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BIOPHYSICAL INVESTIGATIONS OF BONE TRANSPLANTS AND BONE IMPLANTS

AN EXPERIMENTAL STUDY

By

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EJNAR MUNKSGAARD
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Contents

Introduction

Bone grafting has held the interest of investigators for centuries, and a great indebtedness is owed to all those who devoted themselves to investigations on bone transplants. The importance of these studies is now established by the fact that transplantation of bone has become an integral part of modern orthopaedic and reconstructive surgery.

The new danger to-day in the form of bone seeking radioactive elements produced by nuclear fission, such as those in atomic explosions, is no doubt responsible for the modern interest in investigating the ultrastructural organization of bone. The use of modern refined biophysical methods has opened a new avenue for such research on bone. In the study of intact bone by contact microradiography, a quantitative appreciation of the bone salt, and also an insight into the relative age of the inspected structures are attained simultaneously. Moreover by using X-ray diffraction techniques, the possibility of studying the organization of bone on the molecular level is achieved.

The present investigation is based firstly on the application of biophysical methods to the study of the mineralization processes related to the healing and ultimate fate of different bone transplants; and secondly on their application to the study of the resorption rate of bone implants as influenced by their ultrastructural organization.

Historical Resumé of Experimental Examinations of Bone Transplants and Bone Implants

The history of bone transplants is so very extensive that only a brief summary can be given here.

The first reported bone transplantation seems to have been made by MEEK'REN (1682) who filled the defect in a soldier's head with skull bone taken from a dog, but he had to remove the transplant because of religious interference. HUNTER (1728—1793), from his ingenious experiments, recorded among other things the ability of bone splinters to survive, and the resorption and the remodelling of bones. SYME, GOODSIR and FLOURENS (KEITH 1918) in their investigations held diverse opinions as to the importance of the periosteum in healing bone defects; but it was GOODSIR who finally showed the osteogenic properties of bone cells. The earliest published experimental bone transplantation made on animals is attributed to MERREM (1810). OLLIER (1867) reported very extensive investigations on transplantations of periosteum, bone marrow and bones in different animals and it was his opinion that the major part of a bone transplant survives, and he emphasized that what he considered new bone formation arose from the periosteum and the endosteum of the transplant. His theories were opposed by BARTH (1893) and MARCHAND (1899) who stated that the major part of the graft dies when transplanted, and that the periosteum and the endosteum of the graft do not influence on the outcome of the transplantation. Many polemical papers by different authors were written to substantiate these opposing views, and later MARCHAND (1901) (see also SALTYKOW 1909) and BARTH (1908) changed their opinions and admitted the osteogenic properties of the periosteum, and also took back their opinions of the prevalence of a special kind of bone resorption attached to the healing of bone transplants which they called "Schleichender Ersatz". AXHAUSEN (1908) published many thoroughly planned experiments of bone transplantations on animals, and also studied material from human grafts. His findings included the theory that most of the cells in a bone graft die, with the exception of some cells in the periosteum and endosteum and also in the superficial Haversian canals. According to his opinion, the substitution of a transplant when grafted to skeletal surroundings takes place principally by the processes of bone resorption and apposition by vascular tissue from the host and only to a lesser extent by surviving cells in the graft. The present opinion of the fate of bone following transplantation differs little from AXHAUSEN'S views.

The innumerable investigations of later authors have been to a great extent directed towards settling the question of bone transplant survival, and the origin of the cells which give rise to the formation of new bone. MACEWEN (1912) and his followers DAVIS and HUNNICUTT (1915) and GROVES (1920) found the graft viable, and their opposition to AXHAUSEN's findings seem, in part at least, to have been caused by MACEWEN's failure to recognize the cambium layer as part of the periosteum.

BASCHKIRZEW and PETROW (1912) conceived the idea of "metaplasia" of the granulation tissue around a transplant being responsible for the regeneration of the graft. This theory has been accepted by many authors, for example NAGEOTTE (1920), SIMON (1922) and LERICHE and POLICARD (1926). Very thorough experiments directed in order to clarify these questions have been made by DE JOSSELIN DE JONG and EYKMAN VAN DER KEMP (1928) and BULL (1928). The investigation of the former authors disclosed that the possibility of indifferent cells in the granulation tissue around the graft becoming osteogenic by "induction" from existing bone cells could not be denied, while BULL rejected the theory of metaplastic bone formation having any importance in the substitution of a bone transplant.

SULTAN (1902), AXHAUSEN (1908), BASCHKIRZEW and PETROW (1912), HAAS (1923), ROHDE (1925), and WERESCHINSKI (1925), in opposition to BARTH, concluded from the implantation of dead bone in soft tissue parts that no new bone was created around the implant. ORELL (1934, 1937), however, could confirm BARTH's findings, and showed that cooked bone and os purum, when buried in the subcutis on humans, produced new bone formation, but at a much slower rate than fresh autogenous transplants. H. ENGSTRÖM and ORELL (1943) by transplantation of dead bone, killed in liquid air, showed that new bone formation was produced at the same rate as in fresh bone transplants, and they stated that osteogenic properties of a graft were not necessarily bound to viable bone cells, but must have been the result of specific chemical substances in the bone transplant which transform cells in the connective tissue into osteogenic bone cells (see also WILTON 1937). Studies on the osteogenic effects of various extracts of bone and periosteum by LEVANDER (1938), ANNERSTEN (1940), RÖHLICH (1941), BERTELSEN (1944), LACROIX (1951), HARTLEY, TANZ and SCHNEIDER (1949), WILLSTAEDT, LEVANDER and HULT (1950) led to a belief that bone formation was caused by specific substances called osteogenins. Several investigators have studied the heterotopic bone formation by an "in vivo" technique utilizing the anterior chamber of the eye as the site for osteogenesis "induced" by the introduction of various bone substances and bone stimulating agencies (HUTCHISON 1952, URIST and MC LEAN 1952). The results reported by other investigators, however, namely HEINEN, DABBS and MASON (1949), HEGEMANN (1951), LINDAHL and ORELL (1951), RAY, DEGGE, GLOYD and LOONEY (1952)

and MOSIMAN (1952) indicate that no specific chemical osteogenic organizers have yet been isolated (see also WEISS 1950).

The effects of different lime-salts used as grafting material or as means to accelerate fracture healing have been negative according to STEWART (1934), KEY (1934), HALDEMAN and MOORE (1934), HUGGINS, CARROL and BLOCKSOM (1936), BISGAARD (1936), SHANDS (1937), BISGAARD and MACUMBER (1940) and HUDAK, BLOUNT and DARBY (1947), but ALBEE and MORRISON (1920) found an increased callus formation from the use of calcium salts. RAY and WARD (1951) stated that hydroxyapatite crystals acted as an "ossifiable medium", but the healing was never as rapid as when fresh autogenous grafts were used.

The first autogenous transplant used on humans was reported by V. WALTHER (1821). MACEWEN is credited as having been the first to use homogenous grafts on humans in 1880. Following MACEWEN, a great many clinical cases were reported (see STREISSLER 1911). After ALBEE introduced his surgical technique which depended upon a mechanically accurate fit of the graft to its bed, better clinical results were obtained (ALBEE 1921, 1944); and since the early 1920's bone grafting has become so universal that authors have numbered their cases of bone transplants in hundreds. The preservation by refrigeration of homogenous bone for clinical use led to the development of bone banks accredited to INCLÁN (1942), and to BUSH and WILSON independently (1947). Clinical reports by WEAVER (1949), KREUTZ, HYATT, TURNER and BASSET (1951), REYNOLDS, OLIVER and RAMSEY (1951), PATE (1954), CARR and HYATT (1955) seem to indicate that deep-frozen or otherwise preserved homogenous bone grafts give results which are as satisfactory as those obtained from fresh homogenous transplants. Nevertheless, the superiority of fresh autogenous transplants over other kinds of transplants is generally admitted by the above authors and also by BOSWORTH, WRIGHT, FIELDING and GOODRICH (1953). This fact is substantiated by the experimental work of REYNOLDS and OLIVER (1950), CAMBELL, BROWER, MC FADDEN, PAYNE and DOTHERTY (1953), LENTZ (1955) and SIFFERT (1955).

According to the modern literature on bone transplants, the general opinion of the authors is that the transfer of an autogenous cortical bone transplant into a long bone is followed by death of most of the cells in the transplant while the periosteum and the endosteum of the graft survive in part. When the bed for the transplant is cut in any bone, the osteocytes die near the line of the cut. Through the agency of vascular connective tissue which is derived mainly from the periosteum and the marrow of the host bone, the transplant is fixed in its place. Primarily from this tissue, and also from surviving cells in the periosteum and endosteum of the graft, the Haversian canals in the transplant are invaded; and by processes of resorption and new bone formation the transplant is slowly replaced [DE JOSSELIN DE JONG and EYKMAN VAN DER KEMP (1928), BULL (1928), CAMITZ, HOLMGREN and JOHANSSON (1934), ABBOTT, SCHOTTSTAEDT, SAUNDERS and BOST (1947), VAINIO (1950), SIFFERT (1955), CHASE and HERNDON

(1955), HAM and HARRIS (1956)]. Cancellous bone, according to HAM and GORDON (1952), might survive transplantation.

If not in contact with living bone, a bone transplant to soft tissue sites is slowly resorbed. Possible exception is an autogenous transplant grafted in a site such as the subcutaneous tissue of the human nose [CARTER (1932) and MOWLEM (1941)]. Exceptions are also fresh autogenous, septal, nasal and turbinate bone grafts in humans which when buried in soft tissue sites retain their calcified matrix together with the survival of their vascular system (PEER 1955). The healing of a homogenous graft takes place by similar processes as for an autogenous graft, but the rate of healing is usually slower. There seems to be an agreement that the cells in a homogenous graft all die when transplanted. [PEER (1955), HAM and HARRIS (1956)].

Boiled bone, os purum, and ashed bone are replaced in a manner similar to autogenous bone, but there is a variation in the timing of repair and in the manner of bone resorption [e.g. BULL (1928), ORELL (1934) and HUGHES (1943)]. Many investigations have been made in order to discover the cause of the delay in the healing of homogenous transplants. Investigations by CURTISS and HERNDON (1955), CURTISS, CHASE and HERNDON (1956) have shown that homogenous bone acts as an antigen, but a known blood group antigen-antibody antagonism has no influence on the fate of homogenous grafts in dogs. BONFIGLIO, JETER and SMITH (1955) showed a persistance of foreign protein cellular inflammatory reactions by repeated grafting of homogenous bone in rabbits.

Experiments in the study of the uptake of radio-active isotopes by various types of transplants and implants have been reported by KIEHN, FRIEDELL, BENSON, BERG and GLOVER (1950), ODELL, MUELLER and KEY (1951), KIEHN and GLOVER (1953), KARCHER (1953), WOJTA (1953) and KIEHN, GUTENTAG and GLOVER (1954), and their results have indicated that the autogenous transplants have the highest uptake, with the exception of WOJTA who did not find any sure difference between autogenous and homogenous transplants.

Summing up the investigations made on bone transplants, it appears that the riddle buried within the bone graft is the riddle of osteogenesis, and therefore it seems reasonable to believe that questions pertaining to bone transplantation should be approached along modern lines of research on bone. The present work is intended as an attempt in that direction.

CHAPTER II

Material of Investigation

A. Experimental Animals

The experiments were performed on 56 rabbits of mixed breed, and also on two mongrel dogs.

Ten of the rabbits were three months old, and the remaining 46 were young adults and adults six months and older weighing 3—4.5 kg at the time of operation.

The different transplants examined in the rabbits were fresh autogenous and homogenous cortical grafts from the tibial shaft, and also fresh autogenous and homogenous cancellous iliac bone grafts. Cortical and cancellous homogenous frozen bone grafts were also examined. Four different kinds of bone implants were studied, namely calcined cancellous rabbit bone, cooked cortical rabbit bone, glycerol-ashed rabbit bone and os purum implants which were manufactured from human cancellous bone.

One series of rabbits was sacrificed at intervals ranging from 4 days—28 weeks after operation and the remaining rabbits after a longer healing-in time of 48—64 weeks. In order to maintain the uniformity in this investigation seven rabbits were excluded from examination because of fracture in the operated leg. From the remaining 49 rabbits 180 transplants and implants in all were studied. The number of different types of transplants and implants and their various healing-in times is given in chapter V.

Both dogs were nine months old at the time of operation, weighing 12—13 kg; they were sacrificed ten months after operation. One dog had an autogenous cortical transplant, and an autogenous cancellous transplant. The other dog had an autogenous and a homogenous cortical transplant, and also an autogenous and a homogenous cancellous transplant.

B. Preparation of Bone Implants and Frozen Bone Transplants

All the bone implants which were prepared from rabbit bone were removed from the tibia or the iliac wing with a power-driven trephine.

After removing adherent soft tissues, calcined cancellous rabbit bone was prepared by heating the bone specimen to 900° C for 1½ hours.

The implants of cooked bone were obtained by boiling cortical rabbit bone for ten minutes in physiological sodium chloride solution.

Glycerol-ashed bone was prepared according to GABRIEL's method by treating cortical rabbit bone with five per cent KOH in boiling anhydrous glycerol, thereby removing the organic fraction in the bone specimen, which was subsequently rinsed in sterile water for 24 hours before implantation.

The os purum implants in this study were manufactured by Pharmacia Co. from human cancellous bone*. Before being implanted the os purum specimen was cooked for ten minutes in physiological sodium chloride solution. The preparation of os purum includes different chemical "purifying" processes, such as digestion of the bone by different enzymes (buffered pepsin-pancreatin solution at 35—37° C) and treatment with acetone, one per cent potassium carbonate solution, ethylene trichloride and also steam sterilization at 110° C for ½—1 hour (ASKELÖF 1956).

The frozen grafts were taken under sterile conditions either from the tibial shaft or from the iliac wing of a rabbit with a trephine. The piece of bone was then put into a sealed glass tube which was subsequently immersed in a thermos flask containing absolute alcohol at —20° C, and put in cold storage at the same temperature for a period of three days to 18 weeks before being implanted.

C. Surgical Procedures

The animal was anaesthetized with nembutal and ether. The operation was performed under sterile conditions. The soft tissues, including the periosteum, were incised over the anterior border of the tibia, whereafter the periosteum was gently stripped, uncovering the anteromedial surface. A line indicating the longitudinal direction of the leg was scratched with a scalpel along the tibial shaft. Using a power-driven trephine of stainless steel, with an inner diameter of about three mm, the cortical transplant was bored out with its center on the aforementioned line. At some distance from the donor site, the bed for the transplant was prepared with a trephine slightly smaller in diameter than the one first used, and by turning the transplant 90° it was fitted into its bed. By carefully selecting the trephines beforehand, a snug fitting could usually be obtained, and no other fastening of the transplant in its bed was necessary. While using a trephine any undue heating of the bone was prevented by continuous pouring of physiological saline solution.

After operation the soft tissues, including the periosteum, were closed with a continuous nylon suture. The leg was enclosed in a polstered plaster of Paris keeping the joints of the leg in "functional" position. The plaster was removed after three weeks when the risk for spontaneous fractures had passed.

Several transplants or implants of different kinds were placed below each other on the same leg, with a fresh autogenous cortical transplant usually included for comparison.

* My thanks are due to Prof. S. ORELL for kindly supplying the os purum specimens.

The cancellous grafts were taken from the iliac wing by incising the soft tissues over the iliac crest, stripping the fascia and boring through the entire thickness of the ilium with a trephine. The cancellous transplant with one cortical side removed and the other one usually facing outwards was then put into its bed prepared on the tibia.

Only one leg was operated on at a time. The rabbits were kept in separate cages and given a standardized mixed vegetable diet. Apart from spontaneous fracture of the operated leg in seven cases, healing took place by first intention.

Both dogs were operated in the same way as the rabbits. Uneventful healing took place for both. They were allowed to run free three weeks after operation and were given a mixed diet.

D. Preparation of Specimens

After sacrificing the animals with nembutal, the bone specimens were freed from soft tissues. By removing the posterior part of the tibia, the localization of the transplants was facilitated, because traces of the transplants were more evident on the endosteal side of the shaft.

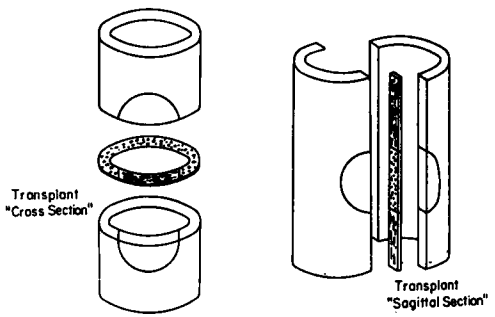


Fig. 1. Schematic illustration showing a cross-section and a sagittal section through a cortical transplant.

Specimens examined for their content of mineral salts were dehydrated in absolute alcohol and thereafter embedded in methyl-methacrylate. After polymerization of the plastic, either frontal, sagittal or cross-sections (see Fig. 1) were sawn out with a power-driven saw in slabs of about ½ mm thickness.

For preparing ground sections, a modification of the method described by HAMMARLUND-ESSLER (1955) was used. The specimen was affixed to a carrier of a glass microscope slide which was covered with double-coated Scotch tape. In order to ensure an even grinding, 38—100 μ thick sheets of stainless steel were placed on the tape on either side of the specimen. The grinding was performed by using silicon carbide abrasive paper with varying grit. The paper was mounted

on the flat surface of an oscillating "Metabo" grinding machine, the table of which rotated at 6,000 r.p.m.

Before finishing the grinding with the abrasive paper having the finest grit, the metal sheets were taken off the carrier, and the grinding continued for a short time in order to remove any metal dust from the surface of the specimen. Polishing was then done with fine emery paper. By grinding both sides of the specimen in this manner it was possible to obtain sections with a very even thickness of 70—80 μ.

After removing the plastic in ethylacetate, the ground sections which were to be examined in polarized light were decalcified in a HCl-NaCl solution according to v. EBNER's method. The specimens were then mounted in ten per cent NaCl solution or in Canada balsam.

CHAPTER III

Techniques for Determination of Mineral Salts

A. Contact Microradiography for Visual Examination

A contact microradiogram is obtained by exposing to X-rays a specimen which is placed in contact with a photographic emulsion. The emulsion is then developed according to routine photographic procedures. As the absorption of the X-rays is related to the chemical composition of the sample, the microradiographic picture will, under specific conditions, give information of a chemical nature for different structures in the sample.

For a survey of the literature pertaining to this method reference is made to CLARK (1955), ENGSTRÖM (1946) and LINDSTRÖM (1955).

The resolution in the obtained microradiogram is dependent on many factors, the graininess of the emulsion used being one of them. The fine-grained emulsions have a resolution of more than 1,000 lines per mm, and it has been shown that the resolution in microradiography can be brought down to the same limit as the light microscope, i.e. 0.2 μ (GREULICH and ENGSTRÖM 1956). The resolution is also dependent on the X-ray geometry for producing the image. Provided the roentgen rays penetrate the whole emulsion, the blurring in the image of a point at the surface of the specimen at the distance c from the emulsion with a target-to-sample distance of b is,

$$B = f(c + s)/b,$$

where f is the size of the target and s is the thickness of the photographic emulsion.

For microradiograms registered in the present investigation, the target-to-sample distance was 10—25 cm, f had a value of 1 mm and the thickness of the specimens was 70—80 μ. The thickness of the emulsion used (Eastman Kodak Spectroscopic Plates GH 649) can be neglected in comparison to the thickness of the specimens. The computed geometrical blurring is then 0.3—0.8 μ.

Photoelectrons emitted when the X-rays pass through the sample is another source for blurring the emulsion. Within the radiation energy used in the present study (12—40 kV) the range of the electrons excited is short (LEA 1946), and their contribution to the blurring effect is small.

The absorption of monochromatic X-rays in matter is expressed by the equation,

$$I = I_0 \cdot e^{-\mu \cdot d},$$

where I_0 and I are the intensities of the incident and the transmitted roentgen-rays respectively, μ (cm^{-1}) is the linear absorption coefficient, d is the thickness of the specimen in cm and e is the basis for the natural logarithm. As the thickness d can be substituted by m/ϱ where m is the mass of the absorber expressed as $g \cdot cm^{-2}$ and ϱ is the density, the formula can also be written:

$$I = I_0 \cdot e^{-\mu/\varrho \cdot m}.$$

In this expression μ/ϱ is called the mass absorption coefficient with the dimension $g^{-1} \cdot cm^2$.

The transmission t of the X-rays is defined as $t = I/I_0$. The extinction of the X-rays is defined as $\log_e I_0/I$ and is thus the same as the absorption index which is $\mu/\varrho \cdot m$ or $\mu \cdot d$.

When a microradiogram of a section of bone tissue embedded in plastic is registered, the X-ray absorption is expressed by the equation,

$$E = \log_e I_0/I = \mu_1/\varrho_1 \cdot m_1 + \mu_2/\varrho_2 \cdot m_2,$$

where the indices 1 and 2 stand for the inorganic and organic fractions in bone respectively. The water content in the specimens is negligible because of dehydration. In the embedding procedure, the plastic might substitute the water, and in the following discussion it is included in the organic fraction. If apatite ($Ca_{10}(PO_4)_6(OH)_2$) represents the inorganic fraction, and nitrogen represents the organic fraction, the ratio of the numerical values of the respective mass absorption coefficients is about ten in the wavelength region used (1.5—0.5 A), which is on the short wavelength side of the K-absorption edge for calcium. The density of apatite is about three and for the organic fraction the density is approximately 1.3. As the respective fractions each occupy about the same volume, it becomes evident that under the conditions used, the absorption of X-rays caused by the organic fraction in bone can be neglected in comparison with the attenuation caused by the inorganic constituents. The change in the total extinction caused by a relative change in the absorption index for the inorganic fraction will be about 30 times greater than from the same relative change in the absorption index for the organic fraction.

