paid to the possible connection between the alkaline phosphatase and the process of bone tissue formation since *Robison* (1923) on the basis of chemical studies discussed a possible significance of the enzyme in the calcification mechanism.

Freeman & McLean (1941) seemed to have been the first ones to apply the classical *Gomori* method (1939) for the visualization of phosphatase activity in undecalcified sections of bone.

Since that time a number of authors have studied the distribution of phosphatase in undecalcified sections of embryonic bone *(Gomori,* 1942; *Bevelander & Johnson,* 1950; *Pritchard,* 1952). Normal and pathologic bone tissue was studied by the same technique by *Freeman & McLean* (1941), *Bourne* (1943, 1948) and by *Follis & Berthrong* (1949). Tooth structures were investigated by *Engel & Furuta* (1942) and *Wislocki & Sognnaes* (1950).

The problem of distinguishing between the preformed phosphate in undecalcified bone and the phosphate liberated from the substrate by enzymatic activity evidently made difficult an interpretation of the findings, and thereby, an understanding of the specific location of phosphatase.

With the knowledge of the sensitivity of the enzyme towards acids (*Robinson,* 1923 and *Gomori,* 1939), *Bourne* (1948) decalcified the specimens e n b l o c after staining of sites of enzyme

activity. Although this procedure might have diminished the difficulties in cutting sections, it certainly did not contribute toward a more adequate method for the exact localization of the enzyme.

Seemingly more reliable results were obtained with *Lorch's* (1947) and *Greep, Fischer & Morse's* (1948) methods for the decalcification of bone before the histochemical test. Similar procedures were used by *Zorzoli* (1948), *Majno & Rouiller* (1951) and by *Siffert* (1951). The essence of the techniques of *Lorch* and of *Greep et al.* was that they utilized acid decalcifying solutions buffered to pH 4.5—5.0, whereafter the enzyme was reactivated in alkaline solutions at a pH about 9.

The results of the investigations of undecalcified bone mentioned above, and the reports of the findings after decalcification of embryonic rat bone (*Zorzoli*, 1948), young rat bone (*Lorch*, 1947; *Greep et al.*, 1948), and animal and human bone (*Majno & Rouiller*, 1951; *Siffert*, 1951) may be summarized as follows:

Phosphatase activity was found in the periosteum and in *Haversian* canals, as well as on the surface of spongious bone. The presence of phosphatase in these tissues appeared to be confined to osteogenic cells and fibers and to vascular elements. Phosphatase activity was absent from the osteoid itself but seemed to occur on the surface of the latter as well as of bone spicules.

Bevelander & Johnson (1951) reported positively reacting matter in the osteoid. A decrease and final loss of phosphatase activity seemed to occur toward the zone of calcification.

Majno & Rouiller (1951) on the other hand, contended that exhibition of positive enzymatic activity outside the osteoblastic layer was caused by diffusion phenomena. *Lorch* (1947) occasionally observed patches of positively stained material in the decalcified bone matrix. This she interpreted as being remnants of cartilage trapped in bone. It should be mentioned also that *Wislocki & Sognnaes* (1950) were unable to corroborate the findings of *Engel & Furuta* (1942) and *Gomori* (1943) that odontoblasts were devoid of phosphatase activity. In other words, they showed that the cells concerned in dentinogenesis displayed the same properties, with regard to alkaline phosphatase, as did the osteoblasts (*Pritchard*, 1952).

On the whole, it may be concluded that the distribution of the enzyme suggested some relationship between alkaline phosphatase and calcified tissues. This relation will be considered in greater detail in the following section.

Mechanism of Mineralization

Rôle of Alkaline Phosphatase

Alkaline phosphatase has been considered with great interest since *Robison* (1923) suggested this enzyme as the specific mineralization enzyme (see *Bourne*, 1956). Its action was thought to be that of hydrolyzing monophosphates, thereby increasing the concentration of phosphate ions in areas of bone tissue formation and of calcification of cartilage. This specific rôle of the enzyme in mineralization was, however, not concidered tenable, because calcification would occur in the presence of inorganic phosphorus (*Shiply, Kramer & Howard*, 1926). Later evidence indicating the lack of suitable substrate for the enzyme (*Roche*, 1950) and the improbability that formation of complex structures like the bone salts could take place by simple precipitation (*Neuman & Neuman*, 1958) have weakened the belief in the specific action of alkaline phosphatase in this regard. In fact, *Robison & Rosenheim* (1934) had searched for a second mechanism which together with phosphatase would account for the selective deposition of inorganic salts in areas of tissue calcification. *Robison* evidently believed that this second mechanism would also be enzymatic in nature, its main contribution being to provide the necessary amount of organic monophosphates.

In 1941 *Gutman & Gutman* demonstrated the presence of a phosphorylasing enzyme in cartilage which, together with the available glycogen (*Harris*, 1932) theoretically would give the substrate needed. The phosphate ions thus released were believed to become temporarily fixed on the pre-osseous matrix (*Roche & Mourge*, 1945) and later in presence of calcium ions to be precipitated as calcium-phosphate.

It should be remembered, as aptly stated by *Gutman & Yu*

(1950) that calcification of cartilage and the enzymatic processes involved might have but little application to the fundamental process of the mineralization of bone. The enzymes may have other functions than to initiate the calcification of cartilage. Thus, *Eeg-Larsen* (1958) contented that the glycolysis in cartilage was entirely related to the proliferative processes and intracellular metabolism, rather than to mineralization.

Finally, *Waldman* (1948) showed that slices of bone would calcify *in vitro* after inactivation of enzymes. This indicated that the "second mechanism" or "the local factor" *(McLean & Urist,* 1955), might have been so stable that it resisted the inactivation processes and made calcification possible in the absence of enzymes *(McLean,* 1950).

Although the rôle played by alkaline phosphatase appeared to be inconspicuous in the case of mineralization, it still might be of importance in the formation of the organic matrix of bone.

It was apparent that there does exist a relationship between fiber formation and alkaline phosphatase in soft tissue (*Danielli,* 1945). In accordance with this, *Follis* (1952) observed that phosphatase was absent in scurvy where fibrogenesis failed. During healing, fine argyrophilic fibers appeared in the vicinity of the osteoblasts. These fibers gave positive reaction for alkaline phosphatase.

Due to the general skepticism as to the specificity of the enzyme, together with the lack of evidence of any direct connection with the calcification process, alkaline phosphatase seemed to have been pushed in the background in the effort to find the "local factor" of mineralization. From time to time, and especially on the basis of histochemical findings, speculations occur as to the significance of alkaline phosphatase in the mineralization process (*Gomori,* 1945; *Sylvén,* 1947; *Lorch,* 1947, 1949a, 1949b; *Pritchard,* 1952).

Rôle of Mucopolysaccharides and Collagen

During the last decade the search for the local mechanism that would cause the selective deposition of inorganic calcium salts in the pre-osseous matrix, has been concentrated more on the chemical and physical properties of the osteogenic fibers

and their interfibrillary substance. Also *Robison* (1923) did not exclude that "the second mechanism" was hidden in these substances.

After their finding of an increased metachromatic reaction in areas about to calcify, *Rubin & Howard* (1950) suggested that this indicated either the formation of a new mucopolysaccharide, or a change in that already present, and that these changes represented the introduction of the local mechanism of mineralization into the bone matrix.

Cobb (1949), from her study of the periodic acid-*Schiff* reaction in epiphyseal tissues, believed that the increasing degree of polymerization of the mucopolysaccharides would increase the concentration of the calcium binding units. This idea was further elaborated by *Engel* (1958), who was of the opinion that the colloids of bone by virtue of the state of aggregation, possess a high density of negative charge. Due to this negative charge, bone matrix would attract and accumulate positively charged substances, thereby producing a calcium-protein complex.

Sobel (1952), studying the calcification of cartilage *in vitro*, found that a compound like chondroitin sulphate might well be the specific target for calcium salts during calcification. This author later (1955) appeared to favor the idea of *Neuman et al.* (1952), viz. that although chondroitin sulphate most likely played an important part in the calcification, the latter authors apparently did not consider chondroitin sulphate *per se* a vital factor, but suggested the importance of a chondroitin sulphate-protein complex that would contribute to the local mechanism.

Sylvén (1947) found that metachromasia disappeared in the vicinity of the osteoblasts. Consequently, he took up the idea put forward by *Hass* (1943) that the absence of mucopolysaccharides was a prerequisite for calcification.

This concept was further supported by *Glimcher, Hodge & Schmitt* (1957) who after experiments *in vitro* proposed a physico-chemical mechanism for calcification involving the state of aggregation of collagen macro-molecules. These authors, like *Hass,* suggested that non-collageneous components in the ground substance were responsible for the "inhibition and control of calcification".

OWN INVESTIGATIONS

CHAPTER III - MATERIAL AND METHODS

Material

The present study is based on investigations of bone from radius, tibia and metacarpals of five normal dogs between four and twelve months of age. The animals were all fed an adequate stock diet for several weeks before being killed. After intravenous injection of nembutal, the pieces of bone were removed while the animals were still alive. After amputation, the bones were cut transversely in small pieces. When the diameter was small enough (approximately 10 mm), no further division was made. In case of greater diametrical dimensions, the pieces of bone were also split longitudinally.

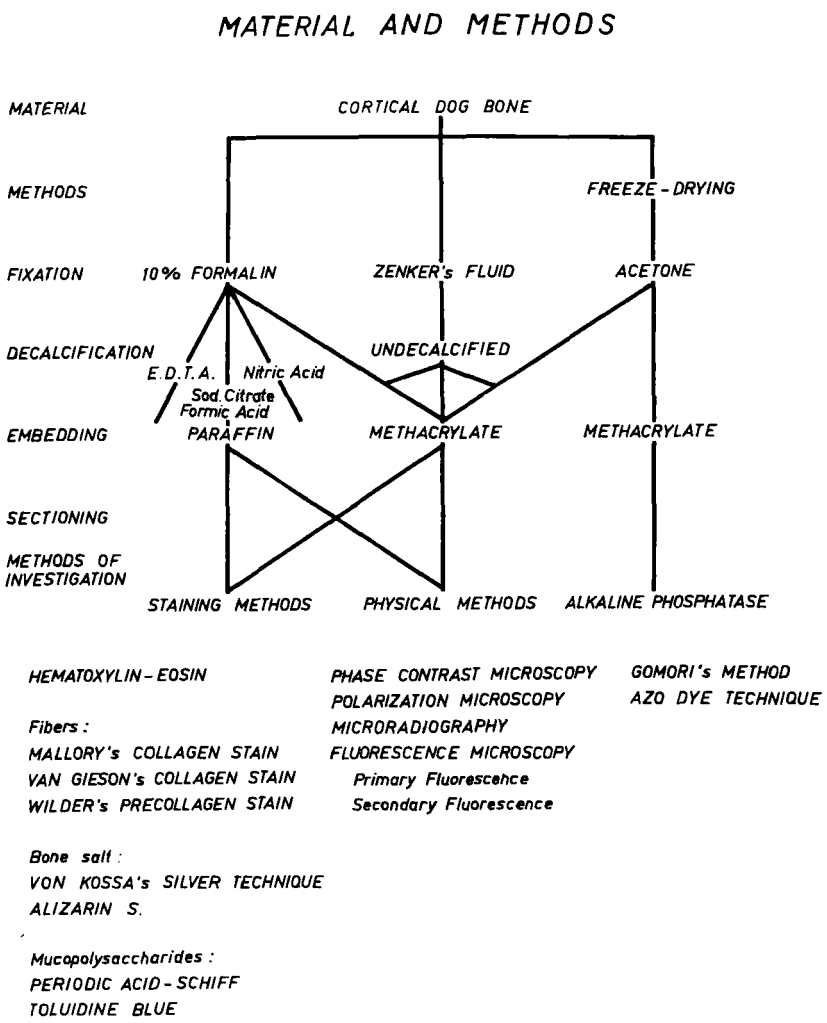
Methods

Fixation

In all instances the specimens were fixed within one or two minutes after amputation.

10 per cent fresh formalin was neutralized according to a formula given by *Langeland* (1957). The specimens were fixed for 48 hours at room temperature, after which they were washed in running water overnight. Other pieces from the same bone were exposed to *Zenker*'s fluid, where the acetic acid had been added shortly before use. The specimens were fixed in *Zenker*'s fluid for 15 hours, and subsequently washed overnight.

Pieces of the same bones were immersed into isopentane chilled in liquid nitrogen for freezing (—150°C) and thereafter dehydrated *in vacuo* at —30°C. The dehydrated material was post-fixed in acetone before embedding.



Textfig. 1. A diagrammatic summary of the material and the methods used in this investigation.

Undecalcified Sections

Embedding

The embedding medium for undecalcified bone consisted of equal parts of poly-methyl and poly-butyl methacrylate, or equal parts of poly-ethyl and poly-butyl methacrylate. The stock solutions were freed from the inhibitor (hydroquinon) by washing in three changes of five per cent sodium hydroxide, followed by washing three times in distilled water. The remaining water was removed with calcium chloride over night. After filtration, the monomer was accellerated (catalyzed) by adding 0.5 per cent Luperco (50 per cent peroxide in dibutyl phtalate). Accellerated monomer was kept in the refrigerator until used.

Formalin and *Zenker* fixed material was dehydrated in ascending alcohols, and allowed to stay in anhydrous acetone over night. Frozen-dried bone was immersed directly into acetone and kept there for an equal period. Infiltration of monomer into the tissues was facilitated by making the infiltrate a mixture of monomer and acetone, starting with 75 per cent acetone. The acetone content of the fluid was daily reduced by 25 per cent. Finally, the specimens stayed in pure monomer for 24—28 hours.

The infiltrates were changed daily and adequate amounts were provided. The infiltration process was run at room temperature, except in case of the frozen-dried specimens which were kept at 4°C.

Embedding syrup was made by letting accellerated monomer start to polymerize in an incubator at 60—70°C. The process was discontinued at the stage at which the polymer was viscous and would allow air bubbles to move to the surface when the flask was turned up-side down. At this point, the partly polymerized polymer was chilled and the flask returned to the refrigerator in order to avoid further polymerization.

Each piece of bone was placed in a small square bottle and covered with the syrup. Before incubation, negative pressure was used to remove the air from the specimen. The final polymerization and embedding took place in a water bath at a temperature of 40°C. Immersion of a maximum temperature thermometer into this methacrylate syrup (*Yaeger*, 1958), showed that

the maximum temperature within the block during polymerization, did not exceed 45°C.

Polymerization was completed over night. The blocks, freed by destruction of the bottles, were trimmed so that only a narrow shell of methacrylate surrounded the specimens. The embedded material was kept at 4°C during storage.

Sectioning

All undecalcified sections used in the present study were cut from methacrylate embedded bone on a metallurgic microtome, *R. Jung*, model K. Among the knives of the standard equipment, Knife No. 1 was found to be of little use in cutting sections of mature cortical bone. Knives Nos. 2, 3 and 4, however, gave equally good results. The angle in the horizontal plane between the specimens and the blade of the knife did not seem to be critical.

Cross-sections of diaphyseal cortical bone were cut at 4 microns. However, as a routine and when serial sections were wanted, sections were usually cut at 6 microns. In order to avoid folding of the sections, pieces of thin paper, (lens or cigarette paper) were glued to the cutting surface of the block with a water soluble glue. This proved to give the necessary support to the section during the passage of the knife through the block. The free end of the paper was held between two fingers of the one hand, while the other operated the microtome.

The knife blade was constantly kept moist with 70 per cent alcohol, a fact which also assisted in keeping the sections from folding during the cutting.

After cutting, the paper-backed sections was transferred to a slide with the section against the glass surface. A few drops of water served to dissolve the glue, whereafter the paper was removed. The section was subsequently kept on the slide while carried through alcohols of increasing strength to acetone in order to remove the embedding material. Usually, the sections were allowed to stay in acetone over night. Sections that were stained already after two hours in acetone did not exhibit any trace of undissolved methacrylate.

Except for the obvious necessity of having a heavy-duty micro-

tome and a firm connection between the specimens itself and the specimen holder, in this case the methacrylate block, the prerequisites for a successful cutting of undecalcified sections of cortical bone seemed to be the support of the section during the moment of cutting, and a slow, even passage of the knife through the specimen.

Apparently, material fixed in *Zenker*'s fluid was easier to cut. Nevertheless, the resulting sections seemed to be inferior to formalin and frozen-dried sections. It appeared as if the inhomogeneous consistency of these preparations might have hindered the knife from passing evenly through the bone.

Greater difficulties were encountered in trying to keep the sections on the slides during the staining processes. Evidently, the fact that the sections had to be kept moist all the time, to prevent breaking and curling up, did not permit the egg-albumin or gelatin solutions to display their adhesive qualities. A protecting collodium film was also tried. It appeared, however, impossible to obtain the same thickness of this cover on two different slides. It was realized that this difference would influence the staining ability of the sections and consequently make comparative studies impossible. The undecalcified sections, therefore, were carried one by one through the different staining solutions with a special section lifter, and thereafter mounted on slides.

Decalcified Sections

Some of the formalin fixed material was demineralized in ample amounts of the following demineralizing agents:

(a) Five per cent solution of nitric acid in water, for 48 hours.

(b) 45 per cent formic acid and 20 per cent sodium citrate in distilled water, according to *Morse* (1945). The pH of this solution was found to be 2.5. Demineralization was completed after seven days.

(c) Saturated solution of the di-sodium salt of ethylene diamine tetra-acetate, buffered with sodium hydroxide to pH 7.2. The tissue was suspended at a distance from the bottom of the jar and exposed to this fluid for three weeks *(Nikiforuk & Sreebny,* 1953).

The decalcification was controlled radiographically.

Demineralization took place at room temperature. After proper washing, the decalcified specimens were dehydrated in increasing strengths of alcohol at room temperature, and embedded in paraffin according to the usual procedure.

Methods of Investigation

Staining Methods

Both undecalcified and decalcified cross-sections of diaphyseal cortical bone were stained for the following purposes:

The *general morphology* was brought out by staining in alun hematoxylin-eosin.

Fibers were demonstrated by *Mallory*'s aniline blue phosphotungstic acid stain, and *van Gieson*'s picric acid—acid fuchsin, counterstained with iron hematoxylin. *Wilder*'s and *Gomori*'s ammoniacal silver techniques were also regularly used *(Mallory,* 1942).

Carbohydrates were demonstrated with the *Schiff* reagent subsequent to the application of periodic acid *(Hotchkiss,* 1948). Reducing rinse was used in all instances. Treatment of the sections with diastase prior to staining served to distinguish other polysaccharides from glycogen. Sections were also subjected to the *Schiff* reagent without preceding oxidation, in order to check whether positive reaction occurred after oxidation only. In addition, acid mucopolysaccharides were identified by staining with aqueous solutions of toluidine blue (National Aniline Division). Concentrations from 0.00001 per cent to 0.5 per cent toluidine blue were used for varying periods of staining. After staining and washing in water the sections were dehydrated in 70 per cent alcohol for one minute, followed by two changes of absolute alcohol, each for 2½ minutes.

Inorganic Mineral Salts. Sections from specimens fixed in *Zenker*'s fluid and undecalcified sections from formalin fixed and frozen-dried material, were stained according to *von Kossa*'s silver technique (1910) for inorganic salts.

For the same purpose an unbuffered 0.01 per cent solution

of sodium alizarin sulphonate in water (*McGee-Russell*, 1958) was applied to the sections for 2.5 and 10 minutes.

Alkaline Phosphatase. Sections of frozen-dried specimens were tested for alkaline phosphatase activity according to the technique of *Gomori* (1939). Incubation with glycerophosphate (pH 9.2) at 37°C lasted for one, three, and five minutes. Control sections were treated with iodine-potassium iodide solution for 20 minutes before incubation. Omission of glycerophosphate from the incubation medium, staining directly with cobalt nitrate-ammonium sulphide, or omission of calcium chloride from the incubation medium, served to distinguish between enzyme activity and preformed phosphates or other anions.

Alkaline phosphatase activity was also investigated with the azo dye coupling technique of *Menten, Junge & Green*, (1944) and *Mannheim & Seligman*, (1949). Incubation in the sodium salt of naphtyl acid phospate mixed with Azo Red RC lasted for 30 minutes at 4°C. Control sections were treated with *Lugol*'s solution before incubation. Sections stained by the azo method were mounted in glycerin jelly.

Physical Methods

Phase Contrast Microscopy. Unstained cut sections from the various series of decalcified and undecalcified bone tissue were examined in the phase contrast microscope. All the sections were mounted in canada balsam. A *Leitz* phase contrast microscope was used equipped with a phase contrast condensor according to *Heine*.

Fluorescence Microscopy. Unstained sections of undemineralized bone were examined in distilled water for their primary fluorescence. For this purpose a fluorescence microscope was used which made possible a radiation of the specimens with ultraviolet and blue light.

By fluorochromation of sections both from formalin fixed and frozen-dried bone, the preparations were investigated for secondary fluorescence.

Among the cationic fluorochromes, acridine orange was used, whereas eosin and thiazin red R were chosen among the anionic ones. Series of buffer solutions were prepared and checked

electrometrically. For pH 1, 0.1 N hydrochloric acid was used. For pH values between 1.7—11.0 phosphate buffers were prepared according to *Strugger* (1949). The concentration of the final staining solutions were 1: 1000.

Prior to fluorochromation, the methacrylate had been removed in the usual manner. The sections were carried through descending alcohols to distilled water, and subsequently kept in the wanted buffer solution for one minute. Following ionization, the sections were fluorochromated in the buffered dye solutions for one minute. After this, the sections were washed in the buffer solution and in distilled water. Finally, the sections were mounted in distilled water and examined immediately in a *Leitz* fluorescence microscope.

The fluorescence colors were recorded on Anscochrome Daylight Film.

Polarization Microscopy. Unstained cut sections were examined in polarized light. As usual, the methacrylate embedding medium was removed, and the sections were studied in distilled water, as well as in canada balsam.

A *Leitz* polarizing microscope was used.

Microradiography. Ground sections were made serially to the sections of the undecalcified specimens which had been cut for histological and histochemical examination. The thickness of the ground sections was 30—40 microns.

Exposures were made as follows:

No.	mA	KV	Filter	Roentgen	Time of exposure
9259	8	12	Be	W	30 min.
9264	8	10	Be	W	15 »
9265	8	8	Be	W	15 »
9266	8	8	Be	W	20 »
9267	8	10	Be	W	20 »
9268	8	10	Be	W	20 »
9269	8	10	Be	W	15 »
9272	8	7	Be	W	35 »

The images were recorded on Kodak Spectroscopic Plates, Emulsion No. 649-GH.

CHAPTER IV - OBSERVATIONS

OSTEOID

Osteoid in Undecalcified Material

Presence of Osteoid

In formalin fixed and in frozen-dried undecalcified sections stained with hematoxylin-eosin, an eosinophilic or partly unstained layer of tissue was observed separating the osteoblasts from the slightly more basophilic calcified bone of the osteons (Figs. 1 and 2).

This layer of tissue was found to occur in a variable number of osteons spread at random throughout the cross-section of the diaphyseal bone. Sections from the different bones of the same animal seemed to exhibit the same frequency of occurrence of this tissue. The number of osteons showing this phenomenon, however, seemed to decrease with increasing age of the animal.

Ground sections exposed to soft x-rays left no doubt that these layers consisted of non-calcified material (Figs. 5 and 6), or at least that they were made up of a tissue containing much less inorganic matter than the surrounding calcified bone.

Sections subjected to the *von Kossa* silver method revealed no stainable matter between the osteoblastic lining and the yellow-brown-black calcified tissue (Fig. 4). When cut and unstained sections were viewed in ultraviolet and blue light, it appeared that the tissue located between the osteoblasts and the calcified bone exhibited a very weak, grey-black primary fluorescence, whereas both osteoblasts and mineralized bone tissue showed a strong, blue-white primary fluorescence (Fig. 25).

In unstained sections viewed in the phase contrast microscope (Figs. 7 and 8) and in sections stained with hematoxylin-eosin

(Fig. 2), *Mallory*'s (Fig. 13) and *van Gieson*'s (Fig. 14) connective tissue stain and others, linear structures originating from the neighborhood of the osteoblasts were seen radiating throughout the uncalcified layer before disappearing into the calcified bone. There is hardly any doubt that these structures were the radiating striations observed in dog bone by *P. C. Meyer* (1956), and which he could not find in human material. Occasionally, osteoblasts were seen embedded, or partially embedded in the uncalcified layer (Figs. 2 and 7).

With the evidence of the correlation between the microradiograms, the fluorescence microscopic pictures and the staining methodes used, it became clear that the layer between the osteoblasts and the calcified bone, as it was seen in undecalcified sections, was osteoid. The linear structures represented the processes of the osteocytes, or the processes of the osteoblasts. That these structures have to be found in human bone tissue is self-evident.

In frozen-dried as well as in formalin fixed specimens these processes of the cells running through the osteoid appeared to arise from the osteoblasts as tapered, bright lines. They could be traced into the calcified bone in their canaliculi. Close to the osteoblasts, the area occupied by these processes in frozen-dried sections was approximately half of the entire osteoid tissue (Fig. 8). After formalin fixation the processes seemed compressed and were only half as wide as in frozen sections (Fig. 7).

Zones of the Osteoid

In hematoxylin-eosin stained sections, it was difficult to determine exactly the end of the osteoblasts and the beginning of the osteoid. This might mainly be due to the eosinophilia of both these structures. However, when stained in *Mallory*'s connective tissue stain (Fig. 13), as well as when viewed in fluorescent light (Figs. 25 through 32), the distinction between the cells and the osteoid became clearly visible.

A slight difference in the appearance of the osteoid was noted when undecalcified formalin fixed and frozen-dried bone stained with hematoxylin-eosin were compared. The osteoid in frozen-dried sections was fairly evenly stained with eosin. In formalin

fixed material the osteoid tissue showed marked eosinophilia only in the area next to the osteoblasts and the degree of eosinophilia decreased peripherally into an unstained area. This turned basophilic near the edge of the calcified bone (Fig. 2).

