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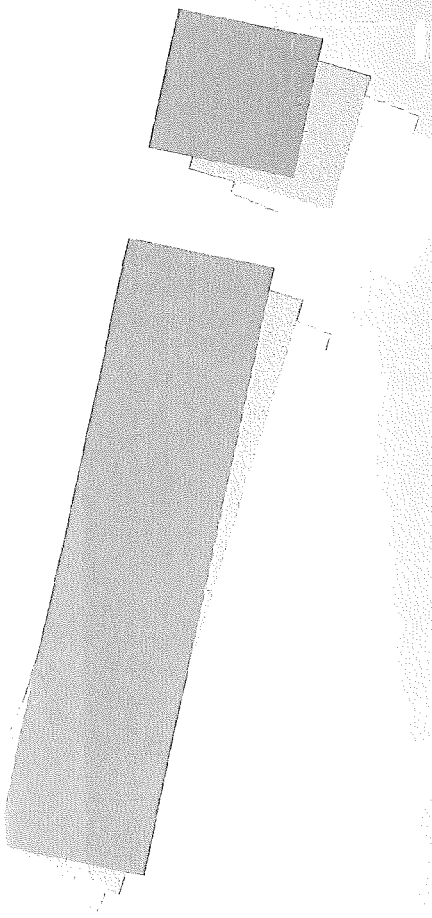

Biomaterials

edited by
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Progress Report on
Biomaterials

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Contents

I. Introduction and summary

Initiated by the Rheinisch-Westfälische Technische Hochschule Aachen national and international activities have been started on a common project on biomaterials. In Germany regional groups as the "Arbeitsgemeinschaft Biowerkstoffe des Landes Nordrhein-Westfalen" and the "Thüringer Arbeitsgemeinschaft Biomaterialien" have been formed including universities as the Rheinisch-Westfälische Technische Hochschule Aachen, the Universität-Gesamthochschule Essen or the Friedrich-Schiller-Universität Jena, hospitals and industrie as Keramed Hermsdorf, for example. Supported by the Federal Government of Germany as well as the State Government of Nordrhein-Westfalen a "Graduiertenkolleg" was established in the frame of the "Arbeitsgemeinschaft Biowerkstoffe des Landes Nordrhein-Westfalen", in which 17 postgraduates together with 10 university-lecturers develop both

- an interdisziplinary teaching program

and

- a research and development (r+d)-concept "biomaterials",

on the basis of which the doctorate projects of the postgraduates are performed.
This program was linked right from the beginning with bilateral cooperation between Germany and former Yugoslavia and internationally extended to Greece and Italy in the frame of Eureka-Project 294 on biomaterials. The present first progress report - which contains english and german written contributions - presents results and describes the above mentioned research concept in its chapter II.
The importance of orthopaedic implants has increased steadily over the last years. Though the success rate of these operations is relatively high, they suffer from one major disadvantage: the life span of, for example, a total hip replacement prosthesis, is only about ten years. Mostly, the reason for failure of orthopaedic prostheses is loosening, due to biomechanical inadequacies and/or factors concerning biocompatibility. Important aspects in biomaterials research are therefore the microstructure and the mechanical behaviour of the bone-implant interface.
The aim of the work presented in chapter III is the combination of preparation methods for light and electron microscopy from materials science and histology to minimize artefacts in the "composite specimens" of hard tissue and different biomaterials. In a first step, hard tissue has been examined on its own.
After a general introduction, chapter III presents basic considerations to be kept in mind when dealing with biomaterials. The most important biomaterials and fundamental aspects of their behaviour when implanted in the human body are shortly explained. The structure and microstructure of bone as one component of the composite system mentioned above are described followed by an introduction to histological preparation focusing on methods for undecalcified bone. Basic features of the scanning electron microscope are explained before the reader is introduced to the materials and methods used. Finally own results are presented taking into account both histological and materials science aspects.
The preparation method best suited for reflected light and electron microscopy of bone is the following:
The fixed bone is embedded in a fast hardening resin - as used in microstructural analysis - for grinding and polishing according to a scheme of preparation in microstructural analysis. Subsequently, the resin is carefully removed and, after dehydration in an ascending alcohol series, the specimens are critical point dried via acetone and carbondioxide. Damaging influences during preparation and examination in the scanning electron microscope are largely excluded.
Scanning electron microscopy can be performed in the backscattered electron mode with an accelerating voltage of 25 kilovolt (kV) or in the secondary electron mode with an accelerating voltage of 5 kV.
A new preparation method for bone for transmission electron microscopy (TEM) -examination by dimpling a bone disc has been tested. Certain areas of special interest can be detected more reliably. First experiments have been promising, further examination is necessary however.
The results presented above show that the used preparation method is an appropriate tool to prepare hard tissue for light and electron microscopical examination. The structural features of bone are revealed. If topographical contrast is excluded, back scattering electron (BSE)-imaging can be used to show differences in calcium content by density contrast. This is of

interest especially at a bone-implant interface to determine the maturity state of the newly developed bone.
The specimens can further be used for energy dispersive x-ray (EDX)- determination of the contents of different elements in the surface range, which again is especially interesting for the examination of the bone-implant interface.
Since for the preparation of interfaces between bone and implanted materials including the interface between both a variety of method should be available bone-bioglass combinations are treated by the gas contrasting method in the last chapter IV.
The present progress report starts a serie, in which information about is spread to those, who are interested. Whoever belongs to this circle should send the coupon below to be kept in mailing list.

I am interested in further
information about biomaterials

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Acknowledgement

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Personal assistance has been given by Dr. A. H. L. Chamberlain University of Surrey, Department of Microbiology, Dr. C. Doyle and Dr. Z. Luklinsky, University of London, Queen Mary and Westfield College, Department of Materials and Dipl.-Ing. Ute Müller, Rheinisch-Westfälische Technische Hochschule Aachen, Graduiertenkolleg der Arbeitsgemeinschaft Biowerkstoffe des Landes Nordrhein-Westfalen. This support is gratefully acknowledged by the editors and authors.

II.Biomaterials - a research concept

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Biocompatibility describes the interaction between a living system and an allotropic material introduced into this system. This interaction first becomes apparent at the interface.

In materials science a differentiation is made between:
- interfaces in nonequilibrium
- interfaces in equilibrium.

Interfaces are thermochemically in nonequilibrium ("unstable") if their constituents interact with each other, i.e. react with each other chemically or are dissolved into each other. The termination of such a process is a materially different, "stable" system.
In contrast, interfaces in equilibrium are formed by constituents which neither react chemically with each other nor are they dissolved into each other.
If these considerations are transferred to allotropic materials in the biosystem then at their interface to the living tissue bioinert materials are "in equilibrium", the interface is stable. As shown in fig. II.1, their bioactivity is 0.

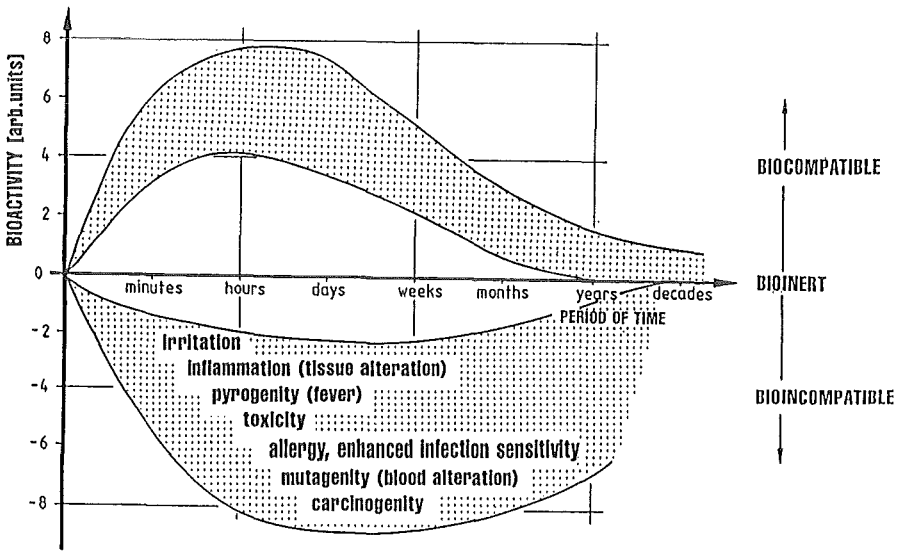

Fig.II.1. Bioactivity and biocompatibility

The expression "bioinert" is used in this context to mean "thermochemically defined" since the Consensus Conference of European Biomaterials has recommended that this term should otherwise not be used.

In comparison to the interface between bioinert materials and the biosystem, the interface between bioactive materials and the living system is subjected to interactions and is therefore unstable. Events at unstable interfaces are generally complex and not restricted to the interface. These events gradually involve the area surrounding the interface and ultimately perhaps the whole system. Frequently it cannot be predicted when stability will be achieved and which status of the system this corresponds to. The upper limiting curve of the positive bioactive (= biocompatible) region or the lower limiting curve of the negative bioactive (= bioincompatible) region in fig. II.1 corresponds to this situation. In contrast, with - for example - resorbent materials, the stable final status is as a rule more easily reached. For biocompatible materials, the lower limiting curve in the positive bioactive region of fig. II.1 corresponds to this situation.

Negative bioactive, i.e. bioincompatible, materials will not be discussed further in this consideration, but for positive bioactive, biocompatible materials long-term kinetics must be taken into consideration requiring many years of observation - not only of the interface. If intermediate layers - such as connective tissue or protein deposits - are formed (cf. fig. II.2), i.e. the situation is biotolerant or - expressed in terms of materials science - metastable, then the interaction between the biosystem and biomaterial is temporarily blocked or at least retarded.

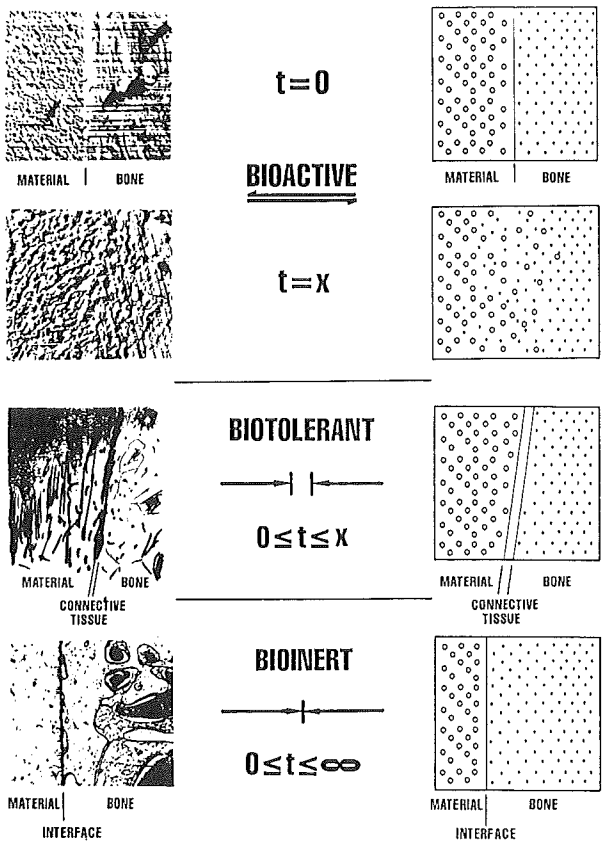

Fig.II.2. Bioactivity and interaction

The most scientifically appropriate and most readily understandable case is therefore that of the stable interface between the biosystem and bioinert material (cf. fig. II.2). It restricts the implant to itself and its sole permanent contact position to the biosystem is the - always controllable - interface. This is therefore the subject of this study. The problem to be investigated is the adhesive strength which can be achieved between known metallic, polymer and ceramic bioinert materials and "hard tissue" (bone or cartilage), and the way in which interface behaviour towards soft tissue and body fluids can be characterized.