A microradiogram of a ground bone specimen registered under the aforementioned conditions will therefore show the distribution of mineral salts (ENGSTRÖM and AMPRINO 1950).

The density of a photographic emulsion with the density of fog subtracted is given by the relation,

$$D = \log_{10} i_0/i_1,$$

where i_0 and i_1 are the intensities of the transmitted light in an unexposed area and an exposed area respectively, in the developed film emulsion. The photographic density response of the X-ray emulsion used in this investigation

(Eastman Kodak Spectroscopic Plates GH 649) was plotted as a function of the incident X-ray energy at an applied potential of 20 kV. The X-ray tube had a copper target and the radiation was filtered through an 18 μ thick Ni-foil. The emulsion was developed in D19 b for five minutes at 18° C. As can be seen in Fig. 2 the plotted curve below a density of 0.7 is straight. Within this density range it can be shown that, for a constant voltage, there is an optimum thick-

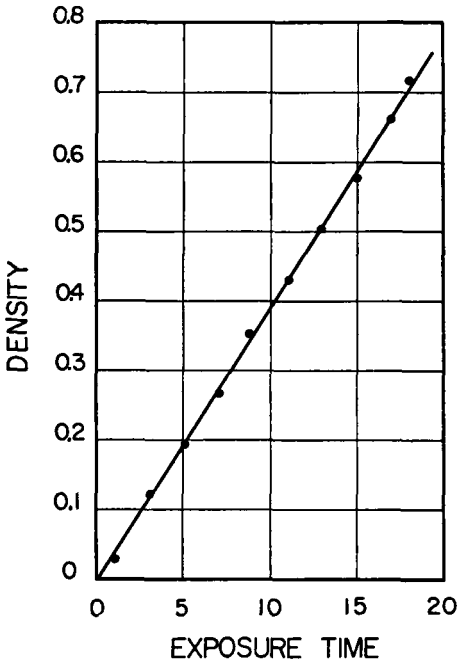


Fig. 2.

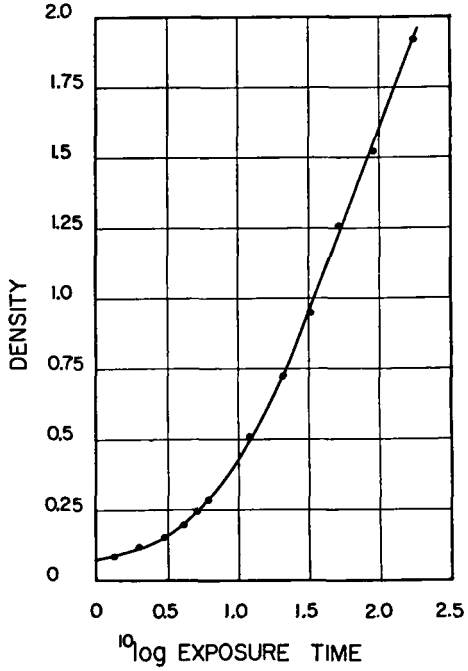


Fig. 3.

Fig. 2. The density as a function of exposure time at constant intensity of 20 kV. Ni-filtered Cu Kα-radiation. Emulsion: Kodak Spectroscopic Plate no. 649 GH.

Fig. 3. The density as a function of $\log_{10}$ exposure time at constant intensity of 20 kV. Unfiltered radiation from Cu target. Emulsion: Kodak Spectroscopic Plate no. 649 GH.

ness of the section, which gives optimum conditions for the visual detection of small differences in mass between structures with the same thickness but with different linear absorption coefficients (ENGSTRÖM 1955). If X-rays of intensity I_0 are incident on the specimen and the X-rays transmitted by two adjacent structures are I_1 and I_2 respectively, the difference in density ΔS can be written,

$$\Delta S = k(I_1 - I_2) = k\, I_0\, (e^{-\mu_1 t} - e^{-\mu_2 t}),$$

where t is the even thickness of the specimen and k is a constant. The thickness t_{max} for which ΔS has a maximum can be shown to be approximately $1/\mu$ where μ denotes the average linear absorption coefficient (ENGSTRÖM 1955).

For visual examination of microradiograms however, in order to give higher contrast, the exposure times are usually kept long enough to give a density within the range where the photographic density response is directly proportional to the logarithm for the incident X-ray energy. In a paper by HENKE, LUNDBERG and ENGSTRÖM (1956) the optimal conditions for visual evaluation of microradiograms were considered. By defining the visibility or contrast as the change in photograhic density for percentage variation in absorption index, it was shown in this paper that within the density range giving optimum contrast there is no optimal sample transmission as such for visual evaluation of microradiograms. If the microradiogram density is kept at a fixed value, the contrast will increase with the absorption index. In the present investigation the microradiograms were registered with a sample transmission value of $1/e$. If the density is plotted as a function of the logarithm of exposure, the contrast function at this transmission is the same as the slope number of the density curve for the X-ray emulsion multiplied by $\log_{10} e$. From Fig. 3 it can be seen that the slope number is about 2 at a density of 1.7 and therefore the contrast function for a sample with a transmission value of $1/e$ is about 1. Differences in density which are visually detectable under optimal conditions are less than one per cent. It is therefore evident according to the definition of the contrast function given above, that in bone samples examined under these aforementioned conditions, differences in absorption index of about one per cent are visually detectable.

As a radiation source to record the microradiograms for visual examination, a Philips diffraction tube (type 25 293/52) with a copper target and a mica-beryllium window and also a Machlett X-ray tube OEG 50 with a tungsten target and a 0.2 mm beryllium window was used. The exposed X-ray emulsions were developed in Kodak D 19 b or DK 50 at 18° C for five minutes. After fixing and washing they were subsequently enlarged by microphotography.

B. Quantitative Microradiography with Thickness Determination

In the present investigation the mineral content in the ground bone specimens was determined by a technique whereby quantitative microradiography was combined with thickness determination of the specimen. An area in the specimen was selected and was cut through with a microdissecting knife. The thickness of the cut edge was measured in a profile microscope at equidistant points. The accuracy of the thickness determined was $\pm$ 2 μ. These points could be quite accurately localized in the microradiogram by making absorption measurements in equidistant areas along the edge of the specimen. By using a micro-dissecting knife to scratch a line along the surface and towards the edge of the specimen, the point for the thickness determination could be exactly localized in the microradiogram because the scratch line showed up clearly. To register the microradio-

grams, a highly stabilized X-ray unit (Philips PW 1010) was used as a radiation source, and a copper target tube was operated at 20 kV. The radiation was filtered through an 18 μ thick Ni-foil. In Fig. 4 are shown the patterns from an X-ray spectrometric analysis of the radiation before and after filtering through the Ni-foil. 80 per cent of the radiation was computed to be within the range

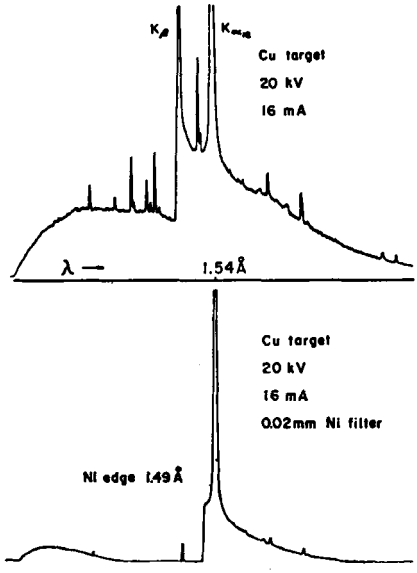


Fig. 4. Spectrometric analysis of the intensity of the X-ray radiation before (top) and after (below) filtering through a 18 μ thick Ni-foil. Philips diffraction tube (type 25 293/52) with a copper target run at 20 kV.

of 1.5—1.6 A, thereby corresponding to the wavelength of a comparatively pure $CuK_{\alpha_1\alpha_2}$ radiation ($\lambda = 1.54$ A).

The microradiograms were registered on Eastman Kodak Spectroscopic Plates GH 649, and simultaneously with the sample a step-wedge made up of aluminium foils was exposed and used as a reference. As mentioned previously the photographic density response was found to be linear in relation to X-ray exposure up to a photometric density of 0.7. The microradiograms were developed for five minutes in D 19 b at 18° C and mounted under cover slips with Canada balsam as the mounting medium. The absorption of monochromatic X-rays in a bone sample is expressed by the equation:

$$I = I_0 \cdot e^{-\mu \cdot d}.$$

The values for I and I_0 can be substituted by their respective densitometric values so that $I = k \log_{10} i_0/i_s$ and $I_0 = k \log_{10} i_0/i_d$ where i_0, i_s and i_d represent the transmitted light intensities respectively in the non-exposed emulsion area,

in the sample area and in the direct X-ray beam area. k is a constant of proportionality. Solving $\mu \cdot d$ one gets:

$$\mu \cdot d = \log_e \left[\frac{\log_{10} i_0/i_d}{\log_{10} i_0/i_s} \right].$$

Knowing the value of d it is possible to compute μ.

The variations of the organic content of old and young regions of bone have been shown to be small (DAVIES and ENGSTRÖM 1954). Referring to the discussion on page 17 it is therefore evident that variations in the absorption index for a bone sample are mainly caused by changes in the absorption index for the inorganic fraction. The mass absorption coefficient of the bone mineral will vary only slightly as the composition of the bone salts can be considered relatively constant (KLEMENT 1938). The computed value of μ will therefore be approximately proportional to the amount of bone mineral per unit area, and the variations of μ will be approximately equivalent to the mass variations of mineral salts in the bone sample. The standard errors associated with this technique of determining the mineral content in bone specimens will include errors arising from the thickness determination and the photographic-photometric procedure, and also from the biological variations in the specimen.

The errors in the photographic-photometric procedure arise from a combination of several factors such as: inhomogeneity in the intensity of the incident X-ray beam; unevenness and artefacts of the photographic emulsion; the granularity of the photographic emulsion and variations inherent in the photometer and uncertainty in reading the photometric values.

In a paper by WALLGREN and HOLMSTRAND (1956) an analysis of the standard errors associated with this technique was made. A "physical" model experiment was designed using a sheet of fused quartz as a test object. The relative standard error in determining the linear absorption coefficient for quartz was shown to be respectively 1.6 or 3.25 per cent, depending upon whether the thickness was measured on easily defined structures or along equidistant points. The sensitivity of the method in determining mass differences was about one per cent at an average thickness of 100 μ. From 100 measurements the standard error for the thickness determination was 1.4 μ at a constant thickness of 161 μ.

In microphotometric measurements of microradiograms the precision is dependent upon the sample transmission and the photographic density used. For maximum precision measurements of the absorption index, it was shown by HENKE, LUNDBERG and ENGSTRÖM (1956), that, assuming a linear density versus exposure curve for the X-ray emulsion, a minimum error existed for transmissions of 20—30 per cent. The increase in error was small for other transmissions provided the base density, i.e. the density in the directly exposed part of the microradiogram was within the range 0.5—1.25. For one per cent error made on the photometer reading for the unexposed emulsion, the minimum

relative standard error in the photometric measurement of the absorption index was about three per cent. In the present investigation the thickness of the bone specimens was 70—80 μ. As the linear absorption coefficient of bone at the wavelength used (1.54 A) is about 130, transmissions of approximately 37 per cent were obtained. These conditions gave optimal contrast for visual inspection of the microradiograms (p. 18), and also secured small errors in determining the absorption index, as the photographic density was kept at 0.4—0.6.

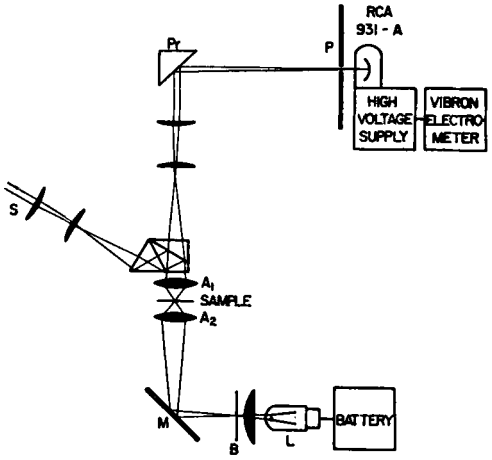


Fig. 5. Schematic representation of high resolution microphotometer. A_1 Objective. A_2 Condenser. L. Tungsten filament lamp. M. Mirror. S. Side tube. B. Iris diaphragm. Pr. Prism. P. Screen with a central pin-hole in the housing of photomultiplier tube (type RCA 931 A).

Part of the densitometric measurements were made using a "Zeiss Schnell-photometer II". The photometric readings were made on a scale with 1,000 divisions, and scale deflections could be read within half a division. By increasing the intensity of the light source, the slit could be closed to include an area corresponding to 900 μ^2 in the microradiogram, while still keeping the value for transmitted light through the unexposed part of the emulsion approximately at 100. The total percentage error in determining the absorption index arising from the error in reading the values for the transmitted light intensities could then be kept small, and was computed to be approximately 1.5 per cent at the photometric densities used.

Measurement of smaller areas gave a larger photometric error as the intensity of the light source could not be increased in order to keep the value of i_0 at 100. Therefore at the fixed magnification 33×, the accuracy of the measurement of small structures was restricted. To determine the amount of mineral salts in areas smaller than 900 μ^2 a microphotometer shown schematically in Fig. 5 was used. In this microphotometer the magnifying system was a Zeiss-Winkel microscope with a 6.8 mm apochromatic objective ([A_1] numerical aperture

0.45) and a periplane ocular (8×). The light source was a 15 watt tungsten filament lamp (L) connected directly to an accumulator by soldered wires. After a warm-up period of 30 minutes under load conditions, the variations in the light intensity from the lamp were considerably less than one per cent during a two hour period. While not in operation the accumulator was loaded with a sele-

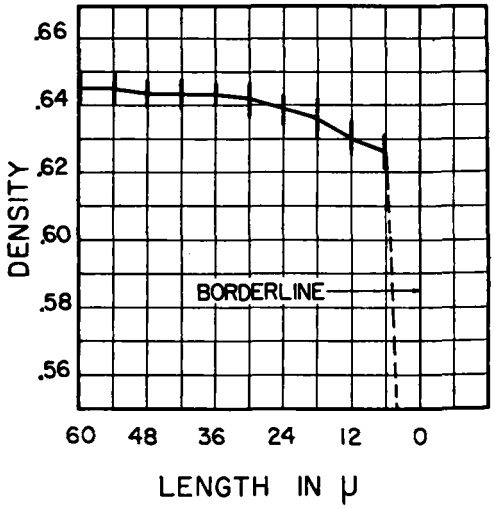


Fig. 6. Diagram demonstrating the influence of stray light on the determined density which is plotted as a function of distance from the border line between exposed and unexposed parts of the X-ray emulsion. (See text.)

nium rectifier connected to the AC line. A 16.2 mm Zeiss apochromate objective ([A_2] numerical aperture 0.30), was used as a condenser for the light in Köhler illumination. An area in the microradiogram was inspected through the side tube (S) and was projected by the prism (Pr) on the photosensitive surface of a photomultiplier tube (RCA 931 A) through a pin-hole (P) (diameter 1.5 mm) in the housing of the photomultiplier tube. The area of the pin-hole corresponded to an area of 75 μ^2 in the microradiogram. A highly stabilized power supply was used for the photomultiplier. The anode current was recorded by a Vibron Electrometer (E.I.L., model 33 b) connected to the photomultiplier over a precision resistance of one megohm. The photometric readings were made on a scale with 50 divisions, and scale deflections could be read within one fourth of a division. To avoid fatigue of the photomultiplier the anode current was kept below 1 μA corresponding to the highest value on the scale. The value for transmitted light through the unexposed part of the emulsion usually had the scale value 40.

In an effort to analyze the errors associated with the densitometric measurements using this microphotometer, absorption measurements were made in ten different points on the plain part of the quartz wedge in nine separate micro-

radiograms with a density varying between 0.45 and 0.6. The mean value of the linear absorption coefficient for the quartz wedge thus determined was 75, with a relative standard deviation of 1.3 per cent. The accuracy was about the same as for the microphotometric measurements being performed in using the "Zeiss Schnellphotometer" under the same conditions, but the systematic error in the value obtained was smaller (see WALLGREN and HOLMSTRAND 1956).

In order to estimate the effect of stray light on the densitometric measurements with this microphotometer, a series of scans were made on an Eastman Kodak Spectroscopic Plate GH 649, the density of which by direct exposure to X-rays was approximately 0.64. The illuminated part of the emulsion had a diameter of 84 μ and was bounded by the image of the iris diaphragm (B) in the lamp condenser. Densitometric values were measured at various distances from the sharp border line between unexposed and exposed emulsion. Five linear scans were carried out in different regions of the emulsion starting 60 μ from the border. Readings were made at intervals of 6 μ, the area measured at each point having a diameter of 10 μ. The mean values of the densities, with their standard deviations, obtained in five separate points at the same distance from the border line were plotted as a function of the same distance. As can be seen from the curve in Fig. 6 the stray light effect is negligible at a distance of more than 30 μ from the border line, but inaccurate density readings are obtained at closer distances. There is slightly more than one per cent deviation in the density values 18 μ from the border line if comparison is made with the mean density values for points more than 30 μ from the edge.

In the present investigation the stray light effect on the densitometric measurements of mineral salts in the specimens was minimized by preferably measuring structures situated more than 30 μ from areas with abrupt density changes.

C. Autoradiography

The uptake *in vivo* of Sr^{90} which has a half life of 25 years was studied by autoradiography. The localization of this isotope in the bone specimen was correlated with the pattern of mineral salts obtained from microradiography of the same specimen.

In one part of the experiments the tracer was injected intravenously on previously operated rabbits, with a single dose of carrier-free Sr^{90} Cl_2 solution, 8—14 days before the animal was sacrificed. The dose given was $^1/_3$—$^1/_2$ mC per kilogram body weight. In another part of the experiments two unoperated rabbits were given a dose of $^1/_3$ mC Sr^{90} at the age of 8 weeks. Their weight was then 900 g and 16 weeks later they were operated simultaneously, and were each given an autogenous cortical transplant and a homogenous cortical transplant from the other rabbit. 28 weeks after operation both rabbits were sacrificed.

Ground bone specimens which had been microradiographed, and which were

incubated with Sr^{90} were mounted for autoradiography by sandwiching the bone specimen between sheets of Agfa Printon Rapid film and were kept in a special holder to ensure good film-specimen contact. Exposure times varied from ten days to three weeks. The films were developed in Kodak D 19 b for three minutes at 18° C and further treated according to standard procedures. The autoradiographs were enlarged by microphotography, Kodak B 40 plates being used.

CHAPTER IV

Techniques for Determination of Ultrastructure

In this chapter a short summary of the different X-ray diffraction methods used in the present investigation is given, including some technical aspects. For detailed information on the subject of X-ray diffraction the reader is referred to the textbooks by CLARK (1955), CULLITY (1956), GUINIER (1952), KLUG and ALEXANDER (1954) and SPROULL (1946).

A. Micro-X-ray Diffraction

Passing through a polycrystalline sample, a narrow beam of monochromatic X-rays will be diffracted at certain angles between the direction of the beam and the atomic planes in the crystallites according to BRAGG'S law which is written:

$$n \cdot \lambda = 2\ d\ \sin \Theta.$$

In this relation λ is the wavelength of the X-rays in A, and Θ is the angle between these planes and the incident X-ray beam, n is a whole number giving the order of the reflection, and d is the spacing between parallel atomic planes in A.

As the wavelength is kept constant, the different reflections recorded on a photographic film will correspond to the spacings between different atomic planes of the substance. For a specific value of d the diffracted beams form a cone which has its apex in the specimen.

If the direction of the incident X-ray beam is perpendicular to the surface of the recording film, and provided the crystallites are sufficiently small and randomly oriented, the reflections will then show up as a set of concentric rings.

If the axes of the crystallites in the specimen are grouped more or less narrowly around certain directions, there will appear preferred orientation manifested in the X-ray diffraction pattern by arcing of the rings.

In this study the ultrastructure of the ground bone specimens was studied in a CHESLEY (1947) microdiffraction camera. The collimators in this camera consisted of exchangeable glass capillaries, the inner diameters of which were either 45 or 95 μ. Under a dissection microscope at about 40 times magnification, the structure for analysis could be selected and adjusted exactly over the collimator opening by moving a cross table. Hence it was possible to record the ultrastructure in areas varying in width between 50 and 100 μ. The diffractograms were recorded with Ni-filtered CuK_α radiation ($\lambda = 1.54\ A$) from a Philips

PW 1010 X-ray diffraction unit at 40 kV, and registered on "Ilford Industrial G" X-ray film and developed in D 19 b. The distance between the film and the specimen was 9.85 mm.

The specimen thickness which produces the maximum diffracted intensity at low diffraction angles is given by $1/\mu$ where μ is the linear absorption coefficient. At the wavelength used the linear absorption coefficient for bone was about 130. Samples which were studied by microradiography and which had a suitable thickness of 70—80 μ were also studied by microdiffraction. These same samples studied by X-ray microdiffraction gave maximum diffraction effects because of the thickness used.