In unstained sections fixed in formalin or by freeze-drying examined with the phase contrast microscope (Figs. 7 and 8), three zones could be differentiated by their difference of optical path. These three zones were seen regardless of the width of the osteoid. The osteoid nearest the osteoblasts started off as a narrow, fairly bright layer. Farther from the cells this layer decreased in brightness, and about half-way through the width of the osteoid, the tissue appeared quite dark. It increased again in brightness, however, and reached a maximal brightness towards the calcified bone. A sudden decrease in brightness appeared in a granular area adjacent to the calcified bone. The border against the calcified bone was fairly sharp.

In sections stained according to *von Kossa* and counterstained with safranin, the peripheral part of the osteoid lost its bright red color, and became more yellowish just next to the edge of the calcified bone. In sections not counterstained with safranin, the inner and the major portion of the peripheral part of the osteoid were unstained, whereas the yellow color appeared adjacent to the edge of the bone (Fig. 3). The brown-black calcified bone did not show a clear-cut edge against the osteoid. Instead, the transition area displayed a granular appearance, the dark brown granules being more or less surrounded by the yellow colored material.

On the basis of the phase contrast microscopic picture (Figs. 7 and 8) (and for the sake of convenience in the following description) an inner and outer zone of osteoid, and a transitional zone between osteoid and calcified bone can be preliminarily defined as follows:

The inner zone, the young osteoid, begins at the apical end of the osteoblasts and comprises the narrow bright and the broader dark layer.

The outer zone, old osteoid, begins at the point where the osteoid starts to brighten again, and comprises the layer of increasing brightness.

The transitional zone, or the area of transition, represents the narrow area of maximal brightness which continues into the layer of granular appearance. Stained according to *von Kossa,* this zone was made partly visible by the yellow color.

As will be seen later, the inner, outer and transitional zones of the osteoid were visualized by a multitude of methods used in this study. It should be born in mind, however, that although the zones were clearly different, the border between them by no means represented sharp lines. Moreover, the individual width of all three zones appeared to vary independently of the width of the whole osteoid layer.

The entire width of the osteoid ranged between eight and fourteen microns.

Demonstration of Fibrous Elements

Phase Contrast Microscopy

In unstained sections examined with the phase contrast microscope the inner zone adjacent to the osteoblastic layer showed loosely packed fibers running more or less in all directions (Fig. 8). In approaching the middle part of the same zone, the fibrous structures seemed to be more densely packed. Most of the fibers in this area, appeared to be in the plane of the section, and parallel to the surface of the calcified bone. As the outer zone brightened peripherally, fewer fibers could be identified and the number further decreased in approaching the zone of transition. The part of the outer zone next to the rather sharply demarked border of the zone of transition, seemed to be devoid of fibers and to be of a more hyaline nature. Nor could any fibers be seen in the transitional zone.

The fibrous structures between the processes of osteoblasts and osteocytes stood out more clearly in frozen-dried acetone fixed specimens (Fig. 8) than after fixation in formalin (Fig. 7). The osteoid in formalin fixed specimens appeared more dense.

Staining Methods

The osteoid next to the osteoblasts within the inner zone stained heavily blue with *Mallory's* solution (Fig. 13). With *van Gieson's* collagen stain the area of the osteoid adjacent to the osteoblasts was almost unstained, but the intensity of the stain increased rapidly toward the middle of the inner zone (Fig. 14). However, the middle area of the inner zone was evenly stained by both *Mallory's* and *van Gieson's* stains. From here on, the amount of staining decreased. The outer zone remained unstained in *van Gieson's* stain and was stained yellow by *Mallory's* stain. Peripherally to the outer zone the area of transition first showed a very narrow rim of red granules whereafter the tissue took on a light blue hue in *Mallory's* and a light red in *van Gieson's* solutions.

Frozen-dried specimens responded somewhat differently. The blue or red staining of the inner zone extended into the outer zone. Therefore, the unstained or yellow part of the outer zone was reduced in width compared with the formalin fixed sections.

When neighboring sections of both formalin fixed and frozen-dried bone were stained for precollagenous fibers according to *Wilder's* and *Gomori's* silver techniques, a narrow black band of reduced silver was consistently found in the inner zone adjacent to the osteoblasts (Figs. 15 and 16). In the rest of the osteoid no argyrophilic fibers could be seen. Part of the remaining osteoid was stained brownish-pink. The distribution of the brownish-pink color was the same as the distribution of the other stains for collagenous fibers and showed the same differences with different fixation.

Polarization Microscopy

When similar sections were examined between crossed Nicols, the inner zone showed birefringence. The outer zone appeared dark at all azimuths. In some instance very weak birefringent structures could be observed radiating across the dark outer zone.

The outer zone was surrounded by a narrow bright band, the birefringence of which sometimes did and sometimes did not exceed that of the inner zone and the rest of the calcified bone.

The behavior of the zones when the stage was rotated is shown in Figs. 11, 12, 17 and 18. These illustrations leave no doubts that the inner and transitional zones show double-refractive qualities and have the same extinction position.

After insertion of the 1st order red gypsum compensator, the inner and transitional zones exhibited increasing interference colors (2nd order blue) in the positive quadrants, and decreasing colors (1st order yellow) in the negative ones. The outer zone revealed a color close to the red color of the isotropy.

Demonstration of Carbohydrate Components

The Periodic Acid-Schiff Reaction

Sections subjected to the periodic acid-*Schiff* reagents (Fig. 24), demonstrated the presence of periodic acid-*Schiff* positiv material in the cytoplasm of the osteoblasts and the inner zone of the osteoid. The presence of reactive material in most osteons diminished from the inner border of the osteoid through the inner part of the inner zone.

The positive reaction continued into the outer zone which showed an intensity of reaction, similar to that of the inner zone. Close to the margin of the bone, the reaction was distinctly diminished. The width of this area of reduced PAS-reaction corresponded to that of the yellow color obtained after staining according to *von Kossa*, and to the birefringent transitional zone.

Diastase-treated sections did not reveal any difference in the location of PAS-reactive material in the osteoid. The intensity of the reaction in the cells, however, was lower after such treatment. In sections where the oxidation was omitted and that were stained in *Schiff*'s solution only, no staining of the osteoid was seen.

Metachromasia

(Table I). When sections stained with toluidine blue were examined before alcohol dehydration, the entire osteoid appeared red or purplish. After dehydration in alcohols, all undecalcified sections showed red metachromasia in the inner zone of the

TABLE I

METACHROMASIA IN OSTEOID OF UNDECALCIFIED SECTIONS*

Concentrations of Toluidine Blue in Distilled Water (%)	Time of Staining	Young Osteoid	Old Osteoid and Intermediate Bone
0.00001	60 Minutes	Unstained	Unstained
	18 Hours	Faint Red	Faint Green
0.0001	60 Minutes	Unstained	Unstained
	18 Hours	Faint Red	Faint Green
0.001	1/2 Minute	Faint Red	Unstained
	3 Minutes	Faint Red	Faint Green
	30 Minutes	Faint Red	Faint Green
	60 Minutes	Faint Red	Green
	18 Hours	Red	Green
0.01	1/2 Minute	Faint Red	Faint Green
	5 Minutes	Red	Green
	10 Minutes	Red	Green
0.02	1/2 Minute	Faint Red	Faint Green
0.05	1/2 Minute	Faint Red	Green
	1 Minute	Red	Green
	5 Minutes	Red	Green
	15 Minutes	Red	Green
0.1	1 Minute	Red	Green
	2 Minutes	Red	Faint Purple
	5 Minutes	Red+	Purple
	15 Minutes	Red+	Purple+
0.3	1 Minute	Red+	Green+
	5 Minutes	Red+	Purple
	15 Minutes	Red+	Purple+
0.5	15 Minutes	Masked by the Blue Color	

* All sections were dehydrated in:
70 per cent alcohol for 1 minute
100 per cent alcohol for 2½ minutes
100 per cent alcohol for 2½ minutes

osteoid and numerous metachromatic granules in the cytoplasm of the osteoblasts (Figs. 19 through 22). The staining of the osteoblasts and of the inner zone of osteoid appeared continuous, and therefore, the border between them was not always distin-

guishable. The intensity of the metachromatic staining decreased from the inner border of the osteoid and through the first half of the inner zone, and disappeared before the outer zone was reached (Figs. 19—21). The shade of the color in this area seemed to be fairly constant below concentrations of 0.3 per cent toluidine blue for staining up to 15 minutes, although the intensity of the color varied with the concentration and staining time.

The outer and transitional zones did not exhibit red metachromasia at lower concentrations or shorter staining time (Figs. 19 and 20). The outer zone either was partly unstained next to the inner zone and light green peripherally (Fig. 19) or was entirely green (Fig. 20). In the transitional zone, close to the calcified tissue, this green color was markedly intensified (Figs. 19 and 20). With increasing concentrations and time, densely packed minute purplish granules appeared in the transitional zone (Fig. 21). With further increase, also the outer zone turned red (Fig. 22). Finally, with concentrations above 0.5 per cent toluidine blue, all three zones appeared orthochromatic (Fig. 23). The orthochromasia evidently had a tendency to mask the metachromasia, and it was nearly impossible to distinguish between zones in the osteoid.

Fluorescence Microscopy

Primary Fluorescence

When unstained and undecalcified, both formalin fixed and frozen-dried acetone fixed sections were viewed in the fluorescence microscope, the osteoblasts exhibited a bright primary fluorescence (Fig. 25). Also the calcified bone fluoresced to some extent. The inner and outer zones of the osteoid showed a very weak fluorescence. Any differentiation between these two zones was impossible. On the other hand, the transitional zone showed a strong blue-white primary fluorescence, which definitely exceeded that of the surrounding bone, thereby making the distinction between bone and osteoid easy. No difference could be observed between preparations fixed in formalin and those subjected to the freeze-drying procedure.

Secondary Fluorescence

Acridine Orange. Some of the results of fluorochromation with different buffer solutions of acridine orange are shown in Figs. 26 through 29.

Already at pH 1.0 (Fig. 26) the osteoblast nuclei and the zone of transition exhibited a faint yellow-brown fluorescence color. It was noted that the color was located at the inner border of the transitional zone. The cytoplasm of the osteoblasts showed a fresh green hue, whereas the inner and outer zones of the osteoid, as well as the mineralized bone appeared dark dull green.

Around pH 2.0 no marked change could be observed. On the other hand, at pH 3.0 the nuclei of the osteoblasts revealed a copper red color and the cytoplasm was yellow (Fig. 27). The area of transition also increased its secondary fluorescence. The green of the inner zone, corresponding to the area of the silver stained fibers, had lost its dullness and had taken on a fresher hue. The rest of the inner zone and the entire outer zone remained dark green.

At pH 4.5 both the nuclei and the cytoplasm exhibited the copper red color. Also, the narrow part of the osteoid adjacent to the osteoblasts, which formerly was fresh green, showed a change to yellow-red. The rest of the osteoid did not show any appreciable change of the fluorescence intensity.

The characteristic picture at pH 5.3 was an intensely red cellular layer bordering on a copper red narrow rim of the osteoid (Fig. 28). The rest of the inner zone as well as the entire outer zone showed a dull green coloring, while the copper red transitional zone marked the border against the bone.

This constellation in the osteoid was drastically changed at pH 7.3 (Fig. 29). The entire osteoid displayed a grey-red fluorescence. Separate zones could not be distinguished.

From this stage up to pH 11.0 the findings did not leave much to say, except that the fluorescence showed a general gradual diminuation. In the extreme alkaline range, no secondary fluorescence could be seen.

A noticeable difference was observed between the secondary fluorescence of preparations from formalin fixed tissue and that

of the frozen-dried acetone prepared specimens. It appeared that the color changes of the various tissues and tissue layers of the frozen-dried sections generally lagged one step behind those of the formalin treated ones. The recording of the secondary fluorescence given above, was made from fluorochromated sections of frozen-dried acetone fixed tissue.

Thiazin Red R and Eosin. When frozen-dried acetone fixed sections were subjected to the anionic fluorochromes thiazin red R and eosin, only the zone of transition and the nuclei of the osteoblasts were unstained below and at pH 2.0 (Fig. 30). The inner as well as the outer zones of the osteoid showed dull color intensities, and consequently weak secondary fluorescence. The same was true for the osteoblastic cytoplasm.

This distribution of the fluorescence remained fairly unchanged until pH 4.5. At this pH, also the osteoid just next to the osteoblasts had ceased to fluoresce, whereas a steady increase of the intensity of the fluorescence had occurred in the rest of the osteoid (Fig. 31).

At pH 5.5 (Fig. 32) and through the neutral range no alteration in the qualitative image could be observed. The color intensities appeared to have reached a maximum, as no further increase was noticed at higher pH values. Thus, the outer part of the inner zone and the entire outer zone showed secondary fluorescence at all pH values, the intensity of which increased with increasing alkalinity of the buffered staining solutions.

Demonstration of Alkaline Phosphatase Activity

The localization of alkaline phosphatase activity was the same with both *Gomori*'s method (Fig. 33) and the azo-technique (Fig. 34), and did not vary with lengthening of the time allowed for enzyme action.

Phosphatase activity was invariably observed in the nuclei, in the perinuclear cytoplasm and in the processes of the osteoblasts bordering upon the osteoid. Also the cells lining completed osteons showed enzyme activity to some degree in the nuclei and cytoplasm.

The osteoid next to the osteoblastic layer was regularly active

up to a circumference corresponding to the peripheral limit of the area of the fibers blackened by silver. The rest of the inner as well as the entire outer zone were inactive. The cell processes were variably stained throughout the osteoid. The ones that were stained, however, could not easily be traced into the calcified bone.

Control sections treated with iodine-potassium iodide were not stained by the azo dye (Fig. 35). Measures of control of the cobalt technique gave the following results:

Treatment of the sections with iodine-potassium iodide before incubation or omission of glycerophosphate from the incubation medium resulted in weakly stained nuclei. The stained material was located close to the nuclear membrane. Sections stained directly with cobalt nitrate-ammonium sulphide also showed this intra-nuclear staining. When calcium chloride was omitted from the incubating mixture, the nuclei were still stained.

The present series was planned for the study of bone tissue formation as it occurred in the formation of osteons. For that reason, little attention was paid to the preservation of the periosteum during the preparatory procedure. Thus, in some instances the periosteum was partly removed together with other surrounding soft tissues before fixation, while in others both the endosteum and periosteum were found *in situ*. What has been described here concerning the presence, the morphology and the histochemistry of the osteoid lining the inner surface of the *Haver*sian systems, was also valid for the non-mineralized tissue between the bone surfaces and the periosteal and endosteal cells.

Osteoid in Decalcified Material

Osteoid which had not been exposed to decalcifying agents differed from osteoid in material in which the decalcification of the mineralized bone was complete, which again differed from specimens where demineralization was incomplete. Therefore, the qualities of osteoid will first be described on the basis of sections

of bone which have been exposed to demineralizing agents for a relative long period of time and following this, a similar description will be given of osteoid from sections of bone which have been subjected to demineralization for shorter periods of time.

Complete Decalcification

The osteoid of bone tissue that had been completely decalcified in nitric acid, in formic acid—sodium citrate, or in ethylene diamine tetra-acetate (EDTA), appeared as a dark layer between the osteoblasts and the slightly lighter decalcified bone matrix, when examined in the *phase contrast microscope* (Fig. 10). The osteoid, however, was not uniformly dark. It increased in brightness from the area adjacent to the osteoblasts towards the bone matrix. This increase in brightness was gradual throughout the osteoid and did not allow any division of the latter into zones. Moreover, as the difference between the optical path of osteoid and that of bone matrix was very small, the border line between these two structures could not be determined with certainty.

In sections decalcified in EDTA, the tissue between the cell processes in that part of the osteoid which bordered the osteoblastic layer appeared to be of a fibrous nature. In the peripheral two-thirds of the osteoid, fibers could not be distinguished.

After decalcification in the formic acid solution, fibrous structures were but rarely seen. The osteoid appeared granular next to the osteoblasts, and became homogeneous towards the bone matrix. Osteoid in specimens which had been decalcified in nitric acid had a hyaline appearance, as if it were composed of a homogeneous structure.

Sections from bone decalcified in the above-mentioned decalcifying agents and stained by the different procedures showed no basic differences as to the staining abilities of the osteoid.

After staining in *hematoxylin-eosin* the entire osteoid was eosinophilic. Occasionally, a stronger basophilic line ocurred at the peripheral limit of the osteoid.

The affinity for both *Mallory's* and *van Gieson's* stains was strong throughout the entire width of the osteoid, and did not

permit the distinction of layers. Although the osteoid was more heavily stained than the bone matrix, a borderline could not be recognized, as the decrease in the intensity of color was gradual.

In sections from bone decalcified in EDTA and stained according to *Wilder*, the silver stained fibers were seen in the inner third of the osteoid only. The outer two-thirds of the osteoid showed a light red-brown color. The rest of the osteon was of a darker red-brown color. After exposure to acids the area which stained black by *Wilder*'s method was twice as wide as in sections exposed to EDTA. A sharp border between the osteoid and the bone matrix could not be seen.

Signs of *metachromasia* were absent in the osteoid of acid demineralized material. Occasionally, metachromatic material was found located in a very narrow zone next to the osteoblasts, but only in sections from EDTA treated specimens, and only after staining with toluidine blue at high concentrations and with a long staining time.

On the other hand, *periodic acid-Schiff* positive material was present. The intensity of the color reaction was fairly even throughout the entire width of the osteoid.

Partial Decalcification in Zenker's Fluid

In sections of bone fixed in *Zenker*'s fluid, and thereby exposed to acetic acid, decalcification had occurred to some extent. The period of exposure to the acid was shorter (15 hours) than that used in demineralizing bone fixed in formalin. In addition, the degree of demineralization in *Zenker*'s fluid varied with the location of the osteon in the block. The degree of decalcification was evaluated in sections stained acording to *von Kossa* and by *microradiography*. It was found that the area adjacent to the periosteal surface showed complete decalcification. This was also true for the bone underlying the cross-cut ends. In other words, the cross-sections obtained in the beginning of the sectioning were completely decalcified. As the cutting continued further into the block, the demineralized areas appeared to be more unevenly scattered in the section. The bone surrounding the *Haversian* and *Volkmann*'s canals, was decalcified to a variable extent, ap-

parently dependent on the size and location of the canals. Furthermore, in osteons which were not completely decalcified, the degree of decalcification decreased from the surface of the *Haversian* canals toward the periphery of the osteon. In contrast to the periosteal bone tissue, the bone tissue adjacent to the endosteum hardly showed any sign of decalcification.

The picture presented by the osteoid was extremely variable, varying with the degree of decalcification of the osteon and, therefore, depending probably on the time of exposure to *Zenker*'s fluid. All variations were found, from the clear-cut picture of osteoid with an inner and outer zone, to the stage at which the osteoid consisted of one more or less homogeneous layer whose outer limit could not be determined.

When unstained sections of this series were viewed in the *phase contrast microscope*, it seemed as if the outer and transitional zones which showed increasing brightness in undecalcified sections, became increasingly darker with increased time of exposure to *Zenker*'s fluid and, therefore, the difference between the inner, outer and transitional zones of the osteoid was progressively blurred (Fig. 9).

In stained sections, osteoid which had been subjected to the fluid for a very short time, as judged by the lack of decalcification of the surrounding calcified matrix, the inner and outer zones stood out clearly due to the characteristic ability or inability to take the various stains. Parallel to the increased time of acid action, the inner zone seemed to widen in its staining reaction at the expense of the outer zone.

In sections stained with *hematoxylin-eosin* the entire osteoid of *Zenker* fixed specimens constantly showed eosinophilia which passed into the more basophilic demineralized bone independent of the length of exposure to acids.

When stained according to *Mallory* or *van Gieson*, the inner zone with its strong affinity for collagen stains increased in width with the increase of exposure time, *e.g.* with increased decalcification, leaving an increasingly narrow unstained outer zone. Finally, after about 12 hours of influence of the decalcifying fixative, *e.g.* in case of the osteons located near the periosteal surface, this unstained outer zone had disappeared, and the entire osteoid was stained.

Periodic acid-Schiff positive material was observed in all areas of sections fixed in *Zenker*'s fluid, and seemed to be evenly distributed throughout the osteoid. This was apparently not influenced by the total period of exposure to *Zenker*'s fluid.

CALCIFIED BONE TISSUE

A thorough analysis of bone matrix seemed outside the scope of a study on the formation processes. Consequently, observations will mainly be recorded which might contribute to the understanding of the morphology and histochemical behaviour of osteoid.

Bone Matrix in Undecalcified Material

No major differences were observed in undecalcified sections of the mineralized part of the bone whether fixed in formalin or after freeze-drying. The following description is, therefore, valid for both types of specimens, and reference to the method of fixation will be made only when deviations from this conformity occurred.

The bulk of the calcified bone matrix stained homogeneously and slightly basophilic in *hematoxylin-eosin* (Figs. 1 and 2). Cementing lines, the lining of the osteocytic lacunae and the canaliculi, and the innermost border of completed osteons showed a stronger basophilia. A basophilic zone also marked roughly the division between the calcified matrix and the osteoid. Lamellar arrangements of the osteons could, however, not be observed in sections stained with hematoxylin-eosin.

In sections subjected to *Mallory*'s collagen stain, a variably wide zone adjacent to the osteoid stained regularly blue. The outer part of the osteon or the core of trabeculae and the entire cross-section of completed osteon stained irregularly. In some areas it stained blue, in others yellow or it remained unstained. The distribution of the staining could not be reproduced in neighboring sections. Lamellation of the calcified bone matrix could

sometimes be noticed in the completed osteons, but it was more clearly seen in the tubular spongiosa, where green lamellae appeared to alternate with the blue-colored ones.

Examination in the *polarization microscope* revealed osteons composed of lamellae of stronger birefringence alternating with weakly birefringent ones (Figs. 11 and 12). Moreover, isotropic lamellae could be seen in between, although these were relatively few. Other osteons or areas of osteons, however, did not show this lamellated pattern but exhibited a more or less diffuse, weak birefringence.

In undecalcified sections stained according to *von Kossa,* the incompleted osteons showed a uniform distribution of the brown silver staining (Fig. 3), which did not permit recognition of differences in the degree of mineralization, or the lamellar patterns. As has been mentioned before, the boundary between osteoid and calcified bone, the zone of transition, stained yellow. In completed osteons, in the tubular trabeculae and in the interstitial lamellae, lighter and darker stained lamellae were arranged in an irregular sequence. A very narrow black line separated the calcified part of completed osteons from the soft tissues of the *Haver*sian canals. A similarly stained line surrounded the osteocytes within the calcified matrix.

In sections stained with *alizarin* the area of transition between osteoid and bone stood out clearly, as already described. The rest of the calcified matrix stained faintly red. The contrast between the red transitional zone and the rest of the osteon appeared greater in frozen-dried specimens than in those fixed in formalin.

Sections stained with *periodic acid-Schiff* reagents assumed a woolly appearance, apparently as a result of an unavoidable slight decalcification in the acid reagent (Fig. 24). In the incomplete osteon the calcified matrix showed a greater affinity for leucofuchsin than did osteoid. In complete osteons the inner border stained more intensely than the surrounding matrix. Both in incomplete and in complete osteons the rest of the calcified matrix was stained fairly homogeneously pink. Only the capsules of the osteocytes stained more intensely. Lamellar structures were usually not seen in the osteons. Occasionally, in fully developed *Haver*sian systems and more often in the tubular spongiosa, the

lamellated structure was brought out by the fact that some lamellae took less color than the others.

Calcified bone matrix did not show *metachromasia* at low concentration of toluidine blue and with short staining time. When sections were stained with dilute dye solutions, only the osteocytes and their capsules and the inner wall of completed *Haversian* systems revealed red metachromasia (Fig. 19). A green stained narrow layer, corresponding to the transitional zone, was, however, observed in the newly calcified bone adjacent to the osteoid (Figs. 19 and 20). With increasing time and concentration this layer became red (Fig. 21). By further increase of the determining factors the layer became wider and turned purple (Fig. 22), and finally all of the bone took on this color. In sections which had been stained in a high concentration and for a long period of time (0.5 per cent toluidine blue for 15 minutes) the bone was almost orthochromatic (Fig. 23).

The bulk of the calcified bone matrix as it appeared in undecalcified sections, exhibited an even, medium strong *primary fluorescence* (Fig. 25). Variations in the intensity of primary fluorescence corresponding to minor differences in the degree of mineralization as brought out by the micro x-ray technique, (Figs. 5 and 6), could not be discerned. The transitional zone, however, revealed a strong primary fluorescence. As a matter of fact, no other areas of bone matrix reached similar intensities. It should be noted that this very zone usually showed low degrees of x-ray absorption.

After fluorochromation with buffer solutions of acridine orange, calcified bone matrix did not show "concentration effect". From pH 1 to the neutral point a steady increase of the freshness of the green color occurred (Figs. 26 through 29). In the alkaline range, a gradual muddling of the green color took place. Finally, at pH 11 the secondary fluorescence was close to that of intercellular substance of bone fluorochromated at pH 1, namely a dull green color.

Throughout the series, both in the acidic range and at higher pH values, the transitional zone exhibited stronger color intensities than the surrounding bone. As already mentioned, part of this zone distinguished itself by showing "concentration effect" in the extreme acidic range.

Fluorochromation with anionic fluorochromes gave no significant fluorescence. Dull and ill-defined color effects were obtained in the bone matrix at all pH values, in contrast to the transitional zone, which appeared light green in thiazin red, and yellow in eosin (Figs. 30 through 32).

Phosphatase reaction of calcified bone as revealed by the azo-method was negative. Neither intercellular bone tissue nor the cytoplasm of osteocytes were stained (Fig. 34). Osteocyte nuclei showed weak color reaction.