The fundamental scientific law for the formation of an interface in equilibrium is given by equation 1

$$G^{if}_{\alpha\beta} - G^{sf}_{\alpha} - G^{sf}_{\beta} = \Delta G^{ad}_{\alpha\beta} \qquad (1)$$

$G^{sf}_{\alpha}, G^{sf}_{\beta}$ = surface enthalpies of the α, ß phases

$G^{sf}_{\alpha\beta}$ = interface enthalpy of the interfaces between the α -phase and the ß-phase

$\Delta G^{ad}_{\alpha\beta}$ = adhesion enthalpy for the interface between the α -phase and the ß-phase.

Accordingly, an interface is formed if - and only if - the total of the surface enthalpies of the two constituents forming the interface is greater than the interface enthalpy, i.e. the value of the adhesion enthalpy according to equation 1 must be negative - and proportional to the adhesion. This should also be valid for interfaces between the biosystem and allotropic bioinert materials.
The adhesion described by equation 1 can only be effective if the atoms in the interface may approach each other at spacings in the order of magnitude of 10^{-10} m. According to fig. II.3 this is the distance below which interatomic forces become effective.

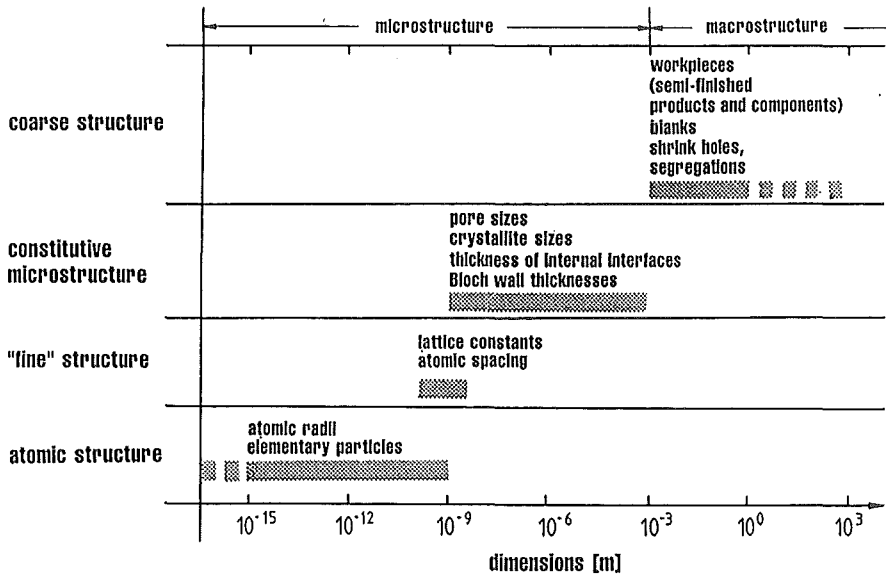

Fig.II.3. Structural division and order of magnitude

Whether they can take on the equilibrium spacings according to fig. II.4 ensuring maximum bonding (adhesion) in this order of magnitude depends on the interface structure.

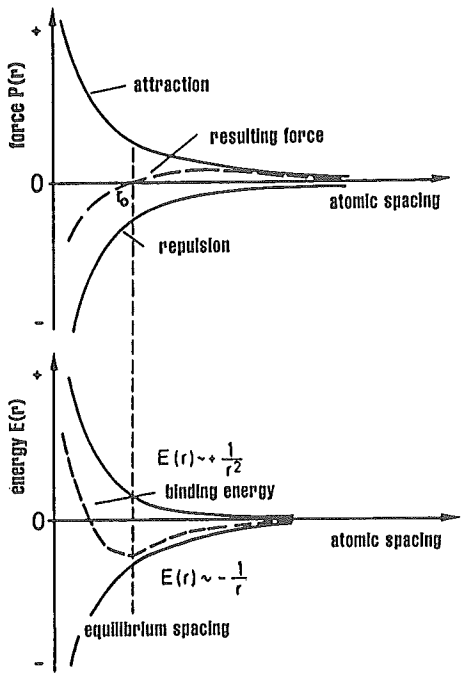

Fig.II.4. Atomistic equilibrium spacing and binding energies

In adherent interfaces (fig. II.5), the atomic spacings are closest to the equilibrium spacing. Whilst partially adherent and inadherent interfaces denote different approaches towards equilibrium spacing. The term "adherent" corresponds to phase boundaries and substitutes "coherent", which can correctly be used only for grain boundaries.

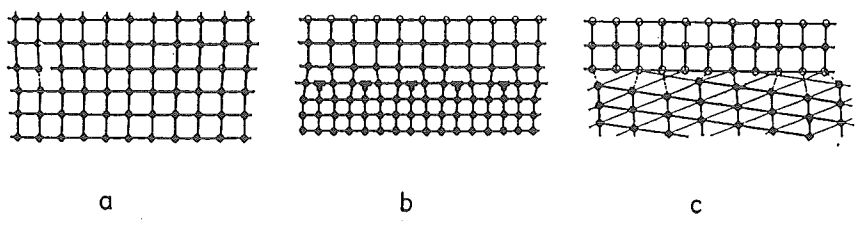

Fig.II.5. Adherent (a), partially adherent (b) and inadherent (c) phase boundary

The interface structure which is formed depends on the x-ray-structure (fig. II.5) of the components forming it or their aggregate status. Interfaces between liquid and solid phases should be able to correspond most closely to the energetic conditions and therefore clearly reflect the influence of surface and interface energies in wetting studies. The wetting of biomaterials by body fluids or their simulates is the major focus of the Eureka Project 294 and the "Arbeitsgemeinschaft und Graduiertenkolleg Biowerkstoffe des Landes Nordrhein-Westfalen" supplemented by boundary layer studies between soft tissue or the body's own secretions (proteins, plaque and similar) (fig. II.6).

As equation 1 shows, a correlation between the surface enthalpy of an implant material alone and the adhesion to the biosystem is not justifiable. Wherever such correlations have been published they either occurred by chance or should be regarded as special cases, not to be generalized. Nevertheless, the surface enthalpies of allotropic materials have a decisive effect on interface formation towards the biosystem and it must be regarded as a disadvantage of most conventional implant materials including ceramic materials such as aluminium oxide, that they are single-phase, i.e. not variable in their composition, since by varying the material composition the surface enthalpy can be varied and thus adapted to the requirements of the biosystem. For this reason, ternary bioceramics are being developed and compared to conventional biomaterials with respect to their interface formation and surface enthalpies within the framework of the Research Association between Eureka Project 294, the International Technical and Scientific Cooperation and the Arbeitsgemeinschaft und Graduiertenkolleg Biowerkstoffe.

The in-vivo formation of interfaces with bone and cartilage in the biosystem is complicated and time-consuming, and should, if possible, be replaced by a simulation, particularly in order to reduce animal experiments. The search for such a simulation is a further objective of work in the above mentioned scientific-technical cooperation.

Cell culture tests have long been a preliminary stage for animal experiments. As a rule, they provide initial qualitative information on biocompatibility or bioincompatibility. Whether they are also able to provide preliminary information for adhesion and interface formation for bioinert materials by correlation to their surface enthalpies is a research topic being dealt with by the above mentioned research association before beginning simulation experiments. The further discussion refers to the simulation of bone and cartilage.

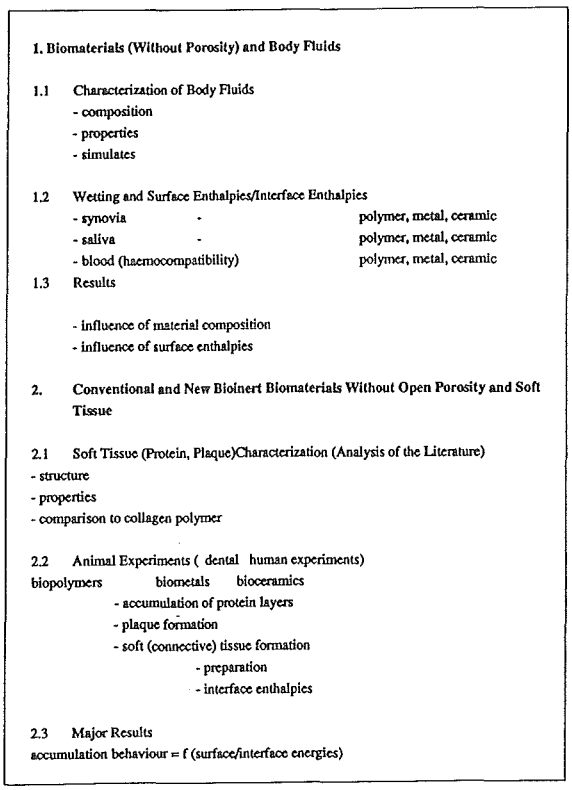

Fig.II.6. Biomaterials without porosity and body fluids

In its structure and properties hydroxylapatite is most similar to bone. In the sense of a first approximation, hydroxylapatite is therefore to be brought into contact with conventional and new biomaterials as a bone simulate. The interfaces between hydroxylapatite and bioinert biomaterials are then to be analysed in comparison to the interfaces between bone and bioinert biomaterials (fig. II.7, fig. II.8).

3. Conventional Bioinert Biomaterials Without Open Porosity and Bone or Cartilage

3.1 Technology and Characterization (analysis of the literature and measurement)
biopolymers biometals bioceramics

- fabrication
- structure
- properties: major priority surface enthalpies, mechanical properties
- cell culture test: hydroxylapatite and bone as standards; dependence on test conditions and surface energies

3.2 Simulation

polymer 1,2...n-hydroxylapatite (HA) metal 1,2...n-HA ceramic 1,2,...n-HA

- sintered, other methods
- constitution: single-phase, multiphase
- interface enthalpies (adhesion, cohesion) and interface structure preparation, measurement or examination
- adhesion: stripping test, splitting test, bending test (fatigue) structure analytical measurement of fractured surfaces and porosities

3.3 Animal Experiments (dental human experiments)

polymer 1,2..n-bone metal 1,2...n-bone ceramic 1,2..n-bone

- preparation
- interface enthalpies and interface structure
- adhesive strength

3.4 Major Results

proportionality adhesion enthalpy-strength hydroxylapatite-bone

- influence of surface/interface enthalpies
- influence of interface structure
- influence of residual stresses

(reproducibility)

reference to cell culture test

Fig.II.7. Conventional bioinert biomaterials without open porosity and bone or cartilage

The load on an interface is the greater, the greater the difference in properties between the phases it joins (coefficient of expansion, modulus of elasticity). Failure to adhere in the interface is the more probable, the "more unfavourable" the geometry of the interface is with respect to the effective stress: smooth interfaces fail more readily than rough, pin implants fail more easily than screw implants. If the idea of roughness is continued to the extreme via surface profiling (screw) then one arrives at an implant material with open porosity into which the bone can grow (fig.II.9).

