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Vital microscopy of bone remodelling in rabbit ear chambers

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CONTENTS

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Oslo, November 1974

Tromsø, June 1975 *Einar Sudmann*

GENERAL INTRODUCTION

Bone remodelling entails the resorption and formation of bone tissue. These complex active cellular processes, fundamental to the integrity and physiology of the skeletal system, have been the object of extensive studies using in vitro and in vivo techniques, but vital microscopy of bone tissue is unique among these in allowing the visualization and instant documentation of the sequence of events.

Vital microscopy in mammals was originated by Sandison, who managed to construct and insert the first successful rabbit ear chambers (Sandison 1924). Later he described the formation and resorption of bone tissue in 1 ear chamber (Sandison 1928). Bone developed in a narrow part of this particular chamber and it was therefore possible to study bone formation, including the process of mineralization, at oil imersion magnification. He also observed bone resorption at cellular level in this chamber. Later Kirby-Smith (1933) and Clark & Clark (1942) studied bone remodelling in 1 and 4 ear chambers respectively.

My own interest in vital microscopy and photomicrography dates back to my student days (Sudmann 1957). Since I was familiar with the ear chamber technique, including Sandison's (1928) vital microscopical studies on bone remodelling, it was natural that later, as an orthopaedic surgeon, I should choose this method, which, according to Ezra-Cohn et al. (1969), is a remarkably convenient in vivo system for bone tissue investigation.

Ear chambers are widely used for vital microscopical studies of the microcirculation (Arfors et al. 1970, Leaf & Zarem 1970), but no reports were available describing the technical difficulties which can be encountered with this technique.

Ear chambers for vital microscopy are easily constructed and inserted as outlined by Leaf & Zarem (1970). It might thus be assumed that vital microscopical investigation of

bone could be easily done. However, bone remodelling takes weeks and months, so that when the time came for me to start the present bone tissue investigations, the ear chambers were no longer of any use owing to mechanical or biological failures. Furthermore no available ear chamber construction was designed especially for use with bone grafts, which are essential for initiating bone resorption and bone formation within the chambers. Thus I soon realized that the methodological problems started with the ear chamber itself, and that a series of ear chamber bone cultures useful for e.g. drug experiments could only be obtained by also revising the technique of bone grafting and bone vital microscopy.

The aims of the present study were therefore to construct ear chambers suitable for vital microscopical investigations of bone tissue (Article I), to establish the essential conditions for bone resorption and bone formation within these chambers (Article II), to study bone remodelling quantitatively and qualitatively in vivo (Articles III—IV) and then finally to assess the effect of indomethacin on bone remodelling in vivo (Article V).

GENERAL DISCUSSION

As a technique of bone research, microscopy of living bone tissue is unique in giving instant visualization and documentation of both bone resorption and bone formation not only at tissue but also at cellular levels. Developed by Sandison (1928), this technique would thus seem to be a promising approach to a number of problems in osteology and orthopaedic surgery. There has, however, been surprisingly little progress in this area of research since Sandison's classical contribution (Kirby-Smith 1933, Clark & Clark 1942, Ezra-Cohn et al. 1969).

Vital microscopy of bone tissue in rabbit ear chambers raises several technical problems mainly related to the design, construction and insertion of the ear chambers. These problems are probably the main reason why so few investigators have used this method for bone research.

A vital chamber is indispensable for in vivo bright field microscopical investigations of bone tissue. Such chambers were not commercially available, but were easily constructed as outlined by Leaf & Zarem (1970) and inserted in rabbits' ears. For the investigation of living bone tissue and not just for a picturesque visualization, however, it was necessary to develop a novel type of rabbit ear chamber (Article I).

An implant designed for permanent anchorage to subcutaneous tissues (including bones) protruding through the skin — like a rabbit ear chamber — is one of the most sought-after devices in orthopaedic surgery today. The implant-skin interface is one of the crucial points, which means that an ear chamber for experimental purposes has to be carefully designed and made. The remodelling of bone tissue — and not the testing of new materials for surgical implants — was the main aim of the present study, and the final chamber type used here was accordingly made of titanium, a commonly used material for making surgical implants — and ear chambers (Brånemark & Lindström 1963, Arfors et al. 1970).

Bone graft resorption was regularly obtained in rabbit ear chambers, but not bone formation. Since bone remodelling includes both resorption and formation of bone tissue, a technique that consistently ensured osteogenesis or bone formation as well as resorption inside these vital chambers was developed (Article II).

Porous implants can be anchored to bones by means of bone tissue ingrowth (Lyng et al. 1973). It is reasonable to assume that for permanent anchorage the diameter of the pores of the implant should be large enough for the formation and remodelling of parallel-fibred bone tissue. Such tissue was observed in 0.2—0.3 mm deep rabbit ear chambers. In the present study no bone entered optical parts that were less than 0.1 mm deep (Article II). The study of bone growth into the narrow optical part of these chambers may thus provide information about the optimum size of the pores in implants for permanent anchorage to bones — especially if current implant materials are incorporated within the chambers. The incorporation of such material into the ear chambers would furthermore make in vivo investigations of the bone-implant interface possible.

Several non-electronic methods for stereometric analysis in microscopy were tried for quantitation of bone area in rabbit ear chambers. It was soon realized, however, that a more convenient method was desirable for routine bone tissue quantitation in vivo. A new method for stereometric analysis in microscopy was developed (Article III). It proved to be a versatile and precise stereometric method for area quantitation in microscopy. This method can be used with any type of microscopical illumination in any light microscope. Provided an area is visible in the microscope — either at the cellular or at the tissue level — it can be measured not only easily, but with a precision better than 1 per cent. With some microscopes the area so measured can be permanently recorded in a photomicrograph.

No reports were available correlating the in vivo observations of remodelling bone tissue in rabbit ear chambers with ordinary histological sections of the same bone tissue when removed from the chambers. It was therefore uncertain which elementary bone types and bone structures were discernable in vivo in the rabbit ear chamber. A study on these lines was carried out in collaboration with Dr. P. F. Marton (Article IV).

Two elementary types of bone tissue, differing both morphologically and physiologically (Frost 1963), are generally recognized. They may be classified according to the texture of the collagen fibres as revealed in plane polarized light microscopy, as woven-fibred and parallel-fibred bone.

Woven-fibred bone is made by disordered osteoblastic activity and parallel-fibred bone by ordered osteoblastic activity (Article IV). Disordered and ordered osteoclastic activity would be their logical counterparts in bone remodelling. After correlating vital microscopical and histological findings, both disordered and ordered osteoclastic and osteoblastic activity were identified in rabbit ear chambers by vital microscopy (Article IV).

The balance between resorption and formation, so essential for bone homeostasis, was easily visualized in rabbit ear chambers in vivo (Article IV).

The purpose of the present study was to investigate bone tissue remodelling in rabbit ear chambers, with a bearing upon current problems in clinical orthopaedic surgery. The revision of the methodology described in Articles I—IV made the assessment of the effect of indomethacin on bone remodelling possible (Article V).

The factors initiating and governing disordered osteoclastic and osteblastic activity may differ both quantitatively and qualitatively from those initiating and governing ordered activity. When investigating the effect of drugs — like indomethacin — on bone remodelling, it may

therefore be important to assess the drug effect on disordered and ordered bone remodelling separately, and this was done when assessing the retarding effect of indomethacin on osteoclastic and osteoblastic activity as described in Article V. Assessing the effect of indomethacin on the balance between resorption and formation — so important for bone homeostasis — was, however, outside the scope of the study.

GENERAL SUMMARY

The present study consists of 5 articles (I—V). The methodology for quantitative and qualitative vital microscopy of bone remodelling in rabbit ear chambers was first revised (Articles I—IV), and thereafter the effect of indomethacin on bone remodelling was assessed in the in vivo culture chamber (Article V).

A novel titanium-glass rabbit ear chamber was devised (Article I). Out of 56 chambers inserted without removing the cartilage on the inner side of ears not operated upon earlier, 44 (79 per cent) became vascularized. In contrast to the other types tested, the titanium-glass chamber proved satisfactory for bright field, fluorescence and polarized light microscopical observations of bone remodelling for half a year or longer.

Bone resorption and bone formation were consistently initiated within the chambers by cortical and cortical cancellous bone grafts (Article II). Spontaneous bone formation within ear chambers was not encountered.

A new method for stereometric analysis in microscopy, called mixed-image planimetry, was developed (Article III). The method proved to be simpler and more precise than any of the usual methods used for area quantitation in microscopy. A precision of less than 1 per cent was easily obtained.

By comparing vital microscopical and histological findings from the same chambers, bone remodelling within the chambers proved in principle in accordance with that of orthotopic bone (Article IV). The sequence of events of such remodelling was remarkably consistent. A balance between resorption and formation was regularly established.

Indomethacin suspension administered per os in a daily dosage of 10 mg/kg body weight retarded both osteoclastic and osteoblastic activity within the chambers (Article V). The clinical implications of these findings are discussed.

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A NOVEL TITANIUM RABBIT EAR CHAMBER FOR VITAL MICROSCOPY OF BONE TISSUE

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Key words: Animal experiments; bone and bones; ear, external; microscopy; models, biogical; rabbits; tissue culture.

Numerous methods have been used for the study of bone tissue remodelling and bone transplants (Frost 1963, McLean & Urist 1968, Trueta 1968), but microscopy of living bone tissue is unique among these in allowing the visualization and instant documentation of the sequence of events.

The technique of vital microscopy of bone tissue is almost as old as that of vital microscopy in mammals, but has only been used for bone tissue studies in a few laboratories (Sandison 1928, Kirby-Smith 1933, Clark & Clark 1942, Ezra-Cohn et al. 1969). An artificial culture chamber developed for insertion in the rabbit's ear made Sandison's (1928) remarkable in vivo microscopical observations of bone tissue possible.

Ear chambers are not commercially available; however, they are indispensable for vital microscopy of bone tissue. The purpose of the present study was therefore to design an ear chamber suitable for vital microscopy of bone tissue.

MATERIAL AND METHODS

In several pilot studies 9 different ear chamber modifications (151 ear chambers) were made and inserted in the ears of 58 rabbits of either sex as outlined by Ebert et al. (1939), Leaf & Zarem (1970), Arfors et al. (1970), Arfors (1971) and Silver (1971). Thirty-seven out of 151 chambers (25 per cent) were vascularized, 114 chambers (75 per cent) were not (i.e. failures). Based upon the results of these pilot studies, the present investigation was carried out as follows.

In all, 78 ear chambers of own design were inserted in 6 female albino and in 35 female sandy half lop-eared rabbits. The animals were 3.5 months and older at the time of chamber insertion. Sixteen of the sandy half lop-eared rabbits were used twice for chamber insertion.

The animals were kept separately in a stable with regulated light, ventilation, temperature and humidity. The room temperature was 22°C. The animals were fed a standard stock diet (No. 176, Møllecentralen, Oslo, Norway) and tap water ad libitum. The lop-eared rabbits were purchased from Hylyne Commercial Rabbits, Northwich, England. They were always kept for at least 3 weeks in our stable before having chambers inserted.

General ear chamber design

A Sandison-Clark round-table regenerating type ear chamber is in principle composed of a transparent peg (or cylinder) and a cover slip in a mounting. The peg is inserted in a circular hole cut right through the rabbit's ear. Ordinary bright field microscopy is thus possible. The top surface of the peg is called the table. Regenerating connective tissue will invade (vascularize) the narrow space (i.e. the optical part of the chamber) bounded by the cover slip and the peg (Figure 1). The mounting anchors the peg and cover slip to the ear and ensures a constant distance (depth) between the cover slip and the table.

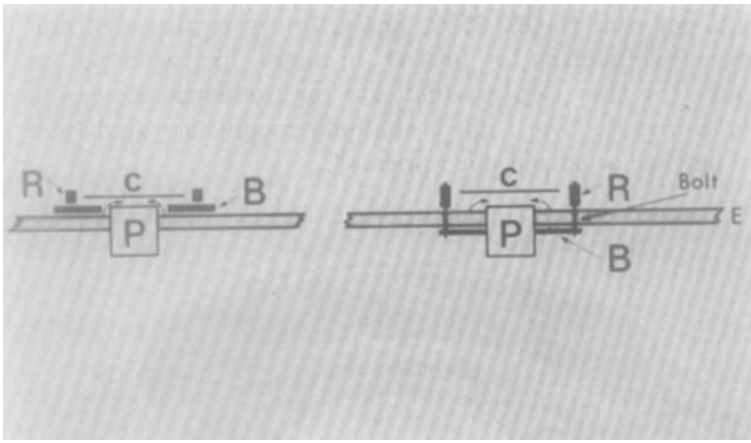


Figure 1. Schematic design of a simple, non-sandwich (left) and a multiple component, sandwich type ear chamber (right). The transparent peg (P) for the optical light beam is located in a hole cut through the rabbit's ear (E). The base disc (B) and the ring (R) are on the same side of the ear in the simple chamber, and on opposite sides and bonded together with bolts and nuts in the multiple component chamber. Regenerated connective tissue will invade (arrows) the slit-shaped optical part of the chamber between the cover slip (C) and the peg. This part of the chamber is accessible for bright field microscopical observations.

Special ear chamber design

The chamber modification used here, called the rosette chamber, was made of titanium (type ATi 24, Avesta Jernverks A.B., Avesta, Sweden) and of optical glass. A central glass cylinder peg (Deutsche Spiegelglas A. G., Grüneplan, Germany), made in accordance with the specifications of Arfors et al. (1970), constituted the base of the slit-shaped optical part of the chamber. The optical part was surrounded by a shallow, annular non-optical part, i.e. the well (Figure 2). Most of the titanium rosette chamber mountings (62 chamber insertions) were anodized (i.e.

oxidized) for 15 seconds by an electrolytic technique (10 weight per cent NaOH, 25°C, 25 V). Ten were annealed and oxidized by heating to 1000°C in moist hydrogen gas for 1 hour (by the Central Institute for Industrial Research, Oslo, Norway). The final 6 chamber mountings used here were neither anodized nor annealed.

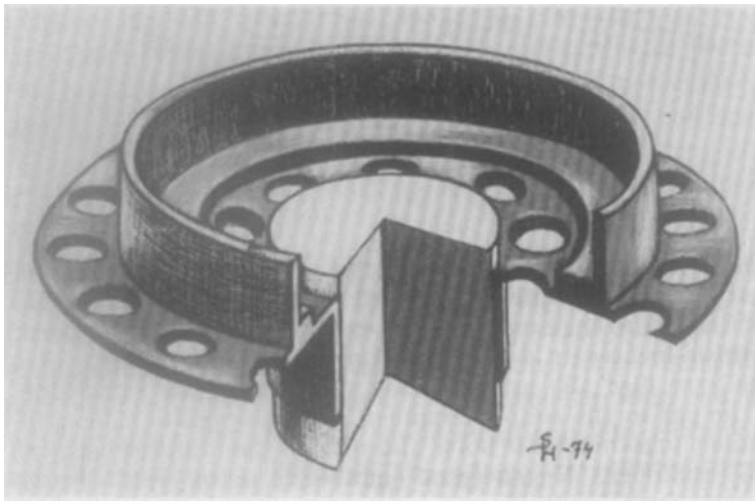


Figure 2. Perspective drawing of the titanium-glass ear chamber of own design. The chamber was constructed of 2 main parts: A central glass cylinder peg 6 mm and a circular titanium mounting 18 mm in external diameter. The mounting was manufactured with a precision better than ± 0.02 internally and ± 0.08 mm externally. The peg was seated flush with the bearing for the standard 12 mm cover slip. The depth of the optical part of the chamber was fixed by a spacer (e.g. 0.13 mm thick) between the cover slip and its bearing. A titanium expansion ring secured the cover slip. Spacer, cover slip and expansion ring are not illustrated here (see Figures 3—5).

By the end of the second postoperative week, reddish regenerated connective tissue filled the vascularization funnels in the well, so that the chamber resembled a small rosette in the rabbit's ear. The chamber was therefore called the rosette chamber.

Spacers for constant chamber depth were made of Teflon (Imperical Chemical Industries Ltd., Welwyn Garden City, England) or of non-reinforced medical grade silicone rubber (Silastic, Dow Corning, Midland, Michigan, U.S.A.). The cover slip was fastened by a metal expansion ring turned from a titanium rod. Cover slips were punched from a 170 μm Melinex sheet (I.C.I.) or standard 12 mm circular cover glasses were used.

Assembly and sterilization

The glass cylinder peg was bonded to the metal mounting with Araldite Standard (Ciba-Geigy, Basle, Switzerland) in a ratio of about 10 parts of resin to 6 parts of hardener. The epoxy resin adhesive was hardened overnight at about 65°C. The optical part of the chambers was 0.05 to 0.13 mm deep.

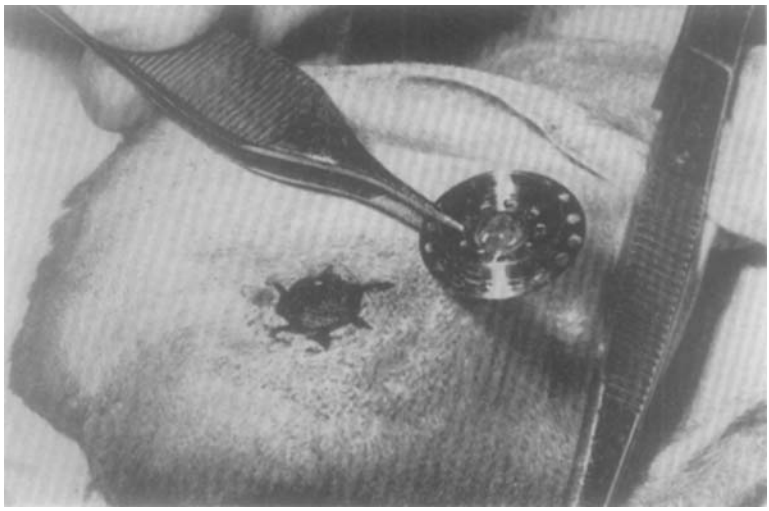
The chambers were sterilized in ethylene oxide on 2 consecutive days and aired at least 48 hours before insertion. The spacers, glass cover slips and surgical instruments were always autoclaved. Prior to sterilization, the chambers were cleaned in an ultrasound bath and their interior surfaces thereafter siliconized in a 0.2 per cent solution (type Z 4141, Dow Corning, Midland, Michigan, U. S. A.).

Anaesthesia and chamber insertion

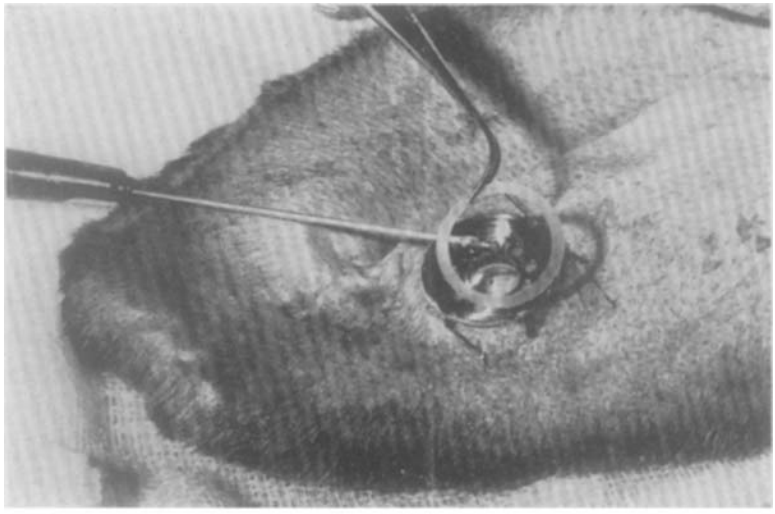
Neurolept anaesthesia was introduced by intramuscular injection of Fluanisone 5 mg/kg and Fentalyl citrate 0.1 mg/kg body weight (Hypnorm Vet., Mecos, Helsingborg, Sweden) (Silver 1969).

The operative technique was based on the methods of Ebert et al. (1939), Leaf & Zarem (1970), Arfors (1971) and Silver (1971). Insertion of a titanium rosette ear chamber is shown in Figures 3—5.

The chambers were inserted either on the inner (70 chambers) or on the outer (8 chambers) sides of the ears. After insertion the chambers were filled either with an



Figures 3—5. Insertion of a titanium rosette ear chamber. *Figure 3.* The circular hole for the peg was always cut from the outer surface of the ear. A subcutaneous pocket was dissected on the inner surface and small skin flaps were raised. *Figure 4.* The inserted chamber was secured post-operatively by a purse-string ligature. The depth of the chamber was fixed by a Silastic spacer. *Figure 5.* The chamber was filled with isotonic saline solution. A water-tight seal was achieved by means of a Silastic washer and a titanium expansion ring. Air bubbles within the chambers should be avoided.