With the *Gomori* method the intercellular substance and the nuclei of the osteocytes showed precipitation of cobalt sulfide (Fig. 33). The border of the calcified bone toward the osteoid appeared uneven, and the color reaction was somewhat diminished in this narrow transitional area. The cytoplasm of the osteocytes, however, was unstained.

Bone Matrix in Decalcified Material

The material for the following series was formalin-fixed prior to complete decalcification in nitric acid, in formic acid—sodium citrate, or by chelation. The bone matrix as a whole appeared slightly basophilic when stained with *hematoxylin-eosin*. Resting and reversal lines, the walls of the osteocytic lacunae and the inner lining of completed osteons, showed strong basophilia. A basophilic layer separating the osteoid from the decalcified matrix was only occasionally found. Lamellar arrangements of the osteons could usually not be seen. Now and then, however, as in completed osteons, the lamellation was brought out by alternating stronger and weaker basophilia. Except for occasional lamellation, the decalcified bone matrix appeared homogeneous.

By examining unstained sections in the *phase contrast microscope,* the cementing lines stood out clearly. The osteocytic capsule and the walls of the *Haver*sian canals of completed osteons showed a difference of the optical path from that of the rest of the matrix. No lamellation of the osteons was observed. The matrix structure itself appeared, however, more fibrous or granular than in the hematoxylin-eosin stained sections.

Decalcified matrix stained according to *van Gieson* and coun-

ter-stained with iron hematoxylin showed a fairly even distribution of the red color in cross-sections. A narrow layer of the decalcified matrix adjacent to the osteoid was, however, more intensively stained than the rest of the osteoid. A thin black line separated the osteoid from the surrounding matrix. In completed osteons the area immediately surrounding the *Haver*sian canals stained more intensively red. A black line covered the inner surface of these osteons. A similar staining reaction characterized the walls of the lacunae.

Cementing lines stained yellow. A lamellar pattern was visible in both the completed and incompleted osteons showing yellow stained lamellae alternating with red ones. Although the lamellar arrangement was not clearly demonstrated in all instances, the staining of decalcified sections in *van Gieson*'s stain appeared to be the best method for bringing out these structures.

After staining according to *Mallory*, the portion of the incomplete osteons, bordering the osteoid, as well as that limiting the fully developed osteons toward the *Haver*sian canals were regularly stained blue. The older parts of the bone matrix remained unstained, or were yellow or red. The cementing lines stained as the bone in which they were found. On the other hand, the capsules of osteocytes stained regularly blue.

Ammoniacal silver stained the bulk of the decalcified matrix red-brown. Cementing lines stained somewhat grayish. The same gray color was found as thin layers between the lamellae of completed osteons, thereby accentuating the lamellar arrangement. In the osteons which were not completed, on the other hand, and especially in their inner halves, this grayish stained material between the lamellae was not seen. Black stain aggregated around the osteocytes and their processes and around the *Haver*sian canals of completed osteons.

Decalcified bone matrix did not show *metachromasia* even when high concentrations of toluidine blue and longer staining times were used. The only recognizable metachromatic material was located in the walls of the lacunae, of the canaliculi, and of the canals of completed Haversian systems. The metachromasia of the capsules of osteocytes which were newly embedded, was weak. It increased, however, peripherally. The amount of metachromatic material seemed more reduced after decalcifica-

tion in nitric acid than in sections decalcified in other media. Cementing lines were consistently orthochromatic, whereas the rest of the decalcified matrix was unstained, apparently independently of the kind of decalcifying agent used.

Periodic acid-Schiff positive material was found throughout the bone matrix. No difference in the distribution or the color reaction was seen after the different decalcification procedures. The decalcified matrix showed a uniform, pale pink staining, whereas the color of the osteoid was more intense. Cementing lines, the osteocytic capsules, as well as the inner surface of the completed osteons showed even greater reactivity. Variations in the color of different osteons did not exist. Neither could any difference in the various part of the decalcified matrix within single osteons, nor any difference in the stainability of the lamellae, be brought out by this method.

CHAPTER V - DISCUSSION

MORPHOLOGY AND HISTOCHEMISTRY OF THE OSTEOID

General Morphology

Osteoid is generally understood as the organic matrix in which the deposition of inorganic salts will take place, giving as the result bone tissue.

Up to now, osteoid has been characterized at the microscopic level as being homogeneous *(Pommer,* 1925), hyaline *(Weinmann & Sicher,* 1955), or edematous *(Pritchard,* 1956).

The present investigation, including examination of unstained section of undemineralized diaphyseal bone in the phase contrast microscope, has disclosed an utterly inhomogeneous osteoid.

On the basis of the phase contrast microscope pictures of such sections (Figs. 7 and 8), the osteoid may be divided into three major zones:

The inner zone, corresponding to the young osteoid adjacent to the osteoblasts,

the outer zone, comprising the middle and the peripheral part of the osteoid, the old osteoid, which in turn, borders on

the transitional zone. This zone is the narrow area of intermediate bone tissue which represents the end of the osteoid and the beginning of the bone tissue.

Also, layers within the zones could be distinguished.

By the definition of osteoid (see above) the latter zone of intermediate bone, does not really belong to the osteoid, as mineralization has already taken place within it.

However, as has been demonstrated, the morphologic characteristics, and as will be seen from the subsequent discussion, the

dynamics of this area favorize the conception of the transitional zone as being a zone of the osteoid.

Different zones in the osteoid have not been described before. The reason why these morphologic features of the osteoid have not yet been observed may be that osteoid of ample width has not been studied in undemineralized thin sections. *P. C. Meyer* (1956) examined osteoid of demineralized bone in the phase contrast microscope and did not find this technique profitable for such material. The present study confirms that the zones of osteoid cannot be seen in sections of completely decalcified bone (Fig. 10).

It has been generally accepted *(Morse,* 1945) that demineralization not only results in removal of the inorganic phase of hard tissue, but also seriously alters the soft tissue constituents. In fact, agents like the demineralizers are used in order to extract and separate the various organic components for chemical analysis.

Accordingly, osteoid of osteons at different stages of decalcification showed a gradual blurring and disappearance of the zones of the osteoid (Fig. 9). Already after a very short time of exposure to the decalcifying fluid, the characteristics of the outer zone were seen in a narrow peripheral layer only. After 15 hours of influence of *Zenker*'s fluid, the zones of the osteoid had disappeared entirely. In other words, an osteoid of three well distinguished zones had been changed into a tissue corresponding to the characterization of a homogeneous, hyaline or edematous osteoid.

At times, the radiating striations of the osteoid effected by the processes of the osteoblasts and osteocytes could not be observed in the demineralized specimens (Figs. 9 and 10). It is conceivable, therefore, that too long time of exposure to demineralizing agents may have caused the disappearance from the osteoid of human bone tissue of the structures on which *P. C. Meyer* (1956) reported.

Major differences could not be observed between undecalcified material fixed by neutral formalin or by freeze-drying-acetone. The same zones and sublayers could be distinguished.

The same subdivisions were also brought out by staining of undecalcified sections.

The optical path differences within the osteoid and the staining differentiation must be due to underlying differences of physical and/or chemical properties of the constituents of the various parts of the osteoid.

The following discussion, therefore, will examine the possible changes to which the major components are subjected in the course of their developing from young to old osteoid, and from old osteoid to calcified bone matrix.

Inorganic Constituents of Osteoid

It is possible that the differences in the different areas of the osteoid could be due to minor variations of inorganic matter. This possibility was tried on the basis of the results obtained with microradiography, the silver staining of *von Kossa*, and alizarin staining.

Microradiography or roentgen absorption technique is probably presently the most reliable and practical method for the determination of inorganic matter in tissues (*Engström*, 1946; *Lindström*, 1955).

Investigations with this method have clearly demonstrated that variations in absorptive qualities exist in different structures of bone (*Amprino & Engström*, 1952), and teeth (*Engfeldt, Bergman & Hammarlund-Essler*, 1954). That the qualitative radiographic image actually represents degrees in the concentrations of calcium and phosphates, was shown by *Lindström*, (1955). Moreover, micro-measurements of hardness have revealed a correlation between hardness and the degrees of x-ray absorption seen in microradiographs (*Carlström*, 1954).

Von Kossa (1901) observed that deposits of calcium phosphates stained with silver. On reduction the silver salt became visible as yellow to brown-black staining, the color depending on the concentration of the phosphates. *Cameron* (1930) found that not only calcium phosphate stains by this method, but that other anions as well as other cations react. In the study of bone tissue, this lack of specificity is not a serious matter as no appreciable amount of compounds other than calcium phosphates and car-

bonates are present which are capable of responding to this technique *(McLean & Bloom,* 1940).

Alizarin has been widely used in the study of bone and bones and is thought to show the sites of calcium salts. The precise action involved is still not known, but it has been suggested that a calcium alizarinate is formed *(Cameron,* 1930). Areas of newly formed bone appears to take more stain than older bone when alizarin was applied to sections of bone, or when administered *in vivo.* The affinity of the dye for newly formed bone may be explained by the availability of calcium in simpler combinations than in fully mineralized bone *(Ham & Harris,* 1952).

The microradiographs (Figs. 5 and 6) showed absence of bone salts in the young and old osteoid. The zone of transition, however, exhibited some, although weak, x-ray absorption, which increased rapidly within a small distance peripherally.

The young and old osteoid did not respond to *von Kossa*'s silver technique either, whereas a yellow color appeared in the area of transition (Fig. 3). This yellow rim was succeeded by the brown-black color of the bone.

As far as the three zones of the osteoid are concerned it appears that there is a corellation between the microradiographic image and the silver stain of *von Kossa.*

Although the osteoid took on a faint red color after staining with alizarin, this tissue did not show more uptake of the stain than other soft tissue constituents (Fig. 4).

On the other hand, the zone of transition distinguished itself by showing a strong red color which markedly exceeded the hue of the osteoid as well as that of the surrounding bone.

The negative results of the staining methods and the lack of x-ray absorption in the young and old osteoid may be held to indicate that the amount of inorganic salts is no higher than that in any other soft tissues.

The weak silver staining and increased uptake of alizarin in the transitional zone, indicate small depositions of inorganic material. This conclusion is in agreement with the microradiographic image.

The absence of mineral salts in the osteoid is not contradicted by the finding that part of the outer zone of osteoid occasionally was stained by *hematoxylin* in undecalcified (Fig. 2) and de-

calcified sections. *Cameron* (1930) and *Schour & Ham* (1934) interpreted the intensive staining of tissues like bone and dentin with hematoxylin as an indication of mineralization. Since this staining occurs in decalcified as well as in non-decalcified material, and therefore does not depend on the retention of the minerals *in situ,* it has been concluded by *Weinmann & Sicher* (1955) that the staining indicates a change in the matrix prior to or simultanous with the precipitation of mineral salts. With this in mind, we may interprete the staining of part of the outer and the entire transitional zone of osteoid as due to chemical or physical changes in the matrix prior to calcification.

These changes postulated by *Weinmann & Sicher* (1955) to have occurred in the organic matrix prior to mineralization are in agreement with the finding of separate zones when phase contrast microscopic examination is employed.

Conclusions

The results of the tests for minerals have demonstrated that the inner and outer zones of the osteoid are devoid of inorganic depositions, but that mineral salts show increasing aggregation through the area of transition towards the bone. The inhomogeneity of the osteoid, therefore, as it appears in the phase contrast microscope, is in the main not caused by differences in mineral content. The explanation of this phenomenon must be sought in differences in the organic constituents.

Charge Conditions of the Osteoid

The histologic determination of the iso-electric point is based on the amphoteric character of the proteins and protein complexes, and the fact that such substances are charged positively below their iso-electric point, and negatively on the alkaline side of the iso-electric point. Consequently, by the use of dissociated dyes in buffer-solutions it is theoretically possible to determine the IEP of such substances. This has been verified in practice by *Zeiger* (1938) in model experiments, and by *Hals* (1953) among

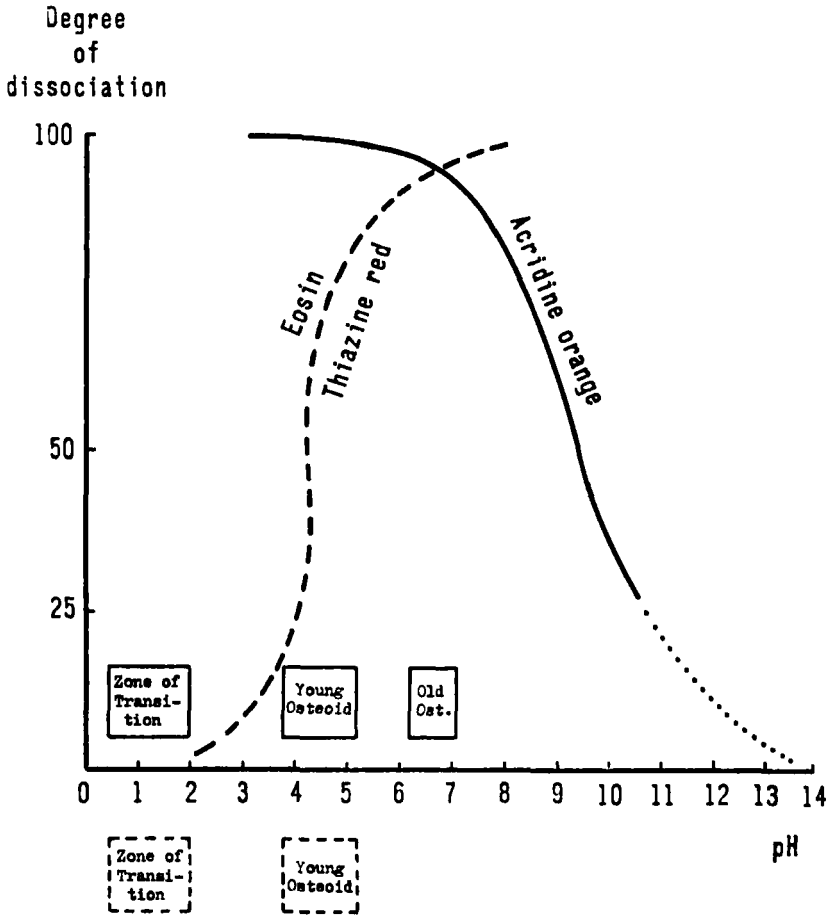
others on biologic material, by the utilization of methylene blue. A different method exists, however, whereby the tissue is subjected to cationic and anionic fluorochromes at various pH values, and subsequently examined in the fluorescence microscope.

Acridine orange, being a cationic fluorochrome, has given evidence of being particularly adapted for the purpose of determining the IEP (*Strugger,* 1949).

Due to the electro-activity of ampholytes, no adsorption will take place by a cationic fluorochrome below the IEP. Above the IEP, on the other hand, the substance will be negatively charged, and consequently electro-adsorption will occur. The fitness of acridine in this context is due to its characteristic change of fluorescence color from green to red. This phenomenon is called "concentration effect" (*Strugger,* 1949) and is caused by the increased concentration of the fluorochrome as a function of the degree of electro-adsorption. *Strugger* contended that acridine orange alone would give reliable information on the IEP of amphoteric elements. In the present investigation, however, parallel series were run with the anionic fluorochromes, thiazin red R and eosin. The mode of action of these fluorochromes is the same as that of cationic ones, except that the scope for action is reversed. In other words, below the IEP, electro-adsorption will occur, whereas theoretically no adsorption takes place above the IEP.

One more point is important in this connection, namely the degree of dissociation of the fluorochromes at the different pH values (Text fig. 2). Watery solutions of acridine orange are dissociated in the entire acid and slightly alkaline range (*Strugger,* 1949). The curve of dissociation is rapidly falling above pH 8.5, and at pH values above 10.5 electroneutrality of the solution is established. Reversely, the anionic fluorochromes show no or only a weak dissociation in the extreme acidic range, the degree of dissociation, however, increasing rapidly from pH 3.5 to a maximum around pH 5.0.

As a whole, the iso-electric points of biologic material are located in the acidic area. Consequently, it appears that cationic fluorochromes would be more adapted for the determination of the iso-electric points of tissues, than anionic ones. This fact has been corroborated in the present study of the osteoid.



Text fig. 2. — Diagram showing the dissociation curve for aqueous solutions of acridine orange (drawn line) and for anionic fluorochromes (dotted line), after *Strugger* (1949). The ordinate represents the degree of dissociation in per cent. At the abscissa the pH is annotated. The pH values at which concentration effect appears after fluorochromation with acridine orange (above the abscissa), and at which pH the eosin and thiazin cease to give secondary fluorescence (below the abscissa) in the different areas of the osteoid, are shown.

Secondary fluorescence as it appears after fluorochromation, represents the total of primary fluorescence of the tissues and fluorescence brought about by the adsorbed fluorochromes.

The primary fluorescence in unstained undemineralized sections was extremely weak in the young and old osteoid. A relatively strong blue-white fluorescence appeared at the area of transition. It was difficult to localize exactly the central limit of this fluorescence as all such images are more or less diffuse having a woolly appearance (Fig. 25). The surrounding mineralized bone, however, showed a smaller degree of primary fluorescence than that located at or very close to the area of transition. The fact that the zone of transition showed strong secondary fluorescence between pH 1 and pH 2 may to some extent have been caused by the primary fluorescence. On the other hand, this cannot account for the concentration effect (copper red color) which was observed to mark the border against the centrally located old osteoid. In these specimens the primary fluorescence could be seen to occupy an area peripheral to the area of the concentration effect (Figs. 26 and 27).

The secondary fluorescence after treatment with anionic fluorochromes in solutions of strong acidity, showed small color contrast. The finding of weak secondary fluorescence in the transitional zone around pH 2 is in keeping with the picture obtained with acridine orange. Surely, the low degree of dissociation of the anionic fluorochromes in this range must be taken into consideration (Text fig. 2). The fact that the rest of the osteoid appeared to have taken some color, however, indicate that the lack of adsorption in the area of transition is not entirely a matter of dissociation of the fluorochrome.

The interpretation of the primary fluorescence picture (Fig. 25) and the correlation of the results after treatment with cationic (Figs. 26, 27 and 28) and anionic (Fig. 30) fluorochromes justify the conclusion that the zone of transition consists of two subdivisions:

1) a narrow rim towards the old osteoid showing concentration effect in acridine orange and left unstained by thiazin red and eosin at pH values just below 2, and which corresponds to the area of maximum brightness in the phase contrast microscope, and

2) a somewhat broader layer located peripherally to the former, showing strong primary fluorescence, but exhibiting green secondary fluorescence. This broader layer corresponded to that part of the zone of transition which appeared granular in the phase contrast microscope.

Below pH 2 the young and the old osteoid neither showed any appreciable degree of secondary fluorescence after treatment with acridine orange nor after treatment with anionic fluorochromes. The explanation of this apparent discrepancy is found in the small degree of dissociation of the anionic fluorochromes, i. e., although the tissue is positively charged in this range as demonstrated by the low color intensities in acridine orange, thiazin red and eosin are incapable of showing strong electro-adsorption because of low degree of dissociation.

Around pH 5, on the other hand, the inner part of the young osteoid adjacent to the osteoblasts started to show concentration effect after fluorochromation with acridine orange (Fig. 28). The area of transition at this point continued to exhibit strong electro-adsorption. As a matter of fact, the area of red secondary fluorescence even appeared to have increased in width. The extinction of secondary fluorescence in the sections subjected to the acridine orange solution around pH 5, is in agreement with the picture obtained after treatment with anionic fluorochomes at pH 4.5. In this range watery solutions of such fluorochromes have reached a considerable degree of dissociation. Accordingly, the entire old osteoid and the peripheral part of the young osteoid showed secondary fluorescence. This picture was fairly unchanged until the neutral point, above which the entire osteoid gave red secondary fluorescence in acridine orange. Theoretically, then, one would expect that from the point of neutrality and through the alkaline range, the entire osteoid would show no secondary fluorescence.

When the osteoid, except for the transitional zone and the narrow layer of the young osteoid adjacent to the osteoblasts, nevertheless, exhibited secondary fluorescence, this is most likely not brought about by electro-adsorptive binding of the fluorochromes, but may have been caused by the ill-defined electroactivity of this tissue.

The utilization of the secondary fluorescence after fluorochro-

mation at varying pH values is no exact way of determining the charge conditions. The relative inaccuracy of the technical procedure does not allow determination of absolute values. The method of registration of the color intensities also seems to be inadequate.

In spite of these reservations, there should be no doubt that this method is capable of demonstrating relative values of the electric charge of tissues.

To the best of the knowledge of the writer the only report available on charge conditions of the osteoid on the basis of fluorescence analyses is that of *Hals* (1953). It appears from his study of osteoid in undemineralized alveolar bone, that concentration effect never occurs in this tissue and that the electric charge of the osteoid is weak.

The discrepancies between these statements and the present findings not only of concentration effect, but of differences of the charge conditions within the osteoid, are most likely to be found in the choice of material. The normal width of the osteoid of alveolar bone may be too narrow to make differentiation of different zones possible.

Conclusions

It is clear from the present series that a mean value for the electric charge of the osteoid does not exist, but that each zone has its characteristic electro-activity. From being fairly strong negatively charged in the narrow part of the young osteoid which borders on the osteoblasts, the rest of the young and the entire old osteoid are very weakly charged. The zone of transition, on the other hand, shows strong negative charge (Text fig. 2).

Fibers of the Osteoid

Fibers of the Young Osteoid

Identification

In undecalcified unstained sections of frozen-dried bone, examination in the phase contrast microscope showed that in the young osteoid, part of the matter which occupies the space be-

tween the cytoplasmic processes of the osteoblasts and the osteocytes consisted of fibers, whereas in the old osteoid no fibrous structures could be recognized (Fig. 8). No fibers could be observed by this method in the transitional zone either. Close to the osteoblasts, the fibers were loosely arranged and oriented at random, but towards the periphery of the inner zone, they were oriented parallel to the surface of the calcified bone and the plane of the section. The diameter of the fibers could be seen to increase in approaching the outer zone. The outer zone itself presented an appearance of increasing homogeneity. The area of transition was first homogeneous and became more granular against the border of the calcified bone.

Mallory's solution stained the inner zone blue from the area adjacent to the osteoblastic cytoplasm on (Fig. 13). The intensity of the color appeared fairly constant throughout this zone. The outer zone was yellow.

The distinctive blue staining of connective tissue fibers by *Mallory*'s solution is well known. It is widely accepted that the intensity of the staining depends on the thickness and packing of the fibers, but that this stain does not differentiate between argyrophilic or precollagenous and collagenous fibers. It is not known whether the uptake of *Mallory*'s or *van Gieson*'s stains by the connective tissue fibers is dependent on their fibrous structure or may also occur in collagen in a nonfibrous state.

Sections stained with ammoniacal silver solutions (*Wilder*'s or *Gomori*'s method) showed the presence of black stained fibers in a narrow area adjacent to the osteoblasts (Figs. 15 and 16), whereas the remainder of the young osteoid as well as the old osteoid were left unstained by silver. The narrow black-stained area corresponded to the bright area in the young osteoid which was observed under the phase contrast microscope.

It is generally believed (*Mallory*, 1942; *Lillie*, 1955) and has also been confirmed by electron microscopic studies (*Irving & Tomlin*, 1954), that the structures which stain black with ammoniacal silver-solutions are reticulin or precollagenous fibers, whereas the reddish fibers are made up of collagen. The relationship between collagen and reticulin is by no means clarified (see *Gustavson*, 1956), and it seems outside the scope of this analysis to discuss this thoroughly. It should be mentioned, how-

ever, that, in electron microscopic studies, *Little & Kramer*, (1952); *Tomlin* (1953), and *Irving & Tomlin* (1954), found that the native reticulin showed precisely the same banded structures as does collagen, but that the diameters of the fibers were smaller. On the basis of reconstitution of collagen, *Nageotte & Guyon* (1930) concluded that the fibers usually called reticulin fibers or precollagenous fibers might represent a stage in the development of collagenous fibers.

Polarization microscopy cannot distinguish between precollagenous and collagenous fibers. The weak positive birefringence of the area of silver impregnation (Figs. 11, 12, 17 and 18), nevertheless, corroborates the observations made in the phase contrast microscope, viz., the presence and the ill-defined orientation of fibers. The increasing double refractive qualities of the rest of the young osteoid, are likewise in agreement with the phase contrast microscopic picture, revealing closer spacing of the fibers and increasing tendency of orientation.

The fibers in the youngest part of the osteoid therefore may be identified as reticulin or precollagenous fibers, more or less without any preferential orientation. The fibers in the rest of the young osteoid, which stain blue with *Mallory*'s solution, red with *van Gieson*'s stain, are reddish in silver preparations, and show increased tendency of orientation, may be identified as collagenous fibers.

Possible Differences between Precollagenous and Collagenous Fibers

By treating collagenous fibers with hyaluronic acid, *Irving & Tomlin* (1954) were able to increase the affinity of the fibers for silver. This led them to suggest that the ability of the precollagenous fibers to take up silver was due to a close relation between these fibers and some mucopolysaccharide. On the other hand, *Partridge* (1948) was unable to separate polysaccharides from collagenous fibers without disrupting either the collagen molecule or the polysaccharide molecule. This goes to show that mature collagenous fibers also have an intimate relation to the polysaccharides, and in fact may have an even closer one than

the precollagenous fibers. It may therefore be that the poor silver uptake of mature collagen fibers is due to the firm relation between the collagen and the polysaccharide in these fibers, rather than to their having a smaller quantity of polysaccharide (*Irving & Tomlin*, 1954) or to their larger diameters (*Nageotte & Guyon*, 1930).