4. New Bioinert Multicomponent Biomaterials: Ceramic/Glass Without Open Porosity and Bone or Tissue

4.1 Constitution and Technology

- temperature-concentration diagram Al_2O_3-SiO_2-TiO_2
- powder preparation (sol-gel etc.)
- compaction and shaping operation
- surface enthalpies: concentration and phase dependence
- cell culture test: hydroxylapatite and
  bone as standards
  dependence on test conditions
  and surface enthalpies

4.2 Simulation

Ceramic 1,2...n-hydroxylapatite

- technology: sintered; other processes
- interface enthalpies (adhesion, cohesion) and interface structure
  preparation, measurement, examination
- cohesion: stripping test, bending test,
  splitting test (fatigue)
  structureanalysis, measurement of
  fracture surfaces and porosities

4.3 Animal Experiments (dental human experiments)

ceramic 1,2...n-bone

- preparation
- interface enthalpies (surface enthalpies) and surface structure
- adhesive strength

4.4 Major Results

proportionality adhesion enthalpy - strength

hydroxylapatite - bone

- influence of surface/interface enthalpies
- influence of interface structure
- influence of residual stresses

(reproducibility)

reference to cell culture test

Fig.II.8. New bioinert ceramic multicomponent biomaterials without open porosity

A bone-biomaterial composite thus arises with maximum mechanical anchorage which distributes the interface adhesion almost homogeneously through the composite material and mechanically supports it. But also other arguments support the composite concept as, for example, concerning the acetabular (hip joint cup). Substituting a natural hip joint the tribological system is changed from one with lubricant (synovia) to an artificial one without synovia. Could the disadvantages with respect to friction and abrasion be reduced if a natural bone forms partly the surface of the acetabular via a composite cup? Does synovia reappear in that case and could wettability take place? In any case the composite material concept provides the theoretical basis for precalculating the porosity structures capable of ensuring an optimum adaptation of properties always proceeding from the bone into the implant. The

production of biomaterials with "tailor-made" open porosity and a study of their healing kinetics is similarly a priority of research team work as described here (fig. II.10).

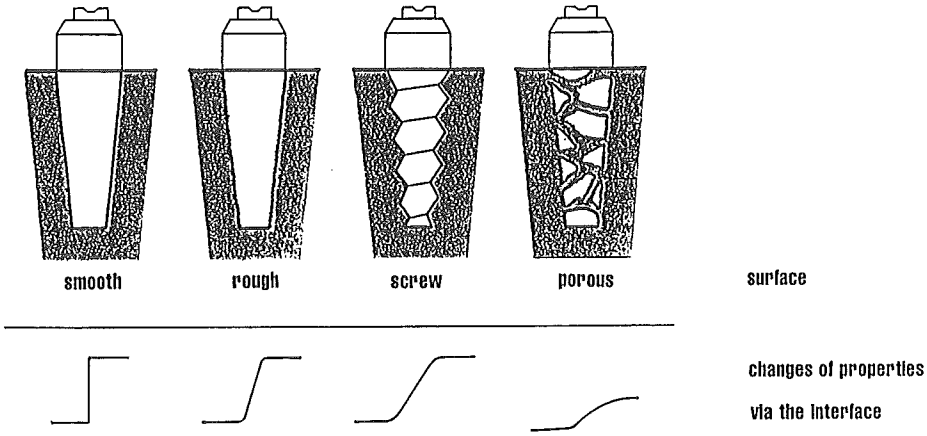

Fig.II.9. Composite material concept and interface integration

In the case of interfaces between solid phases, the kinetics of their origin will be of significance, i.e. the question of which aggregate states of the phases occurred during interface formation; if a phase from the gaseous aggregate state, from melts or solutions is deposited on the surface of the other solid phase then the adaptability of the atoms in the newly formed interface is great ("epitaxi"). In contrast, if solid phases are joined by sintering then the adaptability is less. Reactive layers on the solid surface can temporarily increase atomic mobility and thus optimize the interface structure on the basis of the principle of reaction sintering.

5. Conventional and New Bioinert Biomaterials With Open Porosity and Bone or Cartilage

5.1 Technology and Characterization

biopolymers (conv.) biometals (conv.) bioceramics (conv.+ new)

- fibre characteristics (C fibres)
- mixing, compaction and shaping operation
- structural analysis: pore geometry, porosity

5.2 Animal Experiments (dental human experiments)

biopolymers biometals bioceramics

healing kinetics = f (pore geometry, surface/interface enthalpies)

5.3 Major Results

growth kinetics, anchorage

Fig.II.10. Conventional and new bioinert biomaterials with open porosity and bone or cartilage

If bone and cartilage grow rapidly and without impedance on the bioinert material surface then the interface structure is largely adapted. However, if deposited protein layers or soft tissue occur then the interface adhesion is disturbed. Growth-stimulating resorbent coatings of allotropic implant material may therefore encourage optimum interface structure. This must be studied by a comparison,

- firstly for interfaces of hydroxylapatite (HA in fig. II.11) in contract to bioinert materials coated with materials with which hydroxylapatite interacts, i.e. which it dissolves or with which it reacts (simulation of coated implants),

- secondly for interfaces between bone and bioinert implant materials coated with resorbent materials,

which is the further goal of research team work (fig. II.11).

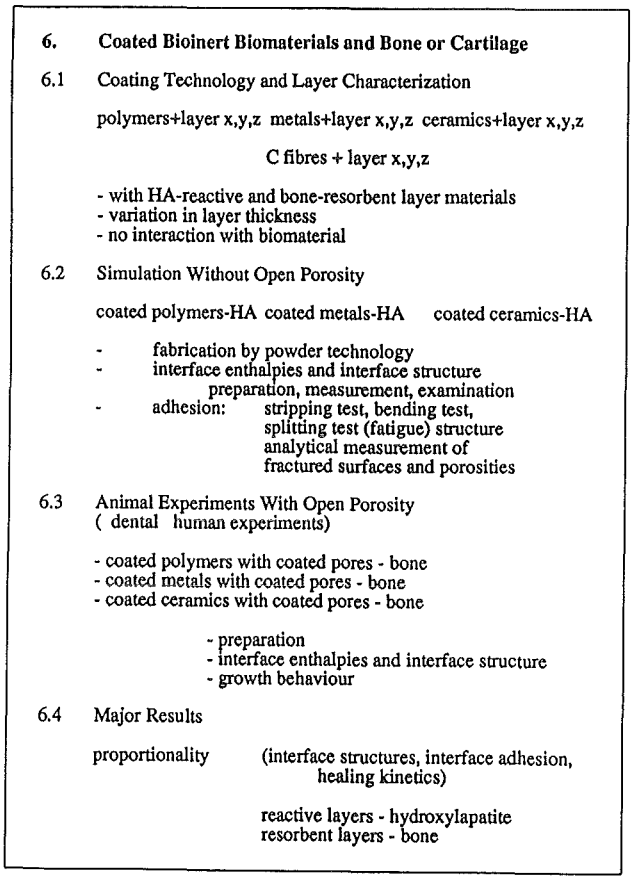

Fig.II.11. Coated bioinert biomaterials and bone or cartilage

Porous materials are weakened by their mechanical properties - precisely by their porosity. After being introduced into the biosystem it takes a certain time until the pores are filled with bone (healing period). On the other hand, load has a positive effect on the formation of bone and cartilage so that this healing period should not be exclusively a period of rest. In order to reduce the risk of a material fracture in spite of porosity, the implants should only display open porosity in their jacket zone. In the core zone, on the other hand, the porosity should be

replaced by a secondary phase which displays adhesion to the implant ceramic and thus modifies its properties in such a way that the desired load during the healing phase and later does not represent an unjustifiable risk (fig. II.12).

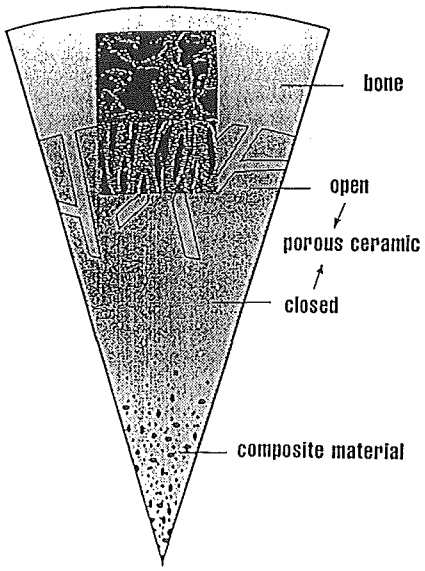

Fig.II.12. Graded implant structure

To calculate such "graded microstructures" by the composite material concept and - together with subsequent realization by powder technology studies on growth behaviour - are a further research topic in the concept (Fig. II.13).

7. New Biomaterials With Graded Microstructure and Bioworkpieces and Bone or Cartilage

7.1 Fabrication of Biomaterials With Graded Microstructure
- calculation according to the composite material concept
- powder technology
- structural analysis

7.2 Workpiece Fabrication
- transfer of laboratory-scale technology to implant forms (new ceramics with and without open porosity)
- transfer of graded structures to implant forms

7.3 Animal Experiments (dental human experiments)

7.4 Major Results
- growth behaviour
- adhesion, strength, "mechanical" compatibility

Fig.II.13. New biomaterials with graded microstructure and bioworkpieces and bone or cartilage

The transfer of laboratory specimen fabrication of multicomponent, multiphase, porous, bioinert biomaterials with graded microstructure and defined surface enthalpies to industrial implant fabrication is - quite literally - a separate field and should be treated as such . Contact to industry is indispensable here. It is therefore of primary necessity that the research programme summarized in fig. II.14 as a jigsaw puzzle includes cooperation with industry.

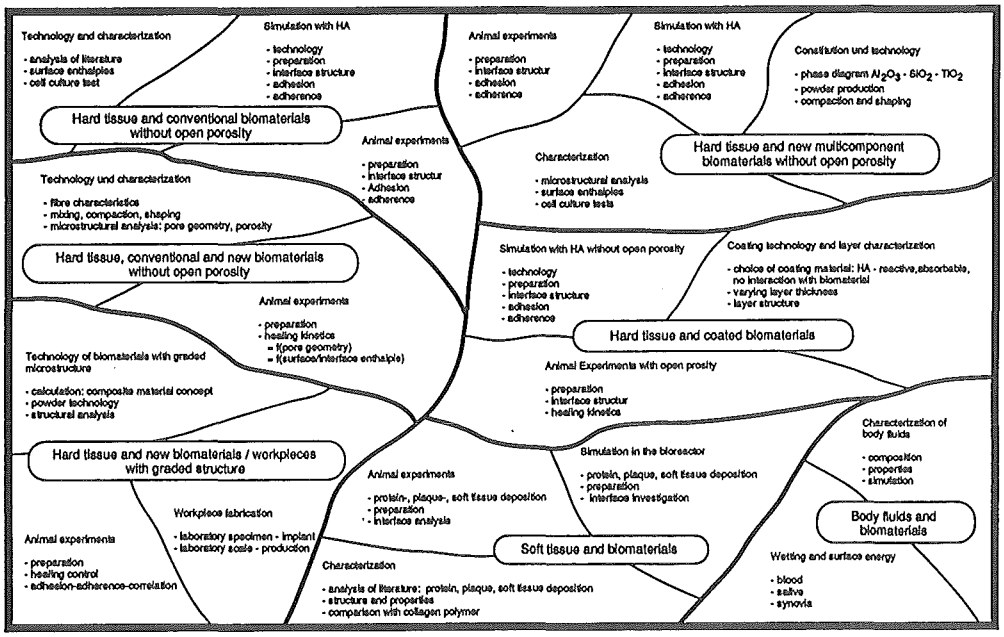

Fig.II.14 Research program of the postgraduate group

However, a different consideration is of basic significance for the interfaculty, interdisciplinary and international organization: after simulation experiments, cell culture test and animal experiments, an examination of material behaviour in the human biosystem is unavoidable since biomaterials are to be introduced into this biosystem as "biological spare parts" in the form of bioworkpieces ("implants"). It is quite obvious that the dental sector serves as the "introductory region" in the human system since body reactions in the dental sector can be directly observed and operations undertaken immediately. Nevertheless, the number of patients with relevant syndromes remains limited ("sample populations" in the sense of material sciences). In order to obtain reproducible results, however, certain "statistical collectives" are necessary.
For a reliable extrapolation of reproducible results it is also necessary that they should reproduce all possible state conditions which may influence the results: nutrition and climate, habits and specific dispositions in various regions of the world. These requirements are accommodated by the organization of the research programme adapted to the research goals: not only various faculties of different german universities are involved (Arbeitsgemeinschaft und Graduiertenkolleg Biowerkstoffe des Landes Nordrhein-Westfalen, Thüringer Arbeitsgemeinschaft Biomaterialien) but also bilateral cooperation with various republics of the former Yugoslavia, Israel and Egypt, as well as cooperation within the framework of the Eureka Project 294 ensures that different ways of life in east and west, north and south are included in the results under real ecological conditions and that their reliability is ensured "statistically". The Biomaterials Project is organized on an interdisciplinary, interfaculty, interlocal and international basis!