B. Determination of Crystallite Sizes with the Geiger-Müller Counter X-ray Diffractometer

In the powder diffraction analysis, the material undergoing examination is reduced to a fine powder. The most common of the powder diffraction methods is the DEBYE-SCHERRER method, whereby a narrow strip of film is curved into a short cylinder with the specimen rotating on its axis. The incident X-ray beam is directed at right angles to this axis. Monochromatic radiation is used. Each particle of the powdered specimen will be oriented at random with respect to the incident X-ray beam. If a sufficiently great number of atomic planes in the different crystallites are in reflecting positions according to BRAGG'S law, the beams reflected will reinforce each other and appear on the film as a continuous line, and cones of diffracted radiation will produce curved continuous lines when they intersect the film. If for any reason the number of orientations of the crystallites irradiated by the X-ray beam is too small, the diffraction rings will be discontinuous and spotty.

The BRAGG law is derived under ideal conditions, namely, a perfect crystal and an incident beam composed of perfectly parallel and strictly monochromatic radiation. These conditions never actually exist, and the reflections will depart from the ideal. For example, the size of the crystallites will influence the width of the diffraction lines. When the grain diameter is in the range 10^{-3}—10^{-5} cm sharp rings are registered. As the particle size decreases below about 10^{-5} cm the diffraction rings will become broader in proportion to the decrease in size, until, in the neighbourhood of 10^{-8} cm, atomic dimensions are reached. This widening of the diffraction lines has led to a method for estimating the size of very small crystallites. And this is done by measuring the diffracted intensity versus 2Θ for the line in question. In the DEBYE-SCHERRER method the intensity of the diffracted X-ray beam is measured by determining the variation of the photographic density across the line on a microphotometer. The density is then plotted versus 2Θ. The particle size can be computed by using the formula of SCHERRER:

$$D = \frac{K \cdot \lambda}{\beta \cdot \cos \Theta}.$$

In this relation D is the average size of the crystallites in A, K is a constant called the crystallite shape factor, λ is the wavelength of the radiation in A; β is the half width of the "pure" broadening of a diffraction line measured in radians, and Θ is the BRAGG angle. The "pure" diffraction width has to be calculated from the observed line width by making corrections for the broadening effects originating from the instrument and the type of radiation used. The corrections can be done by comparing the line width of the sample with the width of a line produced under identical conditions with a material which has a sufficiently large crystallite size (above 1,000 A).

In diffraction analysis for determination of crystallite sizes using an X-ray diffractometer, the photographic film is eliminated, and in place of it a counter-tube detector (as for example a Geiger-Müller tube) is employed to record the intensities of the diffraction lines. The counter is placed on the circumference of a circle which is centered on the powdered specimen, which, in the form of a flat plate is supported on a table. Special slits are used to define and collimate the incident and the diffracted beams. The supports of the counter and the sample are mechanically coupled so that a rotation through a certain angle of the counter is automatically accompanied by a rotation of the specimen through half this angle. This coupling arrangement is necessary to preserve focusing conditions. The counter may be moved by hand to any desired angular position. Because of the relative line intensities being measured, it is imperative to keep the incident beam intensity constant, and a highly stabilized X-ray unit must be used. The intensities of the different reflections from the sample can be electronically measured at fixed angles by a scaler which counts the pulses from the Geiger-Müller detector. As the arrival of X-ray quanta in the detector is random in time, the accuracy of the counting-rate measurement is governed by the laws of probability. The probable error in a single count of N pulses is given by $67/\sqrt{N}$ per cent. As the error depends only on the number of pulses counted, intensity measurements of about the same precision of both high and low intensity beams can be made if the time for a fixed number of counts is measured. This rule is valid only if the counting rate due to the radiation measured is high in relation to the unavoidable background caused by cosmic rays and radioactive material.

In examining a sample, if N is the number of pulses counted in a given time and N_B is the number of pulses due to the unavoidable background during the same time, then $(N—N_B)$ pulses are due to the intensity of the radiation diffracted in the sample. The relative probable error in $(N—N_B)$ is given by:

$$E = \frac{67\sqrt{N + N_B}}{(N - N_B)} \text{ per cent.}$$

In the present investigation the unavoidable background was measured and

found to be about one count per second. The difference in probable error computed from the formula above for high and low intensity measurements amounted to less than 0.03 per cent for the different types of samples examined. The counting rate in a Geiger-Müller tube is limited by its resolving time, which is defined as the shortest interval between the arrival of two successive pulses, permitting detection of both of them. Because of the randomness in time of successive quanta, counting losses will appear at even lower counting rates than given by the resolving time. In this investigation the counting rates were never higher than 300 counts per second, at which rate no counting losses occurred for the Geiger-Müller tube used. For precision measurements of relative X-ray intensities the X-ray diffractometer method is far superior to photographic methods because of its high sensitivity and accuracy.

In the present investigation a Philips GM diffractometer (Type PW 1050) was used to determine the crystallite sizes for different fresh bone samples and for different bone implants. The highly stabilized X-ray unit was run at 42 kV and 20 mA and nickel-filtered CuK_α radiation ($\lambda = 1.54$ A) was used. The specimens were mixed with a suitable glue and flattened into a thin sheet in the specimen holder. All specimens were mounted in the identical manner. Only the intensities of the profile for the (00·2)-reflection were measured, as the intensities for the (*hk*·0)-reflections for all the specimens, with the exception of calcined bone, were too low to be measured accurately. The profiles of the (00·2)-reflections were obtained by plotting the integrated X-ray intensities versus 2Θ with intervals of 0.01°—0.05°. Each point on the profile curve was obtained by counting 12,800 counts and was expressed on the graph in counts per second. To obtain the instrumental broadening effect, the profile for the (00·2)-line of a well crystallized synthetic fluorapatite with a crystallite size above 3,000 A was determined. In order to compute the half width of the "pure" diffraction profiles, corrections were made for broadening effects arising from the instrument and from the radiation. For this purpose the correction curves of ALEXANDER (1954) were used.

A direct method for obtaining the "pure" diffraction profile of a powder X-ray diffraction line, free from the effects of all forms of broadening, has been developed by STOKES (1948). In STOKES' method a FOURIER analysis of a line profile is made by using a direct mathematical treatment of data obtained from the profile of the sample and the profile of a reference substance, the latter with a crystallite size of more than 1,000 A. In the present investigation computation of the "pure" line breadth of the os purum implant was made by STOKES' method. The profile of calcined bone was used as a reference substance. Summation of the FOURIER series was made on a special FOURIER summation equipment (HÄGG and LAURENT 1946).

C. Determination of Crystallite Sizes by Small-angle Scattering of X-rays

X-rays traversing a thin specimen containing submicroscopic particles, will, under specific conditions, be scattered, and on a photographic film produce a halo unresolved from the undeviated primary X-ray beam. This phenomenon called diffuse low-angle scatter can be used for the estimation of particle sizes. The theory and the conditions for its applications have been extensively investigated by GUINIER. It was shown by GUINIER, on the supposition that a system containing submicroscopic particles not too densely packed, and all having a uniform size and shape and oriented at random, there is an approximate linear relationship between the logarithm for the intensity of the scattered radiation ($\log_{10} I$) and the squared scattering angle (ε^2).

If CuK_α radiation is used ($\lambda = 1.54$ A) the radius of gyration ($\bar{R}$) of the particle (defined as the root-mean square of the distances from all the electrons in the particle to its center of gravity) can be computed from the equation,

$$\bar{R} = 0.644 \sqrt{\alpha},$$

where $\bar{R}$ is the radius of gyration in A, and α is the slope of the plotted curve relating $\log_{10} I$ and ε^2, ε being measured in radians.

The method of determining particle sizes by low-angle scatter, apart from being sensitive to a difference of electron density between the particle and the surrounding medium, and naturally also being dependent upon the external dimensions of the particle, is nevertheless independent of the internal crystal structure of the particle. If the shape of the particle is known, the real dimensions can be determined from $\bar{R}$, as the calculation of $\bar{R}$ is classic for a particle of given type and size.

Under specific provisions the shape of the low angle scatter will give a rough image of the particle. For example, with a well-oriented system not too densely packed, containing particles all of the same size and having the shape of elongated elipsoids with their long axis parallel, the shape of the low-angle scatter will be similar to the form of the particle, but turned through a 90° angle; provided the scatter is obtained from a symmetrically collimated X-ray beam incident in a direction perpendicular to the long axis of the specimen.

In systems not too densely packed, with particles of irregular sizes, the plot of $\log_{10} I$ against ε^2 will not give a straight line. The total scattered intensity will then be the sum of different scattered intensities, each intensity corresponding to a set of particles of a given size. In the case of these hetero-dispersed systems, curves with concavity turned upwards are obtained. In this case the particle sizes cannot be determined directly. Different methods have been deviced to determine the distribution of the different particle sizes from the experimental curve, under the assumption that the particles are geometrically all similar, but

this problem does not have a unique solution. On the assumption that the particles, independently of size, can have their scatter treated by GUINIER's formula, a qualitative interpretation of the scatter curve can be made. The slope of the curve close to the undeviated X-ray beam will then correspond to the radius of gyration of the largest particles present in the collection, while the inclination of the tail of the curve is representative for particles of a smaller size.

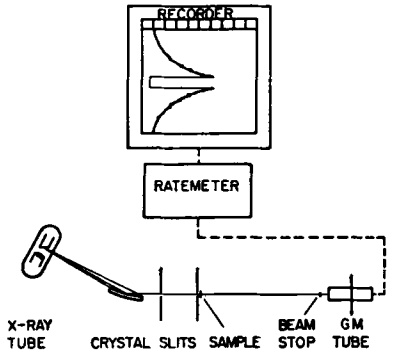


Fig. 7. Schematic representation of the arrangement for slope analysis by small-angle scattering of X-rays.

In the present investigation the low-angle scatter from different powdered specimens of fresh bone and bone implants was studied. The radiation was obtained from a highly stabilized X-ray unit. The X-ray tube had a copper target with a line focus and was run at 42 kV. Pure Cu $K_{\alpha_1\alpha_2}$ radiation ($\lambda = 1.54$ A) was obtained from a curved crystal monochromator of quartz. Two slit apertures (see Fig. 7) defined and collimated the beam, the divergence of which was approximately 0.07°. The slit in front of the specimen had a width of 0.20 mm and its height was 12 mm. The scattered intensities were scanned by the even and slow power-driven motion of a Geiger-Müller tube, the receiving slit of which, having a width of 100 μ, was kept in a plane through the focus of the X-ray beam and perpendicular to its direction. The trace of the direct beam had a width of 250 μ at focus. The pulses from the Geiger-Müller tube were fed to a SC-34 Precision Ratemeter (Tracerlab) and a recorder (Leeds Northrup Speedomax). The number of counts were registered with a time constant of ten seconds. The distance between the sample and the receiving slit in the Geiger-Müller tube was 110 mm. The air scatter was found to be very small as compared to the diffuse scatter from the bone specimens. Measurement of the scatter could then be made in the small angle region outside a distance of 0.8 mm from the trace of the direct beam. To extend the measurements to smaller scattering angles, the scatter was also recorded with the beam stop removed. The scatter from air was recorded, and correction was subsequently made for this parasitic

scatter which was found still to be small as compared with the scatter from the bone specimens (less than five per cent) up to a distance of 0.6 mm from the central beam.

The pulses from the Geiger-Müller tube did not exceed 200 counts per second, thereby corrections for counting losses were obviated.

D. Examination of Collagen in the Polarizing Microscope

In order to determine the orientation of the collagen fibers, the bone transplants were studied in the polarizing microscope.

The specimens were either cross-sections or sagittal sections. By turning the stage of the polarizing microscope, the direction of the optical axis for the collagen fibers in different structures was determined between crossed NICOL prisms, and was referred to the longitudinal direction of the leg. The results from these studies were correlated with the orientation of the bone crystallites as obtained from the same structures by micro-X-ray diffraction.

CHAPTER V

Microradiographic Examination of Bone Transplants and Bone Implants

A. Distribution of Mineral Salts

1. Autogenous Cortical Transplants

In the rabbit series, ground sections from 45 autogenous cortical transplants were studied at intervals ranging from 4 days to 63 weeks after operation.

A microradiogram of a ground cross section from the normal tibial shaft of an adult rabbit reveals a bone tissue mainly composed of lamellae. In this particular type of bone the average difference in mineral content between the various lamellae and the Haversian systems is small in most cases.

A microradiogram of an autogenous cortical transplant with a healing-in time of four days did not show any changed distribution of mineral salts in the transplant nor in the host bone as compared to the normal tibia. Specimens with a healing-in time of eight days showed a callus formation resembling spicules standing out from the surfaces of the host bone and the transplant. This callus formation was usually earliest and most abundant on the endosteal side, but sometimes, especially in young rabbits, was as rich subperiosteally. The deposit of minerals in the callus spicules was relatively high in places and granular in appearance. In Fig. 15 it can be seen that the mineral content in the callus close to the host bone is higher than in the adjacent part of the endosteum. In specimens with a healing-in time of 2—4 weeks, the amount of callus was increased, and a network of trabeculae had formed. In this network, areas with a relatively high amount of mineral salts could be located in the center of the trabeculae. Here the lacunae of the osteocytes were large and had an irregular shape. Resorption cavities were seen within this "primary" callus, and within them bone with a low content of mineral salts had begun to form. This bone often had the shape of Haversian systems, in which the lacunae of the osteocytes were smaller than those of the "primary" callus, and often had their long axis oriented in the direction of the Haversian canal. Changes in mineral content had also taken place in the host bone and within the transplant. A multitude of resorption cavities were seen in the part of the host bone close to the transplant, and in these cavities Haversian systems with a low content of mineral salts were formed. Two weeks after operation a number of resorption cavities had already formed in the transplant, particularly in its periphery. These resorption cavities were the widened canals of preexisting Haversian systems, and within

them the formation of low-mineralized osteons took place quickly. These osteons slowly acquired a higher degree of mineralization, first of all in the part close to the canal and then later centrifugally.

Fig. 18 shows an autogenous cortical transplant seven weeks after operation. Many resorption cavities and some new Haversian systems with a varying degree of mineralization can be seen. In between these newly formed structures there are areas with a high degree of seemingly unchanged mineralization and the highly absorbing characteristic structures of fibrous bone and epiphysial cartilage remnants can still be distinguished. The periosteal callus shows a rich vascular connection with the transplant. The peripheral part of this callus is mostly of the "primary" type with a relatively high amount of mineral salts and with large irregularly sized lacunae.

In specimens with longer healing-in times, a continuous substitution of the original transplant took place, and during the same period rebuilding processes were seen in the periosteal and the endosteal callus (Fig. 16), resulting in longitudinally oriented Haversian systems in the inner part of the callus, whereas the outer part of the callus was lamellar and its periphery usually contained the highest amount of mineral salts. Two rabbits which had received 4—5 autogenous cortical transplants at different heights of the leg, from the metaphysis down to the middle of the shaft, were examined 12 weeks after operation. No major differences in the rate or manner of resorption between these transplants were observed, although these might have been expected considering the different vascularization of the host bone at different heights of the leg. (Compare DE MARNEFFE 1951.)

There was a notable difference, however, in the resorption rate of the transplants in young and old rabbits. For example, in the young rabbits the number of resorption cavities in the host bone and in the transplant were so numerous during the early part of the healing, as to make the bones appear spongious.

The autogenous cortical transplants with a healing-in time of 53 weeks and 64 weeks after operation shown in Figs. 19 and 34 respectively, still show parts of the transplants which have not been absorbed. A great number of Haversian systems with varying mineral content are seen. The callus has not yet reached the high degree of mineralization which can be seen in the host bone and in the remaining parts of the transplants.

2. Homogenous Cortical Transplants

In the rabbit series, ground sections from 25 homogenous cortical transplants were studied at intervals ranging from 8 days to 64 weeks after operation.

The specimens with a healing-in time of eight days showed a callus formation similar in appearance to that of the autogenous cortical transplants. In specimens with a healing-in time of 2—4 weeks the amount of callus had increased,

and showed a network of trabeculae which were commencing to spread over the periosteal and endosteal sides of the transplant. In rabbits having an autogenous and a homogenous transplant placed close to each other, no difference in the amount of callus could be observed.

Two to three weeks after operation resorption cavities were seen mostly in the peripheral parts of the transplant. As in the autogenous transplants, these resorption cavities were the widened canals of the preexisting Haversian systems, and within them formation of low-mineralized bone took place. A comparison between the number of resorption cavities and the amount of mineral salts secondarily put down in the autogenous and in the homogenous cortical transplants did not always disclose a difference at this stage of the healing. Nevertheless, in some of the inspected sections with a healing-in time of 12—28 weeks a difference between the autogenous and the homogenous transplants was noted. In cross sections from the same rabbit, the unabsorbed parts of the transplants were more clearly defined, and fewer areas with low-mineralized bone were observed in the homogenous transplants than in the autogenous. After one year of healing-in time there was no major difference in the amount of Haversian systems put down within the inspected sagittal sections of autogenous and homogenous transplants.

3. Autogenous Cancellous Transplants

In the rabbits a series of ground sections from 14 autogenous cancellous transplants were studied at intervals ranging from 8 days to 64 weeks after operation.

In specimens with a healing-in time of eight days the transplants had a lower content of mineral salts than the host bone. The newly formed callus was most abundant on the endosteal and periosteal side of the host bone, and later coalescing callus spicules were seen to occupy the spaces in between the trabeculae of the transplant. At this stage, 2—3 weeks after operation, the average mineral content in the transplant was higher than in the callus, but the difference was small. Resorption of the trabeculae in the transplant seemed to take place early, and in specimens with a healing-in time of about 16 weeks the resorption of the trabeculae was completed. At this stage, remnants retaining the typical shape of the cortical part of the transplant could be seen within the callus; these remnants had a lower mineralization than the callus. Such remnants at a much later date of 60—64 weeks after operation were few in amount and small in surface area. During this whole observation period, the rebuilding processes in the callus resulted in the formation of Haversian systems containing different amounts of mineral salts, and the majority of the Haversian canals were oriented in the longitudinal direction of the leg. In the peripheral parts of the callus, the rebuilding processes resulted in the formation of lamellar bone which had a structure resembling circumferential lamellae.

In the donor sites from which cortical transplants had been taken, the callus formation extending from the host bone did not fill the defect completely, but otherwise rebuilding processes in the callus followed the same pattern as in cancellous transplants.

4. Homogenous Cancellous Transplants

In the rabbit series, ground sections from 18 homogenous cancellous transplants were studied at intervals of 8 days—64 weeks following operation.

The resorption of the trabeculae of the homogenous cancellous transplants occurred at about the same rate as for the autogenous cancellous transplants. In specimens with a healing-in time of about 16 weeks, the same low-mineralized rectangularly shaped remnants of the cortical part of the transplants were seen in the callus. By comparing specimens of autogenous and homogenous cancellous transplants which had the healing-in time of 60—64 weeks, no discernible difference in the number of remnants of the transplants remaining in the callus could be seen.

5. Frozen Bone Transplants

Ground sections from 14 specimens of frozen bone transplants were examined. The specimens had healing-in times of 8 days—60 weeks, and were cancellous and cortical transplants.

In microradiograms taken after eight days, it was obvious that the comparative content of mineral salts in the transplants and in the host bone was similar to that of fresh transplants. During early healing-in times, the amount of callus that had formed and the rate of resorption were approximately the same for frozen as for fresh homogenous transplants, and the same pattern applied to specimens examined after 21—60 weeks; but in some of these there occurred large resorption cavities within the transplant and the callus, and in the periphery of these cavities there was a narrow zone of low-mineralized bone.

6. Cooked Bone Implants

Ground sections obtained from 14 cooked bone implants were studied over a period of 8 days—60 weeks after operation.

Specimens with a healing-in time of eight days showed a callus formation noticed only on the surfaces of the host bone. The mineral content in the implant was sometimes slightly higher than that of the host bone. Resorption cavities appeared within the preexisting Haversian canals considerably later than for the fresh cortical transplants. A specimen at 27 weeks after operation is shown in Fig. 23, in which it can be seen that low-mineralized Haversian systems are widely spaced, leaving a greater part of the implant unchanged. The cooked bone

implants examined 60—64 weeks after operation had in sagittal sections considerably fewer new Haversian systems than the fresh cortical transplants with the same healing-in time.

7. Os Purum Implants

A series of ground sections from 19 specimens of os purum implants were studied at intervals ranging from 1 to 63 weeks after operation.

In specimens with healing-in times of 1—3 weeks, the central parts of the trabeculae in the implant had a mineralization which was generally lower than that of the host bone. The peripheral parts of the trabeculae showed zones of high X-ray absorption and also streaks of high density traversing the trabeculae. The amount of callus and the time of its appearance did not differ much from that of the other transplants and implants. Evident signs of resorption of the os purum implants were seen in specimens obtained seven weeks after operation. The change in mineralization between the appositional callus and the implant was in some places gradual and in others abrupt. Resorption cavities engaging the callus and the implant were often seen.

Specimens with healing-in times of 12—22 weeks still showed large unresorbed parts of the implants. These remnants often had a high density rim which had an abrupt transition to the relatively low mineral content of the appositional callus (see Fig. 25). In specimens obtained 60—63 weeks after operation, only a slight decrease in the amount of these remnants had occurred (see Fig. 26). The eventual organization of the callus resembled that seen in other cancellous transplants.

8. Glycerol-ashed Bone Implants

Ground sections from 14 specimens of glycerol-ashed bone implants were examined 2—60 weeks after operation.

These implants had a considerably higher absorption of X-rays than the host bone. Callus formed at the same rate as for other implants but there were no signs of resorption until the 13th week, when a few low-mineralized Haversian systems were seen within the implants. Subsequently the resorption rate was very slow. Fig. 27 shows a microradiogram of a glycerol-ashed bone implant obtained 58 weeks after operation. The unresorbed parts of the implant can still be seen to be relatively large. There is a marked difference in mineralization between these remnants and the low-mineralized Haversian systems within them, and this difference also extends to the callus.