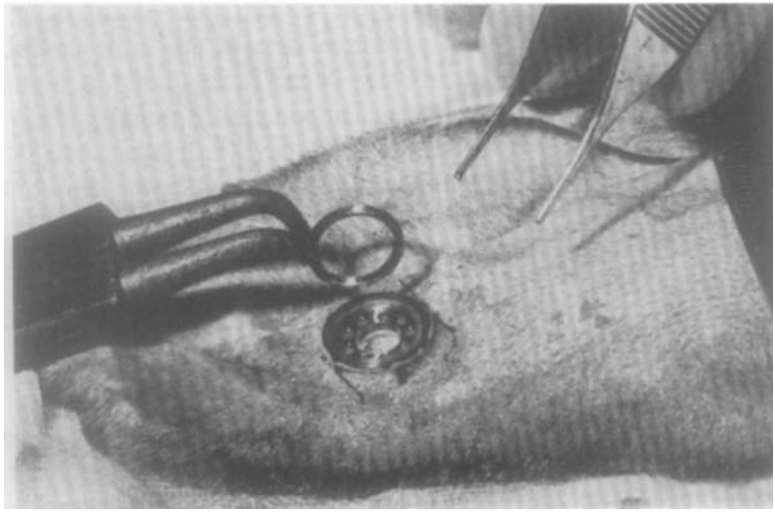


Table 1. Experiments with titanium-glass rosette ear chambers.

Experiment	No. of chambers	No. of rabbits	Side of ear	Penicillin applied topically
1	12[a]	6	Inner[d]	Yes
2	8[a]	5	Outer	Yes
3	16[a]	9	Inner	Yes
4	20[a]	10	Inner	No
5	10[b]	5	Inner	No
6	12[c]	6	Inner	No

[a]Chamber mountings anodized

[b]Chamber mountings annealed

[c]Six chamber mountings anodized, 6 neither anodized nor annealed

[d]Ear cartilage resected corresponding to the width of the chamber (18 mm) on the left ear

isotonic saline solution of benzylpenicillin-natrium in a concentration of 12 mg/ml, or with isotonic saline solution only. The details of 6 experiments using the rosette chambers are given in Table 1.

Vital microscopy and photomicrography

For microscopy the short-eared albino rabbits were tranquilized (0.5 ml Hypnorm Vet., s. c.) and placed on an animal board (Clark et al. 1931) in the supine position. The unanaesthesized lop-eared rabbits were examined in a special restraining box. A long working distance condensor and objectives on an ordinary microscope were used. The chambers were secured in the optical path of the microscope in a special clamping device (Figure 6). The technique of bone grafting and quantitative microscopy of bone tissue is described in detail in 2 other articles (Sudmann 1975 a—b).

Vital photomicrographs (Figure 7) were taken with a Reichert Kam ES system camera for photomicrography (Gabler & Kropp 1969) on 35 mm Ilford Pan F, Agfachrome 50 L Professional, Anschochrome 500 daylight film and on Polacolor Land film type 108. Cine films were taken with a Paillard Bolex cine camera on 16 mm Kodachrome 2 A.

RESULTS

The neurolept anaesthesia gave consistently good results without any per- or postoperative deaths. The chambers were more easily inserted on the inner than on the outer sides of the ears — where the main vessels of the ear are located. Furthermore haemostasis was more easily obtained on the inner side.

Figure 6. Unanaesthesized sandy half lop-eared rabbit in restraining box beside Leitz Ortholux 2 microscope. A special clamping device was used to fasten the rosette ear chamber to the microscope stage.

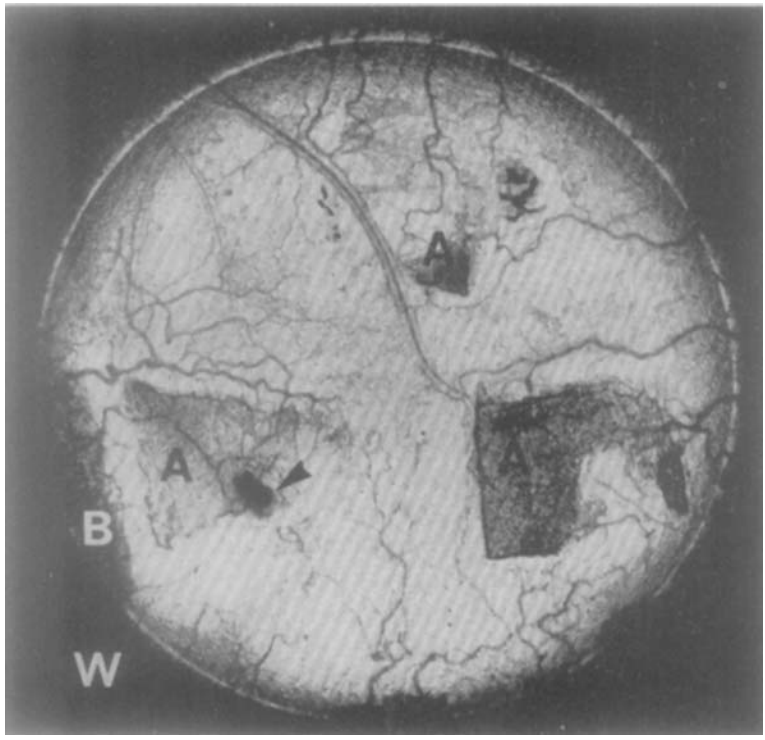


Figure 7. Bright field vital photomicrograph. Rosette ear chamber 21 days after autografting. The areas of the 3 autografts (A) were reduced from 2.2, 1.8 and 2.0 mm^2 to 0.1, 1.3 and 1.0 mm^2 respectively. New bone tissue (B) from the well (W) measured 1.2 mm^2. New bone tissue had just appeared between cover glass and lower left autograft (arrowhead). Field width, 6 mm, objective 1x/0.04.

Experiment 1. All 6 chambers inserted without removing the ear cartilage were vascularized. Small patches of avascular cellular accumulation were observed in some of them. Only 1 out of 6 was vascularized when the ear cartilage was removed. The other 5 were completely filled with avascular cellular accumulation

Experiment 2. Five out of 8 chambers inserted on the outer side of the ear were completely vascularized. Three chambers were filled with avascular cellular accumulation and therefore listed as failures.

Experiment 3. Ten chambers were completely vascularized and 6 were failures.

Experiment 4. Twelve chambers out of 14, inserted in rabbits not operated upon earlier, were completely vascularized within 4 weeks and 2 were failures. Six chambers, inserted in ears previously used for chamber insertion, were all failures.

Experiment 5. Eight chambers were vascularized, but only the last 4 chambers in this experiment were completely without patches of avascular cellular accumulation inside the chambers. Haemostasis had been particularly good in these 4 chambers. Two chambers were failures.

Experiment 6. Eight chambers were vascularized and 4 were failures. Air bubbles were found postoperatively in 2 of the latter chambers.

The rosette titanium-glass chambers gave weaker long-term tissue reactions (crusts, skin necrosis, granulation tissue) than did the methachrylate chambers used in the pilot studies (Figures 8—9).

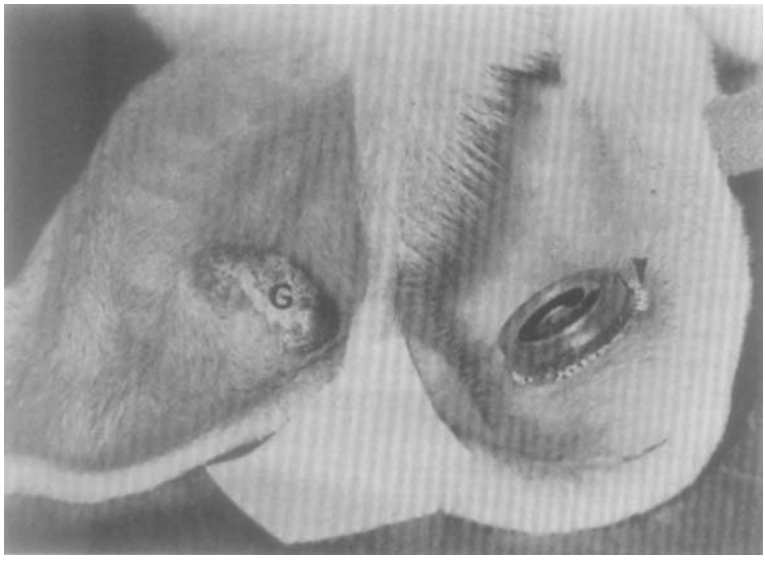


Figure 8. Bilateral methacrylate ear chambers (Leaf & Zahrem 1970) 4 months after insertion. Both methacrylate pegs were completely buried by granulation tissue (G). The mesh-base disc junction was exposed (arrowhead).

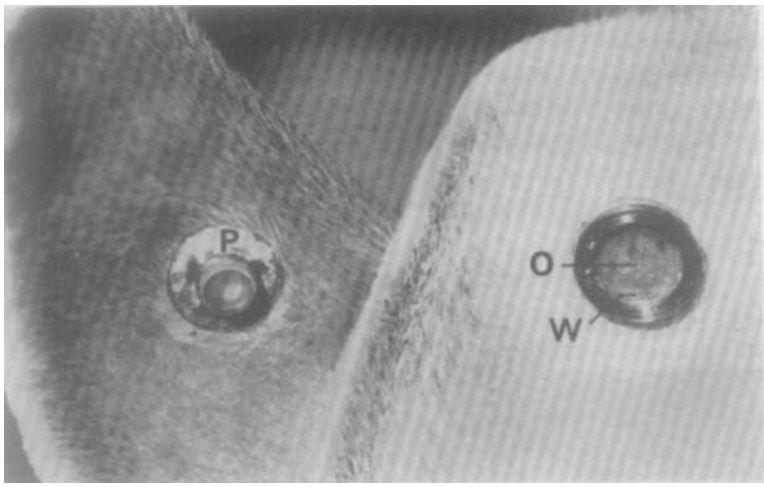


Figure 9. Bilateral titanium rosette ear chambers 7 months after insertion with living bone tissue in both the well (W) and the optical part (O). Both chambers had been used extensively for bone tissue experiments for half a year (Sudmann 1975 d). A paper washer (P) sealed the skin-peg junction.

DISCUSSION

The rabbit ear chamber has definite advantages over other culture methods for the observation of remodelling bone tissue (Ezra-Cohn et al. 1969, Sudmann 1975 c, Sudmann & Marton 1975). The ear chamber technique raised, however, a series of problems of its own — mainly related to the difficulties in securing completely vascularized chambers for subsequent long-term studies. Based upon the results of the pilot studies, the following obvious, but essential facts emerged. In the first place, the chamber components should not fall apart. Secondly, safe anchorage to the ear was essential. Finally, the chamber should accommodate bone grafts within the chamber beside the optical part as well as in it.

The chamber was made of a central glass cylinder peg in a circular titanium mounting, which was turned in one piece. Once bonded to the mounting, the glass peg could only be removed by crushing the glass.

The titanium mounting of the rosette chamber was precision manufactured at a fraction of the cost of the multiple component titanium sandwich type ear chambers of Arfors et al. (1970) used in one of the pilot studies. With an outside diameter of 18 mm, the rosette chamber is — to our knowledge — the smallest ear chamber construction mentioned in the literature. It can be inserted in the ears of any ordinary rabbit. Frequent vital microscopical studies, however, are most conveniently made with a long-eared rabbit in its normal crouching position beside the microscope (Figure 6).

Two concentrically located series of holes were drilled through the metal mounting — one series inside and one outside the chamber — like a rosette (Figure 2). Anchorage to the ear was secured by tissue ingrowth from one hole to another. A completely vascularized chamber could only be removed from the ear of the rabbit with a knife. An incompletely vascularized chamber usually gradually loosened from the ear and was useless for bone tissue studies.

Inside the chamber the tissue anchorage was enclosed within a shallow well (Figure 2). The living connective tissue also vascularized the optical part of the chamber and served as a vascular bed for bone grafts. Bone formation within the chamber was always obtained when autografts were placed in the well (Sudmann 1975 a).

In simple non-sandwich ear chambers (Figure 1) bone tissue can only be grafted onto the optical part, so that the thickness of the graft determines the depth of the optical

part of the chambers. Thick grafts require a deep chamber which is optically undesirable. In contrast, in rosette chambers such grafts can be placed in the well. However, new bone tissue did not enter the optical part of these chambers when its depth was less than 0.1 mm (Sudmann 1975 a).

Forty-four rosette chambers out of 56 (79 per cent), inserted without removing the ear cartilage on the inner side of ears not previously operated upon were vascularized within 4 weeks. Failures (21 per cent) also occurred with this chamber, however, especially when the chambers became blood-filled postoperatively. Neither an oxidized titanium mounting (annealed or not annealed) nor the topical application of penicillin prevented these failures (Experiments 1—6). The rosette ear chamber design made, however, reproducible bone tissue experiments lasting for half a year or longer at last possible (Sudmann 1975 c).

SUMMARY

Seventy-eight small titanium-glass culture chambers of own design for vital microscopy of bone tissue were inserted in the ears of 41 rabbits.

After insertion without removing the cartilage on the inner side of ears not previously operated upon, 44 out of 56 chambers (79 per cent) became vascularized. It proved satisfactory for bright field, fluorescence and polarized light microscopical observations of living bone tissue for half a year or longer.

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OSTEOGENESIS IN RABBIT EAR CHAMBERS

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Key words : Animal experiments; bone and bones; ear, external; models, biological; ossification, physiological; osteogenesis; tissue culture.

Heterotopic osteogenesis — or bone formation — can be initiated by a variety of skeletal or non-skeletal grafts (Ostrowski & Wlodarski 1971) and has been the object of extensive studies using in vitro and in vivo techniques — including vital microscopy in chambers inserted in rabbits' ears.

Vital microscopy of bone tissue as originated by Sandison (1928) in rabbit ear chambers permits not only daily observations of osteogenesis at the tissue and cellular levels but also in vivo assessment of the fate of the grafts which initiated the bone formation (Kirby-Smith 1933, Clark & Clark 1942, Ezra-Cohn et al. 1969).

Rabbit ear chambers are not commercially available so special ear chambers for the study of bone resorption and formation were developed (Sudmann 1975 a). The aim of the present study was to establish the conditions essential for osteogenesis in ear chambers with bone grafts, and if possible to ascertain the source of bone formation in these chambers.

MATERIAL AND METHODS

Eight albino (6 female, 2 male) and 19 sandy half lop-eared (12 female, 7 male) 5 month-old rabbits with 39 ear chambers were used. If not otherwise stated, all chambers were vascularized by regenerated connective tissue at the time of bone grafting. The main components of the chambers were made of either poly methylmethacrylate or titanium (Sudmann 1975 a).

Bone grafts

A spinous process from the middle of the lumbar spine was removed subperiostally through a small midline incision and submerged in cool (6°C) isotonic saline solution. The submerged spinous process was divided into two halves in the sagittal plane. The cancellous bone tissue was removed with a knife from one of these halves leaving a thin cortical flake (CF) about 0.1 mm thick (Figure 1), from which small bone grafts for the optical part of the chambers

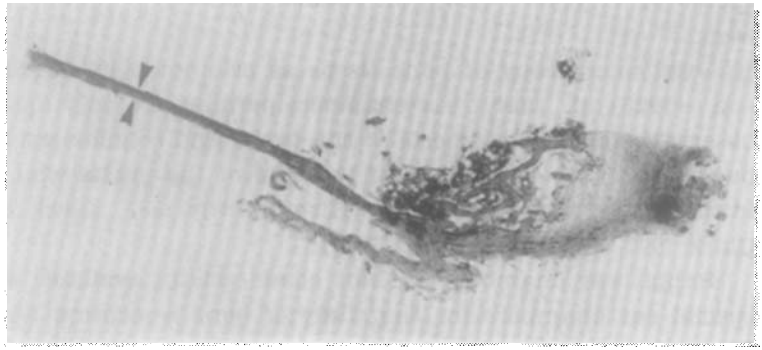


Figure 1. Photomicrograph. Longitudinal section of half of a lumbar spinous process. A bone flake, about 0.1 mm thick (indicated by arrowheads), was prepared by removing cancellous bone tissue including the inner surface of the cortex. Flake grafts for the optical part of the chambers were cut from this part of the spinous process. Decalcified, H + E. Field width, 6.8 mm. Objective 1x / 0.04.

were prepared. The bone grafts were thus about 0.1 mm thick, with areas, assessed by mixed-image planimetry in vivo (Sudmann 1975 b), ranging from 1.4 to 2.5 mm^2 (Figures 2—3). Cortical cancellous (CC) grafts of about the same size, but with intact bone marrow, were prepared from the other half of the spinous process for use beside the optical part.

Cylindrical cancellous (C) bone autografts, measuring 2.5 mm in diameter, were taken from one iliac crest using a punch. These comparatively large grafts were placed beside the optical part in chambers specially designed for this purpose. Through holes in the base of the chamber one end of the graft was in direct contact with the ear tissue beneath the chamber while the other end protruded into the chamber. These grafts were always positioned in the chambers at the time of chamber insertion and the chambers were subsequently vascularized with the grafts in situ.

Bone grafting (Table 1)

The rabbit's ear with the chamber inserted was fastened to a stereomicroscope. The removable cover slip was covered with an isotonic saline solution and carefully lifted off under fluid. The grafts were then placed on the connective tissue inside the chamber under fluid before it was closed with a new sterile cover slip. Drying of the connective tissue and bone grafts was thus avoided. The grafts located in the optical part of the chambers were visible in bright field microscopy. The grafts beside the optical part were only visible in reflected light, macroscopically, or using a low power stereomicroscope. Altogether 5 bone tissue experiments were carried out as specified in Table 1.

Table 1. Bone grafting experiments in 27 rabbits with 39 ear chambers.

Experiment	No. of rabbits	No. of ear chambers	Ear chamber Type	Ear chamber Depth, mm	Grafts Type[a]	Grafts No.	Location	Grafts in all
1	6	8	Meth-acrylate	0.12	CF	3	Optical part	24 auto-grafts
2	5	7	Multiple component titanium	0.05-0.12	CC	3	Beside optical part	21 auto-grafts
3	5	6	Meth-acrylate	0.05	C	3	Beside optical part	18 auto-grafts
4	7	11	Titanium rosette	0.05	CC	9	Non-optical part	99 auto-grafts
5	4[b]	7	Titanium rosette	0.25	CF+CC	3+9	Optical + non-optical part	36 auto-grafts 48 allo-grafts

[a] CF = cortical flake, CC = cortical cancellous, C = cylindrical cancellous

[b] All rabbits had allografts in one chamber and (if available) autografts in the contralateral chamber

RESULTS

Experiment 1. The grafts (all visible) were surrounded by new vascular connective tissue and were thereafter gradually resorbed (Sudmann & Marton 1975). New bone tissue appeared 14—43 days after autografting along or on the remnants of 23 out of 24 grafts. The earlier it appeared the more rapidly and abundantly it formed. The areas of new bone tissue 31 days after autografting ranged from 0 to 7.7 mm^2.

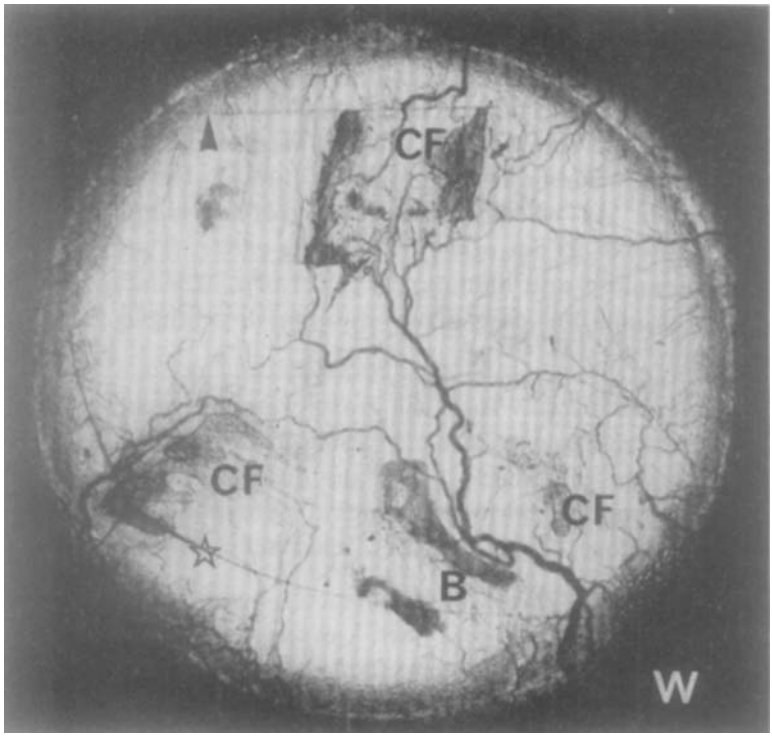
Experiments 2—3. Here the grafts were not visible in bright field microscopy. New bone tissue did not enter the optical part of the chambers. When the chambers were re-opened bone formation beside the optical part was very sparse or absent altogether as observed macroscopically.