III.A critical evaluation of specimen preparation methods for optical and electron microscopy of bone

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III 1. INTRODUCTION

The importance of orthopaedic implants has increased considerably in the last ten to twenty years. More and more people suffer from different forms of joint diseases. Whereas the latter mainly concern elder people, the number of younger people requiring some sort of orthopaedic implant is also on the increase, due to, for example, sport and car accidents.
Replacement of the hip joint is currently one of the standard operations performed by orthopaedic surgery. Most skeletal implants, though usually successful, have one major disadvantage: their average life time is only about ten years. This leaves them suitable for elder patients whose life expectancy does not exceed the life time of one or two subsequent implants. For younger patients this timespan is far too short, especially as re-implanting is not advisable, or even possible (due to bone loss) more than once.

In the majority of cases, failure of bone implants is due to loosening and is caused by the following:

1) biomechanical factors and/or
2) the growth layer of fibrous tissue between the bone and the implant.

ad 1: Bone growth and resorption occur in response to the applied stresses. Under natural conditions this is a very advantageous property, which results in an optimized bone structure and means maximum strength for a minimum amount of materials. However, it can turn into a disadvantage as soon as changes in the stress pattern are induced. Bone will resorb on places where stresses are reduced. This happens by a mechanism known as stress-shielding which occurs when the Young's

modulus of the implant material is higher than the Young's modulus of bone. This results in the removal of bone substance that was planned to give mechanical support for the implant. The Young's modulus of the implant material should therefore match the Young's modulus of bone as closely as possible. There must be a transfer of stress from the material into the bone and vice versa. Under all circumstances, stress shielding of the bone must be avoided, otherwise a stable interface that works over a reasonable amount of time will never develop.

ad 2: Most implant materials get encapsulated by a layer of fibrous tissue. This capsule is usually in some way involved in interfacial failure of implants. Firstly, there is no chemical bonding between the implant material and this layer, so that the contact is purely mechanical; secondly, the mechanical stability of this capsule often deteriorates with time.

This short survey highlights the importance of interface type between the bone and the implant material in relation to the performance of the bone-implant-compound. Unfortunately, up to now there is no generally accepted opinion as to which sort of interface is most suitable. There is agreement, however, that a fibrous tissue layer should be avoided. Other than this, there are the following possibilities: Should the interface just consist of a close contact between the bone and the implant material with or without chemical bonding, or should there be a kind of "interphase" between the two components with gradually changing properties? This review attempts to discuss these points in more detail.
It is necessary, therefore, to judge the behaviour of the implant material and to understand the processes that take place at this interface and the kind of interface which is formed.
There are several characterization techniques that can be applied to examine the bone implant interface. In this work light microscopy (LM), scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were used. As the specimens are composed of biological and non-biological material, histology and materials science have to be combined. Histology is the science of biological specimen preparation and examination.
As we deal with at least two totally different materials of which one is a composite biological material, difficulties during specimen preparation are to be expected. Histological specimen preparation is usually difficult and time consuming. Shrinkage and/or swelling of the biological material occur during different steps of the preparation procedure. This leads to artefacts in the biological specimen itself as well as in the interface between the biological material and the implanted material in the bone-material-specimen.
A preparation method is therefore required which minimizes the number of artefacts and which is as easy and as quick as possible. Fewer artefacts facilitates the interpretation of the features recognized in micrographs and makes the information more reliable.
This work examines critically preparation methods for optical and electron microscopy of bone in order to achieve the goals mentioned above. The examination of the bone implant interface will be the issue of future work.

List of abbreviations

BSE	backscattered electrons	K_2O	potassium oxide
CaO	calciumoxide	MgO	magnesium oxide
CBSE	converted backscattered electrons	Mo	molybdenum
Cl	chlorine	NaOCl	sodium hypochloride
Co	cobalt	Ni	nickel
CO_2	carbon dioxide	PE	polyethylene
CPD	critical point drying	PMMA	polymethylmethacrylate
Cr	chromium	P_2O_5	phosphorus oxide
F	fluorine	SE	secondary electrons
HCl	hydrochloric acid	SEM	scanning electron microscopy/microscope
H_2O	water	TEM	transmission electron microscopy/microscope

III.2. BASIC CONSIDERATIONS

III.2.1 Biomaterials

The term "biomaterial" is often confused with a natural biological material. Of course, biomaterials can be natural materials, for example in the case of bone allografts and autografts. In the majority of cases however, biomaterials are synthetic materials derived from all groups of materials. The prefix "bio-" therefore describes the use rather than the origin of these materials.

Biomaterials are used in nearly all areas of human medicine such as pharmaceutical purposes, implant materials, and medical devices.
Some biomaterials are designed to remain in the human body for the lifetime of the patient, such as total joint replacements, or for as long as the device is designed to function, such as catheters. As mentioned in the above introduction, the former has not yet been achieved.
Other biomaterials degrade in the physiological environment, either to fulfil their function (e.g. degradable polymers for drug delivery systems) or after they have fulfilled their purpose and become unnecessary. Examples of the second group are the commonly used surgical sutures made of degradable polymers, and, not yet fully developed, degradable fracture plates. The latter would make further operations to remove the fracture plate after healing unnecessary. Biomaterials however, are not only materials used in implants in the human body. Materials for medical and surgical instruments also belong to this group of materials.

The following definition for "biomaterial" was agreed on at the Consensus Conference of the European Society for Biomaterials which was held in Chester, England, in 1986 /1/:

A "biomaterial" is a
"non-viable material used in a medical device,
intended to interact with biological systems."

A "medical device" was defined at the same conference /1/ as
"an instrument, apparatus, implement, machine, contrivance,
in vitro reagent, or other similar or related article,
including any component, part or accessory, which is
intended for use in the diagnosis of disease or other
conditions, or in the cure, mitigation, treatment or
prevention of disease in man."

Biomaterials represent all groups of materials, namely ceramics, metals, polymers and composites.
Metals and ceramics are mainly used for hard tissue replacement: metals for parts that are exposed to bending and tensile stresses and ceramics for parts that require especially good wear and friction properties. Polymers are used for both hard and soft tissue replacements due to their wide range of properties.
An example that combines these three main groups of materials is one form of total hip prosthesis. In this case, the stem is made of stainless steel or a cobalt-chromium-alloy, the acetabular cup is produced of alumina or ultra-high molecular weight polyethylene, and the head is made of alumina. A polymer used for soft tissue replacement is polyurethane elastomer for the totally artificial heart. These examples show the stages of development in the biomaterials field: whereas the total hip replacement is already a standard operation, the artificial heart is only in the development stage.

Composite materials are especially interesting for biomaterials applications due to the possibility of tailoring their properties to match the properties of the surrounding tissue. One example in this area is a hydroxyapatite-polyhydroxybutyrate composite for hard tissue applications, which can be designed to match the Young's modulus of bone /2/.

Biomaterials have to fulfil special requirements depending on their application. One requirement however, is crucial to all of them, irrespective of their later use, and that is that they have to be compatible with the physiological environment. Williams /3/ states that biocompatibility is a

"state of mutual coexistence between a biomaterial
and the physiological environment such as neither
has an undesirable effect on the other".

The definition agreed on at the Consensus Conference in Chester /1/ is the following:

"Biocompatibility" is the
"ability of a material to perform with an appro-
priate host response in a specific application."

"Host response" was defined as

"the reaction of a living system to the presence
of a material /1/."

Biocompatibility must always be considered in the light of the special area of application, as the above definition reinforces. There is no biomaterial that is biocompatible for all areas of application. For example, the requirements of a material to be compatible with blood are basically different from those for a material to be compatible with hard tissues.
For orthopaedic implants, the twin co-existence of biomechanics and biocompatibility has to be considered. This leads to the two following co-existing states of compatibility in this special area: biocompatibility and mechanical compatibility. Neither of these is valid without the other.

Furthermore, biocompatibility is also influenced by the surface structure and the geometry of the implant.

Biocompatibility can be tested in vivo or in vitro /4/. In vivo tests means the implantation of the material under investigation into various tissues of animals. After the animals have been killed, the implants and the surrounding tissue are examined using histological and materials science techniques. The common results can be classified into four groups:

1) Toxic material leads to the death of the tissue.
2) Non-toxic but biodegradable material may be replaced by the surrounding tissue or a fibrous capsule.
3) Non-toxic, biologically inactive material leads to the formation of a fibrous capsule around its surface.
4) Non-toxic, biologically active material allows the formation of an interfacial bond between the material and the surrounding tissue.

More recently, cell culture tests have been developed. Toxicity of the tested material is marked by a decrease in the number of cells or an increase in the release of lactate dehydrogenase and/or lysosomal enzymes by cells exposed to the test materials in comparison with control cell cultures. Other in vitro tests are organ cultures.

Drawbacks of cell culture tests are the following /4/:

- the cells used are not typical of tissues of normal implant sites,
- if the test material has only a low level of toxicity, this level may not be sufficient to kill cells or increase their enzyme release, but it may inhibit normal cell functions.

According to Lange and Donath /5/, an accurate judgement of the surrounding tissues and the tissue-implant interface is only possible by microscopic examination. Therefore, examination of implants that have functioned a certain time in an animal is necessary, in addition to clinical observation /5/.

In vitro tests are an interesting and necessary possibility to test the biocompatibility and performance in the physiological environment of future biomaterials, in order to replace and/or complement in vivo tests. One has to bear in mind, however, that especially for hard tissue replacement the mechanical compatibility of implant and host tissue plays an important role. This part of the behaviour of an implant material cannot (yet) be tested by in vitro tests. An alternative in this area is the modelling methods of technical mechanics, especially with the aid of computers. The use of these methods for biological applications has, however, been difficult up to now due to the lack of knowledge about the exact mechanical behaviour of bone.

III.2.2 Healing of bone

Every implantation causes injuries in the surrounding tissue. Injuries cause a stereotype response. To understand the processes that take place at the bone-implant interface the healing processes have to be considered first.
The following explanations follow closely the survey Williams gave in 1987 /6/, unless otherwise stated. If the damage in the tissue is not too great, bridging can take place spontaneously and completely (compare fig. III.1). The first action in the repair of any injury is the forming of an exudate, for example a blood clot. This blood clot is reorganized later on. In bone, this reorganization is the process of osteogenesis.