9. Calcined Bone Implants

Ground sections from 14 specimens of calcined implants were studied at intervals of 1 to 60 weeks after operation.

Specimens studied 1—3 weeks after operation showed a rich callus formation. The trabeculae of the implant had a very high overall absorption of X-rays, indicating a very high density of mineral salts. In some samples, at seven weeks after operation, signs of a slow resorption of the trabeculae were noted. Figs. 28 *a* and *b* show a microradiogram of a calcined implant obtained 22 weeks after operation. Because of the large difference in mineralization between the implant and the callus Fig. 28 *a* was microphotographed in order to show the mineralization of the callus, and Fig. 28 *b* was taken to show the structures in the implant.

The pictures in Figs. 29 *a* and *b* are from a microradiogram of a specimen with 60 weeks' healing-in time and were taken in the same manner. This and other specimens examined about 60 weeks after operation gave evidence of a very slow resorption rate of the calcined bone implants.

10. Specimens from Dogs

The present study of bone transplants in dogs was performed primarily with the purpose of determining the validity of the ultrastructural findings in the series of rabbits. These findings are given in chapter VI under the heading of "Orientation of bone crystallites".

A ground cross section from a shaft of a long bone of a young adult dog differs greatly from that of a rabbit due to the great diversity of mineralization in its Haversian systems. Fig. 30 *a* was taken from a sagittal section through the center of an autogenous cortical transplant in a dog after ten months' healing-in time. The region illustrated here, showing a multitude of cross-cut Haversian systems with different mineralization belonged to the location of the transplant. This could be confirmed by a method employing measurements of the sagittal length of this site, and comparing it with the inner diameter of the trephine which was used to obtain the transplants; and the results of these two measurements showed approximately the same values. In the upper part of Fig. 30 *a* the Haversian systems have a longitudinal orientation, and their average mineralization was equal to that of the adjacent shaft, and it would seem that this new bone was deposited during the growth of the animal. The short dimension of the same site in Fig. 30 *a* agreed with the approximate thickness of the shaft at the time of operation and it was obtained by measuring the thickness of the bone disc which was removed in preparing the graft bed. In the other specimens of cortical transplants, the short dimension of this site was somewhat reduced, indicating resorption of the transplant from the endosteal side, while the sagittal length measurements agreed with the diameter of the original transplant. No difference

in the average mineralization between the autogenous and the homogenous cortical transplants could be observed in the sections inspected. When fitting the graft into its bed the ridges of the fascial attachment on the cortical part of the *cancellous* transplants had been turned at 90° angle with the longitudinal direction of the leg. Later, the difficulty in locating the cancellous transplants was alleviated to some extent by the presence, in sagittal sections, of cross-cut Haversian systems which originated from the cortical part of the transplant. No other traces of the cancellous transplants were evident, because on either side of the cross-cut Haversian systems the osteons had an alignment in the longitudinal direction of the leg similar to those in the rest of the shaft.

B. Quantitative Determination of Mineral Salts

The two different microphotometers described in chapter III were used for measuring the mineral content in the ground bone specimens. With the Zeiss Schnellphotometer, the content of mineral salts was determined in areas of approximately 900 μ^2 at equidistant points along the edge of the cut specimen at intervals of approximately 100 μ. When dividing the specimen, the cut was placed through areas of the transplant, the host bone and the intermediary callus. In this manner the mineral content was determined in 6—7 different places in the transplant and the host bone (see Fig. 31 *a*). To obtain high accuracy in measuring the mineral content in small callus structures, the high resolution microphotometer was used. With this microphotometer the mineral content was measured in six different points within a selected area in the transplant, the callus and the host bone, respectively. These areas were chosen by inspection of the microradiogram for visual examination, and were selected to represent a place where the average mineralization was fairly homogenous, and where the thickness variations were slight according to the previous thickness determination; and care was taken to avoid the inclusion of rebuilding sites in the transplant and the host bone. Each point measured for its content of mineral salts had an area of approximately 75 μ^2. Measurement of mineral salts using the last-mentioned microphotometer will be referred to as the six-point measurement.

The mineral content was determined in the intermediary callus of 17 autogenous and 9 homogenous cortical transplants which had previously been grafted in 20 rabbits. The mean values of linear absorption coefficients for the bone salt were determined with the six-point measurement and are plotted in Fig. 8 as a function of healing-in time. These mean values from the six determinations with their range of variation are given in Table I. The animals examined were about six months and older at the time of operation, and had several transplants and implants in the same leg.

Table I. Mineralization of the intermediary callus for autogenous and homogenous cortical transplants

Rabbit number	Linear absorption coefficients of the bone salt in callus				Weeks after operation
	Autogenous transpl.		Homogenous transpl.		
	Mean value $n = 6$	Range of obtained values	Mean value $n = 6$	Range of obtained values	
28	—	—	92	91—93	1·3
27	93	89—98	—	—	1·3
30	94	86—99	—	—	2
42	110	107—112	—	—	6·5
45	114	112—117	114	112—116	7
53	107	106—110	—	—	7
41	111	108—113	107	105—110	12
34	114	112—116	—	—	12
49	110	109—117	—	—	16
44	118	116—120	112	110—112	21
39	113	110—116	—	—	22
15	114	110—115	120	119—121	27
16	120	119—123	115	112—120	53
50	119	118—120	—	—	58
51	125	116—127	—	—	60
43	118	117—121	—	—	60
52	126	122—127	118	114—118	60
48	—	—	127	124—133	60
55	118	116—119	—	—	63
20	—	—	122	120—126	64

As seen in Fig. 8, the mineralization increased quickly within the first seven weeks after operation, thereafter the increase was very slow. In order to verify if this slow increase was significantly different from zero, a regression line was computed for the values obtained from the callus of the autogenous transplants which had a healing-in time of 6½—63 weeks. The resulting regression line is plotted in Fig. 8 and the difference of its slope from zero was highly significant ($p < 0.1\ \%$; $n = 17$). A regression line computed for the callus of the homogenous transplants with a healing-in time of 7—64 weeks had approximately the same equation as that obtained for the autogenous transplants. (Equation: $Y = 0.001908\,X + 1.096$.) In order to make a comparison between independent series of autogenous and homogenous transplants, six rabbits (numbers 45, 41, 44, 15, 16 and 52) were excluded from the series of autogenous transplants because they were represented in the homogenous series. The regression line computed for the reduced series of autogenous transplants ($n = 8$) had a somewhat lower inclination than that of the regression line for the series of homogenous transplants ($n = 9$). (Equation: $Y = 0.00175\,X + 1.089$.) To determine if this difference had a systematic cause, a Student's t-test was made on the difference between the values of the respective slopes. The probability that this difference

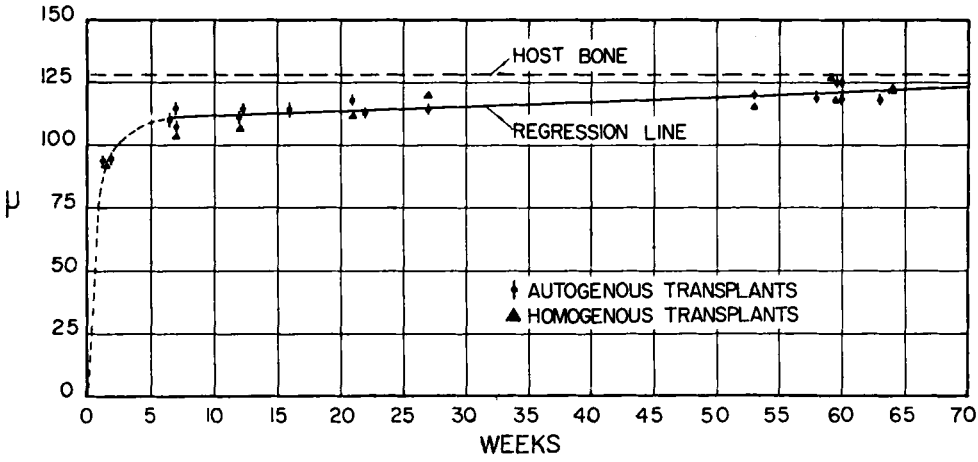


Fig. 8. Mean values of μ with standard deviations, plotted as a function of healing-in time, and representing mineralization in the intermediary callus of autogenous and homogenous cortical transplants. Equation of regression line computed for the mineral content in callus adjacent to autogenous transplants: $Y = 0.001959\,X + 1.095$.

did not have a systematic cause was computed to approximately 85 per cent. In the excluded series a Student's t-test was made on the differences of mineral content in the callus of adjacent autogenous and homogenous transplants ($n = 6$), and the probability that these differences did not have a systematic cause was computed at 25 per cent.

Using the six-point measurement, a total of 18 cross sections taken from the operated legs of 10 rabbits were analyzed within the host bone for their content of mineral salts. The mean value ($n = 10$) with its standard deviation of the average linear absorption coefficient was 128 ± 5. This value represented the average mineral content of highly mineralized host bone in the operated shaft, and it is plotted with a straight broken line in Fig. 8. From a total of six cross sections taken from the unoperated leg of four rabbits, the mean value ($n = 4$) with its standard deviation was 129 ± 3. Assuming that the content of mineral salts in the intermediary callus increases continuously at the same rate as represented by the slope of the regression line in Fig. 8 (the average increase being about 2 per cent in 10 weeks), the time required for the mineral content to reach an average value comparable to that of the tibial shaft was computed at 90 ± 15 weeks. As mentioned before, there is a quicker increase of mineralization during the early months. This could be verified in a rabbit in which the periosteum was stripped twice in the same place on the tibial shaft, with an interval of six weeks between the operations. Six weeks later the animal was sacrificed. A cross section through the tibial shaft showed two adjacent zones of subperiosteal callus with different mineralization. The zones corresponded respectively to 6 and 12 weeks' healing-in time. The mean values with the standard deviations of the linear absorp-

tion coefficients for the bone salt within the respective zones were 110 ± 1 and 114 ± 1 corresponding to an average increase in mineralization of 3.6 ± 1.2 per cent during the last six weeks; or a net increase of bone mineral of approximately 1.2 mg per day in 1 cm^3 of callus.

The mean values of the mineral content in the host bone as obtained by measuring 6—7 different points along the edge with the Zeiss Schnellphotometer were in general about 4 per cent higher, and had 2—3 times larger standard deviations than the values obtained from the six-point measurements; this corresponded with the systematic error for the Zeiss Schnellphotometer previously mentioned (about + 5 per cent see WALLGREN and HOLMSTRAND 1956).

The mean values of μ using the six-point measurement to determine the mineral content in unabsorbed parts of autogenous cortical transplants, varied between 113 and 126 with a mean of 121 ($n = 7$). In relation to the neighbouring host bone these unabsorbed parts contained 2—9 per cent less mineral salts (mean decrease 4 per cent).

The mean values of μ for unabsorbed parts of homogenous cortical transplants varied between 117 and 142 with a mean of 126 ($n = 6$), and in relation to the adjacent host bone these unabsorbed parts had in four of the rabbits 1—6 per cent less mineral content, and in two rabbits the mineral content was respectively 4 and 8 per cent higher.

In the above specimens, which were chosen at random, the determined values of μ in the unabsorbed parts could not be correlated to the healing-in time which extended over a period of 1—53 weeks.

C. Autoradiography with Sr^{90}

About 60 autoradiograms were prepared from specimens which contained different bone transplants and bone implants.

In rabbits injected with Sr^{90} 8—14 days previously the findings were as follows:

Specimens obtained within 2 weeks after operation showed a heavy uptake in the endosteal and periosteal areas of the host bone. A high uptake was also noted in the callus spicules, while the highly mineralized parts of the host bone showed only a diffuse reaction.

In Figs. 32 *a* and *b* are shown a microradiogram and an autoradiogram of an autogenous transplant 12 weeks after operation. In the autoradiogram the "hot" spots in the host bone and in the transplant correspond to the low-mineralized areas seen in the microradiogram. The heavy uptake in the subperiosteal callus is accentuated. The intermediary callus shows only a slight uptake.

In general it was found that the uptake was somewhat higher for the trabeculae in the fresh cancellous transplants, while intact parts of the cortical transplants showed only a slight reaction; and in the implants no reaction was observed.

In both rabbits which were injected at the age of eight weeks, their general growth was retarded, but in other respects the autoradiographic findings were similar to those already mentioned for rabbits injected with Sr^{90} 8—14 days previously. The host bone and the unabsorbed parts of the autogenous and the homogenous transplants showed a slight reactivity, while the uptake was heavy in the low-mineralized Haversian systems within the transplants, thereby indicating a heavier uptake of the mobilized Sr^{90} by the newly formed osteons within the transplants.

It has been shown that a high uptake of Sr^{90} occurs *in vivo* and *in vitro* in low-mineralized areas of bone (ENGFELDT, BJÖRNERSTEDT, CLEMEDSON and ENGSTRÖM 1954) and the uptake therefore follows the same general pattern as that of the radioactive isotopes P^{32} and Ca^{45} (e.g. AMPRINO 1952, ENGFELDT 1953). The results from the present autoradiographic studies are consistent with these findings. No quantitative determinations on the uptake of Sr^{90} were made in the present investigation, but the works of ODELL, MUELLER and KEY (1951), KARCHER (1953), KIEHN and GLOVER (1953) and others have shown that autogenous transplants show the highest uptake of P^{32} and Ca^{45}. The findings of these authors are consistent with the results obtained from the present microradiographic studies, because the uptake by a bone transplant of P^{32} and Ca^{45} is primarily dependent upon its content of areas with a low degree of mineralization, and in the present investigation these areas seemed to be more numerous in the autogenous cortical transplants during the early healing-in period.

CHAPTER VI

Ultrastructural Examination of Bone Transplants and Bone Implants

A. Orientation of Bone Crystallites

In the rabbit series, approximately one hundred X-ray microdiffraction patterns were registered from cross and sagittal sections, mainly from samples with fresh cortical and cancellous bone transplants.

All patterns obtained were identical to the pattern characteristic of hydroxyapatite. In cross-sections of specimens with a healing-in time of eight days, the microdiffraction patterns obtained from the callus spicules had very diffuse lines, indicating a very small crystallite size. The (00·2)-reflection had a faint orientation in the radial direction of the leg. After heating the specimen to 900° C for one hour, the lines in the pattern became sharp due to growth of the crystallites, and the pattern was still of the hydroxyapatite type, and clearly showed that the crystallographic unit cells were mainly radially oriented.

In cross-sections of specimens with a healing-in time of 12 weeks and longer, diffraction patterns registered from places in the periphery of the subperiosteal and endosteal callus, showed an orientation in the (00·2)-reflection indicating a transverse preferential orientation of the unit cells. The same preferred orientation was found in cross-sections from areas in the circumferential lamellae of the host bone.

In specimens with cortical transplants, the callus situated between the transplant and the host bone often showed a radial preferential orientation of the unit cells in cross-sections, as well as in sagittal sections. During the entire observation time in this study, diffractograms registered from *cross-sections* of cortical transplants showed a transverse preferential orientation of the bone crystallites from areas within remnants of the transplant, as well as from areas in the bone substituting the transplant (see Fig. 33).

Diffractograms registered from areas in the callus of *cross-sectioned* specimens with *cancellous* transplants, often showed an unoriented pattern with a faint (00·2)-reflection, indicating an orientation of the crystallites in the longitudinal direction of the leg.

In sagittal sections from specimens taken 12 weeks or later after operation, the microdiffractograms showed a longitudinally preferred orientation of the unit cells in peripheral parts of the subperiosteal and endosteal callus. The same preferential orientation of the bone crystallites was noted in the circumferential lamellae of the host bone and in the host bone itself.

The microdiffraction patterns registered from *sagittal sections* of *cortical* transplants, in areas within remnants of the transplants or in areas of the bone substituting the transplants, were consistent with the findings from cross-sections of cortical transplants, i.e. these patterns had unoriented rings, and the (00·2)-reflection, when not absent was weak, thereby indicating a preferred orientation of the bone crystallites in the transverse direction of the leg (see Fig. 34).

Nevertheless, microdiffractograms registered from the callus in *sagittal sections* of *cancellous* transplants, showed a varying preferred orientation of the unit cells which was often in the *longitudinal* direction of the leg. These results were also obtained from the callus in holes which were left after the taking of transplants.

In conclusion, the cortical and the cancellous transplants showed a difference in their ultimate ultrastructure. The bone replacing the cortical transplants acquired the same ultrastructural organization as the transplant; while on the other hand, the bone crystallites in the bone replacing the cancellous transplants acquired a longitudinally preferred orientation. These results could also be confirmed in microdiffraction studies of cancellous and cortical transplants from dogs. The ultimate ultrastructure of the bone replacing the transplant is therefore influenced by the original arrangement of the transplant and by the type of transplant used.

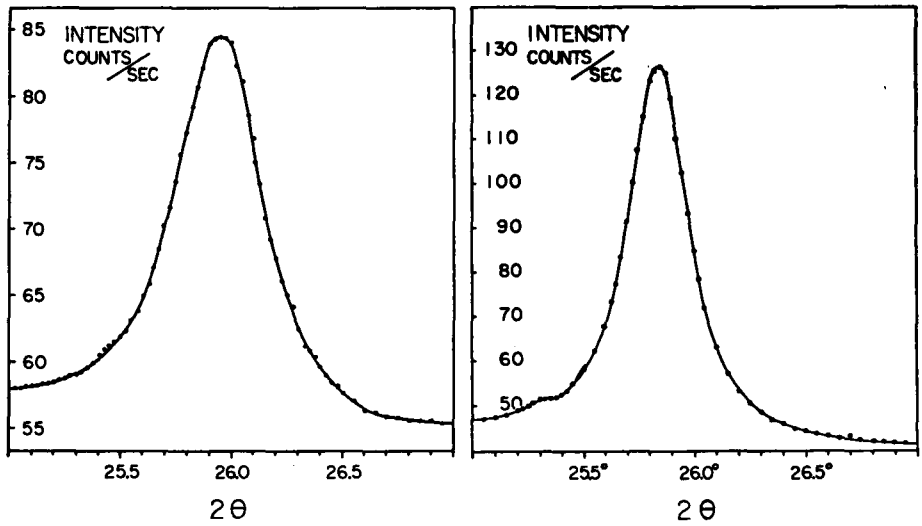
B: Determination of Crystallite Sizes with the X-ray Diffractometer and Geiger-Müller Counter

In Table II are listed the different crystallite sizes determined from the profile of the (00·2)-reflection for different bone specimens which had previously been scanned with the X-ray diffractometer. The patterns obtained from the automatic strip chart recorder were of the hydroxyapatite type in all the specimens examined. The synthetic fluorapatite also listed in the table was used as a reference substance for the computation of the pure line breadth. Some of the line profiles are shown in Figs. 9—13. Every point in these graphs represents 12,800 counts, and is expressed in counts per second. The relative probable error for each point is then approximately 0.6 per cent. The accuracy in measuring the half-maximum width was assumed to be 0.02°. The ratio between the half maximum width of the reference substance and that of the respective samples was low, with the exception of calcined bone. Therefore the precision in determining the pure half-maximum width from the correction curves was assumed to be somewhat better than five per cent (see KLUG and ALEXANDER 1954). The accuracy with which the Scherrer formula (p. 28) can be applied is limited by the uncertainties in β, the pure diffraction broadening, and K, the crystallite shape factor. The value of K depends on many factors such as the shape of the

Table II. Average dimensions along the c-axis for bone crystallites determined from line width measurements of the (00·2)-reflection ($2\Theta = 25.91°$)

Sample	Observed half-max. width	"Pure" width	Particle length (A)
Fresh cortical rabbit bone.....	0.44°	0.36°	215 ± 20
Cooked cortical rabbit bone	0.45°	0.37°	220 ± 20
Human adult cortical bone	0.44°	0.36°	215 ± 20
Human os purum	0.39°	0.30°	270 ± 30
Glycerol-ashed rabbit bone	0.32°	0.22°	380 ± 40
Calcined rabbit bone	0.16°	0.014°	~ 6000
Fluorapatite (reference substance)	0.15°	—	—

crystallites and the adopted definition of D and β. In the present investigation the dimension D is defined as the extension of the crystallite in the direction perpendicular to the reflecting planes (00·2). Under these conditions it has been shown, that, provided β is defined as the half-maximum line width, the value of K is in the neighbourhood of 0.9, and under these conditions there is only a small dependence of K upon the crystallite shape. Because of the residual uncertainty in the value of K, the absolute values for the crystallite sizes, determined in the present investigation, were given with a relative error of ± 10 per cent, with the exception of calcined bone, where the uncertainty was much larger. By comparing the values of the respective half-maximum widths, the uncertainty attached to the absolute values of the crystallite sizes is obviated to some extent. In the present investigation, such a comparison of relative sizes was also justified from the point of view that the samples all belonged to a related series and were examined under approximately uniform conditions.



Figs. 9—10. The profiles of the (00·2)-line of fresh cortical rabbit bone (left) and glycerol-ashed rabbit bone (right).

Nevertheless, in comparing crystallite sizes determined from diffractometer line widths, different factors contributing to the shape and the breadths of the lines have to be considered. Broadened lines are an indiction of either very small crystallite sizes or lattice distortions of some kind, or a combination of both. Lattice distortions originating from internal stress, vacant lattice sites, foreign atoms in substitutional positions and certain kinds of lattice imperfections (WARREN 1941), will have a broadening effect and can also give asymmetry or displacement of the line profiles. The geometrical properties of the diffractometer also modify a pure diffraction profile. The absorption of the X-ray beam can give a pronounced broadening and asymmetry of the profile at lower angles, therefore differences in the line profiles originating from differences in absorption of the X-rays in the samples had to be taken into consideration *(vide infra)*.