Experiment 4. Bone tissue formed a bony ring in the shallow annular non-optical part, i. e. the well, of all the chambers. A thin flake of new bone tissue entered the optical part of 1 chamber. When removed from the chamber this flake was 0.07 mm thick which means that the depth of the optical part of this chamber was not 0.05 mm as intended.

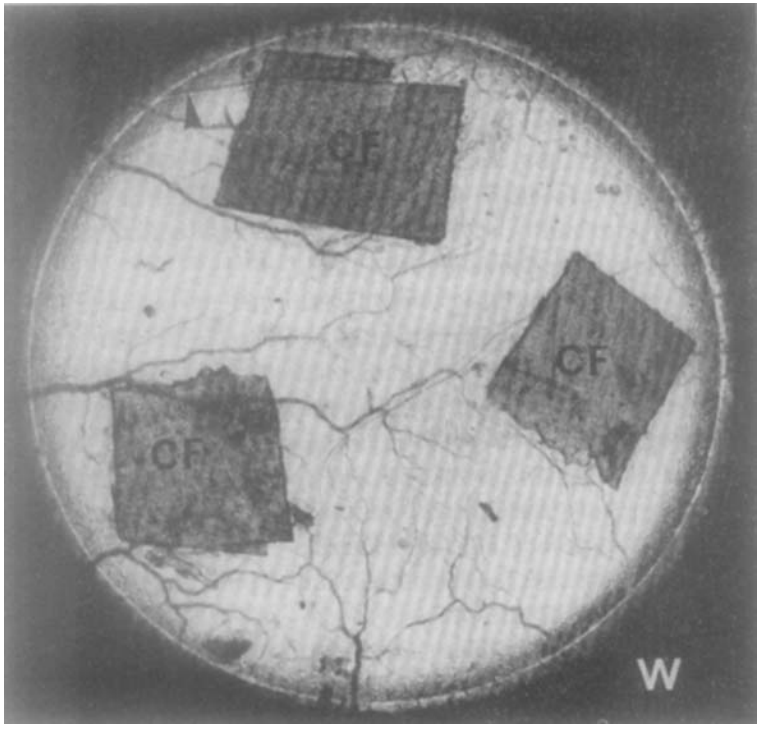
Experiment 5. One chamber (12 allografts) became infected after grafting and these grafts were excluded. One rabbit (2 chambers, 24 grafts in all) died of unknown causes 33 days after grafting.

Visible autografts were gradually resorbed and the autografts in the well were covered with new tissue. New bone appeared in the optical part of the chambers after 15—27 days.

The visible allografts were practically not resorbed and bone formation was not observed by vital microscopy. The allografts in the well — as opposed to the autografts — were unchanged as judged macroscopically. A typical chamber with autografts and one with allografts in the same rabbit are shown in Figures 2—3.



Figures 2—3. Vital photomicrographs. Bone cultures in 2 contralateral ear chambers in the same rabbit 20 days after grafting 3 cortical flake (CF) grafts to each optical part (visible) and 9 cortical cancellous grafts to each well (W) (not visible). Arrowheads indicate system of coordinates in the base of the chambers. Field width, 6 mm. Objective 1x/0.04. *Figure 2.* Left ear chamber. The area of the visible *autografts* was reduced by 82—99 per cent. Bone from the well is just growing (barely visible) into the optical part at several points. Bone tissue (B) had also, after partial resorption of a graft, formed within the connective tissue which had encapsulated the graft. A tiny bone fragment is all that is left of this autograft. The cover glass had unintentionally been broken (asterisk). *Figure 3.* Right ear chamber. The area of the visible *allografts* is unchanged. They measure 2.4, 1.9 and 1.9 mm^2 as assessed by mixed--image planimetry. No bone formation was noted in chambers with allografts.



DISCUSSION

Bone resorption was observed in all autografts, and was thus easily obtained in the rabbit ear chamber in vivo. This did not apply to the formation of bone tissue. In the first place, bone formation was only observed following autografting. Secondly, the size and type of bone graft, the technique of grafting, the type and the depth of the ear chamber were essential for securing consistent bone tissue formation in rabbit ear chambers.

It is generally recognized that heterotopic bone formation is easily obtained in the rabbit. Spontaneous bone tissue formation was occasionally encountered in the orginal Sandison-Clark chambers inserted in rabbits' ears

(Clark & Clark 1942). The appearance of such bone tissue, however, has not been noted in modern ear chambers (Ezra-Cohn et al. 1969) — including the chambers used here.

The tibia, ulna and a lumbar spinous process have been used as donor sites for ear chamber grafts (Sandison 1928, Kirby-Smith 1933, Ezra-Cohn et al. 1969). Of these sites, the spinous process is the most easily approached surgically and provides material both for thin cortical bone flakes and for cortical cancellous grafts.

The size of the graft was determined by its use. Grafts for the study of resorption only need to be as thin as possible for the best bright field microscopy. It is technically difficult, however, to prepare a graft thinner than 0.1 mm. These thin grafts were always placed on vascular connective tissue within the optical part of the chambers. Resorption of autografts regularly followed regardless of chamber type or chamber depth. However, it was osteogenesis inside the shallowest possible optical part which was the main goal of the present study.

Bone formation was obtained on or along 23 out of 24 (96 per cent) of standardized (volume about 0.2 mm^3) autografts in methacrylate-glass chambers 0.12 mm deep (Experiment 1). Smaller grafts were completely resorbed without bone formation (Sudmann, unpublished data). The appearance of new bone tissue (14—43 days) and the quantity of new bone tissue in Experiment 1 varied too much for comparison of groups of animals.

Larger cortical cancellous and cancellous bone grafts were in Experiments 2—3 placed outside but close to a slit-shaped (< 0.1 mm) optical part, as outlined by Sandison (1928) and Kirby-Smith (1933). Neither these cortical cancellous grafts placed in completely vascularized chambers nor the cancellous grafts installed at the time of chamber insertion initiated bone formation within the optical part of the chambers. Furthermore, bone formation was macroscopically irregular and sparse beside the optical part in these methacrylate and multiple component titanium-glass chambers (Experiments 2—3).

The non-optical well of titanium rosette chambers (Experiment 4), however, was always filled with a ring of bone tissue which grew into the optical part of one chamber. The depth of this optical part was — unintentionally — greater than the intended 0.05 mm.

In succeeding studies (Sudmann 1975c, Sudmann & Marton 1975), the depth of the optical part of the rosette chamber was consequently increased from 0.05 to 0.25 mm. This made not only comparison of groups, but also studies of both disordered and ordered (Sudmann & Marton 1975) osteoclastic and osteoblastic activity possible.

Osteogenesis within the rosette ear chambers was thus consistently secured. But what was the initiating source of this bone formation — living osteogenetic cells transferred with the grafts (Chalmers 1959) or the release of an inductor substance (McLean & Urist 1968) ? It is not possible to answer this question unequivocally at present. However, the complete failure of allografts to trigger bone formation may suggest that the cellular component is the essential addition.

SUMMARY

Heterotopic osteogenesis was studied in vivo by grafting 246 bone tissue grafts onto 39 culture chambers inserted in the ears of 27 rabbits. Bone formation was obtained from 23 out of 24 autografts placed inside the optical part ($>$ 0.1 mm deep) of 8 ear chambers. No bone tissue formation was observed in the optical part ($<$ 0.1 mm deep) of 23 chambers in which a total of 129 autografts had been placed in a communicating deeper non-optical part.

Bone formation was always obtained after autografting bone tissue to the non-optical part of a titanium-glass ear chamber of own design, and bone tissue entered the optical part of these chambers when this was deeper than 0.1 mm. The possible source of bone formation in rabbit ear chambers is mentioned. Resorption of bone tissue autografts was regularly obtained.

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MIXED-IMAGE PLANIMETRY

Assessment of a new method for stereometric analysis in microscopy[1]

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Key words: Bone and bones, analysis; methods; microscopy, instrumentation; stereometric analysis.

Assessment of bone quantity is indispensable for the study of bone remodelling (Frost 1963, Schink 1970). True quantitation of mass or volume is impractical or impossible in experimental systems designed for studies of bone remodelling, e.g. in vital chambers inserted in rabbit ears. However, it appears from the findings of other workers (Weibel & Elias 1967, Frost 1969) as well as from own findings (Sudmann & Marton 1975) that in many experimental situations the total area of the bone in one projection, e.g. in bright field microscopy in a rabbit ear chamber, is proportional to the bone mass. Bone area may therefore be used as a parameter of bone mass during resorption and formation (remodelling). There remains the problem of finding a good quantitative method for the measurement of area in microscopy.

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[1]*Presented at the Annual Meeting of the Norwegian Orthopaedic Association, Oslo, Norway, October 28, 1972.*

Several stereometric methods are available for surface area measurement in microscopy, among them the integrating eyepiece, which is widely used. In photomicrographs or outlines of the specimen, the area can be calculated by an ordinary planimeter or by weighing the cut-out areas (Weibel & Elias 1967, Frost 1969, Baylink et al. 1969). Since none of these methods proved entirely satisfactory for the in vivo assessment of the surface area of bone tissue in the rabbit ear chamber, a new method of area quantitation in microscopy has been developed, called mixed-image planimetry.

The new method has proved quicker and simpler than known methods for stereometric analysis in both bright field and fluorescence microscopy. It requires a drawing device for the microscope and a planimeter. These items are easily available and have been widely used for area quantitation by weight and by planimetry. The usefulness of mixed-image planimetry for further routine stereometric analysis thus depended primarily on its precision in area quantitation. The purpose of the present study was to present this method and assess its precision.

MATERIAL AND METHODS

The surface area of 1 bone autograft was quantitated by mixed-image planimetry and by 3 widely used methods of stereometric analysis, i. e. integration, weighing and ordinary planimetry. The graft was composed of compact, cortical bone from a lumbar spinous process of a rabbit. A 0.1 mm thick fragment was heterotopically autografted to a Sandison-Clark round-table type rabbit ear chamber (Figure 1). The estimated surface area of the autografts was in the present study assessed 52 days after autografting in vivo. The bone area was continuous and always within the measuring field.

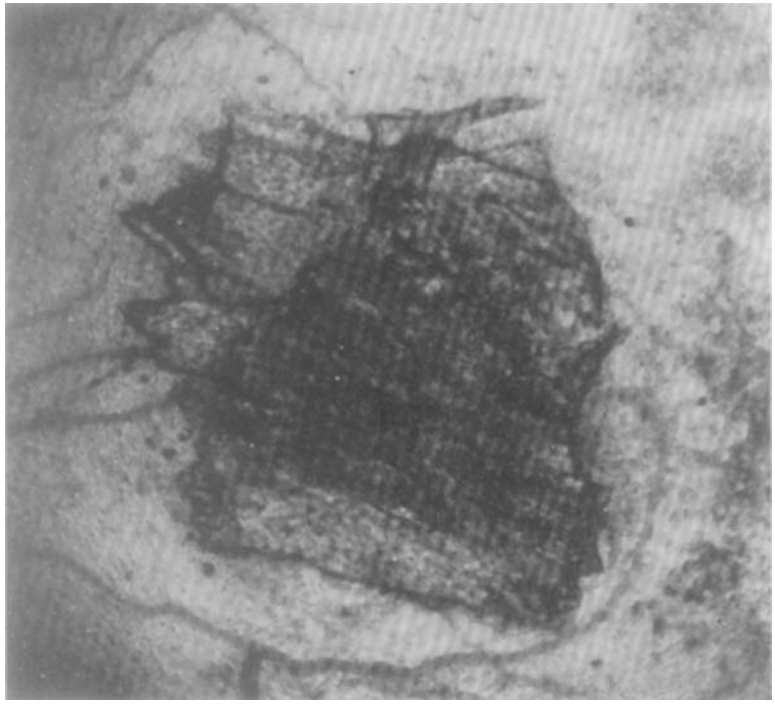


Figure 1. Vital photomicrograph. Bone autograft in rabbit ear chamber used for in vivo stereometric analysis of a finite object (estimated surface area about 1.5 mm^2). Objective 4x/0.1.

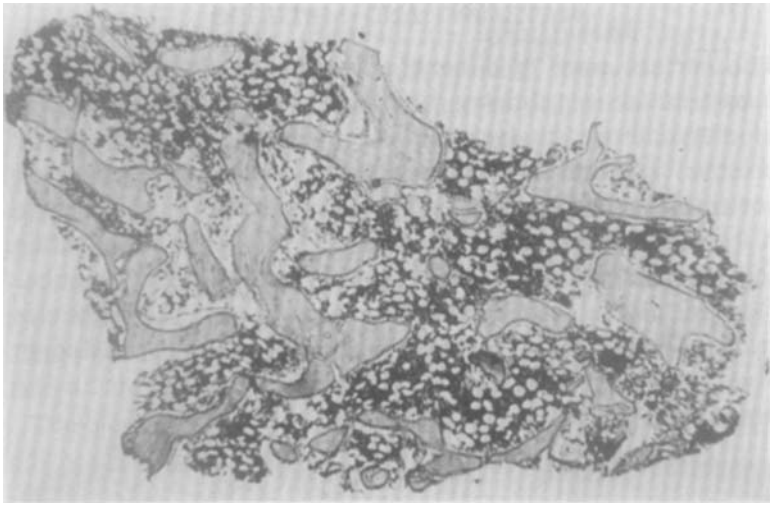


Figure 2. Photomicrograph. Mounted section of cancellous bone used for stereometric analysis of an extensive object. Decalcified section, H + E. Field width, 2.8 mm. Objective 2.5x/0.08.

Bone area quantitation by mixed-image planimetry was also performed in a variety of other ear chambers described in detail in other articles (Sudmann 1975 a—b, Sudmann & Marton 1975). A photomicrograph from one of these chambers is included here (Figure 7).

In addition, the areas of bone and bone marrow in a paraffin section from the iliac crest of a rabbit were assessed by mixed-image planimetry. The biopsy was composed of cancellous bone. It was fixed in buffered formalin and the decalcified sections were stained with haematoxylin-eosin (Figure 2). The estimated surface area of the bone trabeculae in one mounted section was quantitated. The bone area was not continuous and was larger than the measuring field.

Microscopy was done in bright field. The light was adjusted according to Köhler's principles. The magnification changer of the Zeiss Universal microscope was set at 1.25. The autograft was assessed with a Zeiss LD Epiplan

4x/0.1 objective and an 8x ocular. The bone section was estimated with a Zeiss Neofluar 25x/0.60 objective and the Leitz ocular pair Periplan GF' 12.5x M-MF. The MF' ocular incorporates several reticles for photomicrography (e.g. 6.5 x 9 cm).

Surface area of autograft

Method 1. Integrated area of bone. The surface area of the autograft was estimated with an 8x Zeiss integrating eyepiece in accordance with the specifications of the manufacturer (instruction pamphlet G 41-260-d). The eyepiece contained a turret (type 1) which incorporated integrating discs (Figure 3). Discs with 25, 100 and 400 test points were used. Ten different positions relative to the graft were obtained for each of the discs by turning the ocular slightly in increments. The points superimposed on the image of the bone fragment (hits), and every second point superimposed on the rim of the fragment, were counted once in each position.

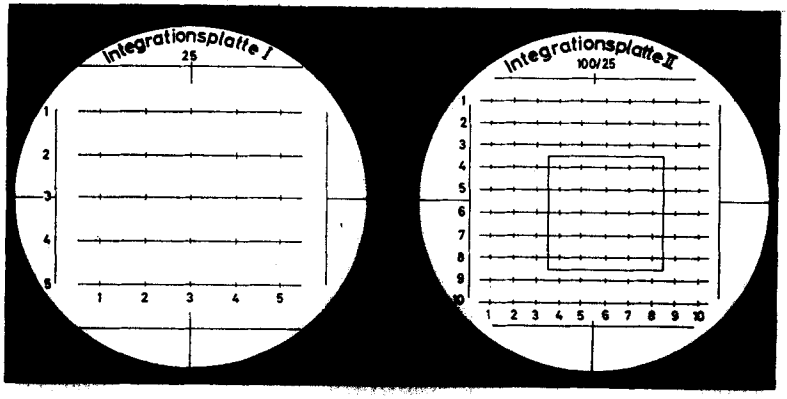


Figure 3. Integrating discs are versatile devices for quantitative microscopy. Discs with 25 and 100 test points are illustrated. These were positioned in an eyepiece and thus superimposed on the image of the specimen.

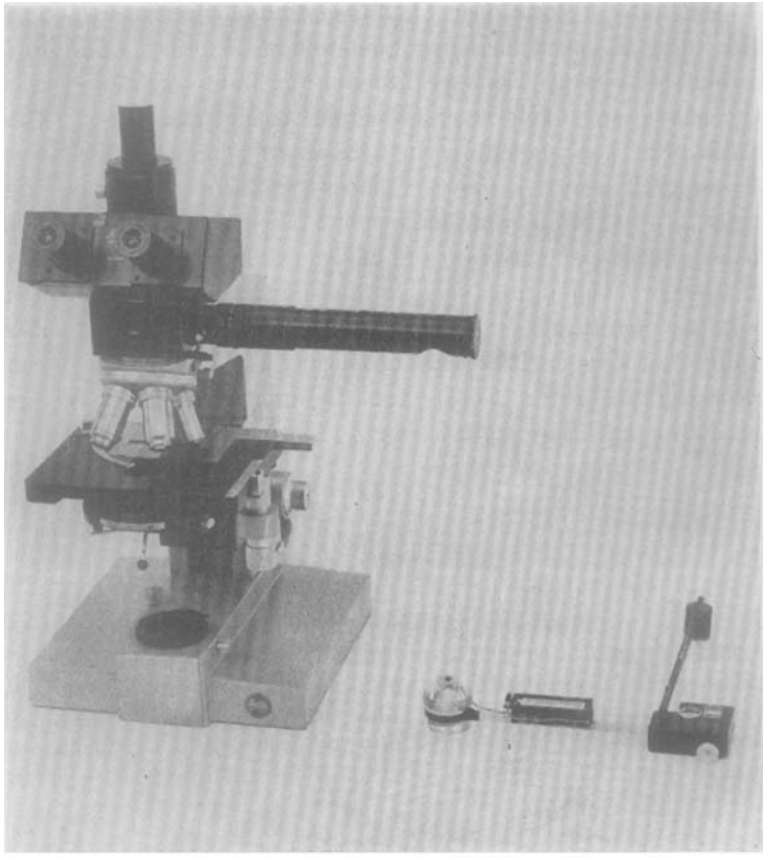
Method 2. Bone area by weight. A monocular Zeiss drawing device, with the observer's eye perpendicular to the drawing surface, was attached to the microscope. Ten enlarged outlines of the bone fragment were traced with an ordinary pencil on semistiff paper with a thickness of 0.19 mm and a weight of 0.018 g/cm^2. Each tracing was cut out by hand using a small pair of scissors and immediately weighed once on an analytical scale (Mettler, type H 16, Zürich, Switzerland). They were thereafter stored at room temperature overnight and weighed once more 24 hours later. The results were identical.

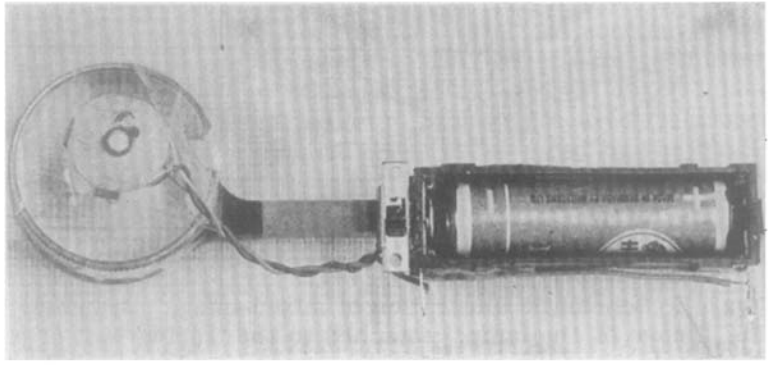
Method 3. Bone area by planimetry. The autograft was photomicrographed on 1 Polacolor Land film type 108 (print measuring 8.5 by 10.8 cm) and 5 outlines were made on semistiff paper, as stated earlier. The bone area was calculated with a Haff planimeter (Type 317, Pfronten, Bayern, Germany). One planimeter reading was recorded for each outline, 5 for the photomicrograph of the graft.

Figures 4—5. Complete outfit for mixed-image planimetry.

Figure 4. Microscope with drawing device and planimeter with a miniature lamp and a battery. The drawing device should permit the planimeter to be used in the horizontal plane. A Zeiss microscope was used for stereometric calculation. A Leitz outfit (illustrated) was used for taking mixed-image photomicrographs (see Figures 7—8).