Osteogenesis can take place in two forms: either as osteoconduction or as osteoinduction. Osteoconduction describes bone growth from the existing bony walls into the reorganising tissue. If osteoinduction takes place new bone grows from isolated areas within the reorganising tissue. This can only happen if the appropriate growth factors and bone cells (osteoblasts) are present.

If a solid material is placed in the gap (compare fig. III.2), healing will take place in the space between the bone and this material. New bone will form if the gap is small depending on mechanical and chemical influences. After a certain time, the gap will be filled, partly with new bone and partly with fibrous unmineralized tissue. Under certain circumstances, the reasons for which are still unknown, the defect will fill completely with bone and an intimate bone implant contact is developed.

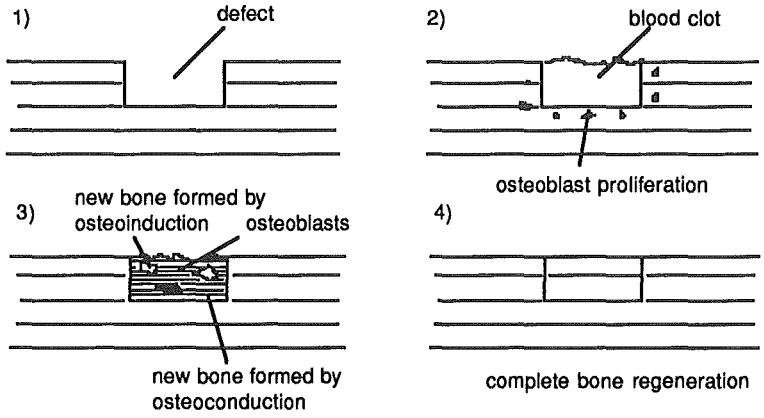


Fig. III.1: Healing without the aid of a biomaterial

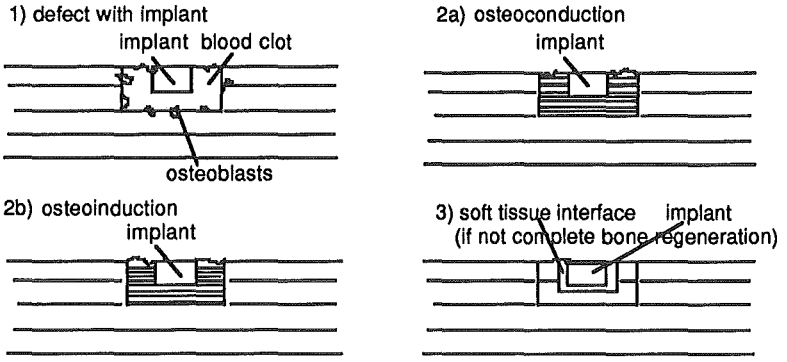


Fig. III.2: Biomaterial placed in the gap

III.2.3 Behaviour of implant materials

Biomaterials for hard tissue applications can be classified in three groups /6/:

- bioinert materials,
- bioactive materials,

and

- materials that allow osseointegration.

This classification has caused controversy. Some authors take issue on this and contest the existence of a bioinert material because there are always interactions between the biomaterial and the physiological environment. Clearly, definition of terms is important here, because to a first approximation if there is no overall reaction then the term "bioinert" is valid. Therefore, bioinert materials are understood here as materials that have neither a positive nor a negative effect on bone growth. Nevertheless, they minimize the formation of a fibrous tissue capsule. To avoid confusion, they could be referred to as "nearly bioinert" /7/.Ono et al. /8/ characterize bioinert materials as materials that do not bind to bone.The Consensus Conference of the European Society of

Biomaterials in 1986 in Chester, England, agreed that the term "bioinert" should be deprecated due to the reasons mentioned above /1/.
The term is used here for reasons of classification; the points discussed above, however, should be born in mind.

Bioactive materials, on the other hand, encourage the formation of new bone at their surface. They induce the process of osteoinduction with bone growth from the implant surface to the surrounding bony walls (compare fig. III.2) /6/.
Other authors see bioactive materials in the light of their property to achieve a direct bone contact at the implant-bone interface and to guide bone formation along their surface /9/ or to bind to bone /8/.
The definition agreed on at the Consensus Conference was the following /1/:

"A bioactive material is one which has been designed
to induce specific biological activity."

The third group of biomaterials mentioned above allow sustained growth from the surrounding bone to their surface with a direct bone-material contact (osseointegration). Osteoconduction takes place, with bone growth from the surrounding tissue towards the surface of the implant material (compare fig. III.2) /6/.
Many authors (/8/, /9/, /10/) do not differentiate between osteoinduction and osteoconduction. They state that bioactive materials, such as calcium phosphate ceramics, lead to a direct bone contact at the implant-bone interface and allow, therefore, osseointegration. These materials guide the bone formation along their surface. This bone-guiding property is often referred to as osteoconduction.
Ono et al. /8/ state that both bone-bonding and non bone-bonding ceramics lead to osteoconduction. Their osteoconductive potential, however, is different. A useful parameter for the evaluation of the osteoconductive potential is the amount of mature bone that is formed around an implant at a certain time after implantation. The amount of primary mineralization, i.e. the formation of immature (woven) bone, is not a useful parameter. Sela and Bab /11/ interpret the primary mineralization as evidence that the implant material does not disturb osteogenesis. The study of Ono et al. /8/ did not show a significant difference in primary mineralization around hydroxyapatite, an apatite-wollastonite containing glass-ceramic (AWGC) (bioactive material) and alumina (bioinert ceramic) implants. A comparable, clear-cut evaluation of the osteoconductive potential of these three materials was possible, however, by examination of the surface area of the granules that was covered by bone. The apatite-wollastonite containing glass-ceramic reached the best values, followed by hydroxyapatite and alumina.
Osborn and Newesely /12/ describe bone formation around bioactive ceramics as bonding osteogenesis and bone formation around bioinert ceramics as contact osteogenesis.
Important for the bonding to bone of bioactive ceramics is the formation of a surface apatite layer. This process is well documented for glass-ceramics, and hydroxyapatite binds to bone by a similar mechanism. The delay in the formation of this surface apatite layer may be the reason for the lower osteoconductive potential of hydroxyapatite in comparison with AWGC in the study of Ono et al. /8/. The following correlation between the formation rate of the surface apatite layer on bioactive ceramics and their osteoconductive potential has been deduced: the osteoconductive potential is stronger if the surface apatite layer is formed earlier /8/.
Linder et al. /13/ describe osseointegration as the formation of an interface with remodelled, viable bone in direct contact with the implant surface with no interposed fibrous tissue. Important is the viability of the bone and the lack of an inflammatory response in such tissue. According to Linder et al., osseointegration does not in itself refer to a specific implant material, implant design, or surface structure. Direct bone-implant contact has been observed in humans with various implant designs and a variety of materials, for example stainless steel, Ti-Al-V alloy, Co-Cr alloy, acrylic bone cement. However, such an interface cannot yet be achieved consistently /13/.

Another classification is given by Ondracek /14/. It is based on the thermodynamical view of the system "material" according to which the state of this system is governed by the state conditions composition, temperature and pressure. The state of the system "biomaterial plus living tissue" is additionally influenced by another state condition: the biocompatibility /14/.
A biomaterial can act biocompatible or bioincompatible (positively or negatively bioactive), or it may be bioinert. These actions have to be seen in dependence on the time after implantation; a specific biomaterial may be positively bioactive for a certain time after it has been implanted. After this time it may be bioinert (compare fig. II.1, page 7).
Negatively bioactive materials are in "negative non-equilibrium" with the biosystem; they cause a reaction of the immune system in varying degrees /14/.
Positively bioactive materials are in "positive non-equilibrium" with the biosystem as long as there are any reactions between the biosystem and the biomaterial. After this time, they are in equilibrium /14/.
Bioinert biomaterials are accepted by the biosystem and no changes can be observed. These materials are in equilibrium with the biosystem from beginning on. The biomaterial does not deliver any substances that are able to activate the immune system, neither due to their quality nor due to their quantity /14/.
A biomaterial may, therefore, according to these definitions, change its qualities from, for example, positively bioactive to bioinert behaviour.
Fig.III.3 (after /7/, /11/, /12/, /14/, /15/, /16/, /17/, /18/) gives examples of each of the groups defined above together with their area of application. In the following, some characteristics of the named biomaterials are explained further.

Group	Examples	Application (examples)
bioactive	calcium hydroxyapatite	ceramic phase in compsite mat. with polymer coatings for metal implants (stabilization) filling materials for cancellous or cortical bone loss
	tricalcium phosphate (ß-whitlockite)	filling materials for cancellous or cortical bone loss
	certain glasses and glass ceramics of controlled activity	repair, modification or reconstruction of skeletal system or teeth
	controlled hydrophilic surface polymers	
osseointegration	titanium	stem of hip prostheses
bioinert	metals: 316L stainless steel	stem of hip prostheses
	Co-Cr surgical alloys (<= 1 % Ni, mainly Co, Cr, Mo)	- " -
	Ni-Cr alloys (69 - 81 % Ni)	dental restorations
	polymers: PMMA	fixation of hard tissue replacements in the surrounding bone
	Dacron (polyester)	coated with degradable polymer for tendons and ligaments
	high density PE	acetabular cup, knee replacement
	ceramics: alumina bioceramics	acetabular cup and head of total hip replacement
	carbon	dental implant artificial heart

Abbr.: Co = cobalt Cr = chromium Mo = molybdenum Ni = nickel
PMMA = polymethylmethacrylate PE = polyethylene

Fig. III.3: Classification of biomaterials

Bioactive materials - calcium phosphate ceramics:
Calcium phosphate ceramics (CPC) are surface-active biomaterials /19/. They build up an interfacial bond with bone (/16/, /17/, /19/). The reports are less equivocal about the biodegradation of these ceramics /17/. This controversy may be due to the use of different procedures to prepare these bioceramics and to an insufficient analysis of the calcium phosphate compounds used by the various authors /17/.
The bioresorbability of CPC-implants is dependent on the chemical composition, the physical forms and the implantation sites of the ceramics. For example, the bioresorption of tricalcium phosphate-hydroxyapatite ceramics is approximately proportional to the tricalcium phosphate content of the compound, as suggested by in vivo experiments /19/.
CPC resorb either through a solution-mediated or cell-mediated process /19/. Solution-mediated resorption refers to the solubility of implants in physiological solutions. Little is known about the cell-mediated process of resorption. The study of Kwong et al. /19/ reinforces the hypothesis that phagocytosis followed by intracellular degradation in phagolysosomes is one of the mechanisms of cell-mediated resorption of CP-implant material. The hydroxyapatite crystals are first phagocytosed by cells. After this, they are solubilized in the acidic environment of secondary lysosomes. The soluble Ca^{2+}-ions either diffuse or are actively pumped from the cells into the surrounding medium. This is supported by the observation that the presence of cells is required for solubilization.
Other studies reinforce the following hypothesis: Bonucci /20/ observed that the site of degradation of calcium phosphate crystals during bone resorption is intralysosomal. Jarcho /21/ showed that the bioresorption of CPC-implants initiates with a microscopic break-up which is secondary to solution-mediated resorption. The fragments or individual crystals are subjected to aggressive cell-mediated removal (phagocytosis).

Calcium hydroxyapatite is one example of bioactive calcium phosphate ceramics. It has a calcium/phosphate ratio of 1.67 /17/ and has the same chemical and crystallographic structure as the hydroxyapatite in bone /22/. It can be produced either as replamineform material or by a sintering process.
Replamineform materials /16/ are made by converting the calcium carbonate skeletons of coral to hydroxyapatite. By hydrothermal chemical exchange with phosphate, a hydroxyapatite replica of the calcium carbonate skeleton is produced. This material is used instead of autografts for cancellous and cortical bone loss either in block or in granular form. The rationale behind this method is that the internal geometry of the synthetic graft should be similar to the trabecular framework of bone.