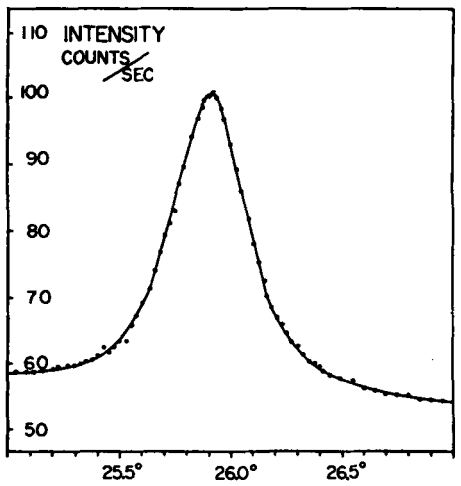


Fig. 11

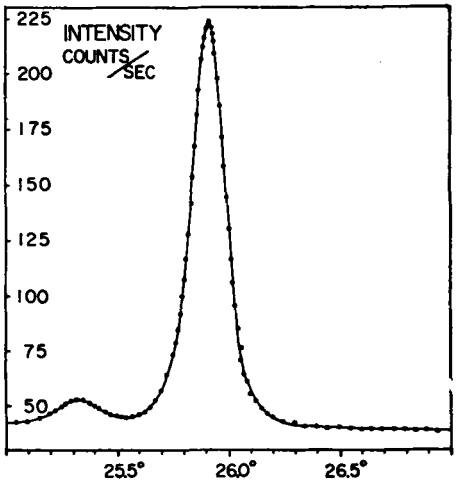


Fig. 12

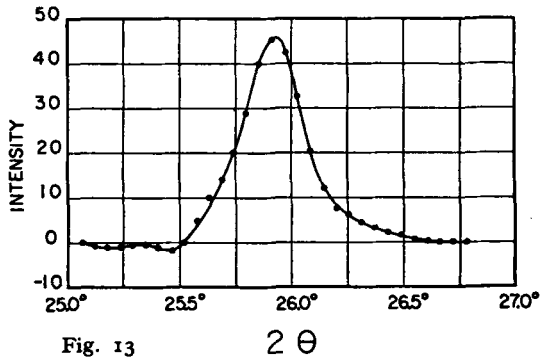


Fig. 13

Figs. 11—13. The profiles of the (00·2)-line of os purum (Fig. 11) and calcined rabbit bone (Fig. 12). In Fig. 13 the pure diffraction profile of os purum as computed by *Fourier* analysis according to *Stokes* from the profiles in Figs. 11 and 12.

Displacement of the line profile can be caused by minute surface imperfections in the mounting of the samples, and it is possible that the small deviations ($< 0.03°$) in the positions of the line maxima for the samples examined originated from surface imperfections.

In the present investigation, the difference in the average particle size between the fresh rabbit bone and the os purum was estimated at 50 A. As it is well known that apatite crystals grow in hot water (HAYEK, MÜLLNER and KOLLER, 1951), it is very probable that this difference can be attributed to a real difference in particle sizes, mindful of the chemical processes in the manufacturing of os purum and especially the sterilization at 110° C. The comparison of the respective profiles with the maximum intensities normalized to the same value, gave a slightly larger breadth of the os purum profile near the base. Provided no lattice distortions were present, this difference in breadth could be interpreted as a size distribution consisting of a small amount of large crystallites, and a large amount of relatively small crystallites in the os purum sample. To determine the shape and the breadth of the pure profile of the os purum implant, a FOURIER analysis according to STOKES' method was made. An unfolding of the observed breadth of the os purum profile was made by reference to the corresponding line profile of calcined bone. The values of the respective diffraction intensities were measured at 30 intervals. The deduced profile is seen in Fig. 13. It has an asymmetrical shape terminating sharply on the low angle side but falling off gradually in intensity on the high angle side. This asymmetry can probably be attributed to a difference in X-ray absorption between the sample and the reference substance. The numerical values for the intensities of the pure profile were checked by folding this profile with the profile of the reference substance. The values of the fold obtained agreed to within 1—3 per cent with the high values of the original os purum profile. The average particle size along the c-axis determined from the integral width of the pure profile of the os purum as obtained by the FOURIER analysis was 245 ± 10 A.

The values for the average particle lengths of the glycerol-ashed bone and the calcined bone respectively, indicate that the heat treatment gave a pronounced increase in the size of the crystallites, but this was not so in the case of the cooked bone, where the heat treatment was inadequate to give an observable increase in the average crystallite size along the c-axis.

C. Bone Crystallites Examined by Small-angle Scattering of X-rays

In Fig. 14 are plotted the curves obtained by measuring the low-angle X-ray scatter of different powdered bone samples. The plotted curves relate the logarithm of the scattered intensity versus the squared scattering angle measured as the distance from the central beam. For all the samples the scattering curves

were curved upwards in such a way that the slope increased with decreasing scattering angle. The tails of the curves were all straight and had a varying inclination. Because of steepness of the curves at small scattering angles, it was not possible by extrapolation of the curves to obtain the slope at zero angle with any accuracy. This was still the case when the stop was removed and correction was made for parasitic scatter. It was therefore not possible to obtain the average radius of gyration for any sample under the conditions used. Nevertheless, the shape of the curves obtained were typical for hetero-dispersed systems containing particles of different sizes, and therefore a qualitative interpretation could be made (see GUINIER and FOURNET 1955). The inclination of the curves at low scattering angles showed an increase which was proportional to the average crystallite size of the respective sample as determined from the wide-angle line width of the (00·2)-reflection. A similar difference was noted in the inclination of the tails of the respective curves. Under the assumption that the particles in the different samples, independently of size, can have their scatter interpreted according to GUINIER'S formula, the following conclusion could be made by comparing the curves: Fresh rabbit bone, cooked bone and os purum contained a mixture of large particles with a large proportion of small particles. The large particles gave rise to the steepness of the curves at low

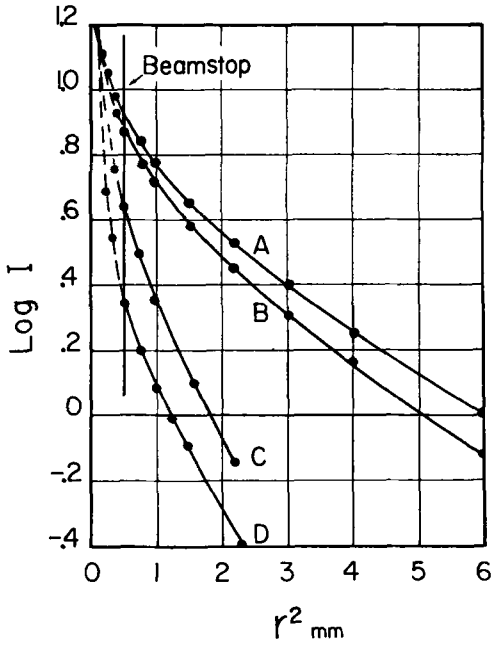


Fig. 14. Diagram relating $\log_{10}$ scattered X-ray intensity versus squared distance from the central beam: A. Fresh cortical rabbit bone. B. Os purum and cooked rabbit bone. C. Glycerol-ashed rabbit bone. D. Calcined bone.

scattering angles, while the small particles gave a comparatively low inclination of the tail of the curves.

By inspection of the curves obtained from the glycerol-ashed bone and the calcined bone, it was concluded that these samples contain many "large" particles and only a minor amount of "small" ones. The possibility of particle interference effects altering the low-angle scatter had to be considered, because the powdered samples examined, especially the glycerol-ashed and the calcined bone, could represent closely packed systems. In case of interparticle interferences, an increase in particle radius would lead to an increase of the smearing of the interference peaks, and would, furthermore, be expected to yield a too low value of the particle radius as obtained from slope measurement at low scattering angles (YUDOWITCH 1949). In this investigation, the slope records were increasing for increasing values of the average particle dimension along the c-axis as determined from the line width measurement of the (00·2)-reflection. Therefore the conclusion was made that variations in the pattern of low-angle scatter for the different samples originated in differences in average particle sizes and their distribution, in accordance with the results obtained from the line width measurements.

D. Orientation of Collagen Fibers

Ground bone specimens were often examined in the polarizing microscope prior to examination by X-ray microdiffraction. This facilitated the choice of areas which were to be chosen for examination by microdiffraction.

After decalcification, the same specimens were examined again with the polarizing microscope and the findings could be summarized in the following manner:

Callus spicules examined in cross-sections from specimens obtained eight days after operation, showed a sparse amount of collagen fibers oriented radially. In cross-sections of cortical transplants, the collagen fibers of the bone deposited within the widened lumens of the preexisting Haversian canals had the same orientation as the collagen fibers in the original transplant. During early healing-in times the collagen fibers occupying the spaces in between the trabeculae of a cancellous transplant were often parallel with the surface of the trabeculae. But later the collagen fibers were arranged in intertwining bundles in the longitudinal direction of the leg. The subperiosteal and endosteal callus contained parallel collagen fibers, which in cross-sections were arranged transversely, but in sagittal sections they were arranged longitudinally.

The orientation of the collagen fibers, as examined with the polarizing microscope, therefore corresponded to the preferred orientation of the bone crystallites as determined by X-ray microdiffraction (see chapter VI: A).

CHAPTER VII

Discussion and Conclusions

The close relationship existing between the mineral crystallites and the collagen in bone was first observed by W. J. SCHMIDT (1923 and 1932) using the polarizing microscope. His views have been confirmed with X-ray diffraction techniques and with the electronmicroscope. The orientation of the crystallites in bone was first detected by X-ray diffraction patterns of long bones by CLARK (1931), who showed that the fiber axis was parallel to the long direction of the bone. STÜHLER (1936) confirmed these findings and showed that the direction of the c-axis of the crystallites was parallel to the collagen fibers. By FOURIER analysis of the (00·2)-line, the dimension of the crystallites in the direction of the c-axis was estimated by CARLSTRÖM (1955) to 230 ± 20 A. By interpreting the diffuse low-angle scatter of X-rays from bone tissue according to the theory of independent particle scatter, ENGSTRÖM and FINEAN (1953), FINEAN and ENGSTRÖM (1953) and CARLSTRÖM and FINEAN (1954) showed that the bone crystallites were elongated, 40—75 A wide, and about 200 A long, and aligned along the collagen fibers in such a way that precise crystallographic planes were produced at sites where they coincided with the fundamental period of collagen at 640 A.

A confirmation of these findings has recently been obtained by electronmicroscopic studies on intact bone, by FERNÁNDEZ-MORÁN and ENGSTRÖM (1957), who found that the apatite particles in bone are rod- or needle-shaped with an average length of 200 A and a diameter of 30—60 A, and arranged along the collagen fibers in a highly ordered manner.

In recent years, special attention has been paid to the tremendous specific surface area of the bone salt, which is due to the extreme smallness of the bone crystallites, and which can explain some physical and chemical properties of the bone tissue; for example the high rate of ion exchange and the uptake of radioactive isotopes (NEUMAN and MULRYAN 1950; NEUMAN and NEUMAN 1953 and CARLSTRÖM 1955). Special interest has been directed to the fixation of the bone-seeking elements produced by nuclear fissions, such as those in atomic explosions. It has been shown that the uptake of radioactive isotopes is greatest in low-mineralized areas in a compact bone, and in the trabeculae of cancellous bone where the rebuilding rate is normally high (ENGFELDT, ENGSTRÖM and ZETTERSTRÖM 1952; ENGFELDT, BJÖRNERSTEDT, CLEMEDSON and ENGSTRÖM 1954). These studies indicate that in these low-mineralized areas there is probably a much larger surface area of the bone crystallites in contact with the aqueous phase than in highly mineralized mature bone (CARLSTRÖM 1955).

The present work has been based on biophysical methods, and from the findings the following conclusions are made:

Microradiography used for visual examination of cortical bone transplants shows that they all have a similar manner of healing. Demineralization processes take place in conjunction with the deposition of low-mineralized bone, which in turn slowly acquires a higher degree of mineral content. These processes are therefore similar to the rebuilding seen in a normal cortical bone (AMPRINO and ENGSTRÖM 1952), but they occur in a much exaggerated manner. During and up to about 28 weeks after operation, autogenous cortical transplants were substituted more quickly than homogenous, but at a later period there was no observable difference between them. The great variations in mineralization of unresorbed parts of the autogenous and homogenous cortical transplants can be explained by the fact that the mineral content varies in different parts of the leg in a rabbit (OWEN 1956) and therefore the transplants taken from the leg might originally contain a varying amount of mineral salts. The difference between the mean values of mineralization for the unresorbed parts of the respective series of autogenous and homogenous transplants was insignificant.

The cancellous transplants have a different manner of healing from that of cortical bone. The difference lies principally in the way in which the callus invades the transplant, and the eventual bearing this has upon the ultrastructure of the callus. Results from the microdiffraction studies led to the conclusion that the structure of the original transplant had a decisive influence on the ultrastructure of the substituting bone.

An interpretation of these findings can be reached in terms of the direction of the ingrowing vessels into the transplant. The preferred orientation of the bone crystallites in a Haversian system in a long bone is in the longitudinal direction of the leg, i.e. in the same direction as the vessel in the Haversian canal. In a cortical transplant the vessels in the intruding granulation tissue show a preference to travel along the preexisting Haversian canals. The same intrinsic factors as in normal growth might then determine the preferred orientation of the bone crystallites in the newly deposited bone, whereby an identical orientation to that of the transplant is achieved. In the cortical transplants, conversion from this abnormal vascular pattern towards the normal was not observed during the whole investigation.

The vessels growing into a cancellous transplant are not limited to one particular direction in order to achieve access to the operated area, and this fact can explain the restoration of the original longitudinal vascular pattern (compare RUBASCHEWA and PRIVES 1932), and thereby also the preferred longitudinal orientation of the bone crystallites in the callus.

From the microradiographic studies it can also be concluded that the manner in which cortical and cancellous *implants* heal is the same, respectively as that of fresh cortical and cancellous *transplants*.

The response of the compact and cancellous bones to mechanical forces has been extensively investigated. That a correlation exists between the amount of osseous tissue formed, and the magnitude of mechanical forces has been demonstrated for example by AMPRINO (1938) and GILLESPIE (1954). Interpretations of the arrangement of the trabeculae in cancellous bone in terms of stress lines (the trajectorial theory) are classical (WOLFF 1892, ROUX 1895, KOCH 1917), but this theory has been subjected to vigorous debate, especially by JANSEN (1920). Recently HIRSCH (1956) has made the interesting observation that cancellous bone does not contribute greatly to the mechanical stability in autopsy specimens of the neck of human femora.

Contradictory results have also been obtained when applying mechanical principles to structures* of 2nd, 3rd and 4th orders in bones. According to GEBHARDT'S opinion (1901 and 1906) structures of 2nd and 3rd orders were deposited in such a way as to yield the best functional resistance of bone to mechanical forces, thereby applying ROUX'S principles on the microscopic structures in bone. PETERSEN (1930) offered a theory for the correlation between the effect of external stress and structures of 2nd—4th orders in bone. Other investigators found no effect of mechanical factors on structures of 2nd and 3rd orders [AMPRINO (1938) and VIGLIANI (1955)]. LAMARQUE (1943) found that the orientation of bone crystallites was independent of the mechanical forces working on different parts in a human femur. ENGSTRÖM and AMPRINO (1950) examined bone structures of 2nd and 4th orders on dogs by microradiography and X-ray diffraction, and they found no difference between active and immobilized limbs.

In the present study, the experimental arrangement of the cortical transplants provided the opportunity to study the influence of mechanical factors on the ultrastructural organization of the substituting bone. The results obtained during the whole observation time of specimens examined in the polarizing microscope, by microradiography and X-ray microdiffraction, all pointed to the replacing bone assuming the same ultrastructure as the original transplant. Therefore the conclusion is made that, contrary to the principles of WOLFF and ROUX, the reorganization of structures of 2nd (Haversian osteons), 3rd and 4th orders *within* a cortical transplant are not dependent upon mechanical factors as stated, e.g., by STREISSLER (1911), KORNEW (1929), and REYNOLDS and OLIVER (1950).

Other investigators (e.g. BULL 1928 and DE JOSSELIN DE JONG and EYKMAN VAN DER KEMP 1928) have reported an almost complete restitution of the operated area within one year after operation. This cannot be confirmed from the present investigation. From the quantitative determinations of minerals in the callus it was concluded that the "complete" acquisition of minerals is a very slow process. The time for equalization of mineral content between the host bone and the

* This nomenclature, applied to different structures of bone, is that of PETERSEN (1930).

operated area was computed at an average of 90 weeks. No significant difference in the calcification of the intermediary callus for autogenous or homogenous cortical transplants could be observed, and it would seem that the initiation and continued maturation of callus is a process which is related to the skeletal trauma and to some extent is independent of the type of transplant used.

Independently of the type of transplant, the subperiosteal and the endosteal callus become the reconstructed circumferential lamellae and assume the latter's appearance and ultrastructural organization. In these callus structures, the mineral content was highest in the periphery, and a parallel might be deduced from the mineralization of Haversian systems. In these, the increase of calcification takes place at first close to the Haversian canal, and later the increase is centrifugal (AMPRINO and ENGSTRÖM 1952). The high calcification of the reconstructed circumferential lamellae in their peripheral parts can be interpreted accordingly, i.e. the calcification is derived from the vessels in the periosteum and the marrow cavity. Changes in bone salt metabolism take place to a considerable extent in the host bone and especially in that part adjacent to the operated area. Formation of resorption cavities in these areas means a decrease of mineral content which is restituted by new Haversian systems which slowly mature. The quantitative determinations of the average content of mineral salts in the evenly mineralized areas of the host bone in the operated leg were similar to the determinations made in the unoperated leg. The increased amount of rebuilding sites in a leg associated with skeletal trauma have been interpreted as resulting from hyperaemia (LEXER 1922 and REMÉ 1953). According to REMÉ (examining rabbits) an increased amount of rebuilding sites could also be seen in the intact leg. Findings in accordance with this view could also be noted in some specimens in the present studies (see Fig. 17).

The suitability of a bone transplant can be defined in terms of the speed with which the transplant is resorbed and followed by an immediate deposition of new bone. In the present investigation the trabeculae of the fresh and frozen cancellous transplants showed a much higher resorption rate than the compact transplants. Normally the trabeculæ in cancellous bone are in the process of being mineralized as shown by the high uptake of radioactive isotopes, while on the other hand highly mineralized compact bone has a low uptake of isotopes. Therefore, there seems to be an inverse ratio between the resorption speed of a fresh transplant and its degree of mineralization which is due basically to a difference in its ultrastructural organization. In young callus tissue the rebuilding rate is high, exhibited by its high uptake of isotopes (see Fig. 32 *b*), and the excellent results obtained by using young callus tissue for grafting purposes in the form of os novum according to ORELL (1934) and STARK (1941) are in accordance with the above view.

The viability of transplants has been vigorously debated. It has, however, been shown that the osteocytes are very susceptible to changes in circulation

(HELLSTADIUS 1942 and MÜLLER 1926) and it may be considered settled that the majority of the cells in a compact bone die when transplanted (HAM and HARRIS 1956). One of the reasons for the previous debates pertaining to the presumed survival of a compact transplant, might have been caused by the simulated survival of the transplant, due to the true imitation of the original by the substituting bone, as has been shown in this work to be the case for compact transplants.

Histological studies of bone transplants have long revealed the differences in cellular patterns and resorption rates associated with the healing of fresh and dead bones (H. ENGSTRÖM 1937, KIND 1951; for a review see WILTON 1937). From the present microradiographic studies of the healing of implants and fresh transplants the following conclusions can be made:

By comparing the resorption rates of cortical transplants and implants, it was noted that the autogenous transplants showed during the early post-operative period (up to 28 weeks) a faster resorption and substitution than did the homogenous and frozen transplants. Definitely slower resorption rates were manifested by glycerol-ashed and cooked bone implants. Comparison between various cancellous transplants and implants showed that fresh and frozen transplants were equally resorbed, but in the case of os purum and especially calcined bone the rate of resorption was much slower. These comparisons show that differences in resorption rates are inherent in a dissimilarity between the organic or inorganic fractions in the transplanted or implanted bones. The manufacturing of os purum tended to dissipate connective tissue from the bone in order to make it more penetrable to invading granulation tissue (ORELL 1934), and the addition of enzymes was used to partially dissolve collagen (ASKELÖF 1956). In the present work, the average particle size determinations for os purum indicated an increase as compared with that of untreated rabbit and human bone; and the microradiographic study showed that the mineral salts in the os purum implants were concentrated to the periphery of the trabeculae. The growth of crystallites is most likely to have occurred in these dense parts of the implant as the heat treatment and the removal of collagen by enzymes, (the latter being active on the surface of the trabeculae), could jointly be responsible for the increase of average crystallite sizes in the os purum. From the microradiograms of os purum remnants, it seemed as if the resorption of the implant was halted at the dense parts of the trabeculae.