The present study contains two observations which support this view: (1) In the sections of decalcified material, there was a widening of the area of black stained fibers adjacent to the osteoblasts. This means that on being exposed to decalcifying agents, the youngest of the collagen fibers acquired the capacity to take up silver. In chemical extraction procedures, acids or chelating agents are used in order to effect a complete separation of collagen and polysaccharides. However, these procedures are applied to unfixed connective tissue. The same agents applied to the fixed tissue of our sections seem to bring about a loosening of the connection between the collagen and the polysaccharides, thus reversing the maturation process and enabling the fibers to take up silver. (2) True metachromasia (Figs. 19 through 21) and the uptake of silver (Figs. 15 and 16) appear together in the region of the youngest osteoid and disappear simultaneously in the slightly older osteoid. The disappearance of metachromasia need not necessarily mean absence of the reacting polysaccharide molecules. It may merely mean that the groups usually reacting metachromatically with the dye are engaged in other interactions. These interactions may consist in the constitution of a chemical relationship between the polysaccharide and the precollagenous fibers, which gives collagenous fibers as its result. The constitution of this chemical relationship may lead to loss of the metachromatic reaction as well as to the inability to bind silver. Before this relation is established, the fibers are able to take up silver and to give a metachromatic reaction, because the reacting groups of the investing mucopolysaccharide are not yet firmly bound to the collagen molecules.

The finding of extracellular alkaline phosphatase at the site of conversion of precollagenous into collagenous fibers (Figs. 33 and 34), agrees with its generally observed occurrence during fiber formation. Its rôle in this connection is not understood. There is, however, no reason to believe that the rôle of the extra-

cellular alkaline phosphatase is different in the osteoid than in other sites of fiber formation (*Bourne*, 1956).

Collagen of the Old Osteoid

Identification

The entire inner zone of the osteoid of undecalcified sections showed fibrous structures in the light microscope, as well as in the phase contrast microscope. Examined between crossed Nicols, the inner zone exhibited positive birefringence. The fibers in the older part of the inner zone showed, with *Mallory*'s, *van Gieson*'s, and the silver methods, the staining reactions which are characteristic of mature collagen fibers.

The outer zone showed no evidence of fibers with any of the light microscopic methods (Figs. 13 through 16) nor under the phase contrast microscope (Figs. 7 and 8). In the polarization microscope, the positive birefringence decreased drastically in passing from young to old osteoid. The peripheral part of the zone remained dark at all azimuths (Figs. 11, 12, 17 and 18).

The picture of the old osteoid in the phase contrast microscope was in sharp contrast to that of the adjacent younger region, regardless of the fixative used. It was this contrast which led to the distinction of the inner and outer zones in the osteoid. At the boundary between inner and outer zones, the increase in brightness was rather abrupt, and from there on the brightness increased gradually through the remainder of the outer zone. This shows distinctly that there exists an optical path difference between the inner and the outer zones. From the phase contrast microscope picture of the inner zone of the osteoid we know that the densely packed collagen fibers appear as the region of maximal darkness. The outer zone, therefore, cannot contain collagen in the same state as the inner zone. The same conclusion follows from the failure of the outer zone to stain with the connective tissue stains.

The best evidence of a change in the organization of the collagen, however, is to be found in the polarization microscopic picture. There are three possibilities that the isotropic area could contain fibrous collagen:

(1) The fibers are completely without orientation.
(2) The positive birefringence of the fibers and the negative birefringence of the crystal depositions are in equilibrium.
(3) All the fibers are cut transversely, i. e. oriented with their optical axes in the direction of the polarized light.

It has already been shown that haphazardly oriented fibers stain with both *Mallory*'s and *van Gieson*'s stains. It has also been clearly demonstrated by means of microradiography and by staining methods that the old osteoid contains no mineral deposits. Therefore, possibilities (1) and (2) can be discarded.

That all the fibers should have been cut neatly perpendicular to their long axes seems highly improbable. Probably no one section was cut at exactly ninety degrees of the osteons, and surely, many osteons were cut obliquely because of the oblique direction of many *Haver*sian systems. Alternative (3), moreover, implies no less than that the fibers be definitely oriented with their long axes perpendicular to the plane of section. On this background it is evident that a fortunate conjuncture of circumstances must be at hand in order to obtain these ideal conditions.

It seems unlikely that such requirements can be consistently fulfilled just by accident, for which reason possibility (3) is discounted.

There is no doubt that the outer zone contains collagen, as it is found both in the outer part of the inner zone, and in the bone matrix. The only possibility to account for the difference in the appearance of the collagen of this area in the phase contrast microscope, in the staining reactions and in the polarization microscope would be that there is a difference in the physical state of the collagen in the young and old osteoid, involving loss of fibrous structure. As a matter of fact, the behaviour of this tissue in polarized light even indicates the absence of oriented fibrillar elements at the submicroscopic level.

Stainability of Fibrous and Non-Fibrous Collagen

The present investigation cannot explain the nature of the changes in the collagen of the old osteoid which cause the demonstrable absence of fibrous structure and the failure to stain with collagen fiber stains. If one is willing to assume that the

three stains in question are not taken up by collagen unless it is in a fibrous state, then the dissolution of the fibers in the old osteoid would explain the observed differences in staining between the young and old osteoid. Such an assumption is neither substantiated nor contradicted by the facts presently known, since the conditions under which collagen loses its ability to stain with connective tissue fiber stains have not been investigated.

The findings of the present investigation are compatible with the above assumption, but suggest that in order for the connective tissue stains to be taken up by fibrous collagen alone, the tissues must be fixed by formalin or a fixative with similar action. This may be inferred from the observations in the frozen-dried sections, which were not subjected to formalin fixation. In these, the collagen of the old osteoid stained with the connective tissue stains.

The same staining of the outer zone was observed in the decalcified sections also, although these had been subjected to formalin fixation prior to the introduction of the decalcifying agents. Decalcifying agents destroy the differentiation which formalin has brought about, however. It should be remembered that decalcified sections show no difference between the inner and outer zones under the phase contrast microscope. Therefore, all collagen stains indiscriminately, just as though formalin fixation had been omitted, as in the frozen-dried sections. This conclusion is in agreement with the polarization microscopic picture of osteoid of demineralized sections, revealing no principle difference from similar pictures of undecalcified sections. In other words, the different response to the staining solutions cannot have been caused by major structural differences of the old osteoid of undecalcified as compared with decalcified sections.

Collagen of the Transitional Zone

Corresponding to the subdivisions of the transitional zone seen in the phase contrast microscope, two sublayers appeared after staining with *Mallory's* stain (Fig. 13). A very narrow layer of red granules was distinguished bordering on the yellow old osteoid, while a light blue layer made up the peripheral limit of the transitional zone against the calcified bone.

The reappearance of the aniline blue color which is characteristic of fiber structures, together with the rise of birefringence (Figs. 17 and 18) and absence of silver reduction (Figs. 15 and 16) in this location, show that collagen fibers are present. The light blue and light red hue after *Mallory*'s and *van Gieson*'s stains respectively, may be due to the presence of fewer fibers per unit area, as compared with the young osteoid and the surrounding calcified matrix.

The variability of the double refraction of this area is in agreement with the concept of a corkscrew fiber orientation in the osteons (*Lacroix*, 1951; *Weinmann & Sicher*, 1955). The reason why the width of the birefringent area in many instances exceeded the usual width of the aniline stained layer, is found in the double refractive character of already calcified surrounding lamellae.

The red granular layer of the transitional zone is a continuation of the yellow old osteoid. In the foregoing section, the conclusion was reached that the collagen of the old osteoid had no fiber structures. Accordingly, the yellow-red color in front of and in between the light-blue stained fibrous elements, indicates the presence of collagen in a non-fibrous form. The strong affinity to orange G of this region is most likely due to increased density of this tissue (*Zeiger*, 1938). This conclusion is in accordance with the finding of strong negative charge and the decreased PAS stainability of the polysaccharide component (see later).

Conclusions

The silver stained fibers of the young osteoid bordering on the osteoblasts are identified as reticulin or precollagenous fibers. They are loosely arranged and oriented at random, although the birefringence indicates that the main direction is parallel to the surface of the calcified bone and in the plane of the section.

The rest of the young osteoid, heavily stained by *Mallory*'s and *van Gieson*'s stains and not blackened by silver, contains mature collagenous fibers. Strong birefringence of this region means that the fibers are arranged with their long dimension in the plane of the cross section and parallel to the calcified bone.

The old osteoid contains collagen, but this is not in a fibrous

state. Parallel with the difference in the picture under the phase contrast microscope and in the polarization microscope goes an equally striking difference in the reaction to connective tissue stains of the undecalcified formalin fixed sections. Neither *Mallory's* nor *van Gieson's* nor the silver method stain the old osteoid. However, none of these negative results can be taken to indicate the absence of collagen. The observed differences between the old and young osteoid are compatible with the hypothesis that the main change in the collagen consists in the loss of fibrous structure.

In the transitional zone the non-fibrous collagen shows increased aggregation, visualized by the uptake of orange G. In the peripheral part of this area, the colors of aniline blue and acid fuchsin respectively reappear. Together with the recurrence of birefringence and absence of silver reduction, this leads to the conclusion that collagenous fibers are present. The orientation of these fibers varies according to the arrangement of fibers in the lamellae of the osteon.

The collagenous fibers which are to appear in the calcified bone matrix thus begin to be demonstrable as precollagenous fibers, mature into collagen fibers, and finally lose their fiber character, which will not reappear until calcification sets in. (Text Fig. 3, page 403).

Polysaccharides of the Osteoid

The Metachromatic Reaction

Acid mucopolysaccharides in the osteoid were demonstrated by metachromatic staining after treatment of the sections with aqueous solutions of toluidine blue of a variety of concentrations and for different lengths of time followed by washing with alcohol (See Table I, page 350).

Much controversy was connected with the acceptance of metachromasia as a histochemical test for polysaccharides since *Ehrlich* (1878, 1879) first described the metachromasia of mast cells. *Ehrlich* stressed that these cells were characterized by a

particular chemical substance rather than by structure or shape. He was unable to identify this particular substance, but he noticed from experiments *in vitro* that solutions of toluidine blue turned red when sulphuric acid was added. This led *Lison* (1936) to believe that the shift in color of the dye which occurred when certain tissues were stained with basic aniline dyes was specifically caused by the presence of sulphuric esters of high molecular weight. This has been found not to be the whole truth, as several authors (e. g. *Wislocki, Bunting & Dempsey* (1947), and *Asboe-Hansen* (1951)) showed that these dyes also stained hyaluronic acid metachromatically. Moreover, *Michaelis* (1947) and also *Lison & Mutsaars* (1950) reported that nucleic acids react in the same way.

Sylvén studied the metachromatic reaction *in vivo* (1941, 1947a, 1947b) as well as *in vitro* (1954). From these studies he concluded that in order to combine with the dyes, a substance must have a minimum number of molecules having charged groups available with which the cationic dye molecules can combine. If the staining is to be metachromatic, the reacting groups must furthermore display a certain degree of aggregation in order to provide the proper intercharge distance so that a polymerization of the dye molecules can occur. This concept explains why chondroitin sulphate stains metachromatically, and also accounts for the fact that hyaluronic acid and the nucleic acids may exhibit metachromasia.

Ehrlich (1877, 1879), using several aniline dyes, found it regrettable that by the use of alcohol the metachromatic staining was removed in some tissue components, while it remained in others. *Sylvén* (1947a, 1947b) elaborated the same experience into the distinction between "true" and "false" metachromasia, true metachromasia being due to the presence of chromotrope material, whereas false metachromasia according to *Sylvén* was caused by unexplained adsorption phenomena, and therefore the dye could be washed off by alcohol.

Lison & Mutsaars (1950) found that on decreasing the concentration of thionine or toluidine, one class of substances retained red metachromasia independent of the concentration of dye. Other substances showed a green color with low concentration of dye, and a red color only with high dye concentration.

They termed the first type positive metachromasia and the second type, viz. the green one, negative metachromasia, the names referring to the direction of shift of the absorption spectra. Positive metachromasia they thought to be characteristic for substances like chondroitin sulphate and hyaluronic acid. Up to now negative metachromasia has only been observed in nucleic acids.

Sections of undecalcified bone stained at low concentrations of toluidine blue showed the entire osteoid red before dehydration in alcohols.

After alcohol dehydration, the red color persisted in the youngest region of the osteoid, corresponding to the area of precollagenous fibers, whereas no trace of the red stain remained in the rest of the osteoid. According to *Sylvén's* classification, this indicates "true" metachromasia in the area of precollagen fibers and "false" metachromasia in the rest of the osteoid.

When slightly higher dye concentrations and alcohol dehydration were used, the area of silver stained fibers was red as before. The area with demonstrable collagen fibers was unstained as before but the outer zone of the osteoid showed a light green color growing in intensity at the edge of the newly calcified bone (Fig. 19).

On further increase of the concentration, the areas showing the red and green colors widened and approached each other (Fig. 20). After staining in 0.1 % toluidine blue for 15 minutes and the usual dehydration, the outer zone was no longer green but purplish, whereas the red color of the inner zone remained unchanged (Fig. 22). Maximal increase of the concentration of the dye solution stained the entire osteoid orthochromatically (Fig. 23). See Table I, page 350.

The occurrence of green metachromasia in bone tissue has not been described before. The correlation between the present findings and the observations by *Lison & Mutsaars* (1950) is striking. The green staining with dilute solutions of the dye and the change to red when the dye concentration was increased correspond entirely to what happened in the old osteoid and in the zone of transition. Attempts to change the red metachromasia of the inner zone into green by diluting the dye solution, failed completely. It was either red or unstained. Indeed, variations in concentrations of the staining solution would cause different in-

tensities of the color reaction, but the shade of the color remained the same. In other words, the area of the precollagenous fibers showed true *(Sylvén)* or positive *(Lison & Mutsaars)* metachromasia, the area of nonfibrous collagen and the area of mineralization, negative metachromasia.

Sylvén would have interpreted the metachromasia of the outer and transitional zones as being caused by truly metachromatic substances if stained e.g. with 0.1 % toluidine blue and dehydrated in alcohol (Fig. 22). Staining with 0.001 % solution for half a minute, i. e., drastic reduction of concentration and time, and subsequent dehydration, would according to *Lison & Mutsaar*, reveal the presence of pseudo-metachromatic substances. It is not clear whether *Lison & Mutsaar* (1950) in their tissue studies made their observations from sections mounted in water or after dehydration in alcohol. From the results of the present study it is evident that standardizing of washing in alcohol as well as variation of the dye concentration are necessary for the differentiation of the degree of positive metachromasia and for the distinction of positive and negative metachromasia.

The finding of true metachromatic material in the area of the precollagenous fibers shows that an acid mucopolysaccharide is present in this area. Chemical analyses of bone *(K. Meyer,* 1956), and autoradiographic studies (*Dzietwiatkowski,* 1951a; *Amprino,* 1956) suggest that this mucopolysaccharide is chondroitin sulphate.

Lison & Mutsaar (1950) do not imply that green metachromasia is confined to nucleic acids only. The results of the present studies permit a tentative identification of the negatively metachromatic material in the old osteoid and transitional zone as well as in the newly calcified bone by drawing on evidence gained from the secondary fluorescence, the periodic acid-Schiff reaction and on the staining reactions of the collagen in this zone. This point will be discussed later.

The Periodic Acid-Schiff Reaction

The periodic acid-*Schiff* technique consists in the treatment of sections with periodic acid (HIO_4) and *Schiff*'s reagent (leucofuchsin). The acid is thought to oxidize adjacent hydroxyl groups

into open chain aldehydes. In the subsequent treatment the colorless leucofuchsin combines with aldehydes into red colored complexes (*Hotchkiss*, 1948). The method is generally accepted as a histochemical test for water-insoluble carbohydrates including glycogen, acid and neutral mucopolysaccharides. Glycogen can be eliminated by incubating the sections with diastase (*Pearse*, 1950).

Sections treated with diastase prior to oxidation showed no difference in the intensity of the periodic acid-*Schiff* reaction in the osteoid, indicating the absence of glycogen. On the other hand, the osteoblasts appeared paler to some extent; therefore, they contain glycogen.

The undecalcified sections which were treated with diastase and subsequently exposed to the periodic acid-*Schiff* reagents showed an intense staining of the area corresponding to the layer of the precollagenous fibers, whereas the rest of the young as well as the old osteoid displayed an even, slightly intense color reaction. Omission of the oxidation procedures and staining directly with *Schiff*'s solution left the sections entirely unstained, indicating the absence of preformed aldehydes.

The osteoid therefore contained carbohydrates of high molecular weight. The reacting groups are affected by the change from precollagenous to collagenous fibers, but unaffected by the changes which occur in the collagen itself during the development from young to old osteoid. The area adjacent to the calcified bone, the transitional zone, which stained yellow with *von Kossa*'s silver stain and red with alizarin, showed an abruptly weakened PAS reaction.

Gersh & Catchpole (1949), in their work on connective tissue, related differences in the intensity of the PAS reaction to the degree of aggregation of the reacting groups, and thereby to the state of polymerization of the polysaccharide molecules. The intensity of staining is thought to be maximal at an optimal degree of polymerization and to decline with further condensation of the molecules because the reacting groups are no longer accessible. Work along the same line by *Cobb* (1949), *Heller-Steinberg* (1951) and *Engel* (1952) has suggested that this applies to the ground substance of bone tissue as well.

Therefore, the weakened reaction in the zone of transition

could be interpreted to mean that further polymerization of the polysaccharides has occurred in this region; in addition it could indicate the masking of some of the reacting groups by the mineral salts (*Yaeger*, 1958).

The Relation between the Metachromatic Substance and the Periodic Acid-Schiff Reactive Compounds

The positive metachromasia diminishes gradually throughout the inner zone, and is absent in the rest of the osteoid, whereas the periodic acid-*Schiff* reaction shows no gradient in intensity peripheral to the region of precollagenous fibers. This indicates that one actually is dealing with two different polysaccharides.

In the section on "Fibers of osteoid" (pp 373—381) it was shown that the decrease in the intensity of metachromasia parallels the development of the precollagen fibers into mature collagen fibers, and that chondroitin sulphate plays a rôle in this development. The more intense PAS-reaction in the youngest osteoid, where the fibers are still precollagenous fibers, is due to the fact that the chondroitin sulphate is not yet involved in the formation of collagen fibers. Its hydroxyl groups are therefore accessible to the PAS-reagents, whereas in the mature collagenous fibers they are no longer reactive. Since chondroitin sulphate is a component of calcified bone, there is no reason to assume that the progressive decrease in the intensity and eventual disappearance of the positive metachromasia are caused by a decrease in the amount of chondroitin sulphate, as claimed by *Sylvén* (1947). It seems far more likely that its anionic groups are no longer accessible to the molecules.

The change in the collagen within the old osteoid does not lead to a liberation of the reacting groups of chondroitin sulphate, since it does not lead to a reappearance of positive metachromasia.

It is possible that the sulphate groups remain interconnected with the collagen and therefore cannot respond to toluidine blue. It is also possible that the change in the collagen fibers has simply caused an increase in the intercharge distance of the

sulphate groups beyond the distance required for metachromasia to occur (*Sylvén*, 1954). However, the fact that positive metachromasia does not recur in the area of mineralization, where the distance between the collagen molecules is minimal, seems to rule out the second possibility. It appears therefore that the intimate relation between collagen and chondroitin sulphate which was established during the development of the fibrous collagen and led to the disappearance of positive metachromasia, has been retained in the nonfibrous state of the collagen in the old osteoid, and is also retained during mineralization. This concept of chondroitin sulphate serving as a cementing substance between fibrous elements is in agreement with what is found in other connective tissues.

The periodic acid-*Schiff* reaction remains constant in part of the young and in the entire old osteoid, exhibiting the same intensity from the area of the precollagenous fibers to a point just next to the margin of the calcified bone, showing that this reaction is somewhat independent of the factors which cause variations in the metachromatic reactions. This suggests the presence of a polysaccharide component other than the sulphated one. This second polysaccharide group does not interact with collagen. The persistence of the periodic acid-*Schiff* reaction in contrast to the loss of metachromasia, which are observed in osteoid after exposure to decalcifying agents, is a further point suggesting the presence of at least two groups of polysaccharides in bone.

Since no *acid* mucopolysaccharides other than chondroitin sulphate have been found in any amount in bone tissue (*K. Meyer*, 1956), the PAS reaction in the osteoid must be due to a different polysaccharide-protein complex. This conclusion is supported by recent findings of *Dische et al.* (1958). They found a neutral polysaccharide-protein complex in bone tissue and considered that this group may constitute the major portion of the polysaccharides of bone. The behavior of this polysaccharide compound throughout the osteoid and especially its independence of the variations of the fibers, suggest that the neutral polysaccharide-protein complex constitutes the colloid part of the ground substance of bone, rather than acting as a cementing substance between the fibrous structures.

Since they have no net negative charge the neutral polysaccharides cannot be responsible for the negative metachromasia of the outer and transitional zones. Therefore, the most probable source of anionic groups for the cationic dye to react with, would be the collagen. The change of the fibers which has occurred in the old osteoid (pp. 373—381) and the increased polymerization of the tissue towards the edge of the bone may be responsible for the negative metachromasia of the outer zone.

The negative metachromasia continues into the newly calcified portion of the transitional zone, as shown by the fact that this area stained green with low concentrations of the dye solution. *Engfeldt & Hjertquist* (1955), *Vincent* (1955) and *Lacroix* (1956) believed that the metachromasia of this region is due to the presence of true metachromatic substances. It appears, however, that they have not reduced the concentration of the dye sufficiently to rule out negative metachromasia.

The increased intensity of negative metachromasia in the transitional zone compared with the old osteoid is the expression of a closer spacing of the collagen. The fact that negative metachromasia is found in the osteoid as well as in the newly calcified bone demonstrates that the staining of the bone is not, or at least not entirely, an adsorption phenomenon as claimed by *Sylvén* (1947a, 1947b), but indicates that nonfibrous collagen is present in the area of newly calcified bone as well as in the osteoid.

The increased density of negative charge in the osteoid resulting from the higher concentration of the nonfibrous collagen and of the polysaccharides is expressed by the maximal intensity of basophilia after staining with hematoxylin-eosin.

Conclusions

Two high molecular polysaccharide components are present in osteoid: (1) An acid mucopolysaccharide, chondroitin sulphuric acid, is closely connected with the development and maintenance of the collagen. (2) A non-acid, presumably a neutral polysaccharide complex, is more independent of the variations of the collagen, and acts as ground substance.

The Distribution of Alkaline Phosphatase in the Osteoid

The histochemical methods for the demonstration of enzymes are somewhat complicated by the fact that as yet enzymes *per se* cannot be visualized. Therefore, the principles underlying the techniques in use are to render visible a product of the reaction of the enzymes with one of its subtrates. *Gomori's* (1938, 1952) method involves the incubation of sections with an aliphatic ester of phosphoric acid as well as with ionized calcium. The resulting precipitate of calcium phosphate is subsequently demonstrated by the replacement of calcium with metals forming insoluble colored phosphates, e.g. cobalt.

Any calcium phosphate contained in the sections is visualized as cobalt phosphate. Consequently, the application of this method to undecalcified hard tissues (*Freman & McLean*, 1941; *Furuta & Engel*, 1942; *Gomori*, 1943; *Wislocki & Sognnaes*, 1950; *Bevelander & Johnson*, 1951) renders the interpretation difficult. However, alkaline phosphatase is not only very sensitive to histologic procedures like fixation and embedding (*Danielli*, 1945), but also to demineralization in acids, since the optium pH of the enzyme is on the alkaline side.

In 1944 *Menten, Junge & Green* demonstrated alkaline phosphatase by using an aromatic ester as substrate and by visualizing the alcoholic part of the substrate-enzyme reaction through coupling with an azo dye. Modifications of this technique were made by *Manheimer & Seligman* (1949). The fact that the reagents do not visualize the inorganic salts made this method seem ideal for the identification of alkaline phosphatase in bone, especially so, as in other areas of research the method seemed to be superior to the cobalt sulphide method (*Mintz & Russel*, 1957). Both techniques were used in the present investigation.

In order to distinguish enzyme activity from preformed stainable material, control sections of both series were inactivated by treatment with iodine-potassium iodide solution before incubation. Omission of glycerophosphate from the incubation medium, or exclusion of calcium chloride from the same, as well as staining directly with cobalt nitrate-ammonium sulphide, served to increase the specificity of *Gomori's* method.

The area of the osteoid adjacent to the osteoblasts stained red with the azo technique (Fig. 34) and black with *Gomori*'s method (Fig. 33) for alkaline phosphatase. Sections treated with iodine-potassium iodide solution and subsequently subjected to the respective methods did not show staining in this region, (Fig. 35) indicating that the matter stained in the intact sections actually was the product of phosphatase activity.

Nuclei and cytoplasm of active osteoblasts were stained by both methods. The cytoplasmic staining included the whole length of the cell processes, as far as they could be followed into the calcified matrix. Resting osteoblasts lining the surface of bone where no bone formation was going on, showed reduced enzyme activity in nuclei and cytoplasm. The extra-cellular staining in the narrow area of the osteoid and the staining in the cytoplasm of cell bodies and cell processes occurred only in the sections tested for enzyme activity and was absent in the control series. Whereas treatment with iodine- potassium iodide solution left an entirely unstained section after incubation in sodium naphtyl phosphate-azo RC, the cell nuclei continued to stain weakly black when different control measures were undertaken to verify the results of the *Gomori* method.

Treatment with iodine-potassium iodide solution, or omission of incubation in glycerophosphate both resulted in faint staining of the area of the nuclei close to the nuclear membranes, indicating that other substances than the products of enzyme activity were stained by the latter method. Staining of sections exclusively with cobalt nitrate—ammonium sulphide gave the same intensity and distribution of staining within the nuclei, as did the previously mentioned control procedures. This confirms the presence of stainable matter other than that indicating phosphatase activity. Finally, the omission of calcium chloride in the incubating mixture gave the same result as inactivation of the enzyme with calcium chloride present, indicating that other substances are able to bind with cobalt.