Figure 5. A spare lamp for a hunting scope (Bushnell, type Scopechief 5, Pasadena, California, U. S.A.) was taped to the tracing lens of the planimeter. A light-emitting diode may also be used. A miniature switch was glued to the battery container enclosing an ordinary 1.5 V battery.





Method 4. Bone area by mixed-image planimetry. The technique is based on the mixed-image principle. A miniature electric lamp was taped to the planimeter's tracing lens and a drawing device was attached to the microscope. The planimeter was placed on the drawing surface. The drawing device reflected the image of the tiny light spot from the lamp into the binocular body tube of the microscope (Figures 4—5). Binocular observation of the superimposed (mixed) images of the light spot and the bone graft was then possible (Figure 6). The tracing lens of the planimeter could thereby be guided along the border of the area to be measured (Figures 7—8). Ten planimeter readings of the graft illustrated in Figure 1 were recorded.

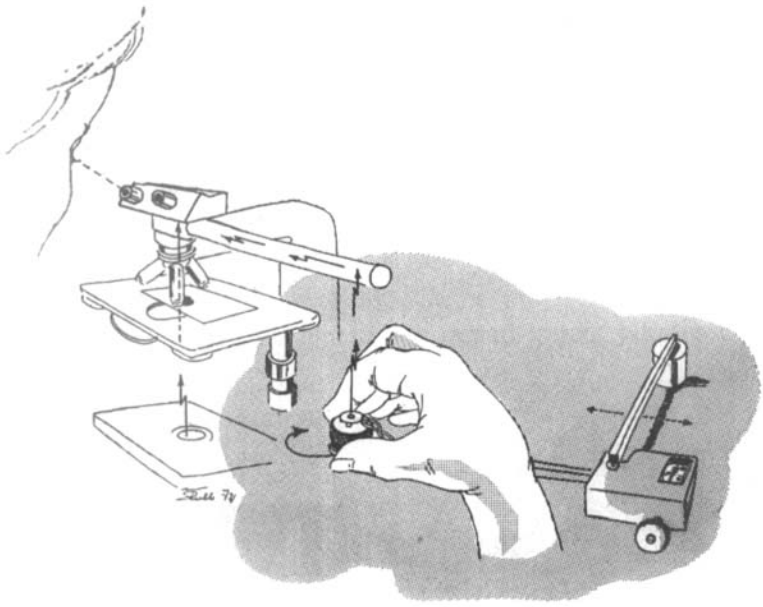


Figure 6. Schematic illustration of mixed-image planimetry. The image of the lamp, attached to the tracing lens of a planimeter, is easily observed superimposed on that of the specimen thanks to the drawing device of the microscope. The intensity of the room illumination makes no difference.

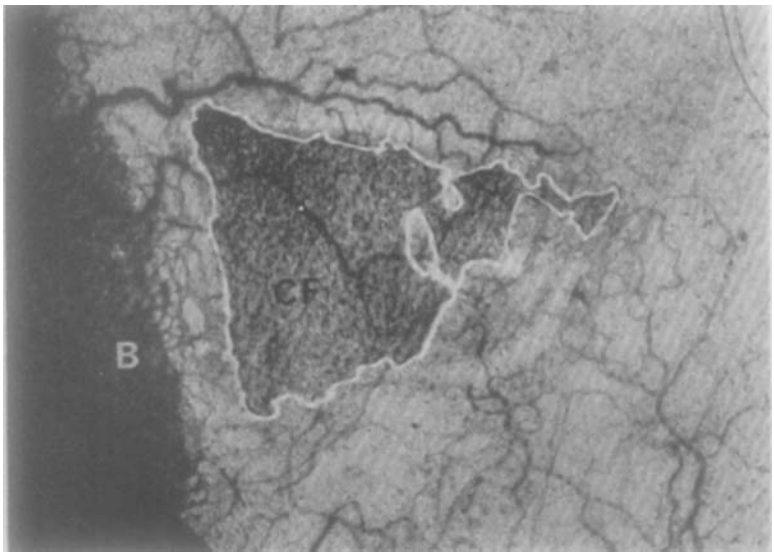


Figure 7. In vivo mixed-image photomicrograph. Partially resorbed cortical flake (CF) autograft in rabbit ear chamber and lamp on the planimeter's tracing lens 20 days after grafting. The autograft was originally rectangular and measured 2.2 mm^2. New bone (B) had grown into the optical part of the chamber from the side (area 0.6 mm^2).

Mixed-image photomicrography : The light intensity of the microscope lamp was reduced until the contour of the bone fragment was barely visible. With open camera shutter, an area of 1.1 mm^2 was slowly assessed by mixed-image planimetry. Additonal exposure time was secured for proper exposure of the specimen. The area traced by the planimeter was thereby permanently recorded in the photomicrograph. Objective 2.5x/0.08.

Calibration. The stereometric methods 1—3 were calibrated with a Reichert object micrometer. Mixed-image planimetric measurements were calibrated by using a Zeiss ocular grid micrometer 10 x 10 mm as an object grid micrometer. Three of these calibration factors are given in Table 1.

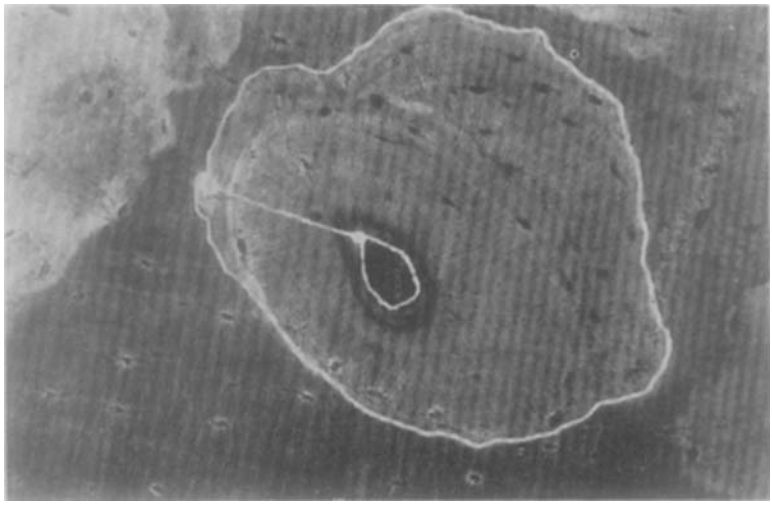


Figure 8. Mixed-image photomicrograph. Cross section of Haversian system and lamp on planimeter's tracing lens. The tracing lens was first guided clockwise along the outside of the Haversian system. A total area of 0.040 mm^2 was calculated.

The specimen was reorientated and the tracing lens thereafter guided anti-clockwise along the Haversian canal. The area of the canal was thereby mechanically subtracted from the total area, giving a net area of 0.039 mm^2 of bone tissue. Objective 25x/0.60.

Table 1. Calibration factors for mixed-image planimetry

Zeiss LD Epiplan objective	Calibration factor
4 x	0.04
8 x	0.01
16 x	0.0025

Planimeter reading x calibration factor = Area in mm^2.

Surface areas in the bone section

The total area framed by the 6.5 x 9 cm labelled reticle of the MF ocular was measured 9 times by mixed-image planimetry. The ocular was then (for best coverage) turned until the short axis of the reticle was parallel to the long axis of the specimen. The surface areas of the bone trabeculae, framed by the 6.5 x 9 cm reticle (Figure 9), were thereafter quantitated by mixed-image planimetry in two ways.

In the first series of recordings the specimen was moved randomly 9 times. Bone surface areas were estimated once for each new position. The planimeter's scale was zeroed after each reading.

In a second series of recordings the specimen was moved a further 8 times, but the scale of the planimeter was not adjusted. To achieve a cumulative reading of bone areas, both the semistiff plastic underlay of the planimeter and the planimeter were moved *en bloc* between each area quantitation. The time required for making this cumulative estimate was noted.

The estimated quantity of bone trabeculae (Vittali 1970) and cellular marrow (Dunnill et al. 1967) in the section was calculated.

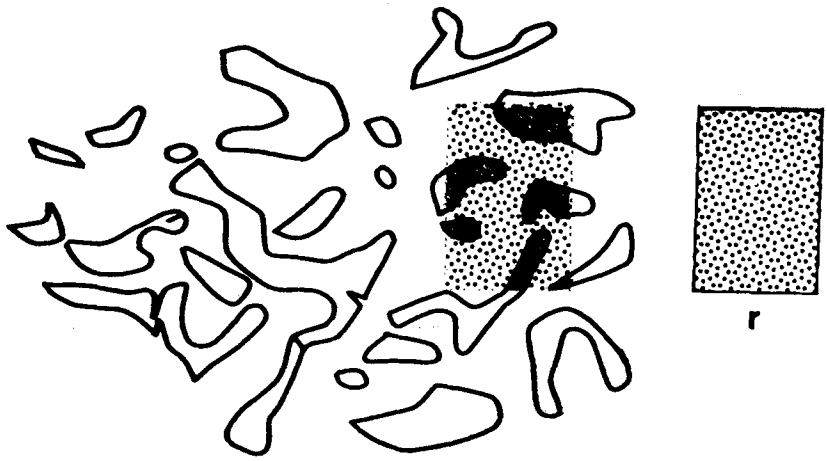


Figure 9. Mixed-image planimetry of bone areas in a histological section of cancellous bone (schematic) : A reticle was attached to the eyepiece of the microscope. The area (r) framed by this reticle was then quantitated by mixed-image planimetry as described in the text.

The section was then moved at random and the bone areas framed by the reticle (double hatched) were estimated as described in the text for each new position.

The following equations were then used for calculation of total measured area (A), of quantity of bone trabeculae (B) and of soft tissue (S) :

$$(I) \quad A = B + S$$

$$(II) \quad A = r \times n$$

$$(III) \quad B = b_1 + b_2 + \ldots b_m$$

(b = bone areas, n = number of positions, m = total number of bone areas measured)

RESULTS

One of the Zeiss drawing devices was attached to the microscope by a simple clamp ring, the other (Zeiss) by a standard Zeiss circular dovetail mounting. Both mountings lacked a positive interlocking with the microscope, so that the position of the drawing devices was easily upset, necessitating control of the position before each measuring session. No lid was provided for closing the drawing devices when not in use. Furthermore it was not possible to attach the binocular body tube to the appropriate drawing device with a standard polarizing filter in between. A drawing device (Leitz) like the one illustrated in Figure 4 eliminates all these difficulties and sources of error.

A light-emitting diode was used instead of a lamp on the first set-up for mixed-image planimetry. Because of its higher light intensity the lamp was chosen for «mixed-image photomicrography» (Figures 7—8). The light intensity of the lamp was, however, too high for area quantitation in fluorescence microscopy (unpublished data). A series-coupled miniature rheostat (0—20 Ohms) was therefore later glued to the battery container.

Surface area of bone autograft (Table 2)

Integrated area of bone. The number of hits ranged from 6 to 121 according to the position and type of integrating disc. When the number of hits on a finite object was increased from 8 to 125, the standard deviation (absolute areas) decreased from 0.2 to 0.03 and the estimate of the area was reduced by 6 per cent. One operation per quantitation was necessary (multiplication with calibration factor excluded). Executed with reasonable care, the procedure was time-consuming and strenuous.

Bone area by weight. The weight of the cut-out paper areas varied from 0.850 to 0.906 g. The standard deviation

Table 2. Results of stereometric analysis of bone autograft illustrated in Figure 1.

			No. of calculations	Calibration factor	$\bar{X}$, mm^2	SDX x 100, mm^2
Method 1.	Integrated area of bone	Disc with 25 test points	9 (74 points)	0.19	1.6	20
		Disc with 100 test points	10 (320 points)	0.04	1.55	8
		Disc with 400 test points	10 (1,251 points)	0.01	1.50	3
Method 2.	Bone area by weight	10 tracings	10	1.77	1.56	3
Method 3.	Bone area by planimetry	1 Polacolor print	5	0.07	1.46	2
		5 tracings	5	0.03	1.54	4
Method 4.	Mixed-image planimetry	1 bone autograft in vivo	10	0.04	1.44	1
		Calibration, Zeiss grid micrometer (1 mm^2)	10	0.04	1.000	0.4

$\bar{X}$ = mean area
SDX = standard deviation. The lower the standard deviation, the better the precision of the method.

was 0.03. Three separate operations, tracing, cutting and weighing, were necessary. The procedure was time-consuming.

Bone area by planimetry. The planimeter readings of the tracings varied from 49.5 to 52.8 cm^2. The standard deviation was 0.04. Readings of bone area in the photomicrograph varied from 19.4 to 20.0 cm^2. The standard deviation was 0.02. The procedure required 2 separate operations and was time-consuming.

Bone area by mixed-image planimetry. The planimeter readings varied from 35.6 to 36.6 cm^2. The standard deviation was 0.01. Only one operation was necessary per area quantitation. One reading was obtained in half a minute. Calibration was easily obtained with a standard deviation of 0.004. This method was therefore in all respects superior to the others tested.

Surface areas in the bone section

The areas framed by the 6.5 x 9 reticle, recorded by mixed-image planimetry, varied from 88.7 to 89.1 cm^2. $\bar{X}$ (mean area) was 88.9 cm^2, SDX (standard deviation)was 0.2 cm^2.

Nine separate mixed-image readings of bone area varied from 10.3 to 54.7 cm^2. $\bar{X}$ was 32 cm^2 and SDX was 15 cm^2. The cumulative reading of bone trabeculae in 8 areas of the specimen was 244.0 cm^2. $\bar{X}$ was 30.5 cm^2. The whole procedure was completed in 4 minutes. The estimated quantity of bone and cellular marrow in the section was about 34 and 66 per cent respectively.

DISCUSSION

Bone mass or volume is the ideal quantitative index of bone resorption and formation. An estimate of the two-dimensional extension was more easily and precisely obtained in the experiments using mixed-image planimetry than by the other methods of area quantitation tested. In addition, in ear chambers an estimate of the bone thickness is necessary for the assessment of bone volume.

Bone quantity in rabbit ear chambers

The upper surface of the bone, i.e. the side next to the cover glass of the ear chamber, was visualized without difficulty using the fine-motion screw of the microscope, but the under surface was not visible. Bone thickness could therefore not be estimated directly.

It sounds feasible to remove the pieces of bone from an ear chamber for volume measurement, but volume quantitation of tiny bone fragments poses technical problems. Furthermore this procedure would automatically have stopped the experiment.

Since the thickness of the tissue to be studied in an ear chamber cannot be measured, area quantitation should only be used with caution as a parameter of volume changes.

However, it appears that bone grows in ear chambers in laminae of surprisingly constant thickness (Sudmann & Marton 1975), which means that volume and area will be proportional.

Secondly, the assessment of vectorial growth of bone as estimated by the increasing area a bone profile occupies in bright field microscopy of ear chambers is in itself a valuable parameter of bone physiology or pathology.

It thus appears that area estimation, as performed in the present study, can supply valuable information on bone resorption and formation in rabbit ear chambers.

Stereometric quantitation of surface area

Two main types of objects, finite and extensive, are encountered in area quantitation. The area of a finite object is entirely within the measuring field. The area, not necessarily continuous, of an extensive (infinite) object is larger than the measuring field (Sitte 1967). A method of stereometric analysis ought to be applicable to both types of objects. The finite object chosen for this study was a small piece of compact bone (autograft) and the extensive object was a histological section of cancellous bone (biopsy).

Generally a total of 100 test points must be counted with the *integrating eyepiece* to arrive at a safe estimate of the object (Frost 1969). The findings of the present study support this contention. An even more accurate and precise estimate can be obtained by increasing the number of hits, but for obvious reasons this would be very time-consuming and laborious. The method may be used with finite and extensive objects for quantitation of tissues of low contrast and in fluorescence microscopy.

Estimation of *bone area by weight* and by ordinary *planimetry* required several separate operations. Area quantitation of a finite object was nevertheless as accurate and precise as the foregoing method. Outlining the object was the crucial factor. High contrast objects are easily outlined, but low contrast objects are difficult or impossible to outline under unfavourable light conditions. Area quantitation of a finite object by these methods was time-consuming. Area estimation of an extensive object, for routine work, would therefore be out of the question.

Mixed-image planimetry, as described here, was more precise than the other methods tested for area quantitation of a finite object. The recordings varied less than 1 per cent. An estimate of the surface area of an extensive object was also obtained without difficulty. Furthermore it could be used for area calculation of low contrast objects and under unfavourable light conditions (e.g. fluorescence micros-

copy) and for photomicrographic documentation (Figures 7—8).

In a finite object mixed-image planimetric measurements required only 1 operation per measurement and this was done in half a minute. The precision of the method was so good that only 1 measurement of the object should be necessary for routine work. This method therefore made area quantitation possible almost instantaneously and without any of the shortcomings of the other methods tested.

By replacing the planimeter with an appropriate instrument, the principle of mixed-image measurement should also apply to the measuring of distances in microscopy, e. g. the length of curved contours lying on a single plane.

Planimetric measurements are ordinarily made by guiding the tracing lens of the planimeter clockwise. The planimeter subtracts when the tracing lens is guided anti-clockwise. The difference between two areas can therefore easily be obtained by guiding the tracing lens clockwise when measuring the largest and anti-clockwise when measuring the smallest area (Figure 8).

Mixed-image planimetry was devised for the assessment of bone tissue remodelling in rabbit ear chambers, but should also find a wider application in quantitative microscopy.

SUMMARY

A new method for stereometric measurement of surface area in microscopy is described, based on the mixed-image principle. The measurement was accomplished by observing the area on which was superimposed the image of a miniature electric lamp attached to an ordinary planimeter.

Mixed-image planimetric measurements were more easily made and required much less time than area calculation by an integrating eyepiece, by weight or by ordinary planimetric measurements of photomicrographs or tracings of the area. Mixed-image planimetric measurements of a finite area varied less than 1 per cent. Such precision was not obtained with the other methods.

The new method was devised for in vivo stereometric measurements of bone tissue remodelling in rabbit ear chambers, but should find a much wider application in quantitative microscopy of both finite and extensive objects.

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BONE REMODELLING IN RABBIT EAR CHAMBERS

A vital microscopical and histological study

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Key words : Animal experiments; bone and bones; bone matrix; bone marrow; ear, external; models, biological; osteoblasts; osteoclasts; tissue culture.

Bone remodelling, i.e. the resorption and formation of bone tissue, is essential for the integrity and physiology of the skeletal system. Experimentally induced bone remodelling can be investigated in vivo in chambers inserted in rabbits' ears (Sudmann 1975 a—d) and several authors have described the morphology and remodelling of bone as revealed in bright field microscopy in such ear chambers (Sandison 1928, Kirby-Smith 1933, Clark & Clark 1942, Ezra-Cohn et al. 1969). Bone tissue from ear chambers has also been studied in histological sections by light and transmission electron microscopy (Ezra-Cohn et al. 1969). To our knowledge no reports are available, however, correlating the in vivo observations with histological sections of bone tissue from the same chambers.

Since vital microscopical and histological findings had not been compared, it was uncertain which elementary types of bone and bone tissue structures could be identified by vital microscopy in rabbit ear chambers. The purpose of the present study was to correlate such findings to obtain a more precise understanding of bone tissue resorption and formation in these chambers.

MATERIAL AND METHODS

Bone tissue remodelling was studied by vital microscopy in 73 ear chambers in 51 rabbits of both sexes, 5—12 months old. Included in this series were animals and ear chambers used for various bone tissue studies described in detail elsewhere (Sudmann 1974 a—d). The findings described and illustrated here, however, represent the typical behaviour of bone tissue as revealed by vital microscopy and histological examination of autologous bone tissue in titanium-glass rosette ear chambers inserted in sandy half lop-eared rabbits (Sudmann 1974 a).

The rosette chambers were composed of 2 communicating compartments. A slit-shaped optical part (depth 0.2—0.3 mm, diameter 6 mm) was surrounded by a shallow (0.7—0.8 mm), annular non-optical part, i.e. the well. The interior of the chamber could be rendered freely accessible for grafting and manipulation by removing the cover glass forming its upper boundary (Sudmann 1975 b). At the start of this study all chambers were vascularized by newly formed connective tissue.

The technical set-up for bright field vital microscopy and photomicrography is described elsewhere (Sudmann 1975 a). The optical field was restricted to the optical part of the chambers (Figure 1). Tissue in the well was therefore examined in reflected light macroscopically or using a low-power stereo microscope. Bone area was assessed with mixed-image planimetry (Sudmann 1975 c).

In plane polarized light microscopy, i.e. with crossed polars, woven-fibred connective tissue and bone appear dark, while parallel-fibred connective tissue and bone appear bright.

Bone resorption and formation were initially studied following bone grafting. The bone grafts were placed on the regenerated connective tissue in the chambers. Three 0.1 mm thick cortical flake grafts, about 2 mm^2 in area, were

placed in the optical part of the chambers (Figure 1), and 9 cortical cancellous grafts of about the same size were placed in the well (Sudmann 1975 b).