To remove the organic part of bone without changing the inorganic fraction has not proved possible. Glycerol-ashed bone is, for example, not accurately representative of the original bone salt (BURNS and HENDERSON 1935). Furthermore a prolonged heating at high temperatures causes growth of the crystallites and encourages reactions between the apatite and ions outside the lattice, resulting in a different composition of the crystallite lattice (CARLSTRÖM 1955). Irrespective of the molecular composition of the inorganic fraction of the bone implants

examined in this investigation, resorption however took place. In the case of glycerol-ashed bone and calcined bone the slowness of resorption was strongly manifested. For these implants the retardation might have a bearing on the specific surface area of the crystallites, as determinations by the gas adsorption technique has shown that, without interference of organic matter, the specific surface area will decrease with increasing crystallite sizes (WOOD 1947 and NEUMAN and NEUMAN 1953).

For the cooked bone implants, the mechanism of resorption was extensively retarded, and this might be due to the change in the ultrastructural organization of the bone resulting from its treatment. Apart from coagulation of proteins, the cooking will convert part of the collagen in the transplant to gelatin. Although this differs only slightly in its chemical composition from collagen, it nevertheless differs greatly in its physical properties (GUSTAVSON 1956). Although there was no notable increase in the average size of the crystallites, the ultrastructural organization between collagen and the bone crystallites in the cooked bone implants was in all probability upset.

In conclusion, the resorption and the substitution of bone transplants is evidently intimately connected with the individual ultrastructural organization of the transplants. In the preservation of bone by freezing, the molecular composition of bone does not seem to be changed to the extent that the processes of resorption and replacement are qualitatively different from the healing of fresh transplants.

CHAPTER VIII

Summary

1. Biophysical techniques, including contact microradiography, X-ray diffraction techniques, the use of the polarizing microscope and autoradiography with Sr^{90} were utilized in the study of about 180 bone transplants and bone implants in a series of 56 rabbits and in two dogs. The study was extended over a period of 64 weeks following operation. The circular transplants and implants were fitted into the tibia, and the fresh cortical transplants were turned at a 90° angle with the longitudinal direction of the leg. The different bone transplants were fresh autogenous and homogenous cortical, fresh autogenous and homogenous cancellous, and frozen cortical and cancellous stored at — 20° C for 3 days to 18 weeks. The implants were cooked bone, os purum, glycerol-ashed bone and calcined bone. A short description is given of the preparation of the specimens, including a grinding technique which was used to obtain evenly ground bone specimens quickly.

2. A short review is given of the microradiographic technique as applied to the study of the relative amounts of mineral salts by visual inspection. The accuracy of this technique with respect to different sample transmissions and photographic densities is considered. It is shown that, under the conditions used, differences in mineralization of about 1 per cent are visually detectable. An account is given of a method for quantitative determinations of mineral salts in bone samples by combining the densitometric measurement of the X-ray absorption in the sample with the thickness determination of the same sample made by using a profile microscope. Different sources of error pertaining to this method are discussed. The relative standard error in mass determinations in surface areas of 900 μ^2 was determined by using a quartz wedge as a test object and was shown to be respectively 1.6 per cent or 3.25 per cent, depending upon whether the thickness was measured on easily defined structures or along equidistant points. By using a high resolution microphotometer in analyzing areas of 75 μ^2, accuracy of the same order was obtained in the mass determinations. Experimental evaluation of the effect of stray light on the densitometric measurements utilizing the high resolution microphotometer was made.

3. A short summary of different X-ray diffraction techniques with some technical aspects is given, which includes the X-ray microdiffraction analysis using the Chesley camera and also the determination of crystallite sizes using the Geiger-Müller counter X-ray diffractometer technique and the slope analysis technique of the small-angle scattering of X-rays.

4. By using microradiography for visual inspection, a detailed description of the changes in mineralization and the rebuilding processes associated with the healing of the different transplants and implants is given for different healing-in times ranging approximately from 1—64 weeks following operation, and special account is taken of the rate of resorption of the transplants and implants. It is concluded that the manner of healing for cortical transplants and implants was similar, but in their resorption and substitution with new bone within the preexisting Haversian canals they differed. The relative resorption-substitution rate was in the following order: autogenous, homogenous and frozen bone, while for cooked and glycerol-ashed bone the rate was very slow.

The healing of cancellous transplants and implants followed a different pattern and took place by callus occupying the spaces between the trabeculae which were in turn resorbed. Rebuilding processes in the callus, with an average increase of mineralization, resulted in the operated area assuming the appearance of cortical bone. The relative resorption rates were about the same for autogenous, homogenous and frozen bone, but rather slow for os purum and extremely slow for calcined bone.

5. Quantitative determinations of the amount of minerals in the intermediary callus for a series of 17 autogenous transplants showed an average increase in the mineralization of about 2 per cent in 10 weeks, and the average time for "full" mineralization of the callus in the rabbit series examined was computed at 90 weeks. No significant difference in the mineral content of the callus for autogenous and homogenous transplants was obtained. The mineralization of unresorbed parts of the transplants showed major variations. Apart from rebuilding sites in the host bone close to the transplant, the average content of mineral salts in the evenly mineralized areas of the host bone did not undergo any changes as compared with determinations made in the unoperated leg.

6. With the X-ray microdiffraction technique, rebuilding processes associated with the healing of the transplants could be followed on the molecular level. The results pointed to the substituting bone assuming the same ultrastructure as a cortical transplant, but with cancellous transplants the bone crystallites in the callus acquired a longitudinally preferred orientation. An interpretation of these findings in terms of the direction of the ingrowing vessels into the transplants is made, and it is concluded that the ultrastructural organization of the bone substituting a transplant is independent of mechanical forces. Studies of the collagen fibers in the polarizing microscope showed that the same rule also applies for structures of 3rd order.

7. From line-width measurements of the (00·2)-reflection using the X-ray diffractometer technique, the dimensions along the c-axis, for the different bone samples examined were the following: fresh and cooked cortical rabbit bone and human adult cortical bone approximately 220 A, os purum 270 A, glycerol-ashed bone 380 A and calcined bone 6,000 A.

The average crystallite size determined for os purum using FOURIER analysis was 245 ± 10 A. The relative particle sizes for the above specimens as obtained from a qualitative interpretation of the small-angle X-ray scattering curves were in accordance with the dimensions along the c-axis obtained from the line-width measurements.

8. Combining the microradiographic findings with the particle-size determinations of the bone specimens, it is concluded that large crystallite units in the os purum implants situated in the periphery of the trabeculae might be responsible for the slow resorption rate of this implant, while the changed ultrastructural organization with a decrease in specific surface area of the bone crystallites might have a bearing on the slow resorption rate of glycerol-ashed and calcined bones.

A direct relationship seemed to exist between the uptake of radioactive isotopes and the resorption rate of a transplant, due basically to a specificity in the ultrastructural organization.

By cooking bone, the ultrastructural disorganization resulted in retarding the resorption, while by freezing bone the resorption rate was approximately the same as for fresh bone.

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[1] With bibliography on bone transplantations up to 1909.

Plates

(Figures 15 to 34)

Figure 15 (top left)

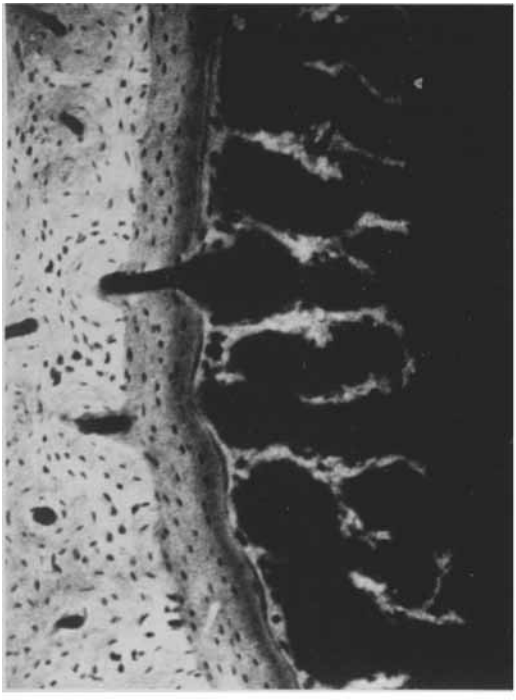
Microradiogram of a 80 μ thick cross-section through the endosteal part of the host bone adjacent to an autogenous cortical bone transplant in a rabbit 8 days after operation. The callus spicules standing out perpendicularly from the endosteal surface show in places a rather high deposit of mineral salts. Recorded at 12 kV. Magnification 138 x.

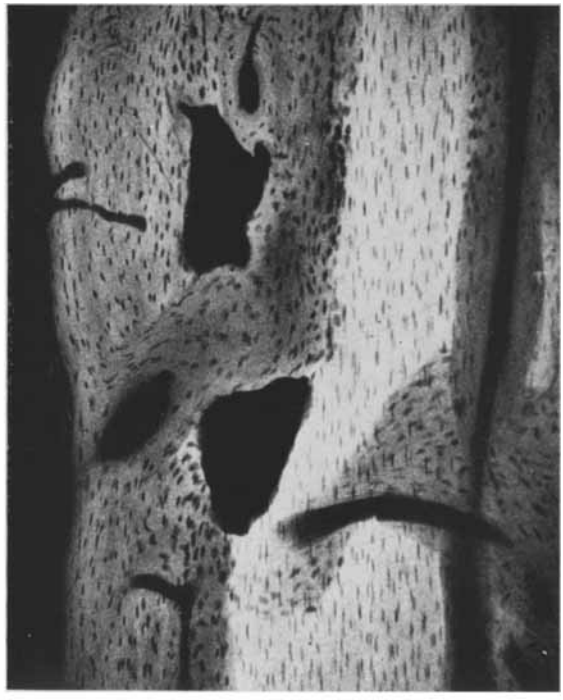
Figure 16 (top right)

Microradiogram of a 70 μ thick cross-section through the peripheral part of an autogenous cortical transplant in a rabbit 12 weeks after operation. Rebuilding processes are seen in the periosteal part of the callus to the left in the picture. The highly mineralized bone to the right in the picture is part of the original transplant and to the extreme right is a newly deposited Haversian system with a low mineral content. This Fig. is a larger magnification of the right upper hand corner of the specimen shown in Fig. 32 a. Recorded at 12 kV. Magnification 114 x.

Figure 17

Microradiogram of a 75 μ thick cross-section through the middle of the shaft of the unoperated tibia in a rabbit 12 weeks after operation. Rebuilding sites are shown containing a varying amount of mineral salts within the different Haversian systems. Adult rabbit. Recorded at 12 kV. Magnification 100 x.





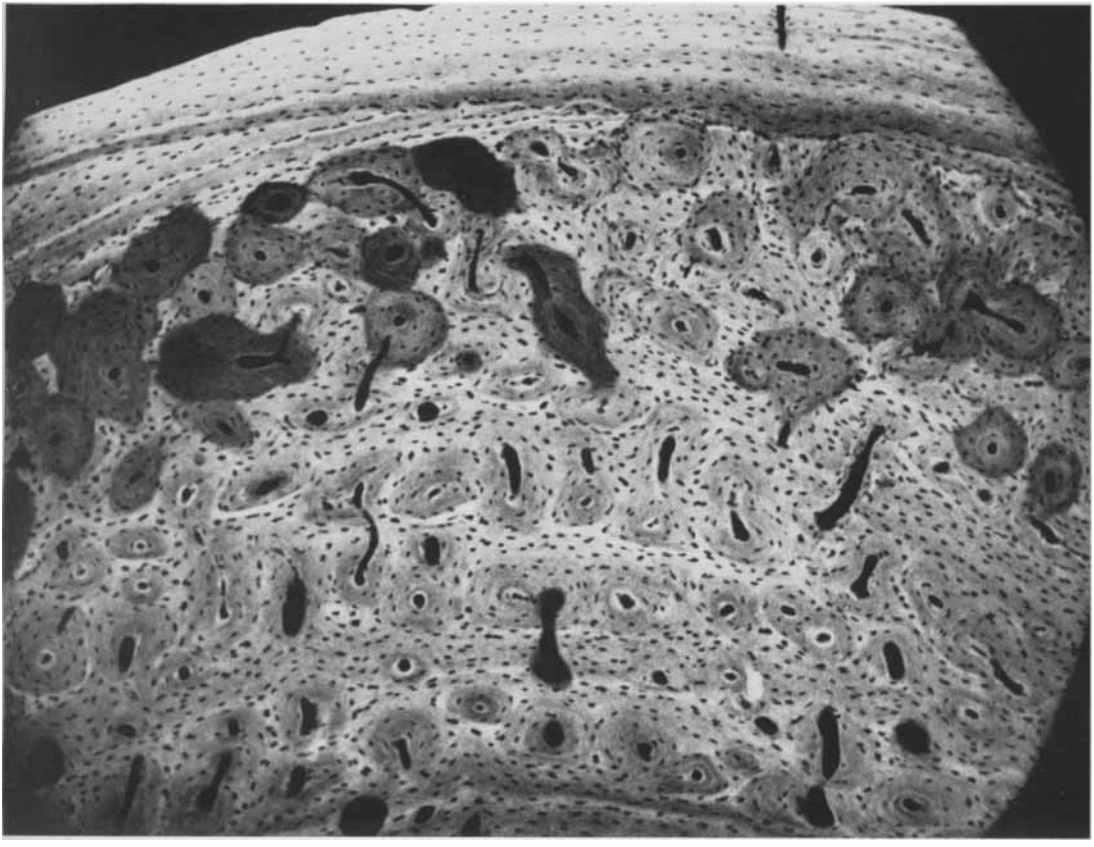


Figure 18 (left)

Microradiogram of a 75 μ thick sagittal section through an autogenous cortical transplant in a rabbit 7 weeks after operation. To the left in the picture is the periosteal callus with a low mineral content. A great number of resorption cavities and some newly deposited young Haversian systems are seen in the transplant (center) and in the host bone (top and bottom). Young rabbit. Registered at 12 kV. Magnification 38 x.

Figure 19 (right)

Microradiogram of a 80 μ thick sagittal section through an autogenous cortical transplant in a rabbit 53 weeks after operation. In the center of the picture are highly mineralized remnants of the transplant, larger part of which is replaced by osteons having a varying mineralization. To the left in the picture is the periosteal callus with a high mineralization in its periphery. In the top of the picture is the host bone which has a considerably higher absorption than the intermediary callus below it. Registered at 12 kV. Magnification 38 x.

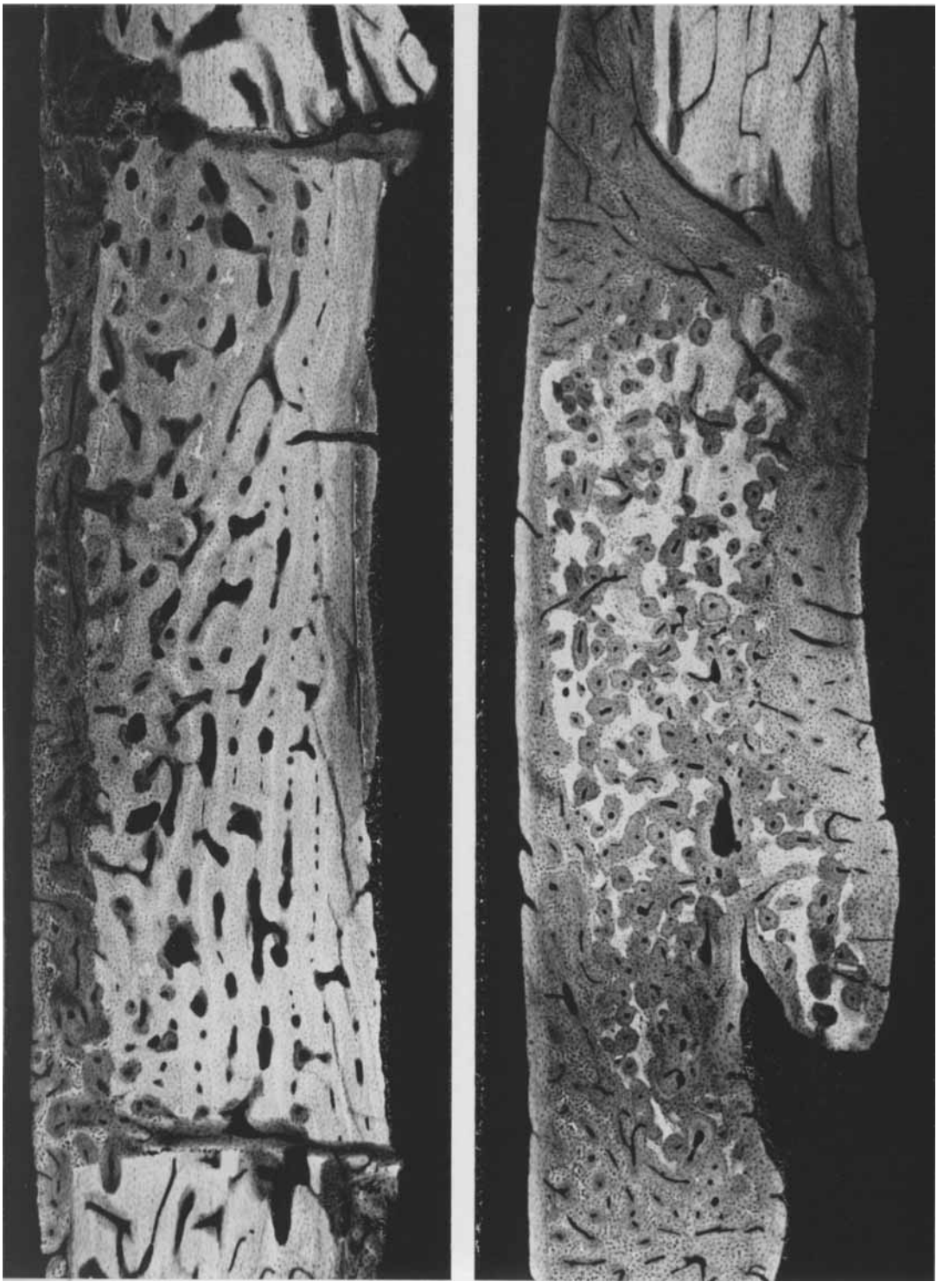
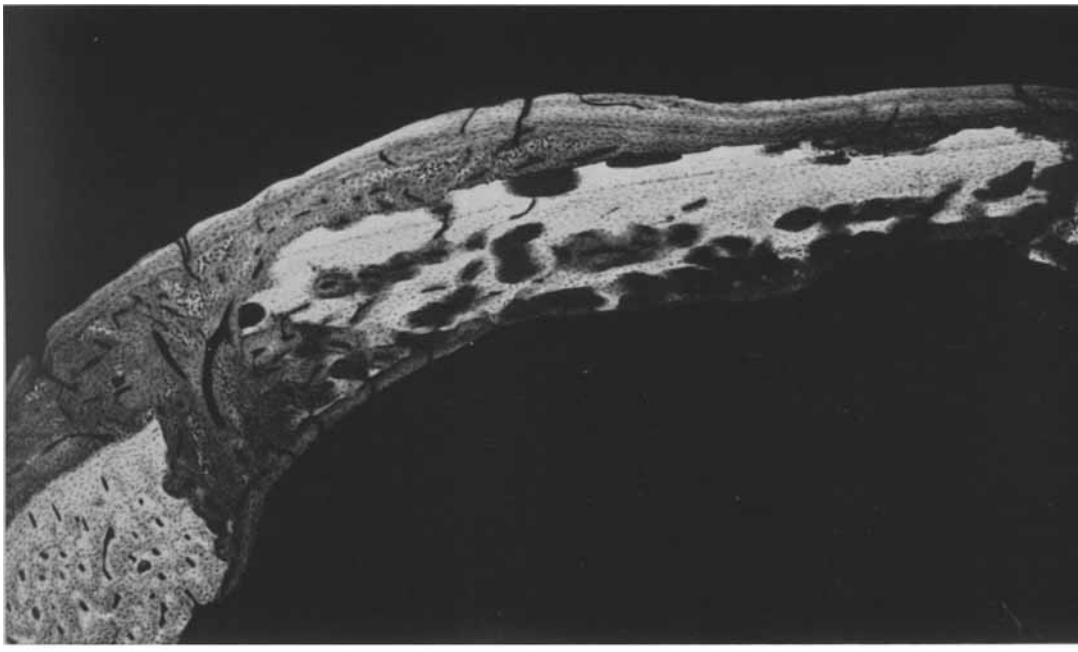


Figure 20

Microradiogram of a 80 μ thick cross-section through a homogenous cortical transplant in a rabbit 28 weeks after operation. Host bone in lower left corner. The transplant (center) is highly mineralized and part of it is replaced by low-mineralized osteons. X-ray microdiffractograms, which were recorded from remnants of the transplant and from the newly deposited osteons, showed a similar asymmetrical arcing of the (00.2)-reflection, indicating that the preferred orientation of the bone crystallites deviated from the transverse direction, because the transplant had not been turned at a full 90° angle (compare Fig. 33). Microradiogram was recorded at 12 kV. Magnification 40 x.

Figure 21

Microradiogram of a 75 μ thick sagittal section through a homogenous cortical transplant in a rabbit 63 weeks after operation. The original transplant shows a high mineral content, and interspersed throughout it are many osteons with varying mineralization. The upper right hand corner of the picture shows part of the periosteal callus. Recorded at 12 kV. Magnification 140 x.