All this shows that also intranuclear preformed phosphates or other anions contribute to the black color when *Gomori*'s method is used. That the preformed substances are not responsible for the entire black stain in the nuclei, appears from the increased intensity of staining after one minute of enzyme action

and also from the results obtained with the azo dye technique.

The consistent finding of an unstained inner zone during these control procedures excludes the presence of preformed insoluble phosphates in this tissue, beyond the amounts found in soft tissue in general.

The possibility that the presence of stain in the osteoid might be due to diffusion artefacts from the osteoblasts into the osteoid (*Majno & Rouiller*, 1951) can be excluded for the reason that the activity was stronger in the osteoid than in the cytoplasm of the osteoblasts. It seems unlikely that an almost complete relocalization of the activity could take place. Moreover, the presence of phosphatase activity was as marked in areas where the osteoblasts had been dislocated from the osteoid as in areas where the osteoblasts were in position. The short period of incubation in glycerophosphate (1—3 minutes) and the incubation in sodium naphtyl phosphate—azo substrate at low temperature (4°C) are probably both apt to reduce the degree of diffusion.

That the black stain in part of the inner zone represented diffusion of mineral salts from the calcified bone can be ruled out because of the strongly alkaline pH of the incubating mixture and because of the finding of azo dye in the same location. Any interaction of this dye with the bone minerals is, as far as can be seen, theoretically impossible.

It was also evident that the azo dye did not stain any part of the calcified matrix. Even if the azo dye technique had not been used, contamination from the mineralized bone would have been unlikely in view of the consistent finding of an unstained portion of the osteoid of approximately 8—10 microns between the area of phosphatase activity, and the black-stained calcified bone.

The only part of the peripheral osteoid which stained with *Gomori*'s method was a narrow band next to the calcified bone. That this did not represent enzyme activity was evident from the fact that this area did not stain with azo dye. It also stained with cobalt in any of the controls of the *Gomori* technique.

Freezing-drying and acetone fixation and the embedding of tissue in methacrylate at a temperature below 45° for 8 hours, seem to have caused little inactivation of the enzyme, as high color intensities were obtained already after very short incuba-

tion periods. The present material does not permit an evaluation of enzyme activity in sections from decalcified material compared with what is found in undecalcified sections. Nor can a direct comparison between the phosphatase activity after paraffin embedding and embedding in methacrylate be made. Nevertheless, considering the time of incubation used for decalcified and reactivated sections (*Lorch*, 1947; *Greep, Fischer & Morse*, 1948; *Wislocki & Sognnaes*, 1950) and for sections of undecalcified bone embedded in paraffin (*Pritchard*, 1952), it seems justified to state that the technique employed in making undecalcified sections of frozen-dried and acetone fixed and methacrylate embedded bone is superior, as far as the preservation of alkaline phosphatase is concerned.

The area of bone used in this study facilitated the interpretation of the results. The occurrence of alkaline phosphatase activity in a narrow layer of the osteoid adjacent to the osteoblasts, and in the cytoplasmic processes and its absence in the major portion (4/5) of the osteoid, have not been clearly shown before. The reason may be that most of the studies of phosphatase in thin sections of undecalcified bone have been confined to spongious bone (*Gomori*, 1943; *Bevelander & Johnson*, 1951; *Pritchard*, 1952). A study of phosphatase in osteoid which is so narrow as to be hardly visible (*Erdheim*, 1914; *Robinson & Watson*, 1953) must be little informative, especially so, as practically all investigators have used the cobalt sulphide method or related varieties.

Decalcified sections of cortical bone for the study of the location of the enzyme activity in relation to the calcified matrix seem to be less appropriate, because in such sections it is rarely possible to determine the exact borderline between osteoid and decalcified bone matrix. It is therefore understandable that less specific statements like "edges of bony trabeculae", "newly formed trabeculae" or "the lining of vascular canals" have been used in describing the localization of phosphatase in bone tissue.

Given the sensivity of the enzyme to acid pH (*Robison*, 1923; *Gomori*, 1939) and the need, in using the cobalt sulphide method, to separate the product of enzyme activity from preformed matters by tedious controls, it appears that the combination of undecalcified sections and the azo dye technique is the method of

choice. The azo dye method enables the investigator to ignore the problem of tissue phosphates. The presence of phenols or naphtols in body tissue under normal conditions is hardly conceivable. In view of the simplicity of the technique involving one substrate-dye solution, the present study corroborates the judgment that this method gives better and clearer results in hard tissues than the cobalt sulphide method.

Conclusions

Alkaline phosphatase is located in the young osteoid, corresponding to the area of the precollagenous fibers. Active osteoblasts show enzyme activity in the nuclei, in the perinuclear cytoplasm and in the cell processes. Resting osteoblasts lining the inner walls of completed osteons show reduced phosphatase activity in the cytoplasm and nuclei. Osteocytes are free of any cytoplasmic alkaline phosphatase activity from the moment they are embedded in the osteoid, and display only weak activity in the nuclei independent of the distance from the surface.

Possible functions of alkaline phosphatase will be discussed in a following section.

MECHANISM OF MINERALIZATION

Calcifiability of the Osteoid

The amount of water found in calcified bone tissue (10 %; *Robinson,* 1958) is smaller than in other connective tissues (60—70 %; *Best & Taylor,* 1954). The water content of the osteoid does not seem to differ from other comparable connective tissues and is much higher than that of bone. This excess water of the osteoid is most likely released just before calcification. By which means water is removed from the area of mineralization is not clear.

The loss of water in the area of mineralization would cause the above-mentioned increase of the negative charge.

Whether reconstitution of collagen fibers is a direct result of the closer spacing of the collagen units due to the withdrawal of water and thus occurs spontaneously, is dependent on the nature of the preceding disorganization of the collagen. If the reconstitution requires energy, the source of energy is most likely located in the osteoblastic processes.

The electron microscopic picture of the earliest calcification (*Robinson & Cameron*, 1956) reveals collagen fibrils and fibers with the characteristic periodicity, to which the inorganic crystals are spatially related. It is always a surprise to see that in the area of calcification the collagen fibers cover only approximately half of the available area, and that relatively large spaces are found between them. A short distance farther into the calcified bone the fibers are more densely packed. The negative metachromasia in the area of earliest mineralization suggests that these spaces still contain nonfibrous collagen, and that this becomes reconstituted at the moment of mineralization. This explains the increase in the amount of fibrous collagen as mineralization proceeds. An extension of the argument gives some reasonable explanation of the finding of thicker fibers in more heavily mineralized bone and with advancing age (*Robinson & Cameron*, 1956).

The fact that the collagen fibers do not reappear before calcification starts, indicates that the reconstitution of fibers takes place simultaneously with or is followed by mineralization after too short a time for a stage of recognizable fibers to occur. In other words, calcifiable bone matrix cannot be visualized because mineralization sets in as soon as calcifiability is conferred upon the tissue. The long duration of the phase of nonfibrous collagen, which occupies the whole outer zone of the osteoid, suggests that calcifiability depends on the restitution of the fibrous state of the collagen, and the increased aggregation of the ground substance. The factors controlling the timing of these processes are not clear. It is, however, evident that calcifiability is not determined by the supply of mineral salts. In order to reach the area of mineralization, the bone salts which are directly or indirectly supplied by the circulation must traverse the entire width of the osteoid, and therefore are close to the organic matrix in all of

its successive stages. Only at the stage of reconstitution of the collagen and the simultaneous changes in the ground substance is there an interaction between the organic matrix and the minerals.

Rôle of Alkaline Phosphatase in Mineralization

Two conflicting concepts exist of the rôle of alkaline phosphatase in the process of mineralization. One is based on the early hypothesis of *Robison* (1923, 1934) supported by *Roche et al.* (1945, 1950), according to which alkaline phosphatase is responsible for a local elevation of the concentration of phosphate ions in areas of mineralization. The other is derived from the observation that cartilage can be caused to calcify *in vitro* after inactivation of the enzyme (*Waldman*, 1948). It has, moreover, been suggested that the concentration of monophosphate esters in the tissue fluids is too low to provide the necessary substrate for alkaline phosphatase. Against this, it was argued that in cartilage the adequate amount of substrate could be obtained by phosphorylation of glycogen (*Gutman & Gutman*, 1941; *Gutman & Yu*, 1950).

The present study shows glycogen to be present in osteoblasts. It is a possibility, therefore, that glycogenolysis and phosphorylation takes place. Furthermore, it is possible that the osteoblasts can take up and phosphorylate circulating glucose. A subsequent hydrolysis of glucose monophosphate can also take place within the osteoblasts, and phosphate ions could be released through the cytoplasmic membranes of the cell processes along the entire width of the osteoid. A polarized activity of the osteoblasts is found in other of their functions also. The present findings on the distribution of alkaline phosphatase activity neither corroborate nor reject the existing theories on the rôle of alkaline phosphatase in the process of mineralization. However, the high level of activity in the cytoplasmic processes tends to show that extracellular enzyme activity in the area of mineralization is not a necessity, nor does the scarcity of monophosphate esters in the tissue fluids preclude the possibility that alkaline phosphatase plays a rôle in mineralization.

Rôle of Collagen and Polysaccharides in Mineralization

Hass (1945) suggested that a protein-carbohydrate complex in connective tissue acts as an inhibitant to mineralization. More recently, studies *in vitro* by *Glimcher et al.* (1957) showed that reconstituted collagen offers less resistance to mineralization than dissected native collagen. Following the suggestion of *Hass*, these authors supposed that during their extraction of the collagen the mucopolysaccharides had been removed and that removal of these substances was the key to mineralization.

The present study shows that mineralization is not inhibited by the presence of mucopolysaccharides. On the contrary, the sudden change in the periodic acid-*Schiff* reaction in the area of mineralization indicates that the neutral polysaccharides may play a positive rôle in the process.

It seems highly improbable that the sulphate groups of the chondroitin sulphate play a decisive rôle in this connection (*Levine, Rubin, Follis & Howard*, 1949; *Sobel*, 1952, 1955), because these groups do not seem to be exposed in the area of mineralization, nor are they available for staining in the bone matrix after decalcification. As a whole, the behavior of chondroitin sulphate from the collagen state in the young osteoid to the area of mineralization indicates that it may be entirely concerned with the collagen as such, in bone as in other mesodermal tissues (*Partridge*, 1948; *K. Meyer*, 1956).

Concerning the rôle of the neutral polysaccharides, the findings of *Yaeger* (1958) suggest a possible interaction between extra crystalline mineral and some polysaccharide.

On the other hand, substantial evidence exists for an orderly arrangement of the crystals upon and perhaps within the collagen fibrils (*Robinson & Watson*, 1953; *Jackson*, 1954, 1957; *Sheldon & Robinson*, 1957). *Neuman & Neuman* (1958) have recently set forth a hypothesis of the crystal formation without a drastic local increase of inorganic ions in areas of bone formation. According to this, in the mechanism of epitaxy or the seeding mechanism a component is necessary which is crystalline by structure and which is sufficiently similar to the structure of the hydroxyapatite. This crystalline material then, would induce a

nucleus of calcium and phosphate ions. Once a hydroxyapatite nucleus is formed, the further growth of the crystal takes place spontaneously.

The only crystalline structure other than the mineral crystals in bone tissue is collagen. Theoretically, the authors just mentioned find it conceivable that collagen or some collagen-polysaccharide complex may play the rôle of host crystal during nucleation.

The present investigation indicates an important rôle of the collagen. At least, there are reasons to believe that the dynamics of the collagen involving formation, dissolution and reconstitution, serve some reasonable purpose in this context. The present study gives no direct clue as to the necessity of such peculiar behavior. It may well be that reconstitution of collagen not only facilitates mineralization, as *Glimcher* and co-workers (1957) suggest, but that reconstitution *in vivo* is a prerequisite for mineralization to take place. It is also possible that the reconstitution involves a rearrangement of the collagen-polysaccharide interrelationship, which is specific for bone matrix and therefore, responsible for the initial nucleation.

CHAPTER VI - SUMMARY AND CONCLUSIONS

Problems

The morphological and histochemical analysis of the processes involved in bone tissue formation has hitherto been performed on either decalcified material or undecalcified sections of embryonic or very young bone. Several authors have shown that the width of the osteoid, the organic precursor of bone, varies greatly in different areas of the skeleton. The purpose of the present investigation was to elaborate a technique for the production of thin undemineralized sections of mature diaphyseal bone in which wide osteoid borders are found. By subjecting these sections to a variety of investigations, it was hoped to disclose the nature of some of the processes which necessarily have to take place in the osteoid prior to mineralization.

Material and Methods

Cortical bone was taken from the diaphyses of radius, tibia and the metacarpals of five normal dogs, aging from four to twelve months. Small pieces of bone were removed while the animals were still alive. In all instances the specimens were fixed within one or two minutes after amputation.

Pieces of the same bone were fixed in neutral formalin, in *Zenker*'s fluid or by freeze-drying. The latter group of material was postfixed in acetone before embedding. The undemineralized material was embedded in methacrylate and cut on a metallurgic microtome. Usually, serial sections were cut at six microns, but thinner sections could be obtained.

Some of the formalin fixed material was demineralized in five per cent watery solution of nitric acid, forty-five per cent formic

acid and twenty per cent sodium citrate in distilled water or in saturated solution of the disodium salt of ethylene diamine tetra-acetate, buffered with sodium hydroxide to pH 7.2.

Both undecalcified and decalcified cross-sections were tested for general morphology, fibers, carbohydrates, inorganic mineral salts and alkaline phosphatase by a variety of staining procedures and histochemical tests. Phase contrast microscopy, fluorescence microscopy (primary and secondary fluorescence), polarization microscopy and microradiography were applied to analogous sections.

Morphology and Histochemistry of the Osteoid

Up to now, osteoid has been characterized at the microscopical level as beeing homogeneous, hyaline or edematous. The present study, including examination in the phase contrast microscope, disclosed three major zones of osteoid:

An inner zone, young osteoid, adjacent to the osteoblasts.

An outer zone, old osteoid, comprising the middle and the pheripheral parts of the osteoid, which in turn, borders on

the transitional zone, i. e. a narrow area of tissue representing the end of the osteoid and the beginning of the bone tissue. Layers within the zones were also distinguished.

Radiating striations of the osteoid are effected by the processes of the osteoblasts and osteocytes.

The parallel series on partially and completely decalcified material leaves no doubt that the reason why these morphologic features of the osteoid have not been seen before, are found in the detrimental effect of demineralizing agents. In principle, no difference is observed after the use of different demineralizers.

The optical path differences in the osteoid which has not been exposed to demineralizing fluids, are consequently due to underlying differences of chemical and/or physical properties.

Inorganic constituents of osteoid. The possibility that the inhomogeneity of the osteoid could be caused by minor variations of inorganic matter was tried by microradiography, the silver staining of *von Kossa* and alizarin staining.

As far as the three zones of the osteoid are concerned, there appeared to be a correlation between these methods.

The results demonstrated that the inner and outer zones of the osteoid are devoid of inorganic depositions but that the mineral salts show increasing aggregation through the area of transition towards the bone.

The inhomogeneity of the osteoid, therefore, as it appeared in the phase contrast microscope, is in the main not caused by differences in mineral content. The explanation of this phenomenon may be sought in differences in the organic constituents.

Charge condition of the osteoid. Prior to the discussion of the organic elements of the osteoid, an analysis was made of the electric charge conditions in the osteoid.

The primary fluorescence in unstained sections and the secondary fluorescence in sections subjected to dilute watery solutions of cationic and anionic fluorochromes at sliding pH values, revealed roughly the same zones in the osteoid as did the phase contrast microscopy.

It is clear from these series that a mean value for the iso-electric point of the osteoid does not exist, but that each zone has its characteristic electro-activity. The iso-electric point of the zone of transition is located in the pH range of 1—2, that of the narrow rim of tissue adjacent to the osteoblasts lies between pH 4 and 5, whereas the rest of the young as well as the entire old osteoid is very weakly charged.

Fibers of the osteoid. The fibrous elements of the osteoid were examined in the phase contrast microscope, by staining in *Mallory*'s and *van Gieson*'s stains and in ammonical silver solutions, and analyzed in the polarization microscope. The silver stained fibers of the young osteoid bordering on the osteoblasts were identified as reticulin or precollagenous fibers. They are loosely arranged and oriented at random, although the birefringence indicates that the main direction is parallel to the surface of the calcified bone and in the plane of the section.

The rest of the young osteoid, heavily stained by *Mallory*'s and *van Gieson*'s stain and not blackened by silver, were identified as collagenous fibers. Strong birefringence would mean that the fibers are arranged with their long dimension in the plane of the section and parallel to the calcified bone.

The old osteoid showed no evidence of fibers. Neither *Mallory's* nor *van Gieson's* method stained the old osteoid, and signs of fibers were not seen in the phase contrast microscope. Furthermore, this area remained dark at all azimuths in polarized light. All of these negative results indicate the absence of collagenous fibers. The polarization microscopic picture even excludes the presence of oriented fibrillar elements at the submicroscopic level.

The transitional zone is characterized by the reappearance of weak color effects after *Mallory's* and *van Gieson's* stains. Between crossed Nicols, this area consistently showed birefringence, the intensity of which might vary. Although individual fibers could not be seen, these findings are taken to indicate the presence of collagenous fibers. The variations in phase difference as revealed by the polarization microscope are caused by the differences in the direction of the fibers, the same phenomenon being responsible for the lamellation of the osteons.

Carbohydrates of the osteoid. Tests for carbohydrates in the osteoid included staining with watery solutions of toluidine blue and the periodic acid-*Schiff* technique. The inner part of the young osteoid corresponding to the silver stained fibers showed red (positive) metachromasia at all concentrations and staining times. The zone of transition showed green (negative) metachromasia at low concentrations and was purplish red at higher concentrations.

The occurence of green metachromasia in bone tissue has not been described before.

On the basis of earlier experiments, the conclusion was reached that in bone tissue positive metachromasia indicates the presence of acid polysaccharides in the form of chondroitin sulphuric acid. The negative metachromasia, being very weak in the old osteoid and strong in the zone of transition, is due to the increasing negative charge of tissue components other than acid polysaccharides. These other elements are tentatively identified as collagen in a non-fibrous form.

The periodic acid-*Schiff* reaction remained constant in part of the young and in the entire old osteoid. In the very narrow area corresponding to the precollagenous fibers, the reaction was somewhat increased. The transitional zone showed an abruptly

weakened reaction, indicating a higher degree of polymerization of the polysaccharides. The constant intensity of the PAS reaction in part of the young and the entire old osteoid, and the persistence of the periodic acid-*Schiff* reaction throughout the inner and outer zones, in contrast to the loss of positive metachromasia after decalcification, suggest the presence of at least two groups of polysaccharides in bone, an acid and a neutral polysaccharide component.

Alkaline phosphatase activity of osteoid. Alkaline phosphatase was demonstrated in undecalcified sections of frozen-dried bone by *Gomori*'s cobalt technique and the azo dye coupling technique. The area of bone used in this study facilitated the interpretation of the results. Given the sensitivity of the enzyme to histologic procedure, frozen-dried acetone fixed specimens and the azo dye technique appears to be the method of choice. Alkaline phosphatase is located in the young osteoid corresponding to the area of precollagenous fibers. The rest of the osteoid, except for osteoblastic processes, is devoid of alkaline phosphatase activity.

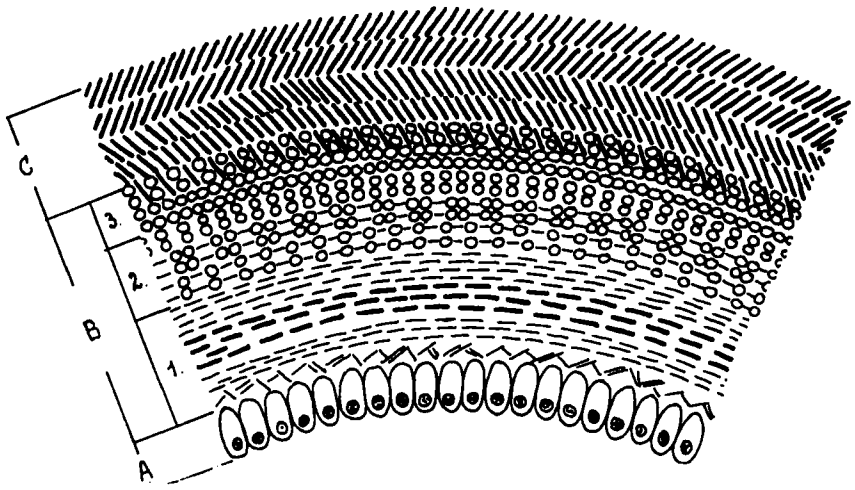
From time to time, both in the written language and orally, the word osteoid is erroneously used in the meaning of demineralized bone matrix. The characteristic morphology and specific histochemical qualities of the osteoid, and the clear differences between bone matrix and osteoid, demand that the word osteoid be reserved for the differentiating organic precursor of bone, in which the deposition of inorganic salts takes place, giving as a result bone tissue.

BONE TISSUE FORMATION

A Concept

Analysis of the histological and histochemical properties of the zones of osteoid permit the distinction of three stages in the process of bone formation (Text fig. 3):

(1) A stage which includes the formation of precollagenous fibers and polysaccharides and ends with the development of



A. Osteoblasts

B. Osteoid
1. Inner zone (young osteoid)
2. Outer zone (old osteoid)
3. Transitional zone (intermediate bone

C. Bone

Text fig. 3.

mature collagen fibers and ground substance, (2) the change of the collagenous fibers into nonfibrous units, and (3) increased degree of polymerization of the ground substance and the negative charge, reconstitution of collagen, and the simultaneous mineralization of the calcifiable matrix.

(1) The present study supports the concept that the osteoblasts play a decisive rôle in bone tissue formation. The presence of glycogen, of metachromatic and periodic acid-*Schiff* positive granules, and the evidence of alkaline phosphatase in the peripheral cytoplasm indicate specific activities of the osteoblasts. It seems reasonable to think of the polysaccharide granules as building blocks in what extracellularly exist as protein-polysaccharide complexes. Signs of fiber production within the cells could not be seen. Nevertheless, the location and the mesodermal nature of the osteoblasts, together with the richness of fibers in their close vicinity, indicate some rôle of the osteoblasts in fiber production similar to that suggested for fibroblasts. Thus, the osteoblasts seem to produce the major constituents of the future bone matrix. Furthermore, the cells are oriented with respect to the prospective calcified bone in the sense that almost all fibers are found between the cells and the bone.

The finding of occasional mature collagenous fibers between the osteoblasts show that the pre-existing fibers of the loose connective tissue are incorporated into the osteoid as the replacement of the former takes place. The fibers formed by the osteoblasts appear as precollagenous fibers, and are vastly more numerous than the pre-existing fibers.

The precollagenous fibers are identifiable by their staining reactions. They are loosely packed and without any strict orientation, although a weak birefringence indicates a preferential arrangement parallel to the surface of the bone and in the plane of the section. A short distance into the osteoid these silver stained fibers become converted into collagenous fibers. At least two constituents of the young osteoid contribute to the conversion into collagen fibers: Chondroitin sulphate and alkaline phosphatase. Chondroitin sulphate is but loosely connected with the precollagenous fibers. The conversion of precollagen fibers into mature collagen fibers consists in the establishment of a firm connection between the precollagen fibers and the chondroitin

sulphate. The nature of the interaction between chondroitin sulphate and collagen is not known, but it appears that it consists in a chemical reaction which involves at least the sulphate groups.

The function of the chondroitin sulphate, therefore, is best understood as that of a *cementing substance*. This is not unique for bone tissue, but is in agreement with what is found in other connective tissues.

The occurrence of alkaline phosphatase in the area of the precollagen fibers corroborates what has been found in other connective tissues during fibrogenesis, namely, that extracellular alkaline phosphatase may be connected with the development of collagen fibers. The reactions catalyzed by the enzyme in this process are not known.

A second polysaccharide component is present in the entire first stage. This is a neutral polysaccharide-protein complex which is unaffected by the differentiation of the fibers, and constitutes *the ground substance* rather than acting as a cementing substance between the fibers.

(2) With the beginning of the second stage, the collagen fibers start to change structurally, and to lose their staining qualities. After a brief transitional stage the fibrous structures disappear. From then on, the collagen does not respond to the conventional stains for collagen fibers and appears structureless. The characteristic birefringence of collagen is also absent. The chemical linkage between collagen and chondroitin sulphate persists in the stage where the collagen is nonfibrous. Judging by the old osteoid, this stage must last for a considerable length of time.

(3) The third stage in the process of bone formation, although brief, comprises a complexity of processes directed toward the final transformation of the osteoid into calcifiable matrix and the subsequent mineralization of the latter. The initiation of the dynamics of the area of mineralization is probably the withdrawal of water. The loss of water leads to a higher concentration of nonfibrous collagen and accompanies increased aggregation of the ground substance. When this highly aggregated state is reached, the collagen fibers are reconstituted. This reconstitution

may be spontaneous or require a specific energy system mediated by enzyme systems of the osteoblastic processes.

With the reconstitution of the collagen and the constitution of the interrelationship between collagen and polysaccharides, which may be specific for bone matrix, this is ready for mineralization. The withdrawal of water and the increase in concentration of the colloid have brought about an increase in the electronegative charge of the area. This would account for the attraction of calcium ions. Phosphates and other anions have reached the area either by diffusion or by enzymatic action of phosphatases in the osteoblastic processes. Whether the actual crystallization is caused by the effect of a booster mechanism or by a seeding mechanism cannot be determined at the morphologic level.

The polymerization of the ground substance, the reconstitution of collagen fibers, and the initial mineralization are most likely simultaneous processes. This means that normally the calcifiable matrix is not demonstrable, because the matrix is mineralized as soon as calcifiability is brought about. The term "calcifiability" should be taken literally as a state of the osteoid which is not dependent on the supply of mineral salts. In order to reach the area of mineralization, the bone salts supplied by the circulation must traverse the entire width of the osteoid in its successive stages. Only at the stage of increased polymerization of the ground substance, increased negative electric charge and reconstitution of the collagen fibers, is there an interaction between the osteoid and the minerals. This applies regardless of whether the mineral ions diffuse in solution as electrolytes, or are released by enzymatic hydrolysis.