Bone formation with subsequent remodelling was later studied following complete removal of all tissue inside the optical part of the chambers (Sudmann 1975 d).

Tissue for histological study comprised : (1) single or repeated biopsies taken from 25 ear chambers and (2) bone cultures in toto obtained from 43 ear chambers when removed from the rabbit's ear.

The specimens were fixed in buffered formalin (Vittali 1970). Undecalcified epoxy resin sections stained with toluidine blue, haematoxylin-eosin or by von Kossa's method were mainly used for the study of osteoid tissue and bone mineralization. Decalcified sections stained with haematoxylin-eosin were used for the rest of the study.

Tissue for epoxy resin embedding was dehydrated in graded water-acetone mixtures, transferred to propylene-oxide and infiltrated with epoxy resin (Epon 812, Ladd Research Industries Inc., Burlington, Vermont, U. S. A.) as described by Luft (1961). Following polymerization at 60°C, the blocks were trimmed and 5 μm sections cut, using a motorized Jung microtome (type 1130, Heidelberg, Germany). The clear and translucent epoxy resin blocks made possible direct microscopy of the embedded ear chamber tissue, allowing the necessary orientation for accurate trimming and sectioning of selected tissue areas for correlation of vital microscopical and histological findings.

Epon sections were either stained with 1 per cent toluidine blue before mounting, or stained with haematoxylin-eosin or by von Kossa's method after dissolving the resin in a saturated solution of natrium-hydroxide in ethanol. Tissue for paraffin embedding was decalcified in Decalc (Bethlehem Trading, Gothenburg, Sweden) for 20 minutes and embedded, sectioned and stained by ordinary methods.

RESULTS

Resorption of bone autografts

Following grafting, the 3 bone flakes in the optical part of the chambers were sandwiched between the cover glass on top and the vascularized connective tissue underneath and were bathed in extravascular fluid. The microcirculation within the chambers was intact, including that underneath the grafts (Figure 1).

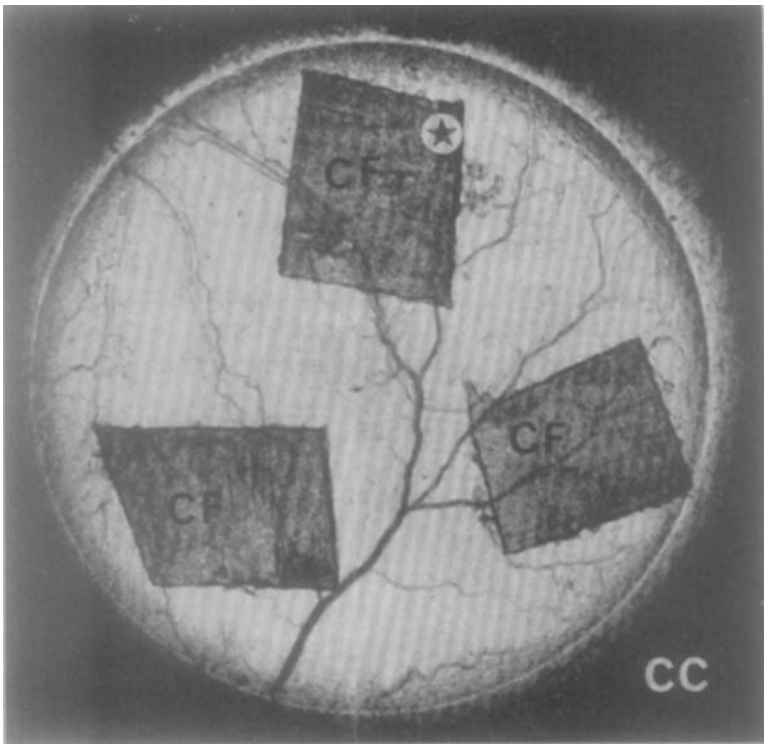


Figure 1. Bright field vital photomicrograph. Titanium-glass rosette ear chamber on the day of bone grafting. Three cortical flake grafts (CF) are visible on vascular connective tissue in the optical part of the chamber. Nine cortical cancellous grafts placed in the non-optical part, the well, are not visible, but one of them is indicated by CC. Asterisk indicates relative position within chamber of detail shown in Figure 2. Field width, 6 mm. Objective 1x/0.04.

During the days following bone grafting the fluid became packed with aggregates of erythrocytes (Figure 2). Within 1 week capillaries were formed between the grafts and the cover glass. During the following weeks the amount of fluid and red cell aggregates around the grafts were gradually reduced and replaced by connective tissue.

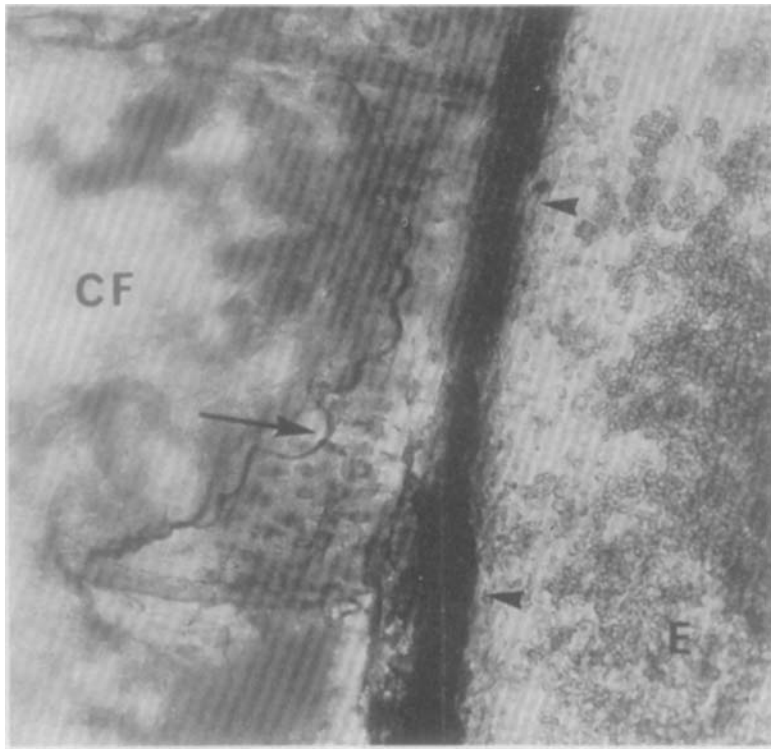


Figure 2. Vital photomicrograph. Detail of partially resorbed autograft (CF) (see Figure 1) in ear chamber 16 days after grafting. Arrowheads indicate edge of bone graft. Arrow indicates one of the Howship's lacunae on the bone surface facing the cover glass. Most of this surface has already been resorbed. Note extravasal erythrocyte aggregate (E). Field width, 0.6 mm. Objective 10x/0.25.

During the second and third weeks small resorption cavities, which formed at irregular intervals, were first noted on the upper and the lower surface of the grafts (Figure 2). After a few more days resorption also appeared at the edges. It was not possible at any time to identify the cellular details of this resorptive activity. By the fifth week a reduction of approximately 50 per cent of a graft area had taken place. This resorptive activity continued until the autograft had completely disappeared, except in cases where bone developed on or near the graft (Figure 6a).

After 3 weeks the 9 cortical cancellous bone grafts located in the well had become buried in newly formed connective tissue and bone.

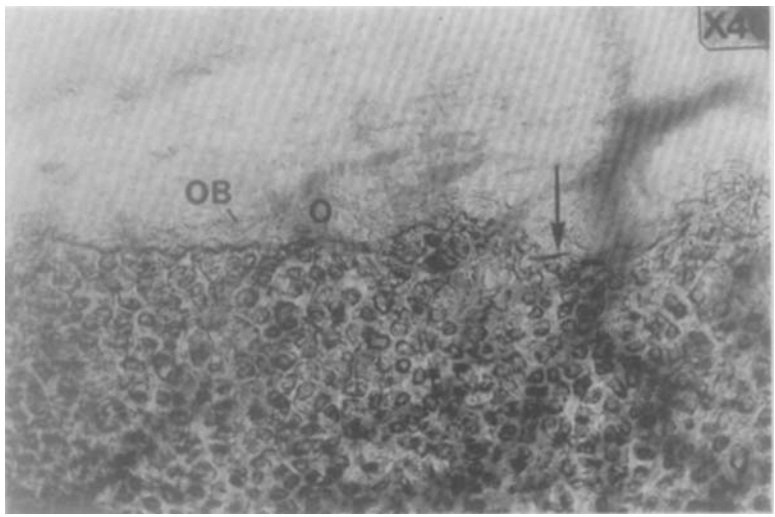


Figure 3. Vital photomicrograph. Part of differentiated type of woven-fibred bone flake, formed underneath the cover glass 36 days after grafting. The flake has grown on a broad front from the well (below) into the optical part of the chamber. The bone rim (arrow) is bordered by an indistinctly demarcated seam of osteoid tissue (O). Both osteoblasts (OB) and lacunae (L) are disordered. Inconspicuous strands of fibres are seen extending from the bone rim. Field width, 0.9 mm. Objective 10x / 0.25.

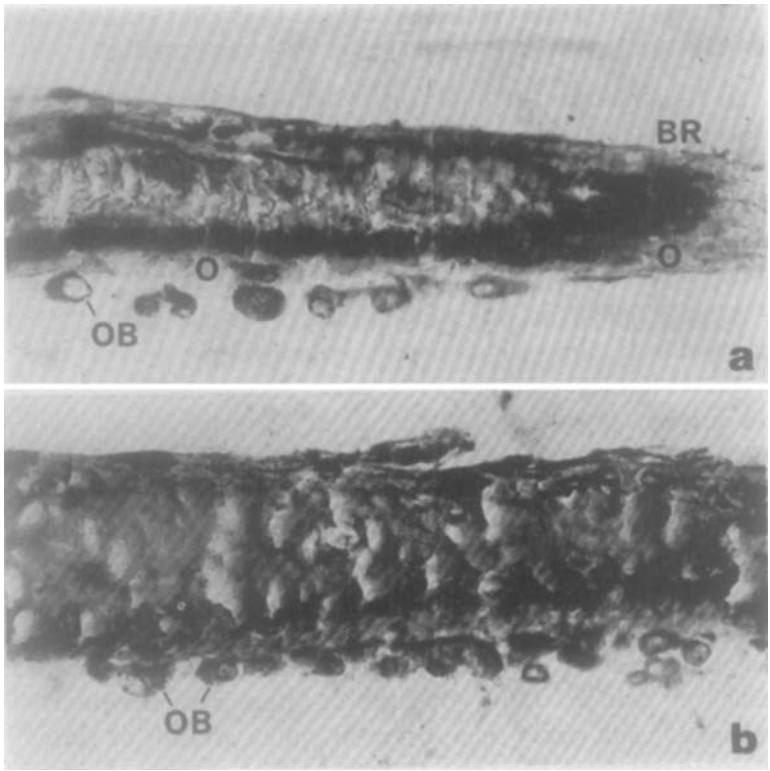


Figure 4 (a—b). Photomicrographs. Histological section of the bone flake shown in Figure 3 36 days after autografting: tip (Figure 4a) and middle part (Figure 4b). Active (disordered) osteoblasts (OB) border the undersurface of the flake. BR = bone rim. O = osteoid tissue. Undecalcified section, toluidine blue. Field width, 0.22 mm. Objective 40x / 0.65.

On histological examination of 14-day-old autografts a varying number of the bone lacunae appeared empty, indicating partial necrosis of the bone graft. Howship's lacunae containing multinucleated osteoclasts were irregularly distributed on the surfaces of the grafts. In the connective tissue of the chambers, which was composed of fibroblasts and conspicuous collagen fibres, were found numerous small vessels, mainly capillaries.

Bone formation from the well following bone grafting

In the third week following grafting budding capillaries advanced from the well and became visible in the optical field just underneath the cover glass. In the wake of these advancing capillaries a flake of new bone tissue grew slowly into the optical part of the chamber, first obliquely towards, later directly underneath the cover glass.

After 60 days varying amounts of new bone tissue were observed in the optical part of the chambers. As quantitated by mixed-image planimetry the bone area occupied from 6 (1.4 mm^2) to 100 per cent (24 mm^2) of the total optical field. The period of most rapid bone growth was between the 20th and 40th days.

The advancing pale brown bone flake was densely and irregularly packed with distinctly delineated lacunae. Canaliculi were barely visible. Between crossed polars these bone flakes appeared dark, i. e. woven-fibred. The bone rim was bordered by an indistinctly demarcated translucent tissue in which faint outlines of numerous irregularly arranged cells were seen, between which strands of fibres extended out from the bone (Figure 3).

As the growth of the advancing bone flake slowed down after a few more weeks, the lacunae near the bone edge were formed with their long axes parallel to the bone rim. Numerous bone canaliculi were now visible interconnecting the lacunae and extending towards the cover glass. The bone edge was framed by a distinct seam of translucent tissue in which a few barely visible cell structures could be seen (Figure 5). Such translucent seams were distinctly demarcated by bone on one side and by a delicate membrane-like structure on the other side (Figures 5, 8b).

Between crossed polars both the bone areas with ordered lacunae and their seams of translucent tissue appeared bright, i.e. parallel-fibred (Figure 8b). In older bone cultures rounded off bone edges were enveloped in connective tissue running parallel to the bone edge (Figure 8b).

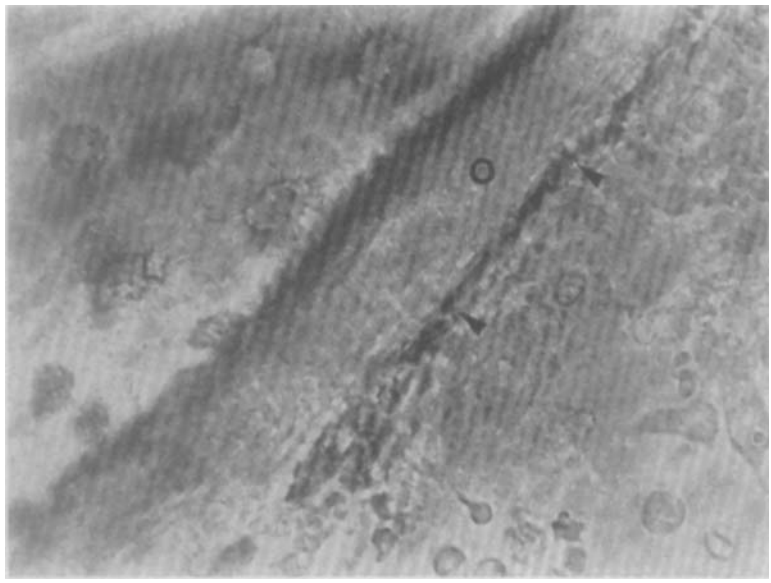


Figure 5. Vital photomicrograph. Edge of parallel-fibred bone flake growing directly underneath the cover glass 33 days after grafting. The lacunae (L) have their long axes parallell to the bone rim and are interconnected by numerous canaliculi. The translucent osteoid seam (O) is distinctly demarcated by mineralized bone on one side and possibly by a lining membrane (arrowheads) on the other side. Field width, 0.17 mm. Objective 40x / 0.75.

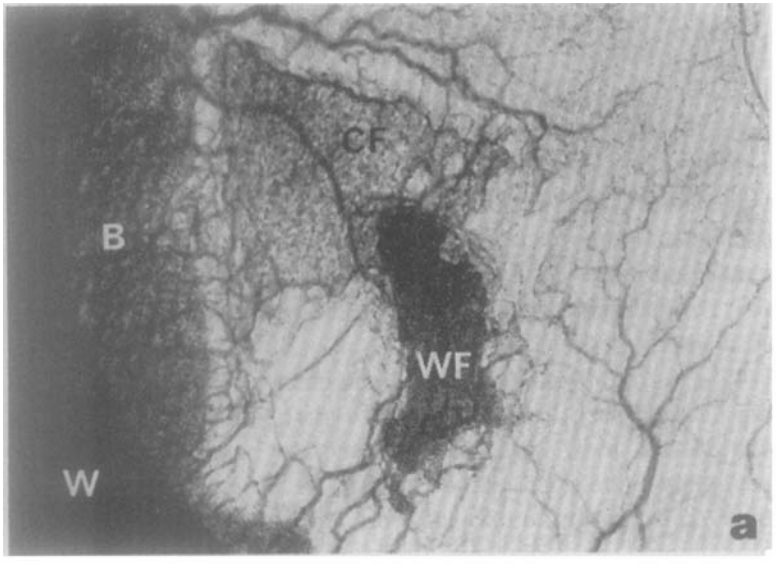
On histological examination the initial bone formed on autografts in the well was of an intramembranous, primitive woven-fibred type (Bernard & Pease 1969, Boyde 1972, Rasmussen & Bordier 1974). This bone formation later changed to a more differentiated type of woven-fibred bone formation. In older bone cultures parallel-fibred bone formed ossicles and hemi-ossicles (Figure 10). From the bone formed in the well bone flakes extended into the optical part of the chambers.

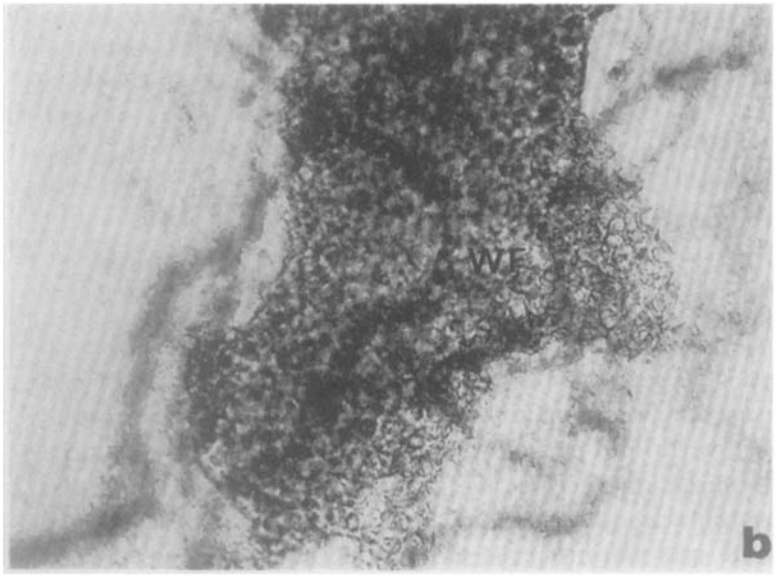
On histological examination (fourth week) the advancing bone flake was composed of a differentiated type of woven-fibred bone tissue. The thickness of the flakes was

remarkably constant, measuring about 60 μm and tapering off towards the bone rim to about 30 μm. The distinctly delineated, disordered lacunae of such bone flakes were surrounded by homogenously mineralized bone matrix. The flat upper surface of the bone flake was bounded by a layer of inactive osteoblasts (Vittali 1970), and the lower surface by an irregular layer of osteoid tissue and active osteoblasts (Vittali 1970) (Figure 4). At the bone edge an irregular zone of osteoblasts and osteoid tissue corresponded to the indistinctly demarcated translucent tissue seen by vital microscopy.

Older bone flakes were usually composed of compact, parallel-fibred bone — at least near the surfaces. Such flakes were framed by a regular seam of osteoid tissue and osteoblasts, and were enveloped in a delicate membrane-like structure (Figure 9b).

Figure 6 (a—b). Vital photomicrographs. Part of bone culture 25 days after autografting. *Figure 6a.* A flake of differentiated woven-fibred bone (B) has entered the optical part of the chamber from the well (W). Resorption of an autograft (CF) stopped at the side facing the advancing bone front. Continued resorption at the other end changed the originally rectangular graft (area 2.2 mm^2) into the shape of a triangle (area 0.8 mm^2). Primitive woven-fibred bone (WF) formed between the autograft and the cover glass. Objective 2.5x / 0.08. *Figure 6b.* Detail from Figure 6a. Primitive woven-fibred bone growing directly underneath the cover glass. The bone is densely packed with indistinctly delineated lacunae. Canaliculi are not visible and there is no osteoid seam. Note the granulated appearance. Field width, 0.8 mm. Objective 10x / 0.25.


CF
B
WF
W
a


WF
b

Bone formation within the optical part following bone grafting

Bone occasionally formed on or beside autografts in the optical part of the chambers. Such new bone grew towards and later directly underneath the cover glass. In contrast to the bone flakes described in the previous paragraph, this bone usually appeared highly cellular, dark brown and granulated. The lacunae were indistinctly delineated. At the bone rim faint outlines of cells could be seen (Figure 6). Bone remodelling started within days.

The same type of bone sometimes developed after complete resorption of an autograft, but then it formed within the new vascular connective tissue which had previously enveloped the autograft. Strands of fibres were observed extending outwards from the bone rim, particularly conspicuous in polarized light. Again bone remodelling started within days (Figures 8a, c).

Parallel-fibred bone formed occasionally on partly resorbed bone tissue (autografts and newly formed woven-fibred bone) in the optical part of the chambers (Figures 8a — b). This was verified histologically (Figure 9 b).