Sintered hydroxyapatite is made of powders that can be produced by one of four routes /23/:

i. precipitation from aqueous solutions,
ii. hydrolysis,
iii. solid state reaction,
or iv. hydrothermal synthesis.

Hydroxyapatite is osteoconductive. It serves as a matrix for the deposition of new bone /10/.
Being a brittle material, solid sintered hydroxyapatite suffers from fatigue failure /22/. Its osteoconductive properties, however, are an attractive possibility to try to avert long-term bone resorption at orthopaedic implants. Porous coatings of hydroxyapatite on metals, preferably titanium due to its high reactivity with hydroxyapatite /9/, have therefore been examined. They avoid the mechanical disadvantages of solid hydroxyapatite and could enhance early bone formation. This would allow full weight bearing much earlier after surgery. The effects demonstrated in different studies vary significantly however. This is due to material-processing induced parametric influences. The ceramic undergoes considerable changes in its physical and chemical characteristics after

deposition. Between the different studies, one or more of the following parameters may have differed, resulting in a different behaviour of the ceramic coating in the physiological environment:

- chemical composition,
- trace ions present,
- phases and their crystal structure,
- macro- and microporosity in the ceramic film,
- specific surface area,
- thickness, size, shape of porous coating and its associated porosity,

and

- chemical characteristics of underlying metal /9/.

Especially suitable for plasma-sprayed coating with hydroxyapatite is titanium. The ideal thickness of the coating has to be determined as a compromise between the following extremes: If the apatite surface is thinner than 10 to 15µm, it will be dissolved during the process of acquiring bone union. If it is thicker than 100 to 150µm, on the other hand, it will suffer from fatigue failure under tensile loading. The optimal thickness, therefore, is about 50µm.

The bone-bonding properties of a plasma-sprayed coating on titanium were examined by Geesink et al. /22/. Cylindrical rods of the material were implanted in the femurs of dogs, protruding from the endosteum to the periosteum. To examine only the bone-bonding properties the implants were allowed to move slightly (0.2mm space).
The bone marrow showed an initial inflammatory response, predominantly due to granulocytes, which ceased 12 weeks after surgery. After this time, a normal population of lymphocytes and plasma cells was observed. This reaction of the bone marrow indicates a good acceptance of the implants.
The periosteal and endosteal bone defects were filled with cortical bone within six weeks. The bone proliferated along the surface of the implant at both the endosteal and periosteal surfaces. Within the cortical bone, a very close bone-implant contact without interposed fibrous tissue was developed. The periosteal tissue, too, showed good bonding with the apatite coating. The new bone was of woven and lamellar structure. Remodelling of bone was obvious and many osteoblasts and osteoclasts could be seen along lacunae /22/.

The bonding between the implant and the bone is thought to be biological and chemical /22/, as the interface shear strength of the bone-implant interface reached the value for cortical bone itself.
Furthermore, these results were achieved without mechanical interlocking of the implant at time of implantation. Signs of biological bonding are the following:

- no interposed fibrous tissue between bone and implant,
- many osteoblasts in direct contact with the implant surface,
- osteoblasts seem to deposit the osteoid on the apatite coating,

and

- increased calcium content in the bone which is in direct contact with the implant.

These studies show that direct bonding between bone and hydroxyapatite can be achieved as well if the hydroxyapatite is applied as a plasma-sprayed coating on titanium implants. Important for the osteoconductive properties of hydroxyapatite are the surface characteristics. These are very similar for plasma-sprayed and sintered hydroxyapatite. Further studies, however, have to be performed under loaded conditions as the mechanical properties of plasma-sprayed hydroxyapatite differ significantly from sintered hydroxyapatite.

Responsible for the biocompatibility of hydroxyapatite is its apatite surface. New bone is deposited epitaxial-like on the surface of the ceramic /5/. According to results obtained by ultra-high resolution TEM /2/, the crystal lattices of hydroxyapatite crystals in new formed bone at the interface to a hydroxyapatite-polyhydroxybutyrate composite align with the crystal lattices of the hydroxyapatite particles.

Another example of bioactive ceramics is tricalcium phosphate or ß-whitlockite with a lower calcium/phosphate ratio of 1.5.
It undergoes a rapid degradation process after implantation, beginning at the periphery of the porous material /17/.

Bioactive materials - glasses and glass ceramics:
(after Hench and Clark /7/ if not otherwise stated)
Bioglasses are defined as glasses "designed to elicit specific physiological responses". To show this property, they provide surface reactive silica, calcium, and phosphate groups, and a slightly alkaline pH at an interface with tissues. A frequently used composition in research is the bioglass 45S5 with 45% SiO_2, 24.5% CaO, 24.5% Na_2O, and 6% P_2O_5 (by weight).
All bone bonding bioglasses show a large increase in surface area when they are exposed to simulated physiological solutions. The formation of a silica-rich gel on the implant surface leads to ultrapores of 3 to 30nm in size. Hydroxyapatite crystals appear to nucleate whether or not P_2O_5 is present in the original glass formula. This formation of hydroxyapatite agglomerates on the implant site leads to the incorporation of collagen, mucopolysaccharides, and glycoproteins within the surface active layer of the implant. The composite organic-inorganic interface appears to be responsible for the mechanically strong bond at the interface exhibited by these glasses, as shown experimentally.
The critical compositional variable in forming the bond with bone appears to be the ratio of total network former (SiO_2) to the total amount of network modifiers (e.g. CaO + Na_2O + K_2O). If this ratio is sufficiently large the glass network becomes three-dimensionally stable so that the ion exchange processes which are necessary for the formation of the active hydroxyapatite surface film are no longer possible. The substitution of K_2O for Na_2O, variations in the alkaline earth/alkali ratio, and the variation of the P_2O_5 content have no significant influence on the bone bonding properties of the glass.

Different experiments, therefore, suggest the following mechanism to produce a stable bond between a bioglass and the bone: The ionic sites on collagen and mucoplysaccharide molecules become cross-linked with the bioactive high surface area hydroxyapatite film. This film forms on the implant surface almost immediately after exposure to physiological solutions. Calcium and phosphorus from these solutions lead to an increase in thickness of the inorganic film that forms at the interface. This film gets hydrated while it is growing. Crystallization takes place to form hydroxyapatite agglomerates which incorporate the organic species into an intimately bonded contiguous structure. This structure stabilizes eventually to a thickness of about 25 to 30µm, merging into the cellular structure of bone. This transition zone between living and nonliving matter has the mechanical strength and compliance that is needed for the interface to transfer stress effectively.

The bioactivity of certain glasses and glass ceramics of controlled surface activity is thought to be due to their specific surface energy. For a long time it has been considered that the surface energy of a biomaterial plays an important role in its performance in the physiological environment, just as surface energy is the determining factor in the development of an interface between two "ordinary" materials. (CF)

Osseointegration - metals:
Titanium is known for its excellent biocompatibility and benign behaviour when implanted in the human body. It is up to now the only material known to allow osseointegration. It is widely applied in the medical and dental field. It is largely accepted now that the immediate spontaneous oxide formation of titanium is the reason for its excellent biocompatibility. This oxide film is formed spontaneously and increases while the metal is implanted. The oxide forms needles with a length of 50nm and a diameter from 7.5 to 20nm /24/.
There is, however, evidence of ion release from titanium and for dissolubility in vivo and in vitro. Released titanium can be accumulated in various organs and large quantities of titanium have been detected in the osseous tissue surrounding a titanium implant /24/. The concentration of titanium ions found in the tissue adjacent to titanium implants is unrelated to wear. The metal or its passivating film must therefore dissolve spontaneously /24/.
Nevertheless, several authors have reported that there is a close contact between the bone and the titanium implant. This was shown by light and transmission electron microscopy of solid titanium implants and titanium coated implants (/24/ and /25/). The bone tissue appears to be normal. Osteocytes are found in close contact with the metal surface. TEM reveals a thin (20 - 40nm) interfacial collagen-free layer of ground substance (i.e. macromolecules consisting of proteoglycans and glucose aminoglycans attached to a backbone of hyaluronic acid). Collagen filaments could be found 20 to 40nm from the metal, the first collagen fibrils at a distance of less than 100nm /25/.
According to Ducheyne /26/, the collagen-free interface layer is related to the minimal biological activity of titanium. He states further that titanium ions, due to their high acidity and the limited coordinating capability to representative cellular delations under physiological conditions, cannot permeate biomembranes and should therefore stay near the implant /27/.

Within this interface, multinucleated giant cells /13/ were observed. The giant cells were normally more numerous around implants with a low ratio of bone-implant contact. They did not seem to behave like osteoclasts. Usually, they were seen where no marrow or organized tissue was present and where no load was applied. Giant cells might therefore be related not to the implant material itself but to the mechanical situation and bone topography at the interface /13/.

The results of several studies (/13/, /28/) propose that a sufficient primary bone-implant contact is necessary for osseointegration and new bone formation. Insufficient primary stability of the implant disturbs the healing process due to micromotions at the interface between the implant and the bone. Instead of viable bone, a fibrous tissue layer will develop in these cases. (CF)

Bioinert materials - metals:
Low solubility and corrosion resistance are the main requirements for metals to be used as biomaterials /24/. Even alloys considered inoxidizable may corrode in a biological environment. Several authors have reported the corrosion of orthopaedic implants or dental restorations. This leads to concern about possible sensitivation and allergies against the released metal ions such as nickel, for example, which is known to cause more allergic reactions than all other metals together /15/. Furthermore, the cytotoxicity of metal salts and pure metals is well known /15/.
The performance of stainless steel and cobalt-chromium alloys is acceptable. Corrosion is indicated by the fact that the levels of ions contained in the alloys are higher than normal in the body, but normally it does not affect the patients adversely. Exceptions however, occur and patients can become hypersensitive to the metals contained in these alloys.

Due to the corrosive degradation of these metals, they are often not considered to be bioinert; many authors include only aluminiumoxide ceramics into this group. Furthermore, as mentioned above, the Consensus Conference in Chester in 1986 advised to deprecate this term.

Bioinert materials - carbon /18/:
Klawitter et al. /18/ have investigated the interface between carbon dental implants and the surrounding bone. They found a bone-carbon interface without an interposed fibrous membrane. Failure of the implant bone compound took place in the carbon or in the bone, but not in the interface. The carbon implants had a microporous spongy surface which apparently provided a substrate capable of forming a mechanical interlocking with the adjacent bone tissue. Klawitter et al. concluded that a load transmitting interfacial bond between the carbon and the bone was formed.

III.3. "BONES" AND "BONE"

III.3.1 Structure of bone

Having talked so much about "bone" - what is it?
First, it is important to distinguish between "bone" as a material, and "bones" as articulating features of the skeleton. The material "bone" builds up the tissue "bone", together with soft vascular interstices and macroscopic pores some of which contain the bone cells.

There are different types of bone present in the human skeleton, namely interwoven (coarse fibred) and lamellar (fine fibred) bone. Both can be organized as compact or cancellous bone. Woven bone is the immature form of lamellar bone. It is less organized than the latter and in the course of time all woven bone is reorganized as lamellar bone.

The specimens used for this work come from the diaphyses of the long bones (femurs) of several species (goat, horse and sheep). The structure of a long bone is shown in figure III.4.

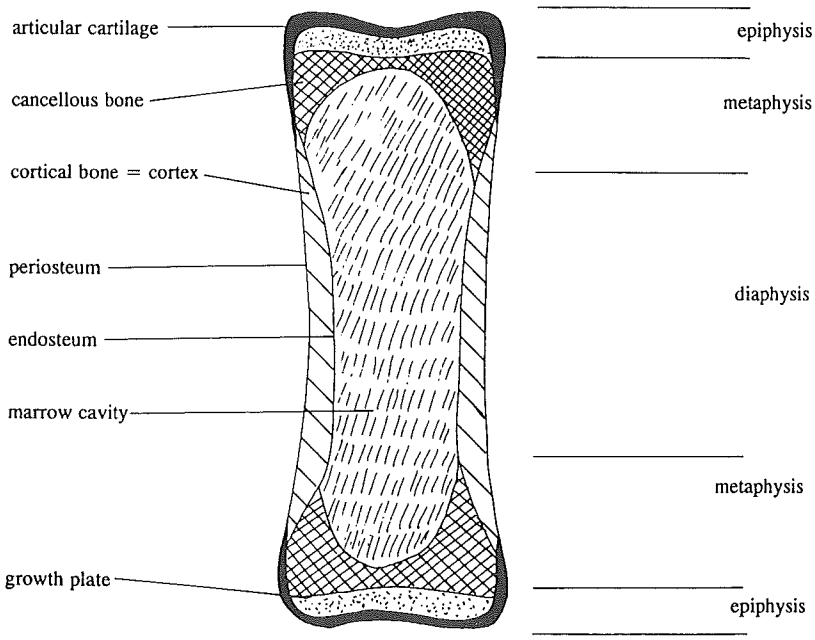


Fig. III.4: Structure of long bones

The diaphysis is the central section of a long bone. It is a cylinder of compact bone, the cortex, surrounded by a vascular, fibrous membrane, the periosteum. On the inner surface of the periosteum are numerous osteoblasts, the bone cells that are responsible for bone formation. The inner surface of the cortex is covered by the endosteum, which is similar to the periosteum. The central chamber contains the bone marrow and is known as the medullary cavity.
The adjacent parts of the bone, near to the ends, are the metaphyses. The cortex becomes thinner and the medullary cavity is permeated by cancellous bone. The ultrastructure of cancellous bone is the same as for compact bone (compare chapter 3.2). Its morphology, however, is different. Trabeculae are arranged mesh-like according to the applied loads (Wolff's law, compare below) /3/.
Bone as a living material has to be delivered with nutrients and waste products have to be transported away. For these purposes, a network of blood vessels goes through the bone. They branch and course through the Haversian and Volkmann's canals /26/. The crucial factor of the organization of the blood supply of long bones is that all vascular flow through the diaphyseal cortex usually appears to be entirely centrifugal from the medulla to the periosteum. There is no effective longitudinal flow of blood for more than one or two millimetres within mature cortical bone. For a short segment of diaphysis, therefore, all blood enters at the endosteal surface and exits at the corresponding periosteal surface /29/.

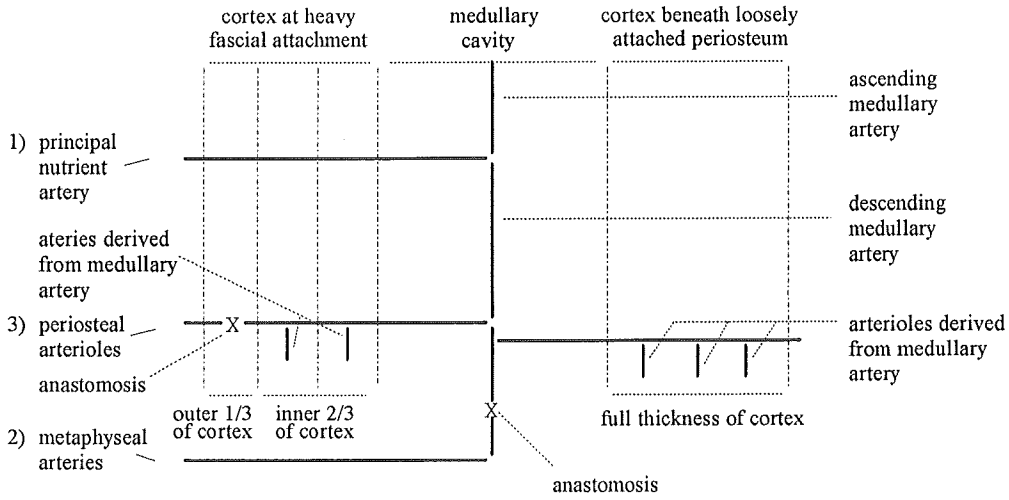
The blood vessels of long bones can be classified into three vascular entities /29/:

- the efferent vascular system,
- the afferent vascular system,

and

- the intermediate vascular system which links the efferent and the afferent vascular systems.

The afferent vascular system comprises the arteries and arterioles. They convey nutrients. The structure of this system is shown in figure III.5. It consists of three primary components: the principal nutrient artery, the metaphyseal arteries, and the periosteal arterioles.
The principal nutrient artery - which may be dual, as in the human femur, for example - traverses the diaphyseal cortex without branching before entering the medullary cavity. Here, it divides into the descending and ascending medullary arteries. These subdivide further and are the major arterial blood supply of the whole diaphyseal cortex.
The proximal and distal metaphyseal arteries supply the metaphyses of a mature long bone. They provide an anastomosis with the terminal branches of the medullary arteries. Under normal resting circumstances, these small-vessel anastomoses are inactive; in the case of obliteration of the nutrient artery by fracture or surgery, however, they are able to expand rapidly to supplement the medullary arterial supply.
The multiple periosteal arterioles enter the diaphysis only at heavy fascial attachments. They supply the outer third of the cortex in the region where they enter. Within the cortex, they anastamose with terminal branches of arterioles derived from the medullary cavity. This normal anastamosis appears unable, however, to supply blood to the medullary cavity when the medullary arterial supply has been disrupted.
The efferent vascular system comprises the veins and venoles that convey waste products. They drain the metaphysis and the diaphysis independently.



1), 2), 3): sources of blood from the general arterial circulation of the animal or man

Fig. III.5: Afferent vascular system of the blood supply of a long bone

The metaphysis is drained through the medullary veins, the diaphysis through separate vascular units for the hematopoietic elements of the medulla (emissary veins and venae eomitantes of the nutrient artery) and for the surrounding cortex (cortical venous channels and periosteal capillaries) /29/.

In surface bone, the blood vessels run in the so-called Volkmann's channels. They join the blood vessels of the Haversian canals of the Haversian bone /30/.

In the cortex of long bones, four structure types of bone (as a material) can be found (compare fig. III.5): periosteal and endosteal bone, Haversian bone, and interstitial lamellar bone.
The periosteal and endosteal bone lies adjacent to the periosteum and the endosteum respectively. Each is formed by several layers of lamellae, concentric to the marrow cavity. They are also known as outer and inner circumferential layers.

The structural units of compact bone between the layers of circumferential bone are the Haversian systems or osteons (compare fig. III.6, III.7). Each Haversian system has a central canal (Haversian canal) that contains one or more blood vessels and nerves. The osteon is surrounded by the cementing line. This line consists of calcified mucopolysaccharides and is devoid of collagen. It is therefore the weakest structure in bone and cracks with a very low crack propagation velocity follow this outermost lamella /31/. Inside the cement line, there are layers of lamellar bone, separated by inter-lamellar cement lines. The lacunae are found here. They contain the osteocytes (bone cells). The osteocytes have prospects which interact with each other through the canaliculi. The length of a Haversian system is several millimetres, the diameter is between 100µm and 300µm.
Until recently, the following scheme of bone organization was widely accepted: the two main types of bone are interwoven and parallel fibred bone. Parallel fibred bone was thought to be organized as either lamellar or non-lamellar bone. According to Gray's Anatomy /32/, lamellar bone consists of thin plates of bone tissue arranged, in the case of an Haversian system, concentrically around the

Haversian canals. The single lamellae are composed of collagenous bundles. These are arranged parallel to each other within each layer, but in different directions in adjacent lamellae.

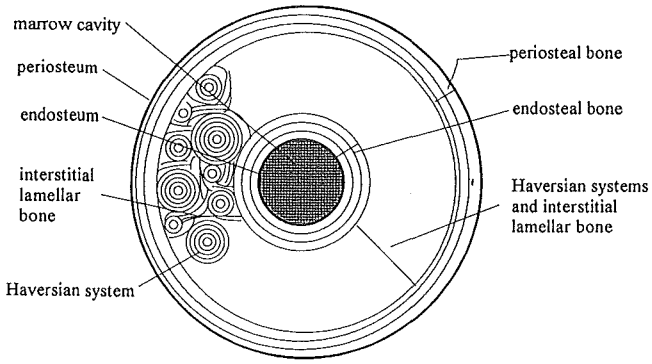


Fig. III.6: Structure of Haversian bone

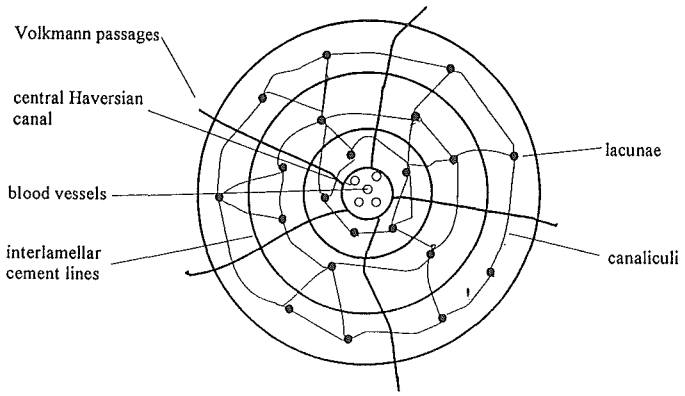


Fig. III.7: Haversian system

The collagen fibres have been thought to change their orientation in adjacent layers by 90° - the layers therefore each consist of parallel fibred bone. This simple perpendicular arrangement of collagen bundles however does not seem to exist /32/; the collagen fibres are rather supposed to be arranged in a helical manner in each layer, the pitch of the helix hereby changing in the bundles of adjacent layers. Nonetheless, cross-sections of stained decalcified bone show lamellae in which the collagen bundles are cut longitudinally and appear striated, therefore, alternating with lamellae in which these bundles are cut transversely with a punctuate appearance /32/.
The single layers are approximately 3µm thick and are separated by "interlamellar cement lines" which incorporate the lacunae. These lines are composed of an amorphous extracellular substance which contains deposits of calcerous salts. The collagen bundles in the lamellae themselves are embedded in this bone matrix too /32/.
Recent results, however, show that this image of bone organization must be changed. The simultaneous examination of a transversal and longitudinal cross-section (cubic bone sample) did not show the expected change in angle of the collagen fibres in adjacent layers. Longitudinally and

transversely cut fibres are seen in each layer. The lamellae seem to be striped in the same manner in all cuts /33/.
This observation leads to the following conclusion: the layers are made up by the interweaving of collagen fibres. The differences in the lamellae visible in micrographs are due to different collagen densities. The space between the collagen fibres is filled with proteoglycans. This allows further confirmation of this interpretation: dense lamellae contain relatively less proteoglycans and more collagen fibres than less dense lamellae. As the bone mineral (mainly phosphorus and calcium salts) is linked to the proteoglycans, the amount of phosphorus and calcium present in a specific lamella can be used as a measurement of the amount of proteoglycans and therefore indirectly the amount of collagen. The denser lamellae should show less phosphorus and calcium than the less dense lamellae. These considerations were confirmed by the observations of Marotti /33/.