As soon as the mineralization is initiated, only a short time is necessary in order to reach a relatively high degree of mineralization, provided the mineral supply is normal. If calcification is delayed by a scarcity of mineral salts, this should be manifested by an increased width of the transitional area. In other words, there should be a wide zone which stains with collagen stains, shows positive birefringence, exhibits strong negative metachromasia and decreased periodic acid-*Schiff* reaction, and shows concentration effect at pH values below pH 2 after fluorochromation with acridine orange, but lacks signs of accumula-

tion of mineral salts and of calcification. Increased width of the zones representing earlier stages in the development of osteoid are not indicative of a shortage of mineral salts.

After the first stage of rapid mineralization is completed, the deposition of inorganic salts continues at a much slower rate. Evidence for continued deposition of mineral salts is found in a higher degree of mineralization in the outer part compared with the inner part of osteons under formation, and in older osteons compared with younger ones. There is also evidence for a thickening of the collagen fibers of bone with age. In the absence of cells capable of synthesizing collagen and ground substance in the calcified bone matrix, it is assumed that these constituents are already present in the interstices of the calcified matrix and are gradually mineralized. This gradual mineralization of residual material may, therefore, be accounted for by processes similar to those preceding the initial mineralization, i.e., loss of water, increased aggregation of ground substance, and reconstitution of collagen fibers.

CONCLUSION ET RÉSULTATS

FORMATION DU TISSU OSSEUX

Etude morphologique et histochimique

Les analyses morphologiques et histochimiques des processus dont l'ensemble constitue la formation du tissu osseux ont jusqu'ici principalement été exécutées après déminéralisation ou sur des coupes non déminéralisées de tissu osseux spongieux embryonnaire ou très jeune. Plusieurs chercheurs ont démontré que la largeur de l'ostéoïde, précurseur organique du tissu osseux, diffère grandement dans les différentes parties du squelette.

Le but de la présente étude a été de développer une technique permettant d'exécuter de minces coupes non déminéralisées de tissu osseux mûr provenant de la diaphyse où l'on trouve habituellement de larges zones d'ostéoïde. En effectuant ensuite divers examens de ces coupes, l'auteur espère pouvoir éclairer quelques uns des processus qui doivent nécessairement se produire dans l'ostéoïde préalablement à la minéralisation. On a prélevé du tissu osseux cortical sur la diaphyse du radius, du tibia et des métacarpiens de cinq chiens normaux. L'âge des chiens variait de quatre à douze mois. Des fragments osseux ont été prélevés sur l'animal vivant. Les tissus ont été fixés sans exception dans un délai d'une à deux minutes après amputation. Les parties d'un seul et même os ont été fixées au formol neutre, au liquide de *Zenker* ou par "congélation-dessiccation". Ce dernier groupe à ensuite été fixé à l'acétone. Ces tissus non déminéralisés ont été inclus dans du méthacrylate et coupés au microtome métallurgique. Les coupes étaient en général d'une épaisseur de six microns, mais on pouvait les faire encore plus minces. Une partie des tissus fixés au formol ont été déminéralisés dans une solution

aqueuse à 5 % d'acide nitrique, dans une solution de 45 % d'acide formique et 20 % citrate de sodium dans de l'eau distillée, ou dans une solution saturée du sel disodique de tétra-acétate d'éthylène-diamine amenée au pH 7,2 par tampon.

Les coupes transversales décalcifiées et les coupes non décalcifiées ont été examinées en ce qui concerne la morphologie générale, les fibres, les hydrates de carbone, les sels minéraux et la phosphatase alcaline à l'aide d'une série de colorations diverses et d'examens histochimiques. La microscopie en contraste de phase, la microscopie par fluorescence (primaire et secondaire), la microscopie en lumiere polarisée et la microradiographie ont aussi été employées pour l'examen de ces coupes.

Au niveau microscopique, le tissu ostéoide a été par des chercheurs antérieurs caractérisé par sa constitution homogène, hyaline ou oedémateuse. L'étude présente a prouvé au moyen du microscope à contraste de phase que l'ostéoïde comprend trois zones principales:

Une zone interne, l'osteoïde jeune, le plus proche des ostéoblastes.

Une zone externe, l'ostéoïde âgé, qui comprend la partie centrale et la partie périphérique de l'ostéoïde. A sa suite vient *une zone de transition,* une aire étroite de tissu représentant la limite périphérique de l'ostéoïde et le début du tissu osseux.

Des couches à l'intérieur des zones ont aussi été trouvées. Des lignes rayonnantes dans l'ostéoïde ont été identifiées comme étant les prolongements des ostéoblastes et des ostéocytes.

Les séries parallèles d'examens sur des tissus partiellement décalcifiées et totalement décalcifiés n'ont laissé aucun doute sur la raison pour laquelle ces traits morphologiques de l'ostéoïde n'ont pas été constatés antérieurement: l'effet destructif des liquides de déminéralisation. Aucune différence notable n'a pu être enregistrée entre les résultats obtenus en utilisant les différents liquides décalcifiants.

En se basant sur les faits mis en évidence indiqués ci-dessus, on doit pouvoir conclure que les zones de l'ostéoïde décelées par la microscopie en contraste de phase de coupes non déminéralisées, sont dues à des différences locales de nature chimique et/ou physique.

Sels inorganiques dans l'ostéoïde

La possibilité que le manque d'homogénéïte de l'ostéoïde soit dû à de petites variations dans le contenu de sels inorganiques a été étudiée à l'aide de la microradiographie, de la coloration à l'argent de *von Kossa* et de la coloration à l'alizarine. En ce qui concerne les trois zones de l'ostéoïde, il semble qu'il y ait corrélation entre ces méthodes d'examen. Les résultats ont montré que la zone interne et la zone externe étaient totalement privées de dépots de sels minéraux, mais que la concentration de ceux-ci allait en augmentant en direction du tissu osseux dans la zone de transition.

Le manque d'homogénéïté du tissu ostéoïde, mis en évidence par le microscope en contraste de phase ne peut donc être dû aux différences du contenu minéral, mais doit être en relation avec les variations dans les éléments organiques.

Charges électriques dans l'ostéoïde

Une analyse des charges électriques relatives dans l'ostéoïde a été effectuée par microscopie de fluorescence. La fluorescence primaire sur des coupes non colorées et la fluorescence secondaire sur des coupes traitées avec des solutions aqueuses de fluorochromes cathodiques et anodiques de pH variables ont dans l'ensemble donné les mêmes zones de l'ostéoïde que celles mises en évidence par le microscope à contraste de phase. Ces séries d'examens ont aussi montré clairement qu'il n'existe pas de valeur moyenne du point isoélectrique de l'ostéoïde, mais que chaque zone présente des valeurs caractéristiques. Le point isoélectrique de la zone de transition se trouve aux environs de pH 1—2. L'étroite aire de tissu la plus rapprochée des ostéoblastes se trouve entre pH 4 et pH 5. Le reste de l'ostéoïde jeune et la totalité de l'ostéoïde âgé sont très près de la neutralité.

Les fibres dans l'ostéoïde

Les éléments fibreux dans l'ostéoïde ont été examinés au microscope à contraste de phase, avec coloration par les méthodes de *Mallory* et de *van Gieson*, avec des solutions ammoniacales d'argent, et analysés au microscope de polarisation.

Les fibres colorées à l'argent se trouvant dans la partie de l'ostéoïde jeune la plus proche des ostéoblastes ont été identifiées comme étant des fibres de réticuline ou pré-collagènes. Le reste de l'ostéoïde jeune, fortement coloré par les solutions de *Mallory* et de *van Gieson*, et non noirci par l'argent, a été identifié comme étant des fibre collagènes. La biréfringence accentuée dans cette région montre que les fibres sont orientées de telle manière que leur dimension la plus longue se trouve dans le plan de la coupe transversale et parallèlement à l'os calcifié.

L'ostéoïde âgé ne présente aucune trace de formations fibreuses. Ni la méthode de *Mallory* ni celle de *van Gieson* n'ont coloré cette partie de l'ostéoïde, et l'on n'a pas non plus au microscope à contraste de phase pu voir d'éléments fibreux ou de fibres. De plus, cette région tissulaire est restée obscure dans tous les secteurs en lumière polarisée.

Tous ces résultats négatifs indiquent l'absence de fibres collagènes. L'image au microscope de polarisation exclut même la présence d'éléments fibrillaires au niveau submicroscopique.

La zone de transition par contre présente une faible coloration par les méthodes de *Mallory* et de *van Gieson*. Cette région présente aussi une biréfringence entre nicols croisés, l'intensité de cette biréfringence étant cependant variable.

Bien qu'on n'ait pas pu voir de fibres individuelles dans la zone de transition, ces résultats indiquent la présence de fibres collagènes. Les variations dans les différences de phase qui ont été mises en évidence par le microscope de polarisation sont dues à des variations dans l'orientation des fibres. La disposition lamellaire des ostéones provoque le même phénomène optique.

Les hydrates de carbone dans l'ostéoïde

Les épreuves histochimiques concernant les hydrates de carbone dans l'ostéoïde ont compris la coloration à l'aide de solutions aqueuses de bleu de toluidine et la réaction de PAS.

La partie de l'ostéoïde jeune, correspondant aux fibres colorées à l'argent, a présenté une métachromasie rouge (positive) à toutes les concentrations et pour tous les temps de coloration.

La zone de transition a présenté une métachromasie verte (négative) aux concentrations basses et rouge pourpre aux concentrations plus élevées. En se basant sur des expériences antérieures, on a conclu que, dans le tissu osseux, la métachromasie positive indique la présence de mucopolysaccharides acides sous forme d'acide chondroitine-sulfurique. La métachromasie négative, très faible pour l'ostéoïde âgé, mais augmentant jusqu'à un maximum dans la zone de transition, est due à une augmentation de la charge négative dans les éléments tissulaires autres que les mucopolysaccharides acides. On a, à titre d'essai, identifié ces autres éléments comme étant du collagène sous une forme non fibreuse.

La réaction de PAS a été constante dans des parties de l'ostéoïde jeune et dans la totalité de l'ostéoïde âgé. Dans l'espace très étroit correspondant aux fibres pré-collagènes, la réaction était quelque peu augmentée. La zone de transition présentait un affaiblissement brusque de la réaction, ce qui indique un degré de polymérisation plus élevé dans les polysaccharides. La constance de l'intensité de la réaction de PAS dans des partie de l'ostéoïde jeune ainsi que dans la totalité de l'ostéoïde âgé, et sa persistence dans toute la zone interne et toute la zone externe après décalcification, en contraste avec la perte de la métachromasie positive, semble indiquer la présence d'au moins deux groupes de polysaccharides dans le tissu osseux, un élément acide et un élément neutre.

L'activité de phosphatase alcaline dans l'ostéoïde

La phosphatase alcaline a été mise en évidence par la méthode au cobalt de *Gomori* et par coloration azoïque sur coupes non décalcifiées. Le choix de tissu osseux provenant de la diaphyse s'est révélé approprié et a facilité l'interprétation des resultats.

Lorsque l'on prend en considération la sensibilité de l'enzyme à la technique histologique, il semble que la congélation-dessiccation suivie de fixation à l'acétone et la technique azoïque soit la méthode la plus avantageuse. La phosphatase alcaline est localisée dans l'ostéoïde jeune, correspondant à l'espace où l'on a trouvé des fibres pré-collagènes. Le reste de l'ostéoïde, excepté

les prolongements des ostéoblastes, ne présente aucune activité de phosphatase alcaline.

En conclusion et en se basant sur les caractères morphologiques et histochimiques de l'ostéoïde, l'auteur présente son hypothèse sur la formation du tissu osseux.

ZUSAMMENFASSUNG UND ERGEBNISSE

DIE BILDUNG DES KNOCHENGEWEBES

Eine morphologische und histochemische Untersuchung

Die morphologischen und histochemischen Untersuchungen der Vorgänge, die insgesamt die Bildung des Knochengewebes darstellen, sind im wesentlichen an entkalktem Material oder an Schnitten von embryonalem oder sehr jungem, spongiösem Knochengewebe vorgenommen worden. Eine Reihe von Forschern haben festgestellt, dass Osteoid, das organische Vorstadium des Knochengewebes, in den verschiedenen Skeletteilen an Breite stark variieren kann.

Der Zweck der vorliegenden Arbeit war, eine Methode zu entwickeln, die es ermöglicht, dünne, nicht entkalkte Schnitte von reifem Knochengewebe der Diaphysen, worin sich relativ ausgedehnte osteoide Zonen befinden, herzustellen. Zunächst hat der Verfasser diese Schnitte mehrfach untersucht, um auf alle Fälle über einige der Prozesse, die sich im Osteoid vor der Mineralisation abspielen müssen, Klarheit zu gewinnen.

Kortikales Gewebe von den Diaphysen von Radius, Tibia und Ossa metacarpalia wurde aus fünf normalen Hunden entnommen. Das Alter dieser Hunde schwankte zwischen 4 und 12 Monaten. Die winzigen Knochenstücke wurden entnommen während die Tiere am Leben waren. Die Präparate wurden ohne Ausnahme innerhalb von 1—2 Minuten nach der Entnahme fixiert. Präparate gleichen Ursprungs wurden in neutralem Formalin, in *Zenkers* Flüssigkeit oder durch "Gefrier-trocknung" fixiert. Die letzterwähnte Gruppe wurde in Azeton nachfixiert. Dieses nicht entkalkte Untersuchungsmaterial wurde in Methacrylat eingebettet und mit einem metallurgischen Mikrotom geschnitten. Die

Schnitte waren in der Regel 6 Mikron, obwohl es möglich war, sie noch dünner herzustellen. Ein Teil des in Formalin fixierten Untersuchungsmateriales wurde in 5 %-iger Salpetersäure, ein anderer in 45 %-iger Ameisensäure, in einer 20 %-igen Lösung von Natriumcitrat in destilliertem Wasser und in einer gesättigten Lösung des Di-natriumsalzes von Aethylen-diamintetra-azetat mit einem pH Wert von 7.2 entkalkt.

Sowohl nicht entkalkte als auch entkalkte Querschnitte wurden mittels einer Reihe Färbemethoden und histochemischen Prüfungen hinsichtlich ihrer generellen Morphologie, ihrer Fasern, ihres Gehaltes an Kohlenhydraten, anorganischen Mineralien und alkalischer Phosphatase untersucht. Die Untersuchung umfasste auch Phasenkontrastmikroskopie, Fluoreszenzmikroskopie, (primäre und sekundäre Fluoreszenz), Polarisationsmikroskopie und Mikroradiographie.

Osteoid ist früher mikroskopisch bezüglich seiner homogenen, hyalinen oder ödematösen Konsistenz charakterisiert worden. Die vorliegende Untersuchung konnte dank der Phasenkontrastmikroskopie feststellen, dass der Osteoid drei Hauptzonen hat:

Eine innere Zone, die jungen Osteoid enthält, an die Osteoblasten angrenzend.

Eine äussere Zone, die älteren Osteoid enthält und den mittleren und peripheren Teil umfasst. Dieser Zone schliesst sich eine *Übergangszone* an, ein dünner Gewebestreifen, welcher die periphere Grenze des Osteoids und den Beginn des Knochengewebes repräsentiert.

Auch zwischen den Zonen wurden Schichten gefunden. Strahlenförmige Streifen im Osteoid wurden als Ausläufer der Osteoblasten und Osteozyten identifiziert.

Die Parallelversuche an teilweise und völlig entkalkten Präparaten lassen keinen Zweifel darüber, dass der Grund, weshalb diese morphologischen Eigenschaften des Osteoids nicht früher konstatiert worden sind, in der zerstörenden Wirkung der Entkalkungsflüssigkeiten zu suchen ist. Es konnte bei Gebrauch der verschiedenen Entkalkungsflüssigkeiten kein nennenswerter Unterschied festgestellt werden.

Auf Grund der obenerwähnten Ergebnisse kann man feststellen, dass die Zonen des Osteoids, die bei nicht entkalkten Schnit-

ten im Phasenkontrastmikroskop in Erscheinung treten, durch Unterschiede chemischer und/oder physikalischer Natur verursacht sind.

Anorganische Salze im Osteoid

Die Möglichkeit, dass die Inhomogenität des Osteoids durch geringe Abweichungen im Gehalt an anorganischen Salzen verursacht sein könnte, wurde mittels Mikroradiographie, Silberfärbung nach *von Kossa* und Färbung mit Alizarin geprüft. Bezüglich der 3 Zonen des Osteoids scheinen diese Untersuchungsmethoden übereinstimmende Befunde zu zeigen, nämlich, dass die innere und äussere Zone keine Mineralsalze enthält, während in der Übergangszone zum Knochengewebe hin eine zunehmende Mineralsalz-Konzentration herrscht.

Die Inhomogenität des Osteoids, wie sie im Phasenkontrastmikroskop demonstriert wurde, kann daher nicht auf unterschiedlichen Mineralgehalt zurückgeführt werden, sondern muss in Variationen des Gehaltes an organischen Komponenten zu suchen sein.

Ladungsverhältnisse im Osteoid

Mittels Fluoreszenzmikroskopie wurde eine Untersuchung bezüglich der relativen elektrischen Ladung des Osteoids vorgenommen. Die Primärfluoreszenz in ungefärbten Schnitten und die Sekundärfluoreszenz in Schnitten, die mit wässrigen Verdünnungen katodischer und anodischer Fluorochrome mit variierendem pH Wert behandelt worden waren, ergaben im grossen und ganzen dieselben Zonen im Osteoid, wie diejenigen, die das Phasenkontrastmikroskop offenbarte. Diese Versuchsreihen zeigten auch eindeutig, dass kein Durchschnittswert hinsichtlich des isoelektrischen Punktes des Osteoids existiert, dass jede Zone vielmehr charakteristische Werte aufweist. Der isoelektrische Punkt der Übergangszone liegt im pH-Bereich 1—2, derjenige des schmalen Gewebstreifens, zunächst den Osteoblasten, zwischen pH 4 und 5, und der Rest des jungen nebst dem gesamten älteren Osteoid liegt nahe dem Neutralpunkt.

Die Fasern im Osteoid

Die fibrösen Elemente im Osteoid wurden im Phasenkontrastmikroskop mittels *Mallory*'s und *van Gieson*'s Färbemethoden, sowie mittels ammoniakalischer Silberauflösung untersucht und im Polarisationsmikroskop analysiert.

Die silbergefärbten Fasern, welche in dem Teil des jungen Osteoids auftreten, der an die Osteoblasten anstösst, wurden als Retikulin oder als präkollagene Fasern identifiziert. Der übrige Teil des jungen Osteoides, durch *Mallory*'s und *van Gieson*'s Lösungen stark gefärbt, indes nicht geschwärzt durch Silberreagenz, wurde als Kollagenfasern identifiziert. Die starke Doppelbrechung in diesem Gebiet zeigt, dass sich die Fasern hier in ihrer Längsdimension in der Ebene des Querschnittes, d. h. parallel mit dem mineralisierten Knochen, lagern.

Das ältere Osteoid wies keine Faserformationen auf. Weder *Mallory*'s noch *van Gieson*'s Methode färbte diesen Teil des Osteoides, und im Phasenkontrastmikroskop waren weder Faserteile noch Fasern zu sehen. Dieses Gewebegebiet verblieb ferner in allen Quadranten des polarisierten Lichtes dunkel.

Alle diese negativen Resultate lassen auf den Mangel von Kollagenfasern schliessen. Das Bild im Polarisationsmikroskop schliesst sogar die Anwesenheit von faserigen Elementen im submikroskopischen Sektor aus.

Die Übergangszone wies dagegen eine schwache Färbung nach *Mallory* und *van Gieson* auf. Dieses Gebiet zeigte auch Doppelbrechung zwischen gekreuzten Nicols — wenn auch von wechselnder Intensität. Obwohl keine individuellen Fasern in der Übergangszone zu sehen waren, lassen diese Befunde auf das Vorhandensein kollagener Fasern schliessen. Die im Polarisationsmikroskop veranschaulichten Variationen des Phasenunterschiedes sind durch Variationen in der Faserlagerung verursacht. Hier handelt es sich um dasselbe Phänomen, welches für die Lammellierung im Knochengewebe verantwortlich gemacht wird.

Die Kohlenhydrate im Osteoid

Die histochemischen Proben bezüglich Kohlenhydrate im Osteoid sind Färbungen mit wässeriger Auflösung von Toluidinblau und Färbung mit PAS.

Der innere Teil des Jungen Osteoides, der den silbergeschwärzten Fasern entspricht, zeigte rote (positive) Metachromasie in allen Konzentrationen und bei allen Färbezeiten. Die Übergangszone zeigte grüne (negative) Metachromasie bei niederen und purpurrote bei höheren Konzentrationen. Ausgehend von früheren Versuchen, wurde der Schluss gezogen, dass positive Metachromasie im Knochengewebe auf das Vorhandensein von sauren Mukopolysacchariden in der Form von Chondroitinschwefelsäure schliessen lässt. Die negative Metachromasie, welche in dem älteren Osteoid sehr schwach, aber in der Übergangszone bis zum Maximum gesteigert war, beruht auf einer Vermehrung der negativen Ladung anderer Gewebeteile als der sauren Mukopolysaccharide. Diese anderen Elemente wurden vorläufig als Kollagen in nicht faseriger Gestalt aufgefasst.

Die PAS-Reaktion war in gewissen Abschnitten des jungen und im gesamten älteren Osteoid konstant. In dem sehr schmalen Gebiet, welches den prækollagenen Fasern entspricht, war diese Reaktion etwas kräftiger. Die Übergangszone zeigte eine plötzliche Abnahme der Reaktion, was dem höheren Polymerisationsgrad der Polysaccharide entspricht. Die gleichbleibende PAS-Reaktion in Teilen des jungen sowie im ganzen älteren Osteoid, ihr Verharren sowohl in der inneren als auch in der äusseren Zone nach der Entkalkung — im Gegensatz zum Verlust der positiven Metachromasie — deutet auf das Vorhandensein von mindestens zweier Gruppen von Polysacchariden, nämlich eines sauren und eines neutralen Komponenten im Knochengewebe.

Die alkalische Phosphatase im Osteoid

Alkalische Phosphatase wurde durch *Gomori*'s Kobaltmethode und mittels Azofärbung nicht entkalkter Schnitte nachgewiesen. Die Verwendung von Diaphysengewebe erwies sich zweckmässig und erleichterte die Deutung der Befunde. In Anbetracht der ausserordentlichen Empfindlichkeit dieses Enzyms gegenüber histologischer Untersuchungstechnik dürfte die Verwendung von acetonfixierten Präparaten, die erst einer "Gefriertrocknung" unterworfen waren, in Verbindung mit der Azotechnik die vorteilhafteste Methode zu sein. Alkalische Phosphatase findet man in

dem jungen Osteoid, was dem Gebiet entspricht, wo man præ-kollagene Fasern fand. Das übrige Osteoid, abgesehen von den Osteoblastausläufern, weist keine Aktivität von alkalischer Phosphatase auf.

Zuletzt legt der Verfasser auf Grund der morphologischen und histochemischen Charakteristika des Osteoids seine Hypothese über die Bildung des Knochengewebes vor.

BIBLIOGRAPHY

Amprino, R. 1956: Uptake of ^{35}S in the Differentiation and Growth of Cartilage and Bone. Ciba Foundation Symposium on Bone Structure and Metabolism. p. 89. Boston.

Amprino, R. & A. Engström 1952: Studies on X-ray Absorption and Diffraction of Bone Tissue. Acta Anat. *15:* 1.

Asboe-Hansen, A. 1951: Om bindevævets mucinøse substanser. København.

Bear, R. S. 1952: The Structure of Collagen Fibrils. Advances in Protein Chem. *7:* 69.

—»— 1956: The Structure of Collagen Molecules and Fibrils. J. Biophys. Biochem. Cytol. *2:* 263.

Bélanger, L. F. 1954: Autoradiographic Visualization of the Entry and Transit of S^{35} in Cartilage, Bone and Dentine of Young Rats and the Effect of Hyaluronidase *in vitro*. Proc. Soc. Exper. Biol. Med. *88:* 150.

Best, C. H. & N. B. Taylor 1954: The Living Body. A Text in Human Physiology. London.

Bevelander, G. & P. S. Johnson 1950: A Histochemical Study of the Development of Membrane Bone. Anat. Rec. *108:* 1.

Bloom, W. & M. A. Bloom 1940: Calcification and Ossification. Calcification of Developing Bones in Embryonic and Newborn Rats. Anat. Rec. *78:* 497.

Bloom, W., M. A. Bloom & F. C. McLean 1941: Calcification and Ossification. Medullary Bone Changes in the Reproductive Cycle of Female Pidgeons. Anat. Rec. *81:* 443.

Bourne, G. H. 1943: Distribution of Alkaline Phosphatase in Various Tissues. Quart. J. Exper. Physiol. *32:* 1.

—»— 1948: Alkaline Phosphatase and Vitamin C Deficiency in Regeneration of Skull Bones. J. Anat. *82:* 81.

—»— 1956: The Biochemistry and Physiology of Bone. New York 1956.

Burstone, M. S. 1952: A Histochemical Study of Irradiated Bone. Am. J. Path. *28:* 1133.

Cameron, L. R. 1930: The Staining of Calcium. J. Path. Bact. *33:* 995.

Carlström, D. 1954: Microhardness Measurements on Single Haversian Systems in Bone. Experentia *10:* 171.

—»— 1955: X-ray Crystallographic Studies on Apatites and Calcified Structures. Acta Radiologica. Supplement 121.

Carlström, D. & J. B. Finean 1954: X-ray Diffraction Studies on the Ultrastructure of Bone. Biochim. et Biophys. Acta *13:* 183.