On histological examination the highly cellular bone formed on partly resorbed autografts proved to be of the primitive woven-fibred type (Figure 7).

Bone formation following bone removal

After removal of all bone and connective tissue from the optical part of the chambers a different type of bone formation was encountered. The optical part was rapidly filled with fluid packed with formed blood elements. Budding capillaries and connective tissue invaded it and it was thus revascularized within a fortnight. This new connective tissue was woven-fibred as assessed between crossed polars in vivo.

During the second week highly cellular dark brown granulated bone tissue formed within this connective tissue. From the edges of the optical field the bone grew rapidly into the central part of the chamber, which was usually completely covered with new bone by the end of the sixth week. The edges of this new bone were ill-defined. Between crossed polars the bone appeared dark, i.e. woven-fibred.

On histological examination this initial rapid bone growth proved to be due to intramembranous, primitive woven-fibred osteogenesis (Figure 10). This scaffold of primitive bone was soon covered by an irregular layer of osteoblasts forming a more differentiated but still woven-fibred bone.

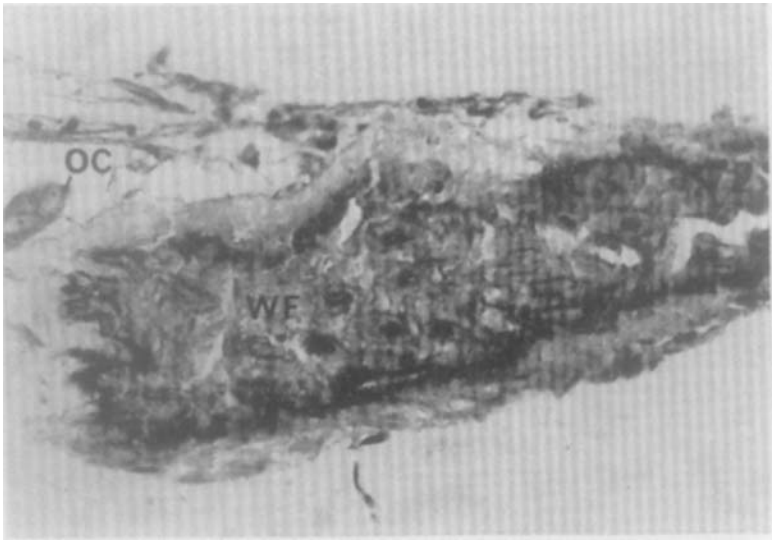


Figure 7. Photomicrograph. Histological section of the primitive woven-fibred bone flake (WF) shown in Figure 6 36 days after autografting. Bone resorption had already started. OC = osteoclast. Undecalcified, toluidine blue. Field width, 0.22 mm. Objective 40x / 0.65.

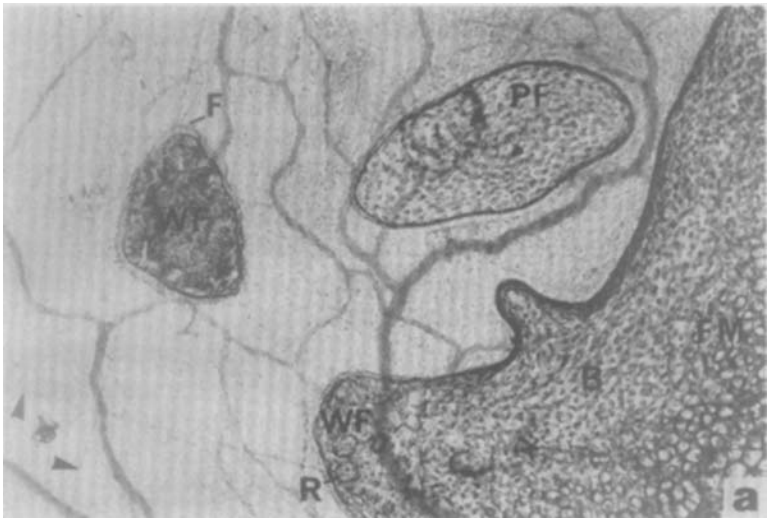
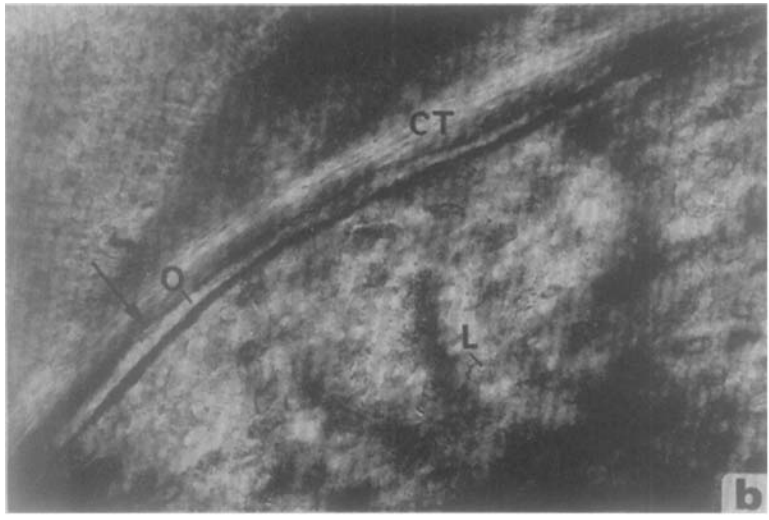


Figure 8 (a—c). Vital photomicrographs. Bone culture 153 days after bone autografting. *Figure 8a.* Bright field. Three bone islands formed after bone grafting, then a flake of bone tissue (B) grew into the optical part of the chamber merging with one of the 2 woven-fibred (WF) bone islands. The third island is composed of parallel-fibred bone (PF). F = strands of collagen fibres, FM = fat marrow, R = resorption cavities (in woven-fibred bone), arrowheads = system of coordinates in base of chamber. Field width, 2.25 mm. Objective 4x/0.12. *Figure 8b.* Crossed polars. Detail of parallel-fibred (i. e. bright) bone island. Arrow indicates ill-defined cellular structure in membrane lining the osteoid seam (O). A connective tissue envelope (CT) runs parallel to the bone edge. L = bone lacuna. Field width, 0.44 mm. Objective 20x / 0.32. *Figure 8c.* Crossed polars. Detail of woven-fibred (i.e. dark) bone island. Conspicuous strands of birefringent collagen fibres (F) extend outwards from the bone edge. Field width, 0.44 mm. Objective 20x/0.32.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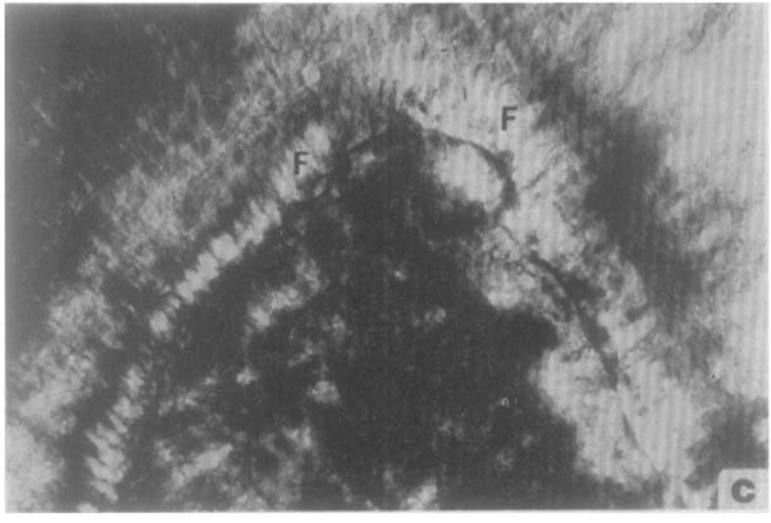
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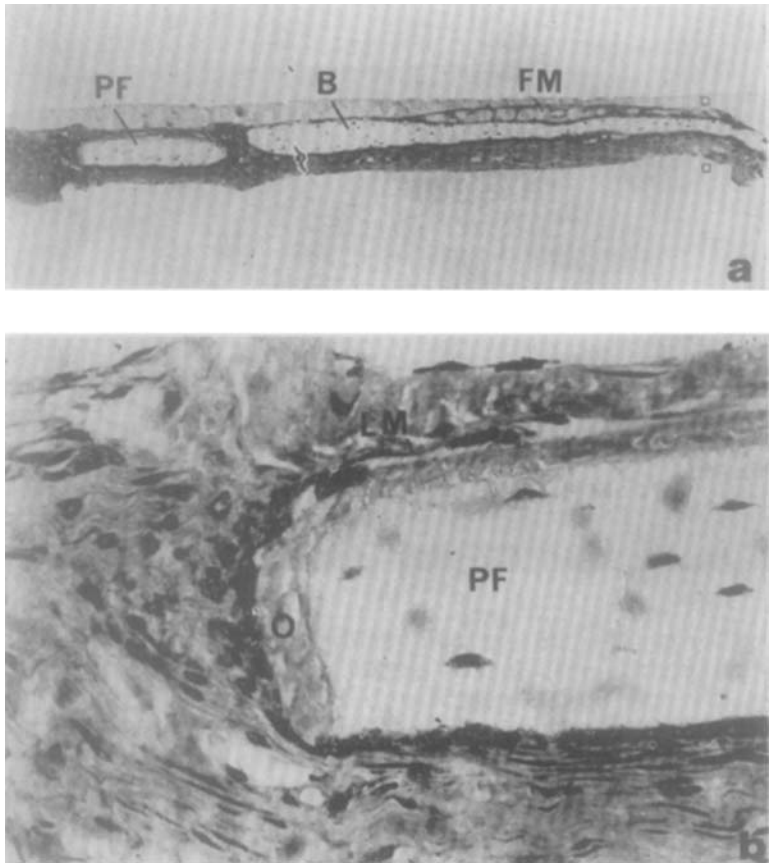
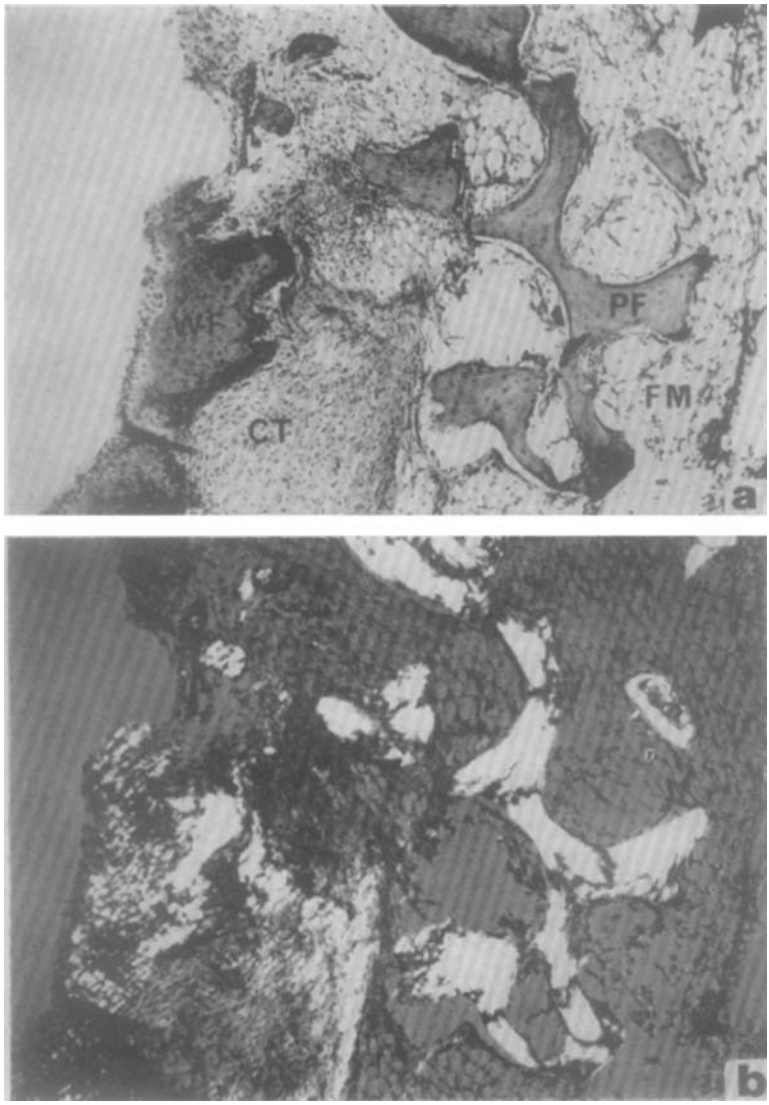


Figure 9 (a—b). Photomicrographs. Histological section of bone flake (B) and parallel-fibred bone island (PF) shown in Figure 8a. *Figure 9 a.* The bone flake had been remodelled into a hemi-ossicle containing fat marrow (FM). □ = Epon embedding material. Field width, 3.6 mm. Objective 2.5x/0.08. *Figure 9 b.* Detail of parallel-fibred bone island. The osteoid tissue (O) is lined by a delicate membrane (LM). Field width, 0.22 mm. Objective 40x/0.65. Undecalcified, toluidine blue.

Figure 10 (a—b). Photomicrographs. Histological section (parallel to cover glass) of bone culture 32 days after removal of all tissue in the optical part of the chamber. Woven-fibred bone (WF) has developed in regenerated



connective tissue (CT) in the optical part. The cancellous, parallel-fibred bone (PF) containing fat marrow (FM) was located in the well of the chamber. Bright field (Figure 10a) and crossed polars (Figure 10b). Decalcified, H+E. Field width, 2.25 mm. Objective 4x / 0.12.

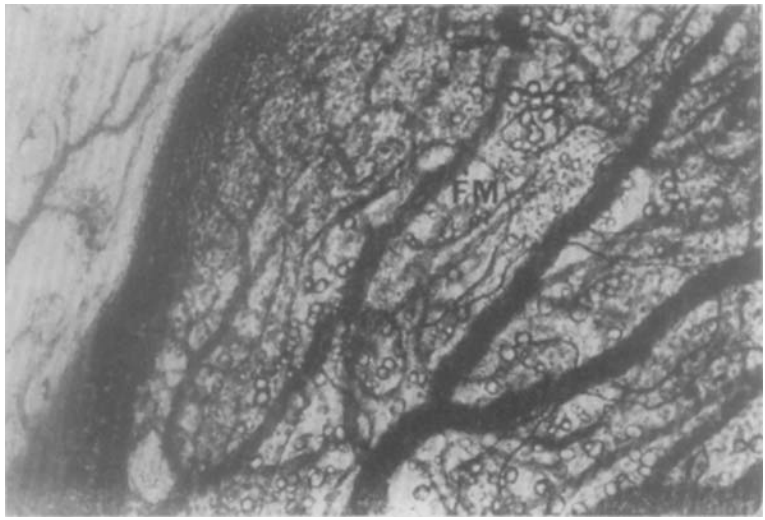


Figure 11. Vital photomicrograph. Part of regenerated bone culture 46 days after removal of all tissue in the optical part of the chamber. Such bone cultures were usually remodelled to cancellous bone containing fat marrow (FM). Field width, 2.25 mm. Objective 4x / 0.12.

Remodelling of bone flakes

Remodelling of the visible part of a bone flake growing into the optical part from the well started when the edge of the flake appeared well within the optical field. Starting from the well, the surface of the bone tissue facing the cover glass was gradually resorbed and replaced by fat marrow (Figure 8 a). Turning the ear chamber upside down on the microscope stage allowed vital microscopy of the other side of the bone flake, which was convex and usually without resorption cavities.

The originally flat bone flake was thus remodelled into a shallow hemi-ossicle containing fat marrow. This remodelling continued as the bone flake grew towards the central part of the chamber, thereby steadily increasing the size of the cavity of the hemi-ossicle. This process was verified on

histological examination, demonstrating osteoclastic activity on the upper, and osteoblastic activity on the lower surface. The bone thickness of the hemi-ossicle varied from 40 to 80 μm, and it was about 40 μm deep (Figure 9 a).

The bone formed after bone removal was gradually remodelled into cancellous bone containing fat marrow (Figure 11). The formation of secondary Haversian systems was sometimes observed in partly resorbed bone tissue (autografts or newly formed woven-fibred bone).

DISCUSSION

The characteristic sequence of events during heterotopic bone remodelling in ear chambers was in principle the same as that of orthotopic bone tissue. A balance between resorption and formation was regularly established and the imbalance in favour of resorption seen in bone cultures in vitro (Rasmussen & Bordier 1974) was not found here. Bone cultures in rabbit ear chambers are accordingly useful for vital microscopical studies of histotypical bone for half a year or longer.

Technical considerations

As reported by Sandison (1928) and Kirby-Smith (1933), the active cells engaged in bone remodelling can be visualized in shallow (probably about 50 μm deep) ear chambers. We did not succeed in obtaining bone formation in such shallow chambers (Sudmann 1975 b). To provide material for the study of both bone resorption and formation, it was necessary to increase the depth of the optical part of the chambers to 0.2—0.3 mm. Cellular details cannot be observed in the depths of such thick preparations and consequently osteoclasts could not be visualized by vital microscopy. However, the presence of osteoclastic activity was easily revealed by the formation of Howship's lacunae

(Figure 2). By repeated observations, supplemented by photomicrographs and stereometric measurements, the progress of the process could be followed.

In the same way the details of new bone formation were concealed in the depths of the ear chamber culture. Bone formation close to the cover glass, however, was clearer. Here the osteoblasts, osteoid tissue and mineralized bone with lacunae could be identified and studied in some detail after correlating vital microscopical and histological findings.

When investigating bone remodelling dynamics by vital microscopy, it should be kept in mind that living cells may be harmed by the microscope light. Goldhaber (1963) reported that vitally stained osteoclasts can actually be killed by the light. During vital microscopy light exposure should therefore be reduced to the minimum necessary for the examination.

The use of polarized light vital microscopy proved to be advantageous in differentiating woven-fibred from parallel-fibred bone in rabbit ear chambers, particularly at low magnification (Figures 8 b—c). However, such observations should be interpreted with caution, since layers of different types of tissue contribute to the picture.

Bone resorption

Two different types of bone resorption were identified in rabbit ear chambers. Autografts were resorbed in a disordered way, i. e. without apparent functional polarity or preference for any particular part of the grafts. In contrast, the new bone flakes growing underneath the cover glass were resorbed in an ordered way and from the upper surface only. To our knowledge this difference between disordered and ordered osteoclastic activity has not previously been described in ear chamber tissues (Sandison 1928, Kirby-Smith 1933, Ezra-Cohn et al. 1969).

In the present study disordered osteoclastic activity was reduced or stopped when bone formed on or near the autografts (Figure 6a). Several factors may be involved in this

inhibition of osteoclastic activity. As reported by Silver (1969) the oxygen tension is reduced in ear chambers along a growing bone front. According to Goldhaber (1963) osteoclastic activity in vitro is reduced or stopped when oxygen tension is lowered. Another important factor influencing osteoclastic activity is the calcium concentration (Rasmussen & Bordier 1974), which may be locally changed by the growth of an advancing bone front.

Bone formation

The formation of 2 characteristic types of woven-fibred bone, primitive and differentiated, and of parallel-fibred bone were encountered in rabbit ear chambers. After correlating vital microscopical and histological findings they were easily recognized in vivo.

By bright field vital microscopy the primitive woven-fibred bone type was characterized by its rapid growth, its dark brown colour and granularity, its numerous, large and indistinctly delineated lacunae and its lack of osteoid borders (Figure 6). Once formed, bone resorption (disordered) started within days (Figure 7).

In contrast, the differentiated type of woven-fibred bone was characterized by its quiescent growth, its pale brown colour, its fewer and distinctly delineated, but disordered lacunae and its indistinctly delineated osteoid border (Figure 3). Flakes of this bone type were slowly remodelled in an ordered way into hemi-ossicles.

Parallel-fibred bone was characterized by its slow, ordered growth, by its distinctly delineated and ordered lacunae interconnected by numerous canaliculi and by a distinctly delineated osteoid seam (Figure 5). Parallel-fibred bone flakes were slowly remodelled in an ordered way into hemi-ossicles.

In the development and growth of the skeletal system there seems to be a correlation between the type of bone, the rapidity of growth and the type of local environment in which it is formed (Frost 1963, Pritchard 1972), and there appears to be a spectrum of bone types from the most

primitive (foetal) to the most differentiated (adult) (Boyde 1972). Depending on the experimental set-up, these various bone types and modes of growth were in principle also encountered in rosette rabbit ear chambers. To our knowledge neither the formation nor the vital microscopical identification of various types of bone tissue in rabbit ear chambers have previously been reported. Sandison (1928), Kirby-Smith (1933), Clark & Clark (1942) and Ezra-Cohn et al. (1969) probably described the formation of woven-fibred bone only, but this fact is not stressed in any of these reports.