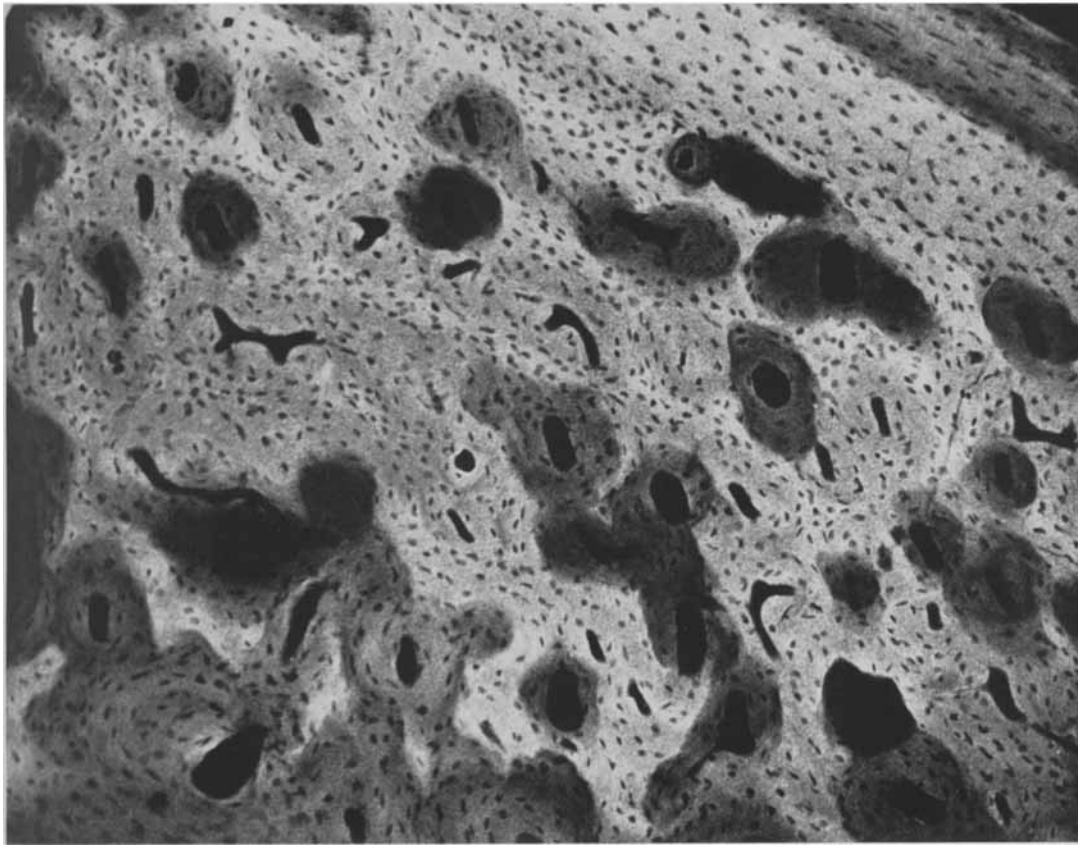


Figure 22

Microradiogram of a 70 μ thick cross-section through a homogenous cancellous transplant in a rabbit 7 weeks after operation. At the left in the picture is the host bone with a multitude of rebuilding sites and the center of the picture shows remnants of the cortical part of the cancellous transplant which have a lower mineralization than the endosteal and periosteal callus. Recorded at 12 kV. Magnification 36 x.

Figure 23

Microradiogram of a 70 μ thick cross section through a cooked bone implant in a rabbit 27 weeks after operation. Only a few newly formed osteons are seen in the implant (center) and its contours are clearly discernable. Recorded at 12 kV. Magnification 41 x.

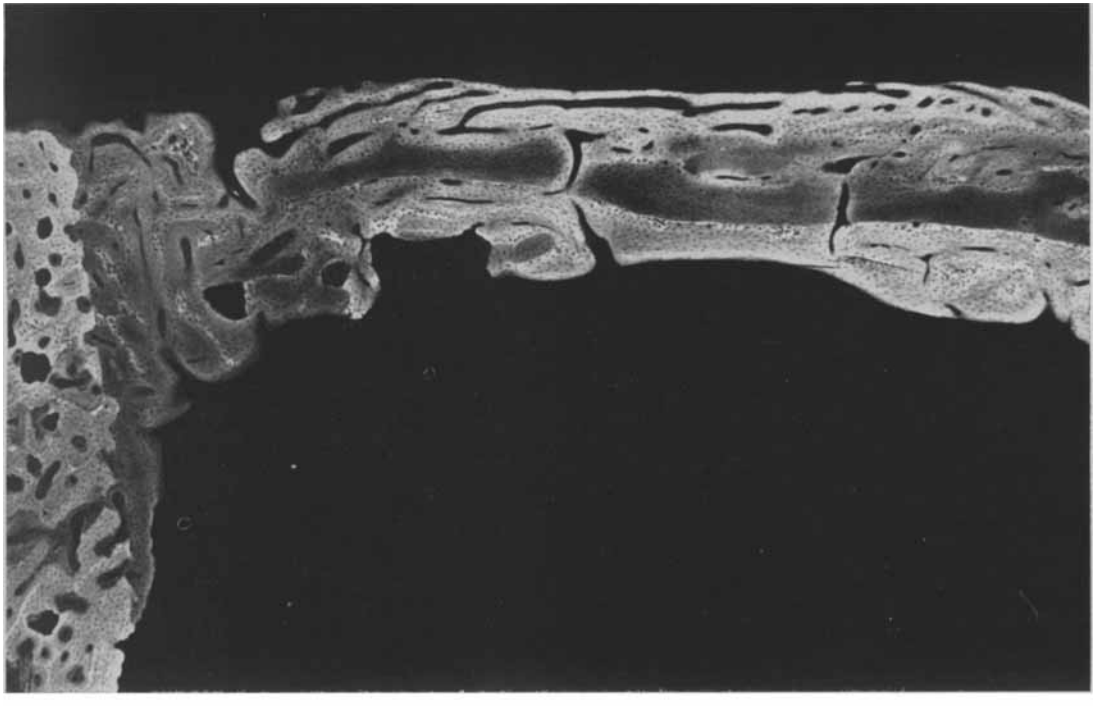


Figure 24 a

Microradiogram of a 75 μ thick cross-section of a frozen cancellous transplant in a rabbit 21 weeks after operation. The transplant was inserted with its cortical side towards the marrow. The elongated dark area in the center of the picture is low-mineralized cortical remnants of the transplant. Most of its trabeculae have been resorbed, and in their place can be seen a callus with many cross-cut Haversian systems. The reconstructed circumferential lamellae are well developed. Registered at 12 kV. Magnification 35 x.

Figure 24 b

Detail of the microradiogram in Fig. 24 a indicated by square, showing rebuilding processes in the host bone adjacent to the healed defect. Magnification 112 x.

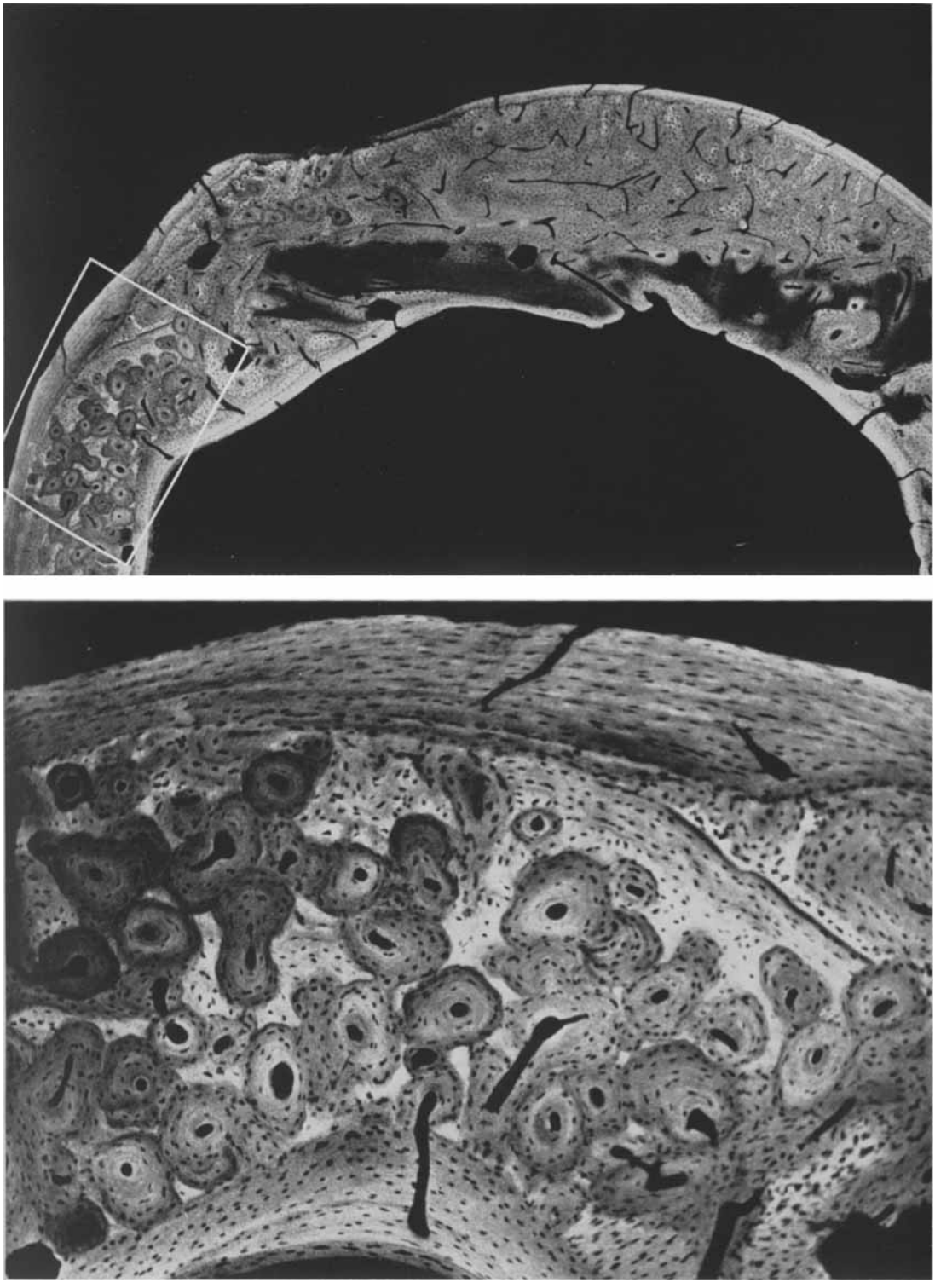


Figure 25 (top left)

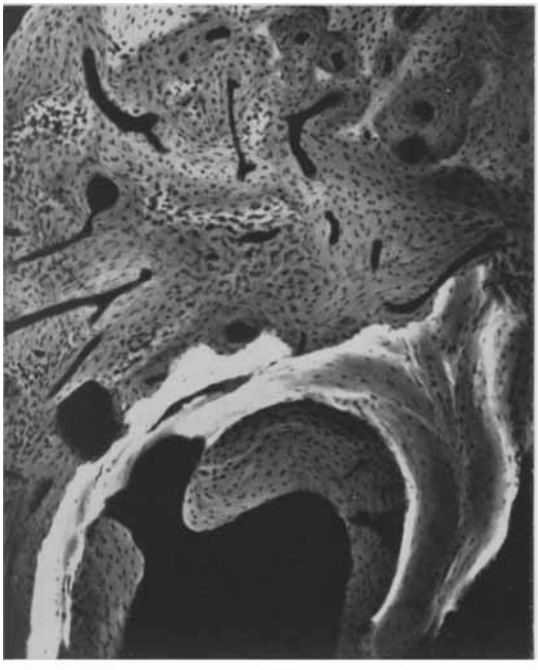
Microradiogram of a 70 μ thick cross-section of a specimen with an os purum implant in a rabbit 12 weeks after operation. In the lower right corner is seen the typical peripheral density of an os purum trabecula. Part of the host bone can be seen in the upper part of the picture. Recorded at 12 kV. Magnification 72 x.

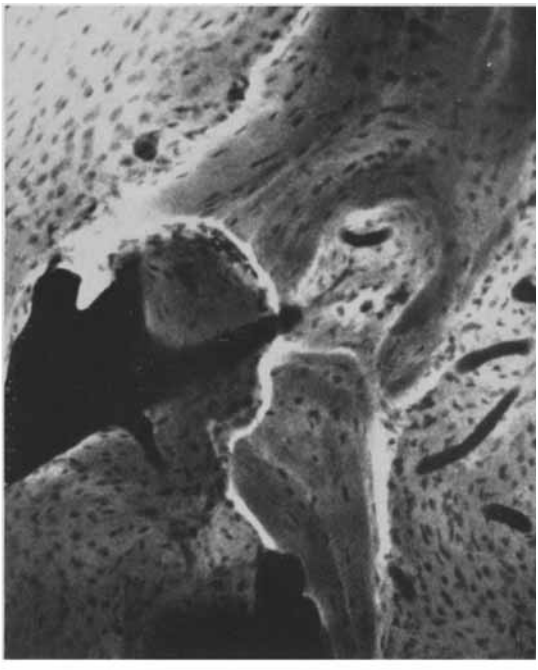
Figure 26 (top right)

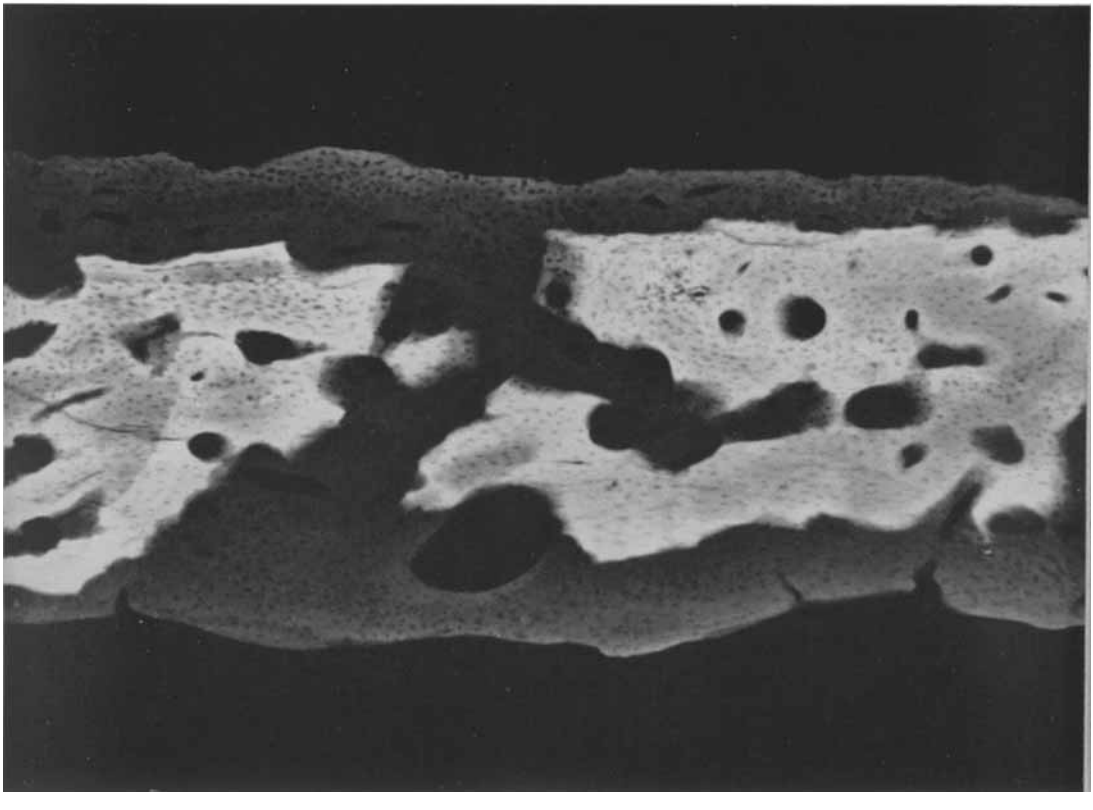
Microradiogram of a 70 μ thick sagittal section of a specimen showing remnants of an os purum implant in a rabbit 60 weeks after operation. The peripheral density of the trabeculae can be seen. The mineralization in the center of the trabeculae is lower than the average mineral content in the surrounding callus. Recorded at 12 kV. Magnification 138 x.

Figure 27 (bottom)

Microradiogram of a 70 μ thick sagittal section of a glycerol-ashed bone implant in a rabbit 58 weeks after operation. The high density in the implant is contrasting with the comparatively low-mineralized callus. Part of the implant is resorbed; widely spaced osteons with a low mineral content have been deposited within the implant. Recorded at 40 kV. Magnification 78 x.





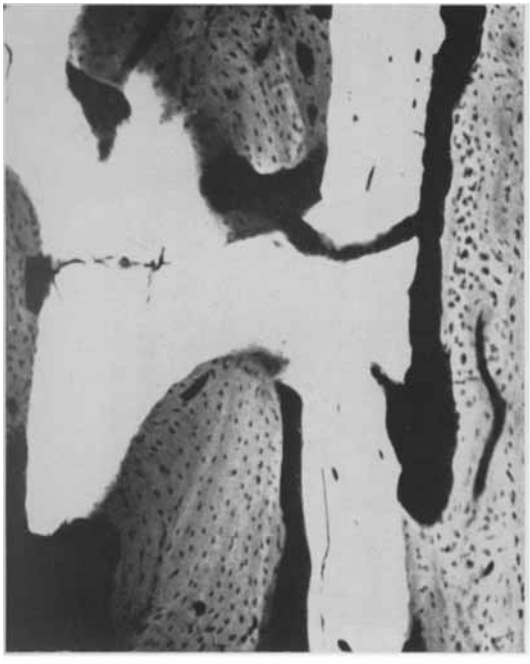


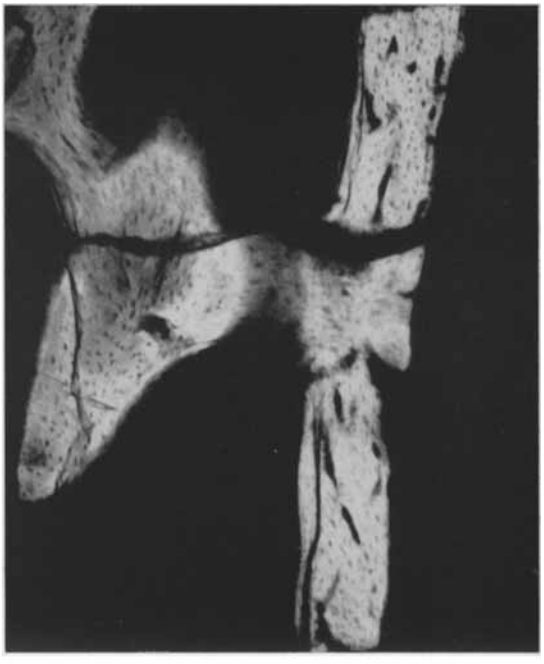
Figures 28 a and b

Microradiogram of a 75 μ thick cross-section through a calcined bone implant in a rabbit 22 weeks after operation. The microphotograph at the left shows the high difference in mineralization between the callus and the implant. The microphotograph at the right was exposed in order to show structures in the implant. The ragged surface of the cortical part of the implant seen to the right in the pictures indicates a lacunar resorption. Recorded at 40 kV. Magnification 80 x.

Figures 29 a and b

Microradiogram of a 75 μ thick sagittal section through a calcined bone implant in a rabbit 60 weeks after operation. Some resorption of the trabeculae to the left in the pictures has evidently taken place, while the cortical part of the implant to the right in the pictures shows a lacunar resorption and resembles that seen in pictures 28 a and b. Recorded at 40 kV. Magnification 74 x.





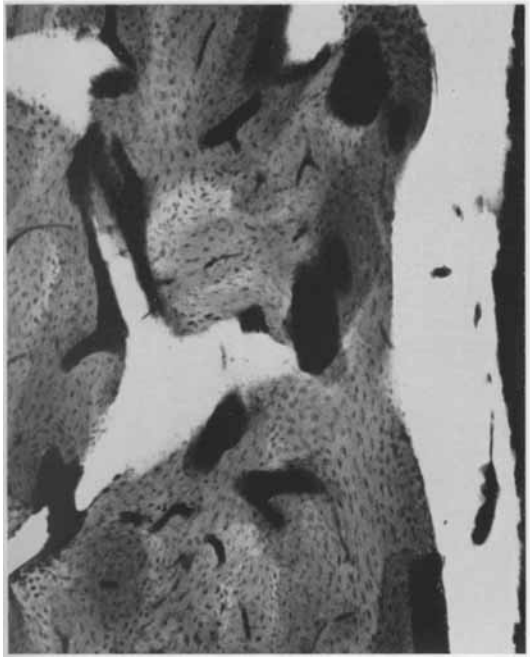


Figure 30 *a*

Microradiogram of a 80 μ thick sagittal section through a cortical transplant in a dog's tibia 47 weeks after operation. Lower part of the picture occupied by the transplant shows up as an area with cross-cut Haversian systems with varying mineralization. In the upper part of the picture are Haversian osteons with a longitudinal alignment. Recorded at 12 kV. Magnification 34 x.

Figure 30 *b* (bottom left)

Detail of the microradiogram in Fig. 30 a indicated by square. Magnification 77 x.

Figure 30 *c* (bottom right)

The same area as in microradiogram 30 b microphotographed in polarized light after decalcification of the specimen. Upper part of the picture shows longitudinal alignment of the collagen fibers in the host bone, and the lower part shows the collagen in some cross-cut Haversian osteons in the transplant. Magnification 77 x.