Carlström, D. & A. Engström 1956: Ultrastructure and Distribution of Mineral Salts in Bone Tissue. In: The Biochemistry and Physiology of Bone. New York 1956. Ed. G. H. Bourne. Chapter VI p. 149.

Cobb, J. D. 1949: The Morphological Distribution of Glycogen and Glycoproteins in the Cells and Extracellular Materials of Growing Bones. Thesis. University of Illinois.

Danielli, J. F. 1945: A Critical Study of Techniques for Determining the Cytological Position of Alkaline Phosphatase. J. Exper. Biol. *3:* 110.

Dische, Z., A. Danilczenko & G. Zelmenis 1958: The Neutral Heteropolysaccharides in Connective Tissue. Ciba Foundation Symposium on Chemistry and Biology of Mucopolysaccharides. Boston. p. 116.

Dzietwiatkowski, D. D. 1949: On the Possible Utilisation of Sulfate Sulfur by the Suckling Rat for the Synthesis of Chondroitin Sulfate as Indicated by the Use of Radiosulfur. J. Biol. Chem. *178:* 931.

—»— 1951 a: Radioautographic Visualization of Sulfur^{35}. Deposition in the Articular Cartilage and Bone of Suckling Rats Following Injection of Labelled Sodium Sulfate. J. Exper. Med. *93:* 451.

—»— 1951 b: Isolation of Chondroitin Sulfates. S^{35} from Articular Cartilage of Rats. J. Biol. Chem. *189:* 187.

Eastoe, J. E. 1955: The Amino Acid Composition of Mammalian Collagen and Gelatine. Biochem. J. *61:* 589.

Eastoe, J. E. & L. B. Eastoe 1954: The Organic Constituents of Mammalian Compact Bone. Biochem. J. *57:* 453.

Eeg-Larsen, N. 1958: Personal Communication.

Ehrlich, P. 1877: Beiträge zur Kenntniss der Anilinfärbungen und ihrer Verwendung in der mikroskopischen Teknik. Arch. mikr. Anat. 1877.

—»— 1878: Beiträge zur Theorie und Praxis der histologischen Färbung Thesis. University of Leipzig. In the Collected Papers of Paul Ehrlich. Comp. and ed. by F. Himmelweit with the assistance of Martha Marquardt. London p. 29.

—»— 1879: Beiträge zur Kenntniss der granulierten Bindegewebszellen und der eosinophilen Leukozyten. Arch. Anat. Phys.

Engel, M. B. 1952: Mobilization of Mucoprotein by Parathyroid Extract. A. M. A. Arch. Path. *53:* 339.

—»— 1958: Integrated Behavior in Connective Tissues. Oral Surg., Oral Med., Oral Path. *11:* 724.

Engel, M. B. & W. Furuta 1942: Histochemical Studies of Phosphatase Distribution in Developing Teeth of Albino Rat. Proc. Soc. Exp. Biol. Med. *50:* 5.

Engfeldt, B. & S. O. Hjertquist 1954: Biophysical Studies on Bone Tissue. X. The *in vivo* and *in vitro* Uptake of Radioactive Isotopes and Ionic Reactions in Bone Tissue. Acta Path. Mictrobiol. Scandinav. *35:* 205.

—»— 1955: Biophysical Studies on Bone Tissue. XV. A. Histochemical and Microradiographic Study of Normal Bone Tissue. Acta Path. Microbiol. Scandinav. *36:* 385.

Engfeldt, B., G. Bergman & E. Hammarlund-Essler 1954: Studies on Mineralized Dental Tissues. I. A microradiographic and Autoradiographic Investigation of Teeth and Tooth Germs of Normal Dogs. Exper. Cell Res. *7:* 381.

Engström, A. 1946: Quantitative Micro- and Histochemical Elementary Analysis by Roentgen Absorption Spectrography. Acta Radiologica. Supplement 63.

Erdheim, T. 1914: Rachitis und Epithelkörperchen. Denkschr. d. k. Akad. d. Wissensch. Math-naturw. Klasse *90:* 363.

Fawns, H. T. & J. W. Landells 1953: Histochemical Studies Of Rheumatic Conditions. I. Observations of the Fine Structures of the Matrix of Normal Bone and Cartilage. Ann. Rheumat. Dis. *12:* 105.

Follis, R. H. Jr. 1952: Cartilage and Bone Matrix. Chemical Structure, Formation and Destruction. In: Metabolic Interrelations. Trans. of Conf. Josiah Macy, Jr. Foundation *4:* 11.

Follis, R. H. Jr. & D. A. Jackson 1943: Renal Osteomalacia and Osteitis Fibrosa in Adults. Bull. Johns Hopkins Hosp. *72:* 232.

Follis, R. H. Jr. & M. Berthrong 1949: Histochemical Studies on Cartilage and Bone. I. The Normal Pattern. Bull. Johns Hopkins Hosp. *85:* 281.

Freeman, S. & F. C. McLean 1941: Experimental Rickets; Blood and Tissue Changes in Puppies Receiving Diet Very Low in Phosphorus with and Without Vitamin D. Arch. Path. *32:* 387

Gardner, E. 1956: Osteogenesis in the Human Embryo and Fetus. In: The Biochemistry and Physiology of Bone. New York 1956. Ed. G. H. Bourne. Chapter XIII p. 359.

Gersh, I. 1952: Ground Substance and the Plasticity of Connective Tissues. The Harvey Lectures Series XLV. The Harvey Society of New York.

Gersh, I. & H. R. Catchpole 1949: The Organization of Ground Substance and Basement Membrane and Its Significance for Tissue Injury, Disease and Growth. Am. J. Anat. *85:* 457.

Glimcher, M. J., A. J. Hodge & F. O. Schmitt 1957: Macromolecular Aggregation States in Relation to Mineralization. The Collagen-Hydroxy Apatite System as Studied *in vitro.* Proc. National Acad. Sci. *43:* 860.

Gomori, G. 1939: Microtechnical Demonstration of Phosphatase in Tissue Sections. Proc. Soc. Exper. Biol. Med. *42:* 23.

—»— 1943: Calcification and Phosphatase. Am. J. Path. *19:* 197.

—»— 1952: Microscopic Histochemistry. Principles and Practice. Univ. of Chicago Press.

Greep, R. O., C. J. Fischer & A. Morse 1948: Alkaline Phosphatase in Odontogenesis and Osteogenesis and its Histochemical Demonstration after Demineralization. J. Am. Dent. Ass. *36:* 427.

Gustavson, K. H. 1956: The Chemistry and Reactivity of Collagen. New York 1956.

Gutman, A. B. & E. B. Gutman 1941: A Phosphorylase in Calcifying Cartilage. Proc. Soc. Biol. Med. *48:* 687.

Gutman, A. B. & T. F. Yu 1950: A Conception of the Role of Enzymes in Endochondral Calcification. In: Metabolic Interrelations. Trans. Conf. Josiah Macy, Jr. Foundation *2:* 167.

Hals, E. 1953: Fluorescence Microscopy of Developing and Adult Teeth. Supplemented by Investigation With Ordinary, Polarizing and Phase Contrast Microscope. Norwegian Academic Press. Oslo.

Ham, A. W. 1953: "Histology", Sec. Ed. Philadelphia.

Ham, A. W. & W. R. Harris 1952: Histologic Technique for the Study of Bone and Some Notes on the Staining of Cartilage. In: McClung's Handbook of Microscopical Technique. Ed. 3. New York. p. 269.

Harris, H. A. 1932: Glycogen in Cartilage. Nature *130:* 996.

Hass, G. M. 1943: Studies on Cartilage. Arch. Path.

Heller-Steinberg, M. 1951: Ground Substance, Bone Salts and Cellular Activity in Bone Formation and Destruction. Am. J. Anat. *89:* 347.

Hotchkiss, R. D. 1948: A Microchemical Reaction Resulting in the Staining of Polysaccharide Structures in Fixed Preparations. Arch. Biochem. *16:* 131.

Irving, E. A. & S. G. Tomlin 1954: Collagen, Reticulum and Their Argyrophilic Properties. Proc. Roy. Soc. London. Ser. B. *142:* 113.

Irving, J. T. & J. P. Weinmann 1948: Experimental Studies in Calcification. VI. Response of Dentin of the Rat Incisor to Injection of Strontium. J. Dent. Res. *27:* 669.

Jackson, S. F. 1957: The Fine Structure of developing Bone in the Embryonic Fowl. Proc. Roy. Soc. London. Series B *146:* 270.

Jackson, S. F. & J. T. Randall 1956: Fibrogenesis and Formation of Matrix in Developing Bone. Ciba Foundation Symposium on Bone Structure and Metabolism. p. 47. Boston.

Jackson, S. F. 1958: Personal Communication.

v. Kossa, J. 1901: Über die im Organismus kunstlich erzeugbaren Verkalkungen Beitr. path. Anat. Physiol. *29:* 163.

Lacroix, P. 1951: The Organization of Bones. Philadelphia.

—»— 1956: The Histologic Remodelling of Adult Bone. An Autoradiographic Study. Ciba Foundation Symposium on Bone Structure and Metabolism. p. 36. Boston.

Langeland, K. 1957: Tissue Changes in the Dental Pulp. An Experimental Histologic Study. Oslo University Press.

Leblond, C. P., G. W. Wilkinson, L. F. Bélanger & J. Robichon 1950: Radioautographic Visualization of Bone Formation in the Rat. Am. J. Anat. *86:* 289.

Lehnerdt, F. 1910: Warum bleibt das rachitische Knochengewebe unverkalkt? Ergebn. d. innere Mediz. u. Kind.-hlk. Bd. *6:* 120.

Levine, A. & M. Schubert 1952: Metachromasy of Thiazine Dyes Produced by Chondroitin Sulfate J. Am. Chem. Soc. *74:* 91.

Levine, M. D., P. S. Rubin, R. H. Follis Jr. & J. E. Howard 1949: Histochemical Studies on Calcinosis Universalis with Respect to the Possible Relationship between Normal and Patological Calcification. In: Metabolic Interrelations Trans. Conf. Josiah Macy. Jr., Foundation *1:* 41.

Lillie, R. D. 1955: Histopathologic Technique. Philadelphia.

Lindström, B. 1955: Roentgen Absorption Spectrophotometry in Quantitative Cytochemistry. Acta Radiologica. Supplement 125.

Lison, L. 1936: Histochimi Animale. Paris. p. 236.

Lison, L. & W. Mutsaars 1950: Metachromacy of Nucleic Acids. Quart. J. Micr. Sci. *91:* 309.

Little, K. & H. Kramer 1952: Nature of Reticulin. Nature *170:* 499.

Lorch, I. J. 1947: Localization of Alkaline Phosphatase in Mammalian Bones. Quart. J. Micr. Sci. *88:* 367.

—»— 1949 a: Alkaline Phosphatase and the Mechanism of Ossification. J. Bone and Joint Surg. *31:* 94.

Lorch, I. J. 1949 b: The Distribution of Alkaline Phosphatase in Relation to Calcification in Scyliorhinus Canula Development of the Endoskeleton. Quart. J. Micr. Sci. *90:* 381.

Majno, G. & C. Rouiller 1951: Die alkalischen Phosphatase in der Biologie des Knochengewebes. Virchow's Arch. Path. Anat. u. Physiol. *321: 1*

Mallory, F. B. 1942: Pathological Technique. Philadelphia.

Manheim, L. H. & A. M. Seligman 1949: Improvement in the Method for the Histochemical Demonstration of Alkaline Phosphatase and Its Use in a Study of Normal and Neoplastic Tissues. J. Nat. Cancer Inst. *9:* 181.

Masamune, H., Z. Yosizawa & M. Maki 1950: Biochemical Studies on Carbohydrates. Tohoku J. Exper. Med. *53:* 237.

McGee-Russell, S. M. 1958: Histochemical Methods for Calcium. J. Histochem. *6:* 22.

McLean, F. C. 1950: Discussion on the Local Factor. In: Metabolic Interrelations. Trans. Conf. Josiah Macy Jr., Foundation *2:* 215.

McLean, F. C. & W. Bloom 1940: Calcification and Ossification. Calcification in Normal Growing Bone. Anat. Rec. *73:* 333.

McLean, F. C. & M. R. Urist 1955: Bone. An Introduction to Physiology of Skeletal Tissue. The University of Chicago Press, Chicago.

Menten, M. L., J. Junge & M. H. Green 1944: A Coupling Histochemical Azo Dye Test for Alkaline Phosphatase in the Kidney. J. Biol. Chem. *153:* 471.

Meyer, K. 1947: The Biological Significance of Hyaluronic Acid and Hyaluronidase. Physiol. Rev. *27:* 335.

—›— 1956: The Mucopolysaccharides of Bone. Ciba Foundation Symposium on Bone Structure and Metabolism. p. 65 Boston.

Meyer, K., E. Davidsen, A. Linker & P. Hoffmann 1956: The Acid Mucopolysaccharides of Connective Tissue. Biochim. et Biophys. Acta *21:* 506.

Meyer, P. C. 1956: The Histological Identification of Osteoid Tissue. J. Path. & Bact. *71:* 325.

Michaelis, L. 1950: Reversible Polymerization and Molecular Aggregation. J. Phys. & Coll. Chem. *54:* 1.

—›— 1947: In: Cold Spring Harbor Symp. *12:* 131.

Mintz, B. & E. R. Russel 1957: Gene Induced Embryological Modification of Primordial Germ Cells in the Mouse. J. Exper. Zool, *134:* 207.

Morse, A. 1945: Formic Acid-Sodium Citrate Decalcification and Butyl Alcohol Dehydration of Teeth and Bones for Sectioning in Paraffin. J. Dent. Res. *24:* 145.

Nageotte, J. & L. Guyon 1930: Reticulin. Am. J. Path. *6:* 31.

Neuman, R. E. 1949: The Amino Acid Composition of Gelatines, Collagens and Elastines from Different Sources. Arch. Biochem. *24:* 289.

Neuman, W. F. & M. W. Neuman 1958: Chemical Dynamics of Bone Mineral. University of Chicago Press. Chicago.

Neuman, W. F., E. S. Boyd & I. Feldman 1952: The Ionbinding Properties of Cartilage. In: Metabolic Interrelations. Trans. Conf. Josiah Macy, Jr., Foundation. *4:* 100.

Nikiforuk, G. & L. Screebny 1953: Demineralization of Hard Tissues by Organic Chelating Agents at Neutral pH. J. Dent. Res. *2:* 859.

Partridge, S. M. 1948: The Chemistry of Connective Tissues. 1. The State of Combination of Chondroitin Sulphate in Cartilage. Biochem. J. *43:* 387.

Pearse, A. G. 1953: Histochemistry. Theoretical and Applied. London.

Pommer, G. 1885: Untersuchungen über Osteomalacie und Rachitis nebst Beitragen zur Kenntniss der Knochenresorption und Apposition in verschiedenen Altersperioden und der durchbohrenden Gefässe. Leipzig.

—»— 1925: Zur Kenntniss der mikroskopischen Befunde der Knochenanbildung und ihrer Untersuchungsmethoden (nebst Bemerkungen zur Osteoklastenlehre). Ztschr. f. d. ges. Anat. *75:* 382.

Pritchard, J. J. 1952: A Cytological and Histochemical Study of Bone and Cartilagen Formation in the Rat. J. Anat. *86:* 259.

--»— 1956: The Osteoblast. In: The Biochemistry and Physiology of Bone. New York. Ed. G. H. Bourne. Chapter VII. p. 179.

Robinson, R. A. 1958: Personal Communication.

Robinson, R. A. & D. A. Cameron 1956: Electron Microscopy of Cartilage and Bone at the Distal Epiphyseal Line of the Femur in the Newborn Infant. J. Biophys. Biochem. Cytol. *2:* 253.

—»— 1957: The Organic Matrix of Bone and Epiphyseal Cartilage. Chir. Orthopaed. *9:* 16.

—»— 1958: Electron Microscopy of the Primary Spongiosa of the Metaphysis at the Distal End of the Femur in Newborn Infant. J. Bone & Joint Surg. *40:* 687.

Robinson, R. A. & M. L. Watson 1953: Electron Micrography of Bone. In: Metabolic Interrelations Trans. Conf. Josiah Macy, Jr., Foundation. *5:* 72.

Robison, R. 1923: The Possible Significance of Hexose Phosphoric Esters in Ossification. Biochem. J. *17:* 286.

—»— 1926: The Possible Significance of Hexose Phosphoric Esters in Ossification. Biochem. J. *20:* 388.

Robison, R. & A. H. Rosenheim 1934: Calcification of Hypertropic Cartilage *in vitro*. Biochem. J. *28:* 684.

Roche, J. & G. H. Deltour 1945: The Role of Phosphatase in the Calcification of Bone *in vitro* and the Theory of "Calcium Trapping Agents". In: Medical Research in France during the War 1939—1945. Ed. J. Hamburger.

Roche, J. & M. Mourge 1945: Premiérs étapes de l'ossification et formation du sel de l'os. Comptes-rendus Soc. Biol. Paris. *137:* 451.

Rogers, H. J. 1951: The Polysaccharide Associated with the Organic Matrix of Bone. Biochem. J. *49:* 12.

Rogers, H. J., S. M. Weidmann & A. Parkinson 1952: Studies on the Skeletal Tissues. Biochem. J. *50:* 537.

Rubin, P. S. & J. E. Howard 1950: Histochemical Studies on the Role of Acid Muco-Polysaccharides in Calcifiability and Calcification. In: Metabolic Interrelations. Trans. Conf. Josiah Macy, Jr., Foundation. *2:* 155.

Schaffer, J. 1888: Die Verknöcherung der Unterkiefers und die Metaplasiefrage. Arch. mikr. Anat. *32:* 266.

Schmidt, W. J. 1938: Polarisationsoptische Analyse des submikroskopischen Baues von Zellen und Geweben. In: Abderhaldens Handb. d. Biol. Arbeitsmethoden. Abt. V. Teil 10 (1. Hälfte). p. 435.

Schour, I. & A. W. Ham 1934: The Action of Vitamin D and of the Parathyroid Hormone on the Calcium Metabolism as Interpreted by Studying the Effect of Single Doses on the Calcification of Dentin. Arch. Path. *17*: 22.

Sheldon, H. & R. A. Robinson 1957: Electron Microscopic Studies of Crystal-Collagen Relationship in Bone. IV. The Occurrence of Crystals within Collagen Fibrils. J. Biophys. Biochem. Cytol. *3:* 1011.

Shipley, P. G., B. Kramer & J. Howard 1926: Studies upon Calcification *in vitro*. Biochem. J. *20:* 379.

Siffert, R. S. 1951: The Role of Alkaline Phosphatase in Osteogenesis. J. Exper. Med. *93:* 415.

Sobel, A. E. 1952: The Local Factor in Calcification. In: Metabolic Interrelations. Trans. Conf. Josiah Macy, Jr., Foundation. *2:* 113.

—»— 1955: Local Factors in the Mechanism of Calcification. Ann. of N. Y. Acad. Sci. *60:* 713.

Sognnaes, R. F. 1955: Microstructure and Histochemical Characteristics of the Mineralized Tissues. Ann. of N. Y. Acad. Sci. *60:* 545.

—»— 1958: Personal Communication.

Stoltzner, W. 1902: Fetal Bone Disease. Brit. Med. J. September.

Strugger, S. 1949: Fluoreszenzmikroskopie und Mikrobiologie. Hannover.

Sylvén, B. 1941: Über das Vorkommen von hochmolekulären Esterschwefelsäuren im Granulationsgewebe und bei der Epithelregeneration. Acta Chir. Scandinav. *86.* Supplementum 65.

—»— 1947 a: Cartilage and Chondroitin Sulphate. I. The Physiological Role of Chondroitin Sulphate in Cartilage. J. Bone & Joint Surg. *29:* 745.

—»— 1947 b: Cartilage and Chondroitin Sulphate. II. Chondroitin Sulphate and the Physiological Ossification of Cartilage. J. Bone & Joint Surg. *29:* 973.

—»— 1954: Metachromatic Dye-Substrate Interactions. Quart. J. Micr. Sci. *95:* 327.

Tomlin, S. G. 1953: Reticulin and Collagen. Nature *171:* 302.

Vincent, J. 1955: Recherches sur la constitution de l'os adulte. Bruxelles.

Virchow, R. 1851: Die Identität von Knochen-, Knorpel- und Bindegewebskörperchen, sowie über Schleimgewebe. Verhandl. physikalmedic. Gesellsch. in Würzburg. *2:* 150.

—»— 1853: Das normale Knochenwachstum und die rachitische Störung desselben. Arch. Path. Anat. Physiol. *5:* 409.

Waldman, J. 1950: Effect of Inactivation of Enzymes on Calcification of Cartilage *in vitro*. In: Metabolic Interrelations. Trans. Conf. Josiah Macy, Jr., Foundation. *2:* 203.

Wallgren, G. 1957: Biophysical Analyses of the Formation and Structure of Human Fetal Bone. Acta Paediatrica. Supplement 113.

Wassermann, F. 1951: Electron Microscopic Study of the Submicroscopic Network of Fibrils as a Component of Connective Tissue. Anat. Rec. *111:* 145.

Watt, J. C. 1928: The Development of Bone. Arch. Surg. *17:* 1017.

Weidenreich, F. 1923 a: Knochenstudien. I Teil. Über Aufbau und Entwicklung des Knochens und Charakter des Knochensgewebe. Ztschr. Anat. Entwickl.-gesch. *69:* 382.

—»— 1923 b: Knochenstudien. II. Teil. Über Sehnenverknöcherung und Faktoren der Knochenbildung. Zschr. Anat. Entwickl.-gesch. *69:* 558.

Weinmann, J. P. & H. Sicher 1955: Bone and Bones. Fundamentals of Bone Biology. Sec. Edition. St. Louis.

Wieland, E. 1909: Klinische und anatomische Untersuchungen über sogen. angeborene und über frühzeitig und erworbene Rachitis. II. Teil. Jahrb. Kinderhlk. *70:* 539.

Wislocki, G. B., H. Bunting & E. W. Dempsey 1947: Metachromasia in Mammalian Tissues and Its Relationship to Mucopolysaccharides. Am. J. Anat. *81:* 1.

Wislocki, G. B. & R. F. Sognnaes 1950: Histochemical Reactions of Normal Teeth. Am. J. Anat. *87:* 239.

Wislocki, G. B., M. Singer & C. M. Waldo 1948: Some Histochemical Reactions of Mucopolysaccharides, Glycogen, Lipids, and other Substances. Anat. Rec. *101:* 487.

Woodruff, L. A. & W. P. Norris 1955: Sectioning of Undecalcified Bone: With Special Reference to Radioautographic Applications. Stain Techn. *30:* 178.

Yaeger, J. A. 1958 a: Methacrylate Embedding and Sectioning of Calcified Bone. Stain Techn. in Press.

—»— 1958 b: Thesis. University of Illinois.

Zorzoli, A. 1948: The Histochemical Localization of Alkaline Phosphatase in Demineralized Bones of Mice of Different Ages. Anat. Rec. *102:* 445.

Zeiger, K. 1938: Physikochemische Grundlagen der histologischen Methodik. Dresden und Leipzig.

PLATES

Fig. 1. Undecalcified cross-section of cortex from dog radius. Formalin fixed, methacrylate embedded and cut on a metallurgic microtome. Hematoxylin-eosin. A few osteons under development adjacent to the periosteal surface (top). Osteoid borders lining the Haversian canals. Farther from the periosteum several fully developed Haversian systems (bottom).

Fig. 2. High magnification of an incomplete osteon shown in Fig. 1. Hematoxylin-eosin. Osteoblasts with strong basophilic nuclei and eosinophilic cytoplasm border on a nearly unstained osteoid. Basophilia of the osteoid increases to a maximum at the edge of the calcified bone. Radiating striations caused by osteoblast and osteocyte processes. × 1250.

Fig. 3. Von Kossa's silver technique. Frozen-dried specimen. Young and old osteoid unstained. Transitional zone yellow. Surrounding mineralized bone brown. × 1250.

Fig. 4. Alizarin red R. Frozen-dried material. Weak staining of osteoblast nuclei, young and old osteoid. Heavy accumulation of stain in the zone of transition (intermediate bone). × 1250.

Fig. 5. Microradiogram no. 9268. Frozen-dried, 30 microns thick ground section of dog metacarpal. Four Haversian systems under development. Completed osteon in the upper left corner. Volkmann's canal traversing the left part of the section.

Fig. 6. Microradiogram no. 9269. Frozen-dried, 30 microns thick ground section of diaphyseal bone. Organic material of the Haversian canal, young and old osteoid show low x-ray absorption. Intermediate bone exhibits higher roentgen absorptive qualities. Calcified bone with lacunae surrounding the zone of transition is bright yellow.

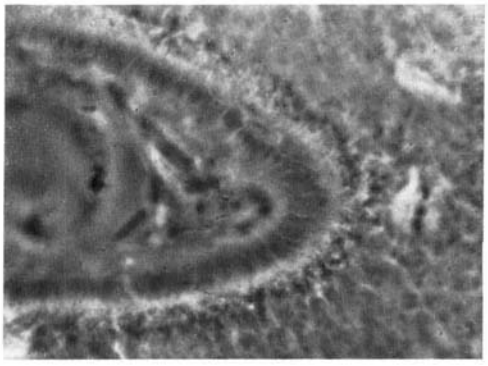


Fig. 7.

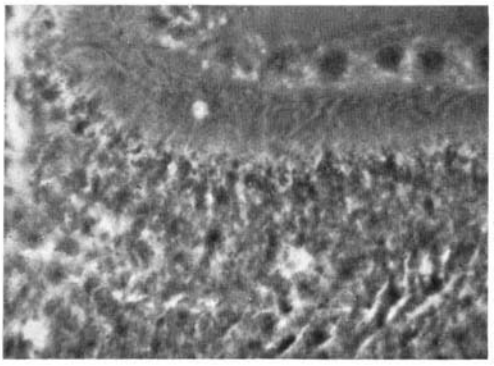


Fig. 8.