Varying amounts of the environmental connective tissue are incorporated during bone growth (Pritchard 1972). This may account for the varying amounts of strands of collagen fibres (Ezra-Cohn et al. 1969) seen extending from the rim of woven-fibred bone in rabbit ear chambers (Figures 3, 6b, 8).

The phenomenon that parallel-fibred bone is only laid down on a previously formed bone surface was also observed in rabbit ear chambers. Rasmussen & Bordier (1974) have stressed the importance of the mesenchymal cell envelope covering skeletal surfaces in both the remodelling and the metabolism of bone tissue. As documented in the present study, this physiologically important structure can be visualized in vivo in rabbit ear chambers (Figures 5, 8a—b).

Remodelling of bone flakes

There was a definite tendency towards ordered growth and remodelling of the bone flakes within the optical part of the ear chambers, oriented in the direction of the cover glass. This affinity for the cover glass was one of the most puzzling phenomena encountered in the present study. Such bone flakes were remodelled in an ordered way into shallow hemi-ossicles containing fat marrow. The fat marrow was regularly located underneath the cover glass in a depression in the bone flake, made by osteoclastic activity (Figure 9a). No explanation has yet been found for this pronounced

polarity of the osseous tissue, with bone resorption on the side facing the glass and bone formation on the opposite side, away from the glass.

The thickness of such bone flakes — as measured in histological sections — was remarkably constant (about 60 μm). The bone area, as assessed by mixed-image planimetry (Sudmann 1975 c), can therefore be considered a reliable parameter of mass or volume in bone flakes formed in rabbit ear chambers.

Experimental implications

Vital microscopy of living, histotypical bone tissue and bone marrow in rabbit ear chambers is unique in giving instant visualization and documentation of dynamic events at tissue and cellular levels. Such bone cultures are freely accessible for manipulation and for taking biopsies. Bone remodelling can be experimentally influenced in the chamber by physical and chemical means. Drugs and hormones can be administered in the chamber, by perfusion of the ear vessels or by parenteral and oral routes, and the rabbit's normal hormonal balance can be changed. In repeated experiments the animals can serve as their own controls (Sudmann 1975 d).

As shown, ordinary bright field microscopy can be supplemented by polarized light microscopy and changes in bone area can easily and precisely be quantitated (Sudmann 1975 c). Bone mineralization and matrix formation can be visualized using bone-seeking fluorochromes (Sudmann 1972, unpublished data).

Bone-seeking gamma-emitting radio-isotopes can be directly quantitated in the rabbit ear chamber in vivo (Sudmann 1972, unpublished data). Finally vital microscopical observations can be correlated to findings obtained by ordinary histological and electron microscopical techniques.

In conclusion we find vital microscopy of bone tissue in rabbit ear chambers a useful method for dynamic studies related to bone resorption and formation.

SUMMARY

Heterotopic autologous bone tissue was studied by vital microscopy in rabbit ear chambers and correlated with the findings from histological sections prepared from the same tissue. The in vivo morphology and remodelling of different bone types were thereby characterized.

Autografts were resorbed in a disordered fashion by osteoclasts and woven-fibred bone tissue formed on such grafts. Later parallel-fibred bone, bounded by a seam of osteoid tissue, formed on this scaffold. Bone flakes growing underneath the cover glass were remodelled into shallow hemi-ossicles containing fat marrow, which were always oriented towards the cover glass.

Following *en bloc* removal of the central part of the bone culture, woven-fibred bone rapidly formed within the new connective tissue, filling in the empty space. Such bone was remodelled into cancellous bone containing fat marrow.

A balance between resorption and formation was regularly established in the chambers. The bone cultures were thus useful for vital microscopical studies for half a year or longer.

In the present study bone remodelling in rabbit ear chambers closely resembled that of orthotopic bone. Ear chamber bone cultures are therefore a versatile in vivo biological model for dynamic bone tissue studies at both tissue and cellular levels.

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EFFECT OF INDOMETHACIN ON BONE REMODELLING IN RABBIT EAR CHAMBERS

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Key words: Animal experiments; bone and bones — drug effects; hip-joint; indomethacin; joint diseases; models, biological; ossification, pathological; tissue culture.

Indomethacin medication is widely used in the treatment of musculoskeletal disorders, including degenerative joint disease (osteoarthritis). However, according to Arora (1968), Desproges-Gotteron et al. (1971) and Solomon (1973), the drug may induce a disabling arthropathy, especially in weight-bearing joints. If this is so the indications for using this potent anti-inflammatory drug may have to be reassessed.

As is well known, arthropathy invariably entails bone remodelling, i.e. bone resorption by osteoclasts and bone formation by osteoblasts. This factor must therefore be taken into account when considering the pathogenesis of drug-induced arthropathies.

Bone resorption and formation can be studied by quantitative and qualitative microsopy in vivo in rabbit ear chambers (Sudmann 1975 c, Sudmann & Marton 1975). The purpose of the present study was to assess by vital microscopy the effect of indomethacin on the resorption of autografts and on bone formation in this in vivo culture chamber.

MATERIAL AND METHODS

Twelve female sandy half lop-eared rabbits with a total of 18 titanium-glass rosette ear chambers of own design (Sudmann 1975 a) were used. Altogether 5 experiments were carried out over a period of 6 months (Table 1). Each animal was used 3 times without change of ear chambers, and served as its own control (Table 2).

All the chambers were vascularized by new connective tissue. The depth of the optical part of the chambers was 0.13 before and 0.2—0.3 mm after bone grafting.

Resorption of the bone autografts and bone formation in the optical part of the chambers were assessed in vivo, both quantitatively, by mixed-image planimetry (Sudmann 1975 c), and qualitatively (Sudmann & Marton 1975). The precision of the mixed-image planimetry method is better than 1 per cent.

Bone tissue experiments (Table 1)

Experiments 1—3. At the start of each of these experiments 12 autografts were placed on the regenerated connective tissue in chambers not previously used. Three cortical bone grafts (0.1 mm thick) were placed in the slit-shaped optical part (Figure 7), whereas 9 cortical cancellous grafts were placed in the shallow, annular non-optical part of the chambers, i.e. tne well (Sudmann 1975 b). The grafts in the well were only visible in reflected light. All the rabbits used in Experiments 1—3 except two (see legend to Table 2) were reused in Experiment 4.

In a concurrent study (Sudmann & Marton 1975) the sequence of events following bone autografting to rosette ear chambers in animals not given any drugs was as follows : Within a week capillaries were formed between the visible grafts and the cover slip, and bone resorption started during the second and third weeks following grafting. In the third week differentiated woven-fibred bone tissue grew into the optical part of the chambers from the well.

Table 1. Bone tissue experiments in 12 rabbits with 18 ear chambers.

Experiment	Drug	Animals: No. of	Animals: Age, months	Weight $\bar{X}\pm$SDX, kg	No. of chambers	Visible bone area $\bar{X}\pm$SDX, mm^2	Autografts in all
1	Indomethacin	2	5.5	3.7±0.2	2	2.2±0.4	24
	Placebo	3			4[a]	2.2±0.7	48
2	Indomethacin	3	5.5	3.4±0.3	3	2.0±0.3	36
	Placebo	4			4[a]	2.1±0.5	48
3	Indomethacin	2	7	3.6±0.4	2	1.8±0.2	24
	Placebo	2			2	2.0±0.3	24
4	Indomethacin	5	7.5-9	3.8±0.4	8[a]	9.2±7.5	None, bone removal
	Placebo	5			7[b]	10.0±3.2	
5	Indomethacin	3	9.5-10.5	3.9±0.3	4[a]	11.7±8.5	None, bone removal
	Placebo	3			3	11.6±5.7	

[a] One chamber was later excluded, see text

[b] Three chambers were later excluded, see text

$\bar{X}$ = mean, SDX = standard deviation

Bone area = Area of each bone graft (Experiments 1-3) or new bone tissue in optical part of chambers (Experiments 4-5)

Experiments 4—5. All the rabbits had been used in the previous experiments (1—3), thus all chambers contained a living bone culture. At the start of Experiments 4—5 the complete contents of the optical part of each chamber were removed *en bloc*. Experiment 4 was carried out twice (Table 2). Six of the rabbits used in Experiment 4 were reused in Experiment 5.

Following such *en bloc* tissue removal, rosette ear chambers are revascularized within 14 days and in the second week primitive woven-fibred bone tissue forms within this new vascular connective tissue (Sudmann & Marton 1975).

Drugs (Table 2)

As specified in Table 2, 1 dose of indomethacin (Indocid Oral Suspension, Merck Sharp & Dohme, Haarlem, the Netherlands) or placebo (suspension without indomethacin) was administered every morning for 3 weeks, except on the seventh and the fourteenth days, when no drug was given. The first dose was administered on the day of grafting or removal of bone tissue from the chambers. Usually the animals served as their own controls and the drugs were accordingly changed from one experiment to the other. The animals were weighed once a week and the dosage adjusted accordingly.

The drugs were either squirted into the mouth by means of a syringe coupled to a 5 cm long plastic tube (oral feeding), or administered through a stomach tube 20 cm of which was inserted through the animals' mouths (tube feeding).

Neither sedation nor restraint was used for oral feedings, but both (sedation : 0.3 ml i. m., Hypnorm Vet., Mecos, Helsingborg, Sweden) were necessary for tube feeding the rabbits (Experiment 1). Tube feeding was abandoned after 4 days in Experiment 2 and in all further experiments.

Table 2. Indomethacin and placebo administration to 12 rabbits.

Experiment	Rabbit identification		Daily dose, mg/kg body weight	Method of administration	Ear chambers (no.)	Termination of experiment (days)
	Indomethacin	Placebo				
1	B, D	A, C, E	5[a]	Tube	All (6)	79
2	G, J, L	F, H, K, M	5	Per os[b]	Left ear (7)	48 (H, J) 62 (F, G, K, L)
3	F, K	G, L	10	Per os	Right ear (4)	61
4	C, E, H	B, D, J	10	Per os	All (7)	47
	G, L	F, K			All (8)	65
5	B, D, J	C, E, H	10	Per os	All (7)	32-65

[a] 10 mg/kg first week

[b] tube first 4 days

One chamber (A) removed due to infection, one rabbit (M) died, see text

Laboratory procedures

Blood samples (5 ml) for spectrofluorimetric indomethacin assay (Gribnau et al. 1973) were drawn twice from each rabbit (Experiments 2—5). In Experiment 2 the samples were taken on the eighth and the eighteenth days 20 hours after the last oral drug feeding. In Experiments 3—5 one sample was drawn 4.5 hours after the last oral feeding on the seventeenth and one after 24 hours on the nineteenth day of the experiment. The heparinized plasma samples were frozen and stored until assayed for indomethacin by F. W. J. Gribnau, Nijmegen University, the Netherlands. Undecalcified and decalcified bone sections were prepared for microscopical study as reported by Sudmann & Marton (1975).

RESULTS

The animals moped and ate less than usual and their droppings were sparse when tube fed. One rabbit (Experiment 2) died after accidental intrabronchial tube feeding of placebo. Tube feeding was then abandoned and the drugs administered per os. The animals' general condition was not noticeably affected when the drugs were orally administered. The rabbits' weight loss or gain during the experiments — with themselves as their own controls — did not differ to any appreciable extent whether they were fed indomethacin or placebo suspension. The results of the spectrofluorimetric indomethacin assay are given in Table 3.

Bone tissue experiments

In the present experiments the sequence of events in the chambers following bone grafting or bone removal with placebo administration was the same as the findings from concurrent experiments in which no drugs were used (Sudmann & Marton 1975).

Table 3. Results of spectrofluorimetric indomethacin assay.

Experiment	Daily dosage, mg/kg body weight	Groups, reading µg/ml Indomethacin	Placebo
2 (8^{th}/18^{th} day)	5	0.014/0.04 0.10/0 0.13/0.05	0/0
3 (4.5/24 hours)	10	2.57/0.68 1.41/0.09	0/0
4 (4.5/24 hours)	10	2.27/1.46 4.42/0.32 0.88/1.01 1.61/1.36 0.63/0.46	0/0 0.04/0.01
5 (4.5/24 hours)	10	1.12/0.07 0.95/0.42 1.06/0.10	0.05/0 0.05/0 0.04/0.02

Experiment 1 (dosage 10 and 5 mg/kg). In the placebo group capillaries formed within 7 days between the cover glass and the autografts in the optical part of the chambers, and Howship's lacunae developed at irregular intervals on the surfaces of the grafts after 10—14 days. In the indomethacin-treated animals, however, capillaries were observed after 10—16 days and Howship's lacunae after 24—38 days. The size of the autografts — as assessed by mixed-image planimetry — was barely reduced during the period of indomethacin administration. In contrast, the control autografts gradually decreased from the tenth day until bone had formed on or near the grafts.

In the placebo group bone tissue entered the optical part of the chambers 14—18 days after autografting. In the indomethacin-treated rabbits, however, bone entered the optical part 24—30 days after autografting.

In both groups, differentiated woven-fibred bone tissue (Sudmann & Marton 1975) grew from the well into the optical part of the chambers like a pale brown flake underneath the cover glass, bounded by a disordered arrangement of osteoblasts in an indistinctly demarcated osteoid tissue. As it grew towards the central part of the chambers the new bone tissue was gradually remodelled into a shallow hemi-ossicle containing fat marrow. Bone formation was not observed or was sparse on or along the autografts in the optical part of the chambers.

One chamber containing 12 autografts was removed after 20 days because of pus formation underneath it. These grafts were excluded. The stereometric data from one typical chamber in each group are illustrated in Figure 1.

Experiment 2 (dosage 5 mg/kg). No differences were noted between the indomethacin group and the controls with regard to the appearance of new capillaries, Howship's lacunae, decrease in the size of the grafts and bone formation. After 80 days the new bone area varied from 2 to 16 mm^2. One typical chamber (placebo group) is illustrated in Figure 2.

Experiment 3 (dosage 10 mg/kg). Vital microscopy revealed no differences between the indomethacin group and the control chambers with regard to the appearance of capillaries on the autografts and Howship's lacunae. There was, however, as illustrated by the stereometric data from one typical chamber presented in Figure 2, obvious inhibition of autograft resorption in the indomethacin group as compared with the controls. In both groups new bone appeared at the same time, but the quantity was sparse during indomethacin treatment.

Experiments 4—5 (dosage 10 mg/kg). Eleven chambers were revascularized after the removal of all tissue in the optical part of the chambers, 4 were not (Experiment 4). Six chambers were revascularized when the same procedure was repeated in 6 animals with 7 chambers (Experiment 5). New vascular connective tissue gradually invaded the optical part. This vascularization was complete within 14—16 days in the indomethacin groups and within 14 days in the controls.

Primitive, woven-fibred bone tissue (Sudmann & Marton 1975) arose in a disordered way within this new vascular woven-fibred connective tissue. Bone formation of this type started after 9—20 days ($\overline{X} = 18$, $SDX = 3$) in the indomethacin groups (10 chambers) and after 7—14 days ($\overline{X} = 10$, $SDX = 2$) in the placebo groups (8 chambers).

Indomethacin medication thus delayed such bone formation for about a week. This was most obvious when the animals served as their own controls. Stereometric data from one typical group of animals are shown in Figures 3—4. Stereometric data from two typical chambers are illustrated in Figures 5—6 and photomicrographs of one of these chambers in Figures 7—9.

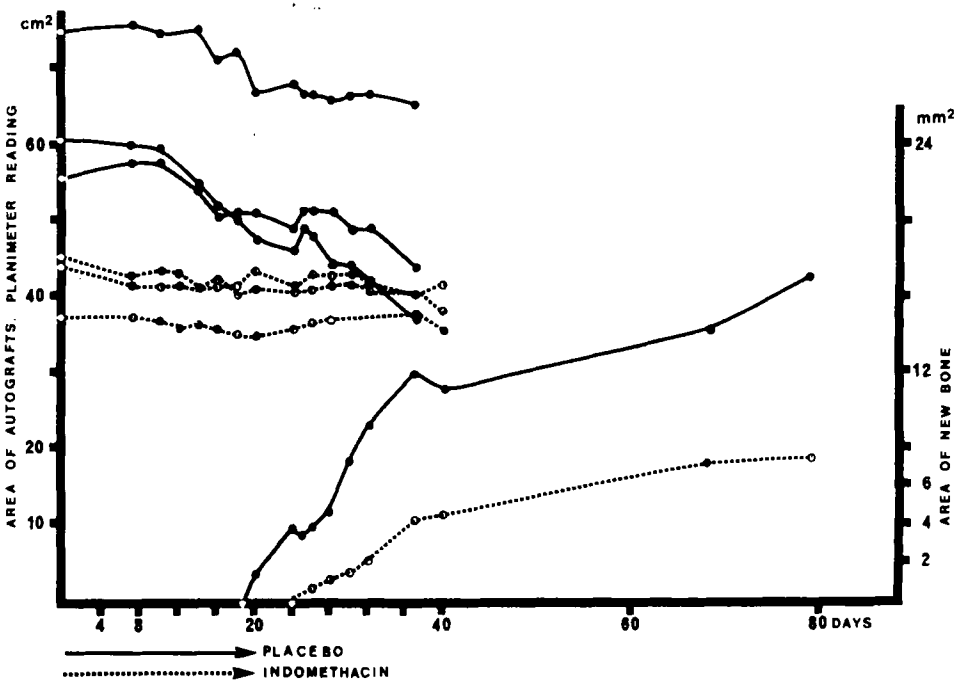


Figure 1. Experiment 1. Effect of indomethacin and placebo medication on osteoclast and osteoblast activity. The sterometric data from one typical chamber in each group are shown. The autografts did not decrease in size during indomethacin medication as assessed by mixed-image planimetry.

The white dots on the ordinate indicate the area of each (visible) autograft in the optical part of the chambers minutes after autografting. The white dots on the abscissa indicate the day when mineralized new bone tissue entered the optical part.

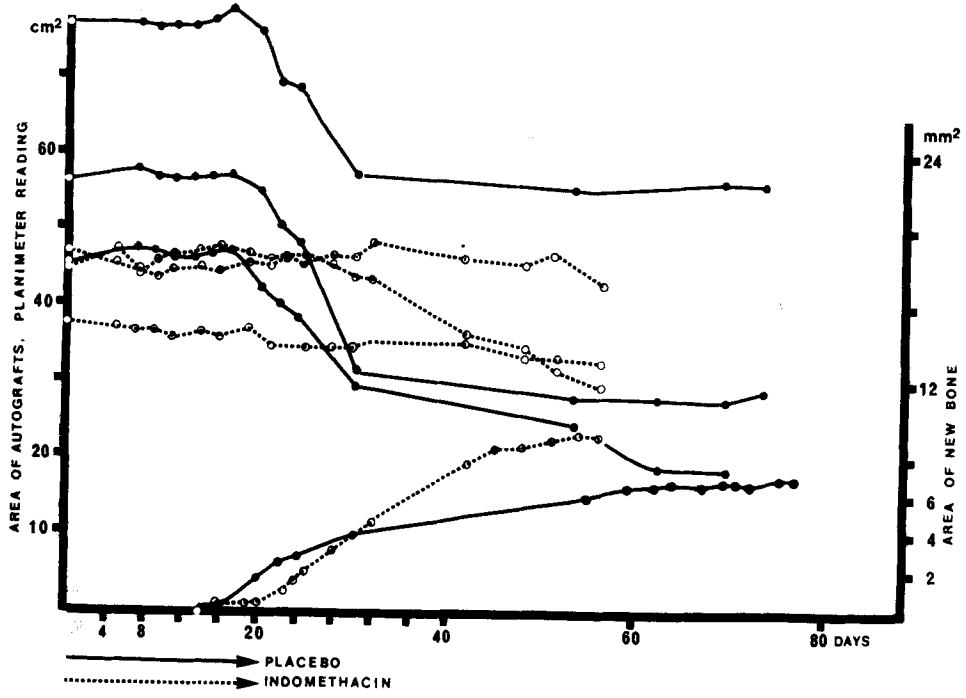


Figure 2. Experiments 2—3. Effect of placebo (left ear chamber, Experiment 2) and 10 mg/kg indomethacin administration (right ear chamber same rabbit, Experiment 3) on osteoclast and osteoblast activity. The rabbit thus serves as its own control. The stereometric data from two contralateral chambers indicate inhibition of bone resorption, possibly also of bone formation. Bone resorption was reduced or stopped when bone formed on or near the grafts.