Coming back to the structure types of bone, the interstitial lamellar bone remains to be explained. It fills the space between the osteons, as those, due to their roughly cylindrical shape, cannot fill the entire space.

Bone undergoes a steady process of remodelling during which the primary osteons are reorganized and the secondary osteons develop. Secondary Haversian bone is less uniformly organized and contains more interstitial lamellar bone.

The above mentioned pores contained in bone as a tissue are the Haversian and Volkmann's canals, the canaliculi, and the lacunae. The first three have been explained above.
The lacunae contain the bone cells of which three types exist: osteoblasts, osteocytes, and osteoclasts.
The osteoblast is a mononucleated cell of variable shape. It is between 20 and 30µm in length. It has a well-developed Golgi-apparatus with few mitochondria, but an extensive granular endoplasmatic reticulum. Fine cytoplasmic processes project out to contact neighbouring cells. The osteoblasts lie adjacent to the surface of the bone matrix. As they do not divide, new osteoblasts must be derived from some other cells /3/.
These cells become buried in the bone matrix that they themselves have produced. They then develop into osteocytes and many of the characteristics of an osteoblast diminish with time.
The osteoclasts are large multinucleated cells with approximately 20 nuclei. They have extensive Golgi membranes, and numerous and varied lysosomal bodies and mitochondria. They are responsible for bone resorption. They derive themselves either from precursor osteogenic cells or from macrophages /3/.

III.3.2 The Ultrastructure of bone

Bone as a material is a complicated composite material of organic and inorganic components. Typically, dry mammalian bone has a weight ratio of inorganic to organic components of 75 to 25. For fresh bone, the weight percentage of inorganic, or mineral, components is 65%; 88 to 90% of the organic matter is collagen, the remainder being non-collagenous proteins such as glycoproteins, sialoproteins and various others. The organic components give bone its toughness and elasticity. The inorganic components are responsible for the characteristic hardness of bone. They are hydroxyapatite crystals and a non-crystalline composite phase of calcium salts, mainly magnesium and sodium. In the following section, the single components are explained in more detail.

Organic components - bone collagen /3/:

Bone collagen consists of up to three different sequences of amino acids, the so-called α-chains (α1 and α2). Each one has a slightly different composition, but their molecular weight is around 95,000. Three α-chains make up the tropocollagen molecule, in most cases two α1- and one α2-chain. Each of these chains is twisted in a left-hand helix and the three chains are wound around a common axis in the form of a right-hand superhelix. The molecular weight of this molecule is about 300,000. It is a long rod with a length of 2,800Å and a diameter of 14Å. The helix is largely held together by hydrogen bonds, but some covalent crosslinks exist between the α-chains forming ß and γ dimers and trimers.

The tropocollagen molecules are arranged into fibrils in a manner that is not entirely clear. It results, however, in a very specific and characteristic appearance in the electron microscope as a banded pattern.

Organic components - non-collagenous organic matrix /3/:

Principally, this phase contains sialoproteins, anionic glycoproteins, proteoglycans, glycosaminoglycans, less acidic glycoproteins, collagenase-related proteins, and some plasma proteins. These are contained within the amorphous continous phase, the so-called bone cement. In this bone cement, the collagen fibrils and calcium hydroxyapatite crystals are embedded.

The cement lines are visible microscopically, but often they are of minimal thickness between the collagen fibrils.

Inorganic components - bone mineral: (after /3/ unless otherwise stated)

The main component of bone mineral is a very common apatite, namely calcium hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$).

Apatites have the general formula $M_{10}(RO_4)_6)X_2$. M is predominantly calcium, or more seldom magnesium, R is most commonly phosphorus, and X can be fluoride or hydroxide. The precise name of an apatite comes from this constituent X. The crystal structure of hydroxyapatite is given in figure III.8.

Besides calcium hydroxyapatite, bone mineral contains several other types of hydrated calcium phosphates. The composition of bone mineral is best seen as the equilibrium between the three components of the calcium-phosphate-water system.

Calcium hydroxyapatite exists in direct equilibrium with the tissue fluids. It may be partly substituted by other calcium phosphates and may contain small amounts of other cations, principally sodium, magnesium and potassium.

The typical composition of the mineral phase of human femoral cortical bone is given in table III.2. The numbers are given as weight percentages, after removal of organic components by glycerine per 5% KOH.

Calcium hydroxyapatite forms as crystals which are rarely stoichiometric or structurally perfect. They are typically 450 to 500Å in length and up to 75Å in width. The exact location of these apatite crystals is vague: some authors state that the long axis of the crystal is parallel to the collagen fibres and that the crystals are arranged at 640Å intervals around (and not in) the fibrils. Other authors state that the crystals nucleate within holes in the tropocollagen.

The interface between the matrix and the fibres is crucial for the properties of a composite material.

Composite materials have interesting possibilities as their properties can be tailored by combining two or more materials with different values for a special property. The value of this property for the composite material lies between the values for the single materials and can be controlled by several parameters - structure, orientation of the fibres or particles, and concentration of the phases.

Bone can be described as composite material on whichever level and magnification one considers it: at macroscopical level, the cross-section of a long bone consists of fibres - the Haversian systems -

embedded in a matrix - the interstitial lamellar bone. The whole structure is surrounded by an inner and outer layer also consisting of lamellar bone.
On microscopical level, each Haversian system itself is made up as a composite: collagen bundles in a certain orientation are embedded in a matrix of ground substance. Additionally, hydroxyapatite crystals are embedded in this matrix, along or within the collagen fibres. The collagen bundles themselves, again, are composites, as can be easily understood from the description of their structure above.
The properties of bone are directly influenced by this composite structure; due to this structure, bone is ideally adapted to its function. Only by the combination of materials with different properties and by arranging the components, which are made of this composite material, according to the applied stresses it is possible to achieve maximum effectivity - maximum strength in the required directions with minimum consumption of material.

cation	CaO		P_2O_5	Na_2O	CO_2	H_2O	MgO	K_2O	F	Cl
wt.%	51.31	36.65	1.04	5.86	3.78	0.77	0.32	0.23	0.01	

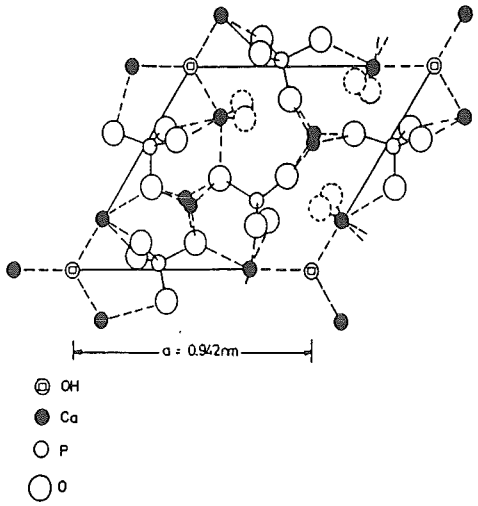


Fig.III.8: Typical composition of the mineral phase of bone (human femoral cortical bone; compare table above) and crystal structure of hydroxyapatite /23/ (below)

III.4. HISTOLOGICAL PREPARATION SCHEME

III.4.1 Introduction

The standard histological preparation scheme consists of the following five steps:

- fixation,
- dehydration,
- infiltration / embedding,
- sectioning,

and

- staining.

The aim of the whole procedure is basically to replace the water in the biological specimen by a non-volatile molecule and to make the specimen therefore more stable. Some soft tissues would otherwise not be handable at all.
In the following, the five steps of histological specimen preparation are explained in detail.

With time however, and with the development of new examination methods (scanning electron microscopy, for example) new methods for the preparation of biological specimens have been developed. These are adapted to the new examination methods and have been found to avoid the withdrawals of the classical methods in some cases.
If a material is handable and stable enough embedding is not urgently necessary. This is true especially for the examination of hard tissue with the scanning electron microscope. Water, though, has to be removed from the specimens, or it has to be made immobile by examining the specimen in the frozen state. The latter makes also possible the examination of soft tissue without embedding. As this method has not been used in the work described here, it is referred to the literature for further information. Water can be removed from the specimens by air-drying, freeze drying and critical point drying. These three methods are also explained in the chapters following the classical methods.

III.4.2 Fixation

III.4.2.1 Purpose of fixation

Definition of fixation is difficult. Most authors therefore give a negative "definition" by description what fixation should prevent. Baker /34/ said in 1960 tissue fixation should prevent autolysis, attack from bacteria and changes in the volume and shape of the tissue. Hayat /35/ and Hopwood /36/ agree so far that the aims of fixation are the rapid preservation of the structure with minimum alterations from the living state and the protection of the tissue during embedding, sectioning, and subsequent treatments. Hopwood however, states additionally that no loss of tissue constituents should occur.
Fixation has the aim to stabilize the cellular organization to such an extent that ultrastructural relations are preserved despite the subsequent rather drastic treatments of dehydration, embedding, and, in the case of electron microscopy, exposure to the electron beam /37/.

Most authors, therefore, agree that, in essence, fixation should enable examination of tissues with the least artefactual change as possible, as Anderson stated /38/. One has to bear in mind, however, that fixation itself already is a major artefact. It causes coagulation of tissue constituents, so that the cell, which is fluid or semi-fluid in its living state, is hardened and made more or less solid /39/.

The theoretical, more or less ideal, aim of fixation is the preservation of cells and tissue constituents in a condition that is identical to that existing during life. This should be done in a way that allows the preparation of thin, stained sections /39/ as most histological examinations are done by transmission light or electron microscopy.

This aim however, can never be completely fulfilled, from the simple reason that histology deals with

material that is removed from a living body. The available methods are therefore compromises between the limitations of the technique and the desire to preserve and demonstrate every tissue component in a completely life-like manner /39/.
The realistically and in practice achievable purpose of fixation comprises the following aspects:

- To prevent or arrest autolysis and bacterial decomposition and putrefaction:
  Autolysis - self-destruction - occurs after the death of cells. It is caused by the action of intracellular enzymes whose normal behaviour is altered. It leads to the breakdown of protein and the eventual liquefaction of cells. The autolytic changes are independent of any bacterial action. They are retarded at low temperatures, greatly accelerated at 37°C, and almost inhibited by raising the temperature to 57°C /39/.
  Highly specialized cells of complex organs are more rapidly and seriously affected by the autolytic changes than, for example, elastic fibres and collagen.
  Under the microscope, autolytic tissue shows characteristic changes: the cell nuclei may be condensated, fragmented or undergo lysis, until they eventually disappear. The cytoplasm may become swollen and granular. Subsequently, the whole tissue converts to a granular, homogenous mass with loss or great alteration of the usual staining reaction.
  Bacterial decomposition causes changes in the tissue that are very similar to those caused by autolysis. It is brought about by the presence of multiplying bacteria in the diseased tissues at the time of death or by bacteria that are normally present in the body in life.

- The loss of easily diffusable substances shall be prevented.

- The tissue shall be fortified against the deleterious effects of the various stages in the preparation of sections (tissue processing i.e. dehydration, cleaning, embedding).

- The tissue shall be left in a condition that facilitates differential staining with dyes or other reagents.

All the aspects mentioned above can be realized with appropriate fixation /39/:

- to inhibit or stop autolysis and bacterial decomposition;
- coagulation of tissue proteins and constituents;
- hardening of the tissue;
- to fortify the tissue against the damaging effects of dehydration and embedding procedures;
- improve in optical differentiation of cells and tissue constituents;
- marked influence on the staining properties of the tissue.

Coagulation of tissue proteins and constituents prevents their loss or diffusion during tissue processing. Together with