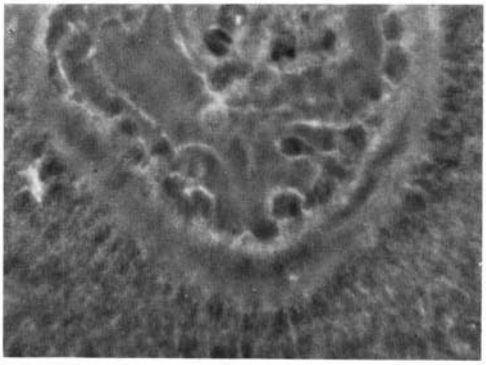


Fig. 9.

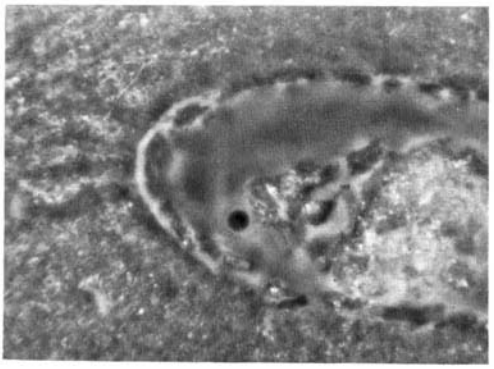
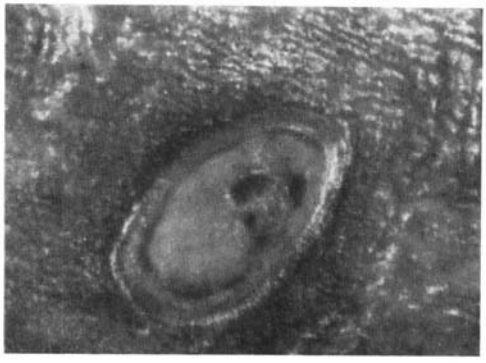


Fig. 10.



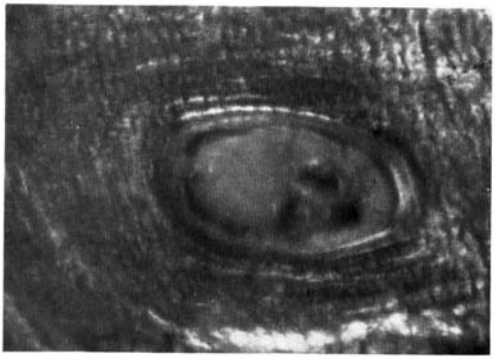


Fig. 7. Phase contrast photomicrograph. Formalin fixed, undecalcified, cut and unstained section of diaphyseal bone. Three zones of the osteoid. Osteocyte with processes in the old osteoid. × 1250.

Fig. 8. Phase contrast photomicrograph. Frozen-dried, undecalcified, cut and unstained section. In the young osteoid, fibrous matter loosely arranged between radiating cell processes. In the old osteoid and in the transitional zone, the tissue is denser and less fibrous. × 1250.

Fig. 9. Phase contrast photomicrograph. Osteoid and surrounding bone tissue after exposure to Zenker's fluid (partial decalcification). Blurred osteoid. Cell processes invisible in the osteoid. × 1250.

Fig. 10. Phase contrast photomicrograph. Specimens decalcified in formic acid–sodium citrate subsequent to formalin fixation. Unstained paraffin section. Peripheral limit of osteoid nearly indistinguishable. Zones cannot be seen. Cell processes invisible. × 1250.

Fig. 11. Frozen-dried, undecalcified, cut cross-section imbibed in distilled water and examined in polarized light. Young osteoid weakly birefringent (lower left corner). Old osteoid isotropic. Intermediate bone tissue birefringent. × 100.

Fig. 12. Same section as in Fig. 11 in extinction position (left).

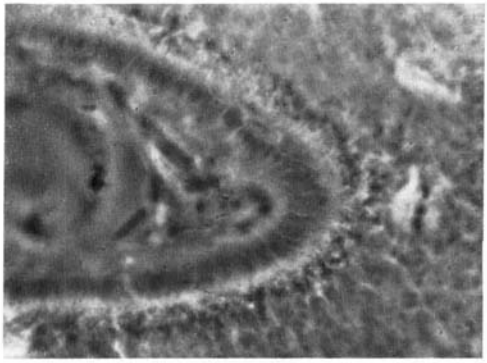


Fig. 7.

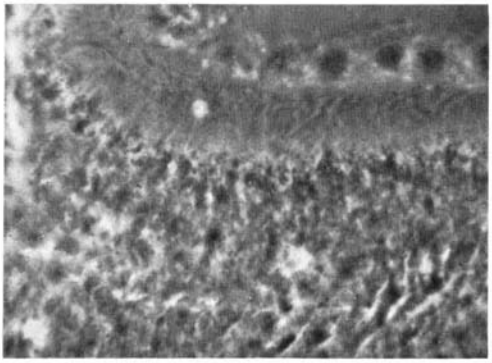


Fig. 8.

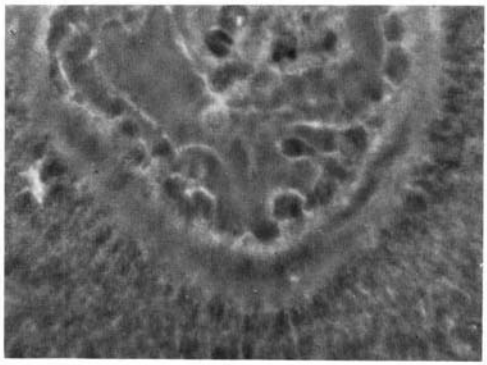


Fig. 9.

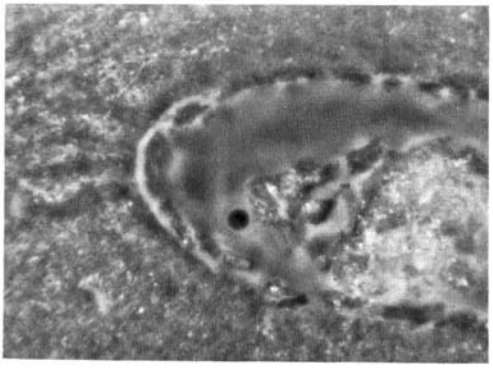


Fig. 10.

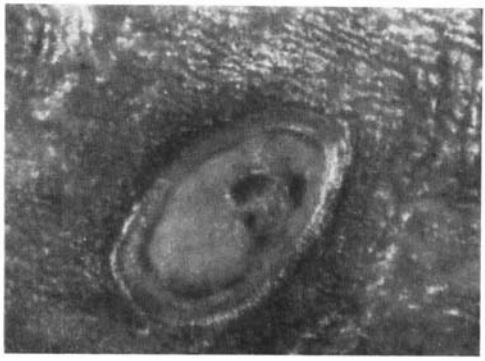


Fig. 11.

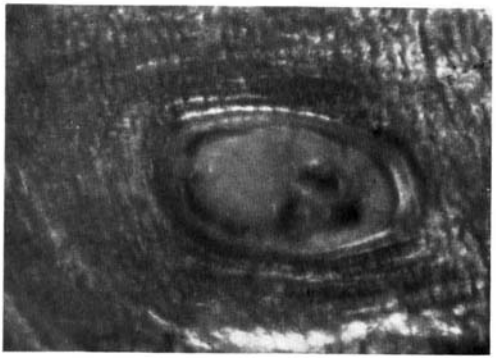


Fig. 12.

Fig. 13. Mallory's connective tissue stain. Formalin fixed, undecalcified, cut cross-section. Young osteoid stained by aniline blue. Old osteoid stained by orange G. Transitional zone is divided in a red, granular inner layer and a weakly blue stained border against the blue stained calcified bone. × 1250.

Fig. 14. Van Gieson's connective tissue stain. Formalin fixed, undecalcified cut cross-section. Young osteoid stained red by acid fuchsin. Old osteoid unstained or weakly stained by picric acid. The zone of transition appears as a faint red layer bordering on the red stained calcified bone. × 1250.

Fig. 15. Gomori's ammoniacal silver technique for fibers. Frozen-dried, undecalcified cross-section. Silver reduction occurs in a narrow layer of the young osteoid adjacent to the osteoblasts. Rest of the osteoid and calcified bone stained red. × 1250.

Fig. 16. Wilder's ammoniacal silver technique for fibers. Formalin fixed, undecalcified cross-section. Silver precipitate in the inner part of the young osteoid. Rest of the osteoid and calcified bone stained red. × 1250.

Fig. 17. Frozen-dried, undecalcified cut section imbibed in distilled water and examined in polarized light after insertion of the 1st order red gypsum compensator (addition position). Increasing positive birefringence peripherally through the young osteoid. The old osteoid is isotropic. Zone of intermediate bone shows positive birefringence.

Fig. 18. Same section as shown in Fig. 17 in subtraction position.

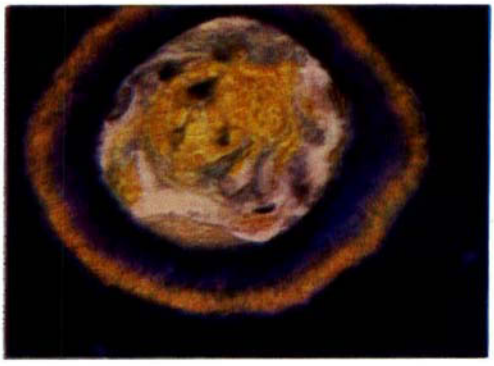


Fig. 13.

Fig. 14.

Fig. 15.

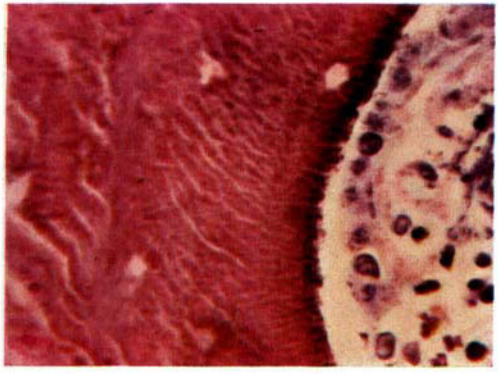


Fig. 16.

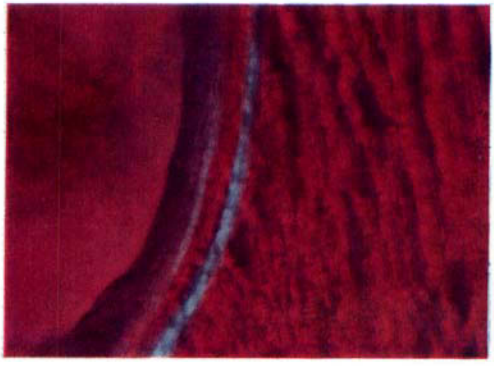


Fig. 17.

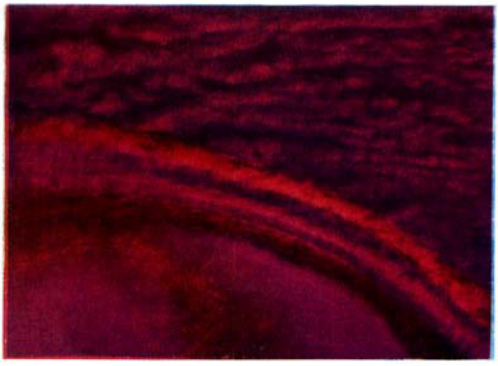


Fig. 18.

Fig. 19. Formalin fixed undecalcified section stained with dilute solution of toluidine blue (0.01 per cent for $^1/_2$ min.) prior to washing in alcohol. Osteoblast nuclei blue stained. Cell cytoplasm and processes as well as young osteoid show positive metachromasia (faint red). The old osteoid and intermediate bone show negative metachromasia (faint green). Osteocytes with positively metachromatic capsules scattered throughout the unstained calcified bone. × 900.

Fig. 20. Undecalcified cut section from the same series as that shown in Fig. 19, stained with slightly stronger solution of toluidine blue (0.02 per cent for $^1/_2$ min.). Osteoblast nuclei blue. Cell cytoplasm and processes as well as young osteoid positively metachromatic (red). The old osteoid shows negative metachromasia (faint green) which increases towards the intermediate bone (green). × 1250.

Fig. 21. Undecalcified cut section of the same series as those shown in Figs. 19 and 20, stained with 0.1 per cent toluidine blue for two min. Osteoblast nuclei blue. Cell cytoplasm, cell processes, and inner part of the young osteoid show positive metachromasia (red) which decreases in passing into the nearly unstained old osteoid. The area of transition contains faint purplish granules (negative metachromasia). Osteocyte nuclei are blue, cytoplasm positively metachromatic. Calcified bone unstained. × 1250.

Fig. 22. Frozen-dried cut cross-section stained with 0.1 per cent toluidine blue for 15 min. and dehydrated in alcohol. Osteoblast nuclei intensely blue stained. Osteoblast cytoplasm and processes as well as inner part of the young osteoid are red (positive metachromasia). The old osteoid also shows the red color which continues into the purplish intermediate bone (negative metachromasia). Calcified bone stains purplish or blue. × 1250.

Fig. 23. Frozen-dried cut cross-section stained with 0.5 per cent toluidine blue for 15 min. Positive metachromasia of the inner zone masked by the blue (orthochromatic) color. Old osteoid and intermediate bone are stained with red and partly blue. Surrounding bone blue-black. × 1250.

Fig. 24. Formalin fixed, cut cross-section subjected to the periodic acid–Schiff technique subsequent to diastase treatment. Osteoblast nuclei and cytoplasm unstained. Young and old osteoid show fairly even PAS positive reaction. A sudden decrease in the color reaction occurs in the area of transition, after which the intense red color of the calcified bone is reached within a short distance peripherally. No stainable matter showing in osteocytes except for some aggregation at the periphery of the nuclei. × 1250.

Fig. 19.

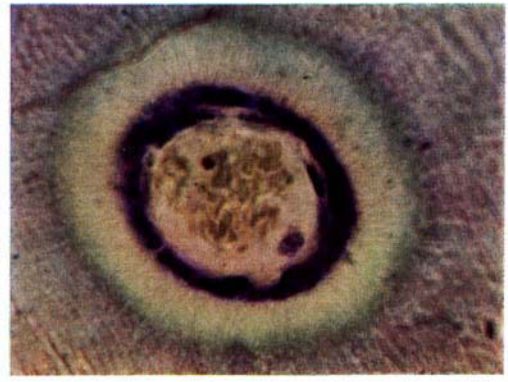


Fig. 20.

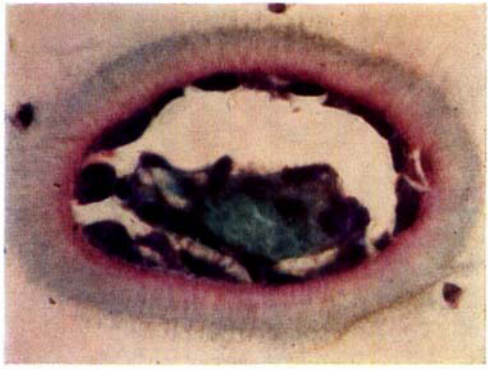


Fig. 21.

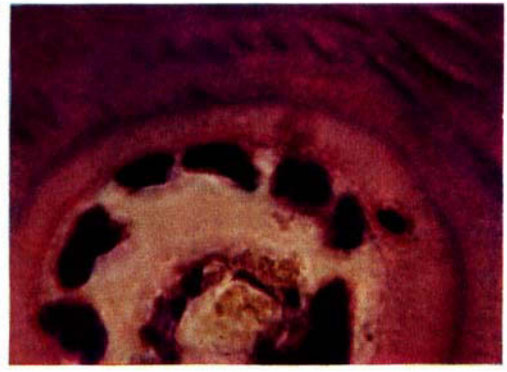


Fig. 22.

Fig. 23.

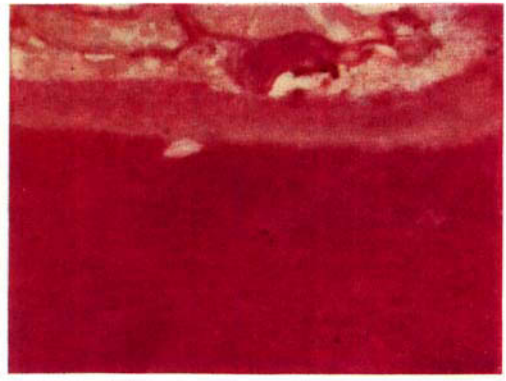


Fig. 24.

Fig. 25. Frozen-dried, undecalcified unstained cross-section in distilled water viewed in ultra-violet and blue light (UG filter 1). Osteoblasts show intense primary fluorescence. Young and old osteoid very weakly fluorescent. Intermediate bone exhibits strong primary fluorescence, thereby accentuating the border between osteoid and calcified bone tissue.

Fig. 26. Frozen-dried, undecalcified cross-section viewed in fluorescent light subsequent to treatment with watery solution of acridine orange pH 1.7. Strong secondary fluorescence in osteoblasts and osteocytes and in the zone of transition (yellow). A narrow rim of the transitional zone towards the old osteoid and the cell nuclei show concentration effect (copper red).

Fig. 27. Acridine orange pH 3.0. Cell nuclei and inner part of zone of transition show concentration effect (copper red). Young and old osteoid and calcified bone tissue show weak secondary fluorescence (dull green).

Fig. 28. Acridine orange pH 5.3. Cell nuclei and cytoplasm, young osteoid adjacent to the osteoblasts, and the inner part of the zone of transition show strong concentration effect (red secondary fluorescence). The old osteoid and the peripheral part of the young osteoid show weak secondary fluorescence (dull green).

Fig. 29. Acridine orange pH 7.3. All three zones of the osteoid and the cells with their processes show concentration effect (red). The calcified tissue exhibits some secondary fluorescence (green).

Fig. 30. Thiazin red R pH 1.7. Very weak secondary fluorescence in the zone of transition. Somewhat stronger color effects (reddish) in the old and young osteoid.

Fig. 25.

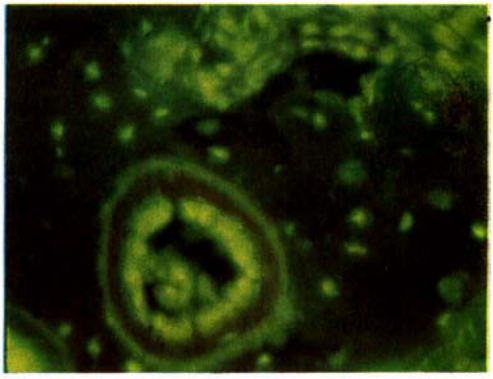


Fig. 26.

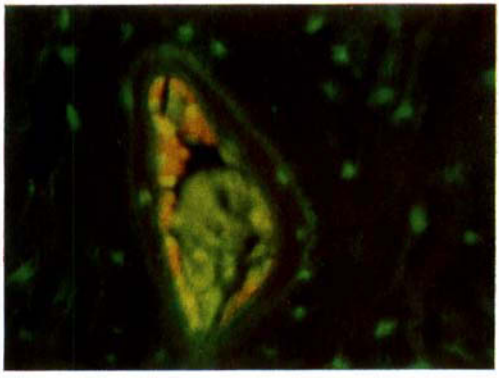


Fig. 27.

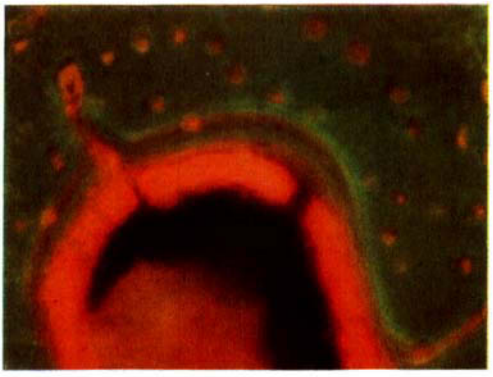


Fig. 28.

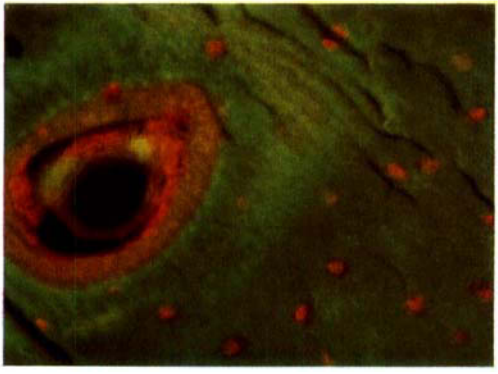


Fig. 29.

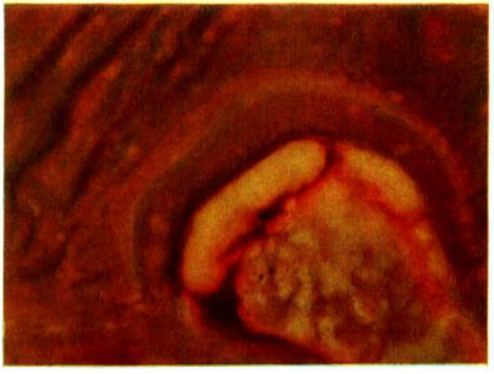


Fig. 30.

Fig. 31. Eosin pH 4.5. The entire old osteoid and the peripheral part of the young osteoid show secondary fluorescence (red). The osteoid adjacent to the osteoblasts as well as the zone of transition and the cells exhibit weaker absorptive qualities.

Fig. 32. Thiazin red R pH 6.8 showing similar distribution as with eosin pH 4.5 (Fig. 31). Old and peripheral part of young osteoid exhibit secondary fluorescence (red). Inner part of young osteoid adjacent to the osteoblasts and intermediate bone tissue show weak secondary fluorescence (dark red).

Fig. 33. Gomori's cobalt technique for alkaline phosphatase. Frozen-dried material. Enzyme activity is seen in the osteoblasts and in the inner part of the young osteoid adjacent to the osteoblasts. Calcified tissue surrounding the osteoid is stained black. × 1250.

Fig. 34. Azo dye technique for alkaline phosphatase. Frozen-dried material. Localization of enzyme activity in the cells and osteoid is the same as that shown with Gomori's method (Fig. 33). The red-brown color of the osteoblasts and their processes and in the inner part of the osteoid represents the phosphatase activity. The rest of the osteoid and the calcified tissue unstained. × 1250.

Fig. 35. Sections subjected to the azo dye technique subsequent to inactivation of alkaline phosphatase by treatment with iodine-potassium iodide. Absence of stained material in the cells and in the osteoid. × 1250.

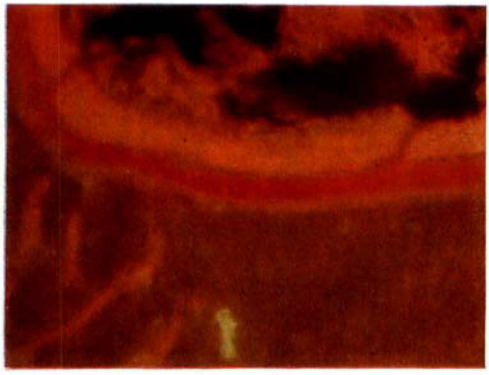


Fig. 31.

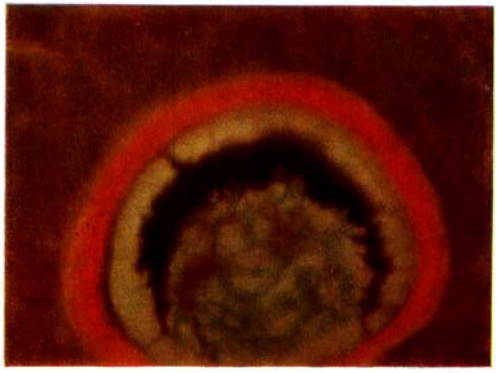


Fig. 32.

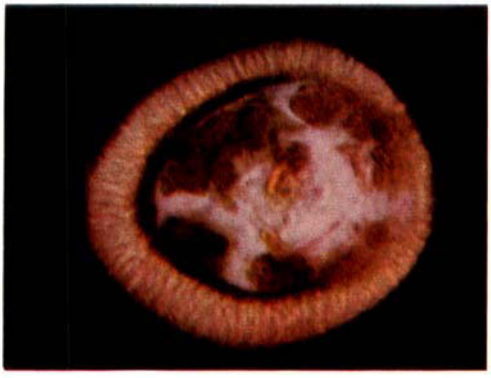


Fig. 33.

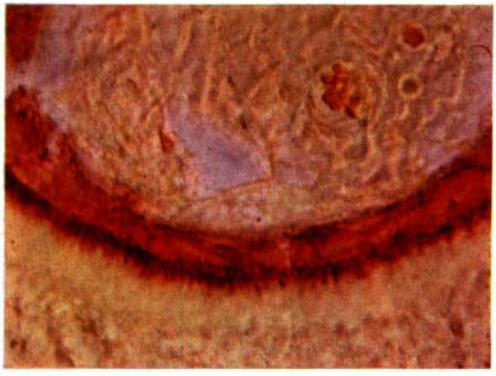


Fig. 34.

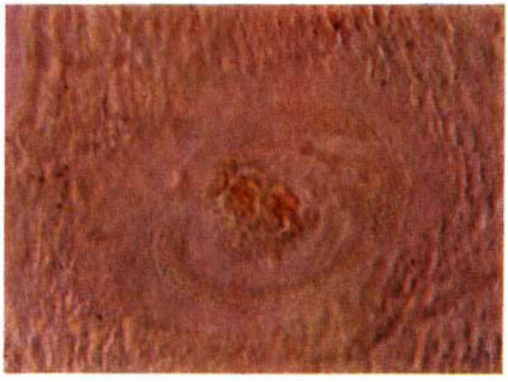


Fig. 35.