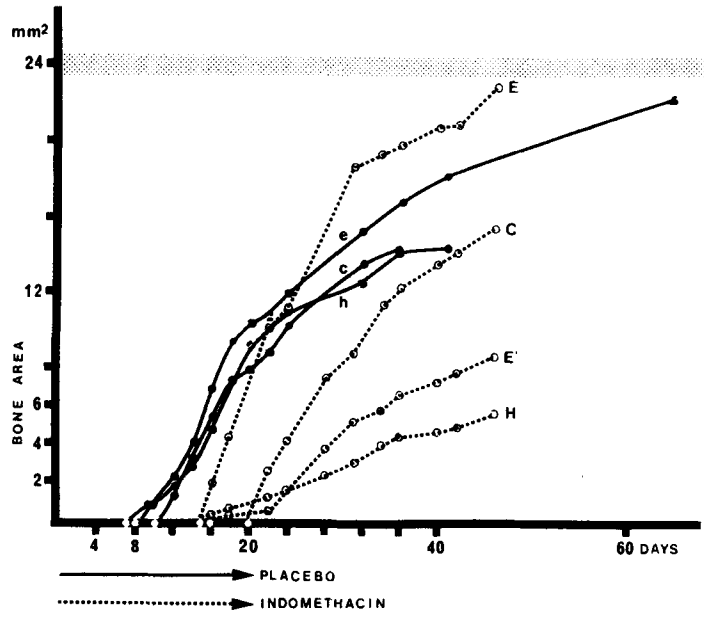


Figure 3. Experiments 4—5. Stereometric data from 3 rabbits (C, E, H) with 4 ear chambers. The rabbits were fed indomethacin in Experiment 4 and placebo in Experiment 5. Note the difference in bone formation in 2 contralateral ear chambers in the same rabbit (E, E'). One of these chambers was not revascularized in Experiment 5. The area of the 6 mm wide optical part of the chambers was about 24 mm².

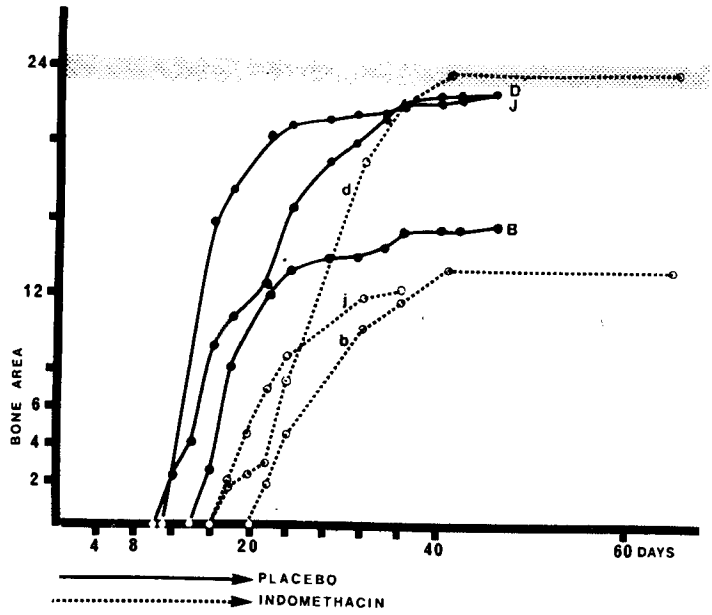


Figure 4. Experiments 4—5. Stereometric data from 3 rabbits (B, D, J) with 3 ear chambers. The animals were fed placebo in Experiment 4 and indomethacin in Experiment 5. Indomethacin administration (10 mg/kg) always retarded bone formation in Experiments 4—5.

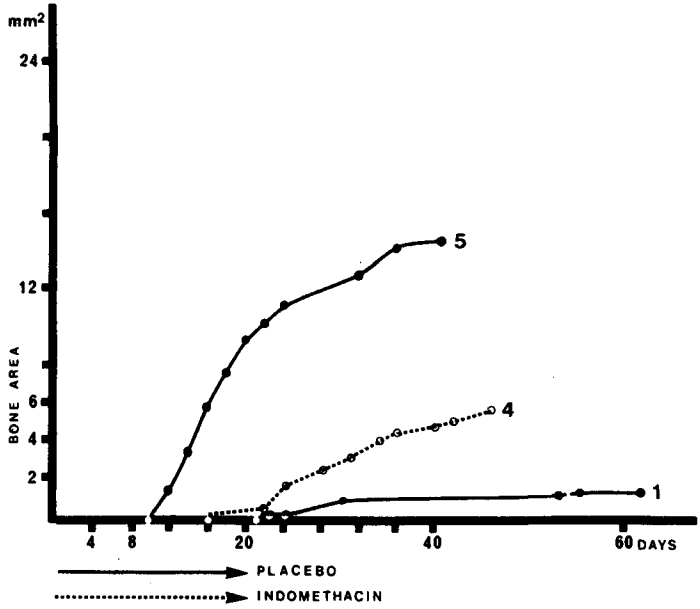


Figure 5. Experiments 1, 4—5. Stereometric data from an ear chamber with moderate osteoblastic activity, rabbit H. Experiment 1 was carried out quite differently from Experiments 4—5 (see text). The rabbit was fed placebo (1), indomethacin (4) and placebo (5) in that order. Indomethacin treatment (10 mg/kg) obviously retarded bone formation.

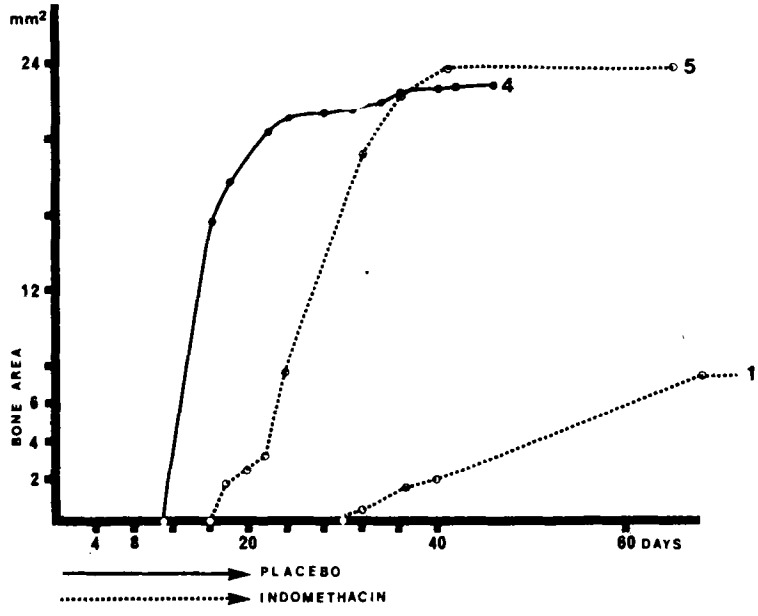
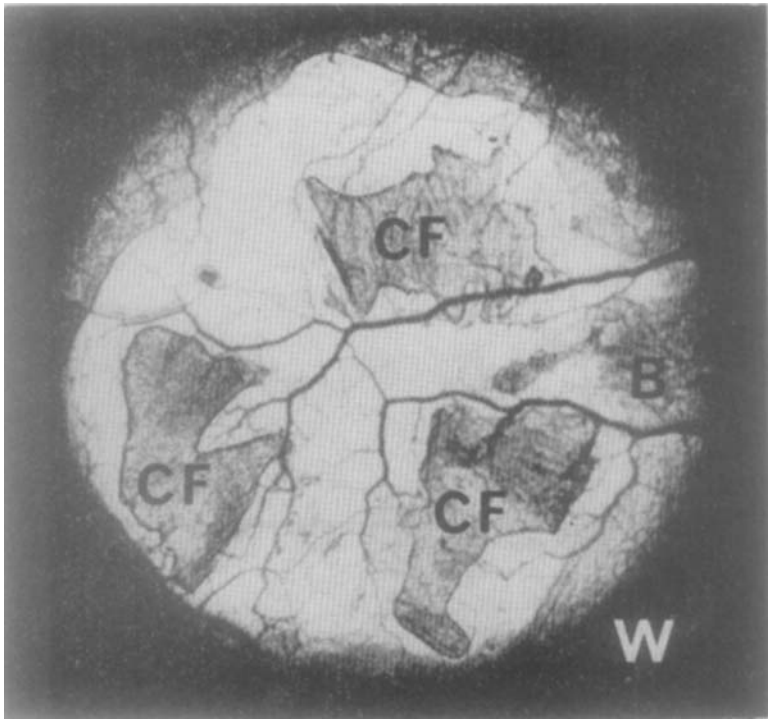
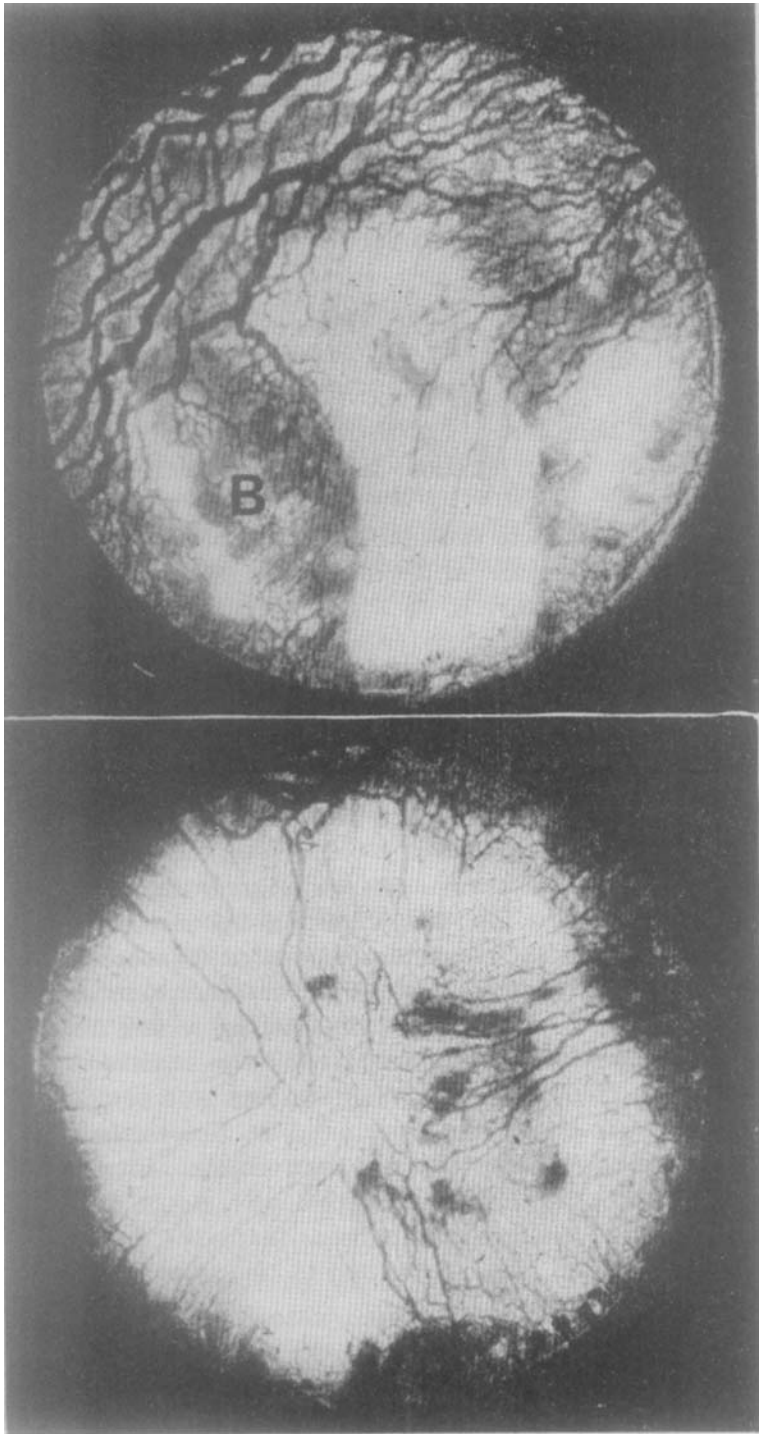


Figure 6. Experiments 1, 4—5. Stereometric data from an ear chamber with high osteblastic activity, rabbit D. The rabbit was fed indomethacin (1), placebo (4) and indomethacin (5) in that order. Indomethacin treatment (10 mg/kg) obviously retarded bone formation. Photomicrographs from this chamber are shown in Figures 7—9.



Figures 7—9. Experiments 1, 4—5. Bright field vital photomicrographs. Same chamber (D) as Figure 6. Field width, 6 mm. Objective 1x/0.04. *Figure 7.* Experiment 1, indomethacin administration. Bone culture 68 days after bone grafting. Three partially resorbed autografts (CF) in the optical part of the chamber (visible) are surrounded by an area of 7.4 mm^2 new bone (B) initiated by the 9 cortical cancellous bone autografts (not visible) in the annular, non-optical part of the chamber, the well (W). No decrease of autograft area occurred during the 3-week period of indomethacin administration. *Figure 8.* Experiment 4, placebo 10 mg/kg, 18 days after start of experiment. Eight-day-old bone culture (B) measures 17.7 mm^2. Note dilated blood vessels. *Figure 9.* Experiment 5, indomethacin 10 mg/kg, 16 days after start of experiment. New bone tissue has just appeared.


B

DISCUSSION

The sequence of events after experimentally induced bone resorption and formation in rosette rabbit ear chambers is remarkably consistent (Sudmann & Marton 1975). Indomethacin administration retarded both bone resorption and bone formation in such chambers.

Technical considerations

As outlined by Danckwardt-Lilliestrom et al. (1972), the use of a stomach tube for the administration of drugs to rabbits may in itself reduce experimentally induced (periosteal) bone formation. In the present study this type of inhibitory effect of tube feeding on bone remodelling was not seen.

Rabbits respond to subcutaneous doses of 2.25—9 mg/kg indomethacin in an antipyretic test (M.S.D. Research Laboratories, Rahway, N. J., U. S. A., personal communication). In the present study an oral dose of 5 or 10 mg/kg was therefore used (Table 2).

The indomethacin plasma readings varied (Table 3), but bone resorption was markedly delayed in rabbits with an indomethacin concentration higher than 0.1 μg/ml 24 hours after oral feeding. The same applied to primitive woven-fibred bone formation.

In the last lot of plasma samples sent for indomethacin assay, fluorescence was also found in the placebo group (Table 3). Since these readings amounted to about 5 per cent or less of the readings in the indomethacin group, this phenomenon should not invalidate the results. In our laboratory the same phenomenon was also observed in a concurrent study in rats given the same type of placebo suspension per os. On the other hand, no fluorescence was detected in the undiluted placebo suspension assayed (by F. W. J. Gribnau) directly or mixed with NaOH or ethyl acetate. These findings may indicate that some ingredient of this suspension was metabolized to fluorescent products in the animals.

Bone resorption

Two types of osteoclastic activity are regularly observed in rosette rabbit ear chambers (Sudmann & Marton 1975). The resorption of autografts is disordered, but the resorption of a new bone flake growing into the optical part of the chambers from the well is ordered.

The appearance of Howship's lacunae on the visible autografts and their decrease in size thus reflected disordered osteoclastic activity. In a dosage of 10 mg/kg indomethacin administration clearly retarded such osteoclastic activity; in a dosage of 5 mg/kg it did not.

Indomethacin inhibition of bone resorption was assessed in 4 rabbits only. The inhibitory effect was, however, reproduced in 2 experiments carried out on different occasions. In one of these experiments the effect of indomethacin on bone resorption in 2 ear chambers was compared with the effect of placebo in 2 contralateral chambers in the same rabbits, which thus served as their own controls (Figure 2). Placebo administration had no effect. In conclusion, 10 mg/kg body weight indomethacin suspension given per os daily for 3 weeks retarded disordered osteoclastic activity in rabbit ear chambers.

As reported by Klein & Raisz (1970), prostaglandins induce bone resorption in vitro. According to Tashjian et al. (1974), prostaglandin E_2 (PGE_2) is the potent bone resorption-stimulating factor produced by the transplantable mouse fibrosarcoma $HSDM_1$. Indomethacin was found to be a potent inhibitor of the production of PGE_2 in vivo. Indomethacin administration retarded disordered osteoclastic activity in vivo in the present study. These findings may indicate a possible experimental approach towards the factors that initiate disordered osteoclastic activity in vivo.

The effect of indomethacin on ordered osteoclastic activity can also be quantitated in rabbit ear chambers. Since, however, hemi-ossicles, remodelled by ordered osteoclastic activity, developed after indomethacin administration had

been discontinued, this effect could not be assessed in this study.

Bone formation

Two types of woven-fibred bone formation (primitive and differentiated) are regularly encountered in rosette rabbit ear chambers (Sudmann & Marton 1975). Indomethacin administration in a dosage of 10 mg/kg for 3 weeks retarded the growth of the primitive woven-fibred type, although it did not altogether prevent such bone formation. In these experiments 6 rabbits and 6 ear chambers served as their own controls. The retarding effect of indomethacin on primitive woven-fibred bone formation thus seems well documented.

Indomethacin administration in a dosage of 10 mg/kg also retarded the appearance of differentiated woven-fibred bone (Figures 1—2), whereas a dosage of 5 mg/kg did not. As autografts initiate a primitive type of woven-fibred bone tissue in the well (Sudmann & Marton 1975), this retarding effect was in accordance with the effect on woven-fibred bone formation in the optical part of the chambers in Experiments 4—5.

Growth of differentiated woven-fibred bone in the optical part of the chambers would probably be retarded by indomethacin administration. As this bone type grew into the optical part of the chambers in the third week following bone grafting, 3 weeks of indomethacin administration was too short a period to study this type of growth.

Parallel-fibred bone develops after bone grafting to rosette rabbit ear chambers (Sudmann & Marton 1975), but not within the first 3 weeks after grafting. The effect of indomethacin on parallel-fibred bone formation was thus not assessed here.

Clinical implications

Bone resorption and formation comparable to that of orthotopic bone tissue was experimentally induced in rabbit ear chambers. A balance between resorption and formation

is regularly encountered in such chambers (Sudmann & Marton 1975). The effect of indomethacin on bone remodelling in such an in vivo system may thus have greater clinical significance than findings obtained in vitro, where an imbalance in favour of resorption is found (Rasmussen & Bordier 1974).

As reported by Tashjian et al. (1974) and Brereton et al. (1974) indomethacin may reduce or correct tumor-induced hypercalcaemia. The complexity of this effect is outlined by Newcombe et al. (1974). In the present study indomethacin inhibited osteoclastic activity, and this may explain the correcting effect of indomethacin on the hypercalcaemia in patients with tumour-induced osteolysis.

The cause of para-articular ossification after arthroplasty in the hip-joint has been the subject of much speculation and several measures have been proposed for its prevention (Jakobsen 1957, Nollen & Slooff 1973). Para-articular ossifications are composed of woven-fibred bone tissue (Nollen & Slooff 1973). Formation of this type of bone tissue was clearly retarded after indomethacin administration in the present study. Indomethacin treatment of patients for the prevention of para-articular ossifications — as outlined by Dahl (1975) — therefore seems rational. The use of this drug might also be considered for the prevention of other types of heterotopic bone formation where woven-fibred bone tissue prevails.

Orthotopic woven-fibred bone formation constitutes an important part of fracture repair and indomethacin medication may thus delay fracture healing.

Idiopathic, aseptic, ischaemic necrosis of the femoral head in adults is a well-known disabling hip disease (Zinn 1971). As reported by Arora (1968), Desproges-Gotteron et al. (1971) and Solomon (1973), secondary aseptic necrosis in the femoral head may be ascribed to indomethacin medication. As outlined by Solomon (1973), the pathogenesis

of drug-induced arthropathy is still a matter of considerable controversy. One factor only, microfractures, will be considered here.

As reported by McFarland & Frost (1961), Radin et al. (1972, 1973) and Pugh et al. (1973), microfractures are continually produced in the subchondral cancellous bone of weight-bearing joints. These microscopic cracks are repaired by callus formation (Pugh et al. 1973, Radin et al. 1973). Woven-fibred bone tissue constitutes an important part of fracture callus. Indomethacin retarded woven-fibred bone tissue formation at the microscopical level in rabbit ear chambers and presumably this also may occur at the microscopical level in weight-bearing joints. Retarded healing of microfractures may thus, as stated by McFarland & Frost (1961), be of importance in the genesis of drug-induced arthropathies — especially in patients with subchondral osteoporosis.

SUMMARY

The effect of indomethacin medication on bone tissue resorption and formation was assessed by qualitative and quantitative microscopy in vivo in 18 rabbit ear chambers in 12 rabbits. Five bone tissue experiments were carried out during half a year using the rabbits as their own controls.

One daily oral indomethacin dosage of 10 mg/kg body weight given for 3 weeks inhibited resorption of bone autografts in this type of vital chamber and thus inhibited disordered osteoclastic activity. The effect of indomethacin on ordered osteoclastic activity was not quantitated, nor was its effect on parallel-fibred bone tissue formation assessed.

The same dosage of indomethacin clearly retarded woven-fibred bone tissue formation (by 1 week) as compared to control experiments, in which the chambers were used as their own controls. It is concluded that indomethacin treatment of patients liable to develop para-articular ossifications therefore seems rational. Indomethacin and drug-induced arthropathy of the hip-joint is discussed in relation to the presented results. A final appraisal of the role of indomethacin in this complex process must await additional material.

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