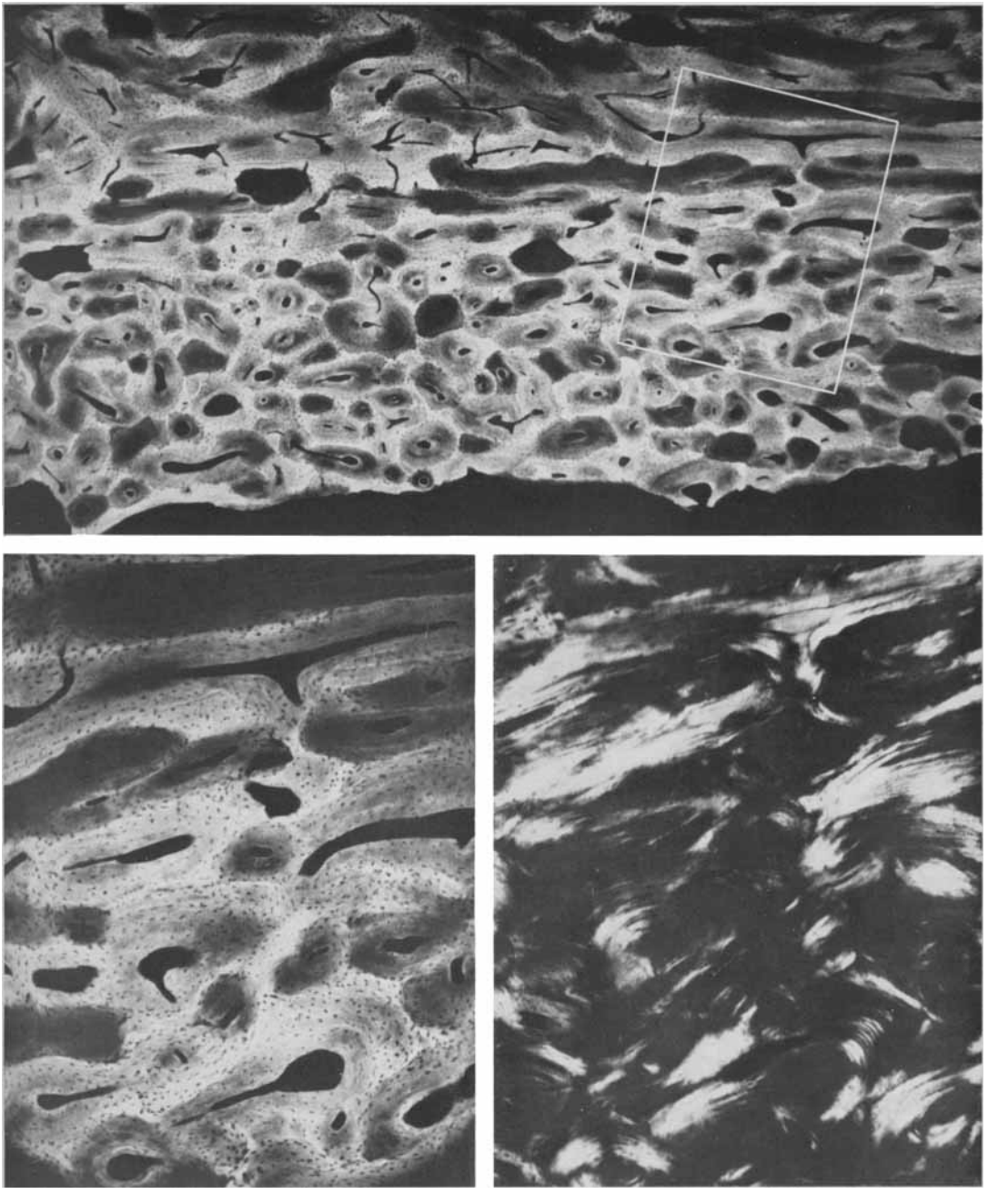


Figure 31 *a* (top)

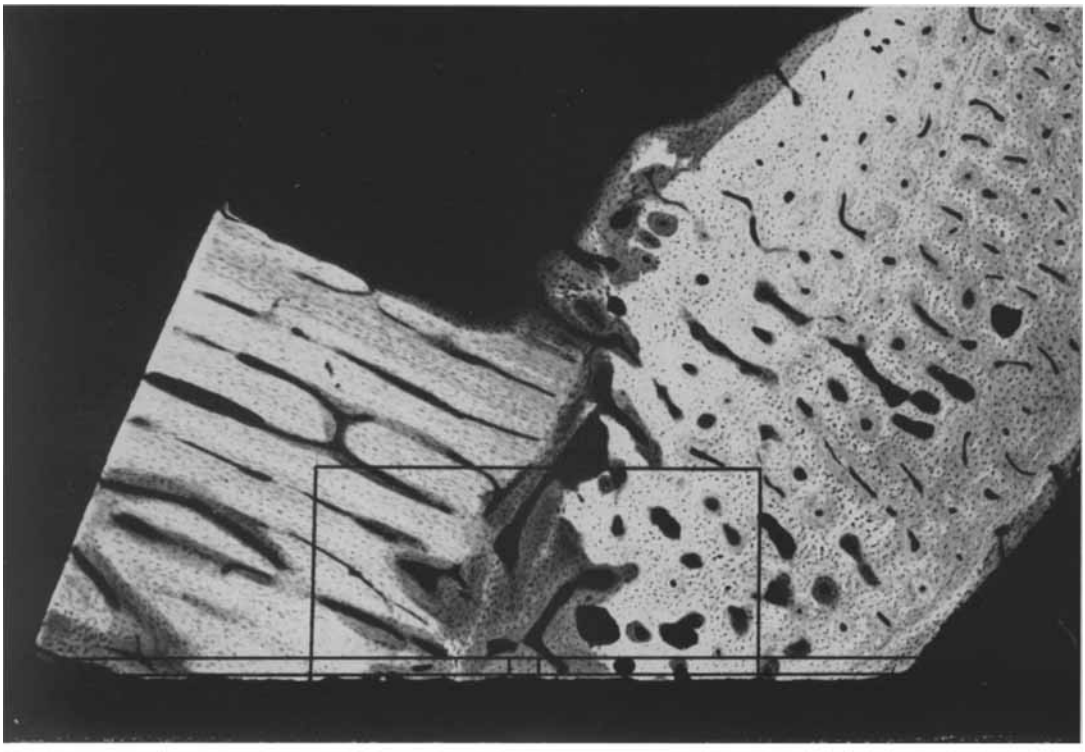
Microradiogram of 75 μ thick cross-section of an autogenous transplant in a rabbit 7 weeks after operation. Determination of mineral salts was made in places within the area indicated by the parallel black lines at the edge of the cut specimen. Recorded at 12 kV. Magnification 49 x.

Figure 31 *b* (middle)

Detail of microradiogram in Fig. 31 *a* indicated by square. To the left in the picture is part of the transplant; to the right in the picture part of the host bone is seen. Within an area indicated by the square in the intermediary callus, determinations of mineral salts were performed in six points according to the "six-point measurement" (see text). Magnification 108 x.

Figure 31 c (bottom)

The 75 μ thick edge of the cut specimen as seen in the profile microscope. Magnification 147 x.



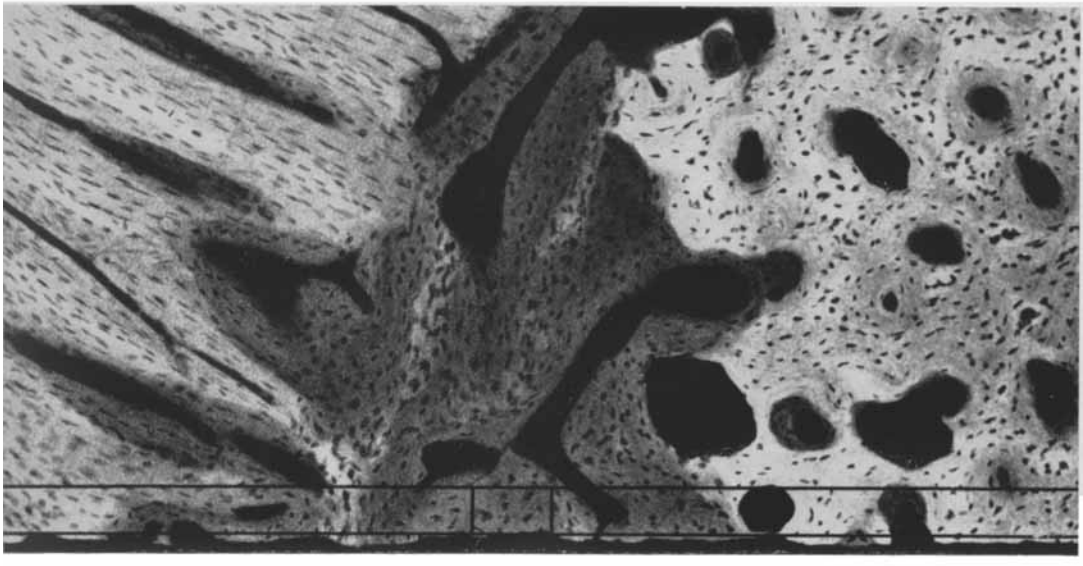


Figure 32 a

Microradiogram of a 70 μ thick cross-section of an autogenous cortical transplant in a rabbit 12 weeks after operation. Host bone to the left. Transplant to the right contains many low-mineralized Haversian osteons. Low mineralization of periosteal and intermediary callus. Upper right hand corner of this fig. is seen in larger magnification in Fig. 16. Registered at 12 kV. Magnification 37 x.

Figure 32 b

Autoradiogram obtained from the same specimen as in Fig. 32 a. The rabbit was injected with Sr^{90} 10 days before being killed. It can be seen by comparing Figs. 32 *a* and *b* that a high uptake of Sr^{90} has taken place in low-mineralized newly built areas, especially in the subperiosteal callus. Magnification 37 x.

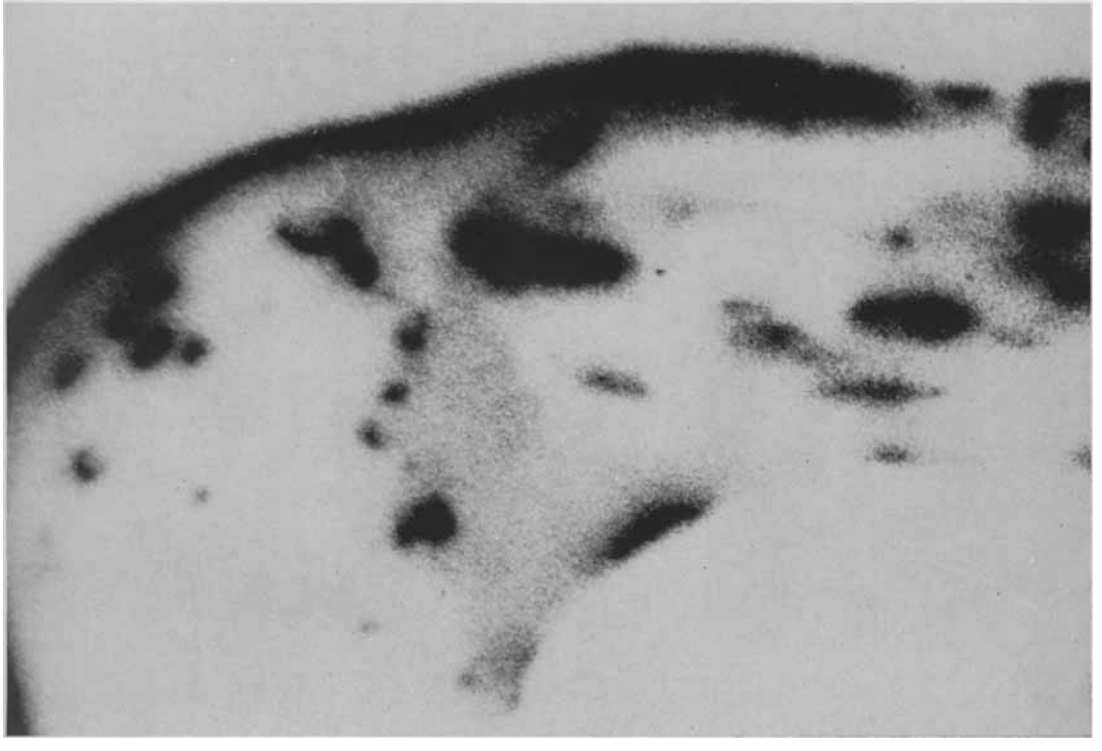


Figure 33

Microradiogram of a 75 μ thick cross-section through a homogenous cortical transplant of a rabbit 21 weeks after operation.

The X-ray microdiffractograms *A*--*E* all show the hydroxyapatite pattern with diffuse diffraction rings, indicating an extremely small crystallite size. Diffractogram *A* was taken from the periosteal callus, diffractogram *B* from remnant of the transplant, and diffractogram *C* from bone substituting the transplant; all these diffractograms show a preferred orientation of the rings, which is particularly well manifested in the (00.2)-reflection, indicating a transverse alignment of the bone crystallites. Diffractogram *D* was recorded from the intermediary callus and indicates a radially preferred orientation of the bone crystallites. In the diffractogram *E*, which was taken from the host bone, the (00.2)-reflection is faint, and indicates a longitudinally preferred orientation of the bone crystallites (perpendicularly to the picture plane). Diffractograms were recorded at 40 kV, and the diameter of the examined areas was 50 or 100 μ. Microradiogram recorded at 12 kV. Magnification 45 x.

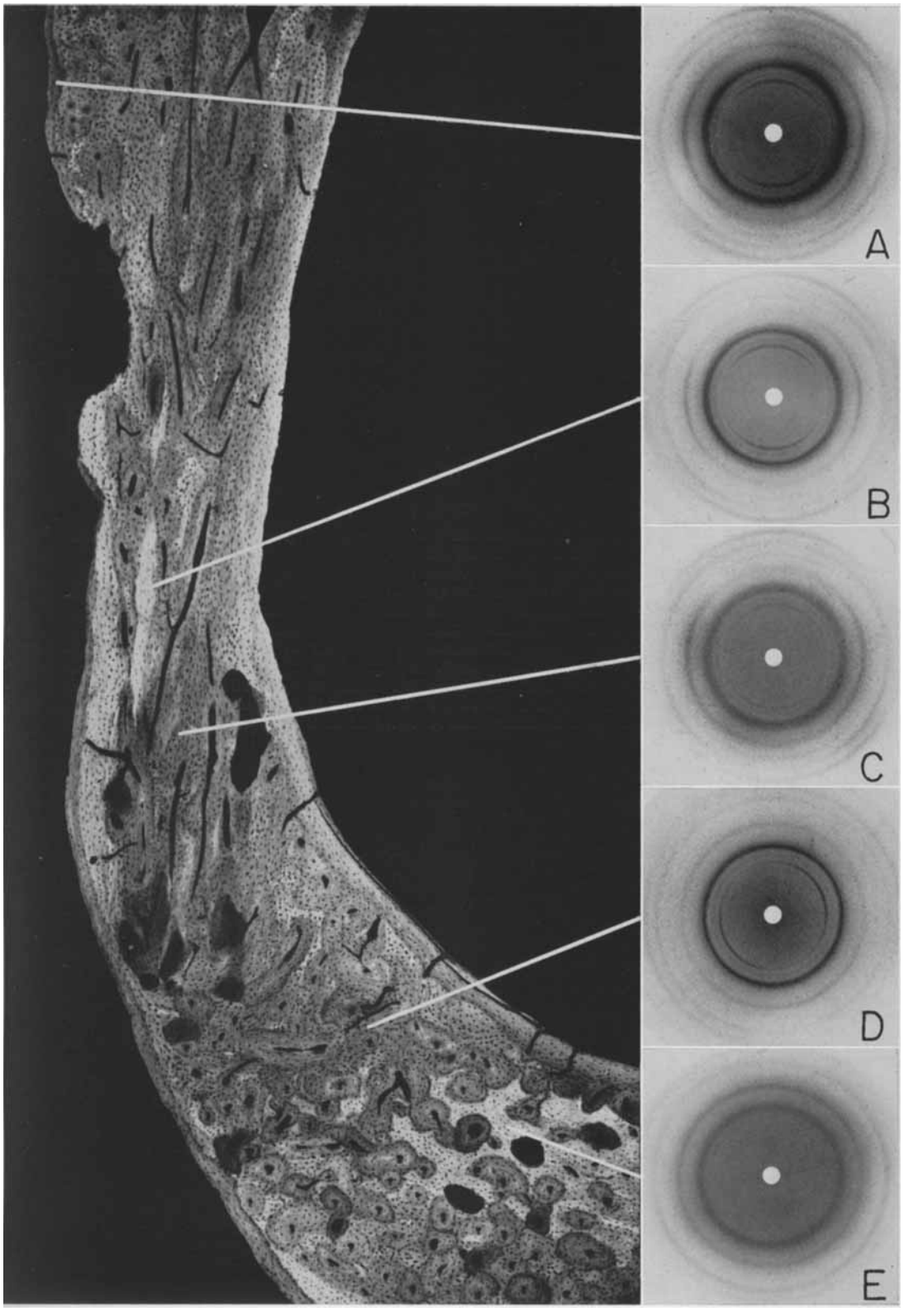
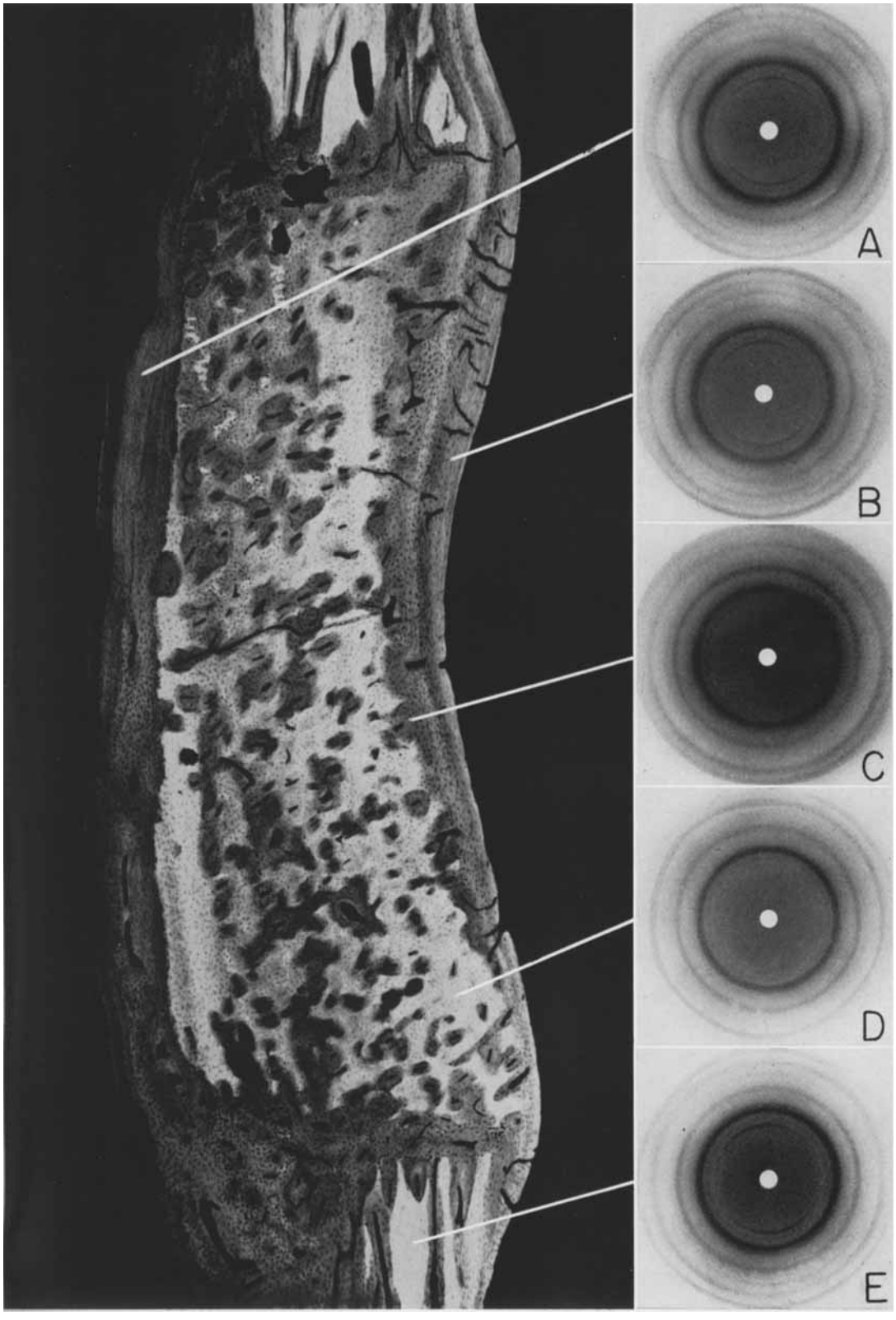

A
B
C
D
E

Figure 34

Microradiogram of a 75 μ thick sagittal section through an autogenous cortical transplant in a rabbit 64 weeks after operation.

Diffractograms A and B were recorded from the periosteal and the endosteal callus, respectively, and indicate a longitudinally preferred orientation of the bone crystallites in the reconstructed circumferential lamellae. Diffractograms *C* and *D* were registered from bone substituting the transplant, and from the original transplant, respectively. In both diffractograms faint (00.2)-reflections indicate a transversely preferred orientation of the bone crystallites (perpendicularly to the picture plane). Diffractogram *E* was taken from the host bone and shows a longitudinally preferred orientation of the bone crystallites (parallel to the picture plane). Diffractograms were recorded at 40 kV and the diameter of the examined areas was 50 or 100 μ. Microradiogram recorded at 12 kV. Magnification 42 x.


A
B
C
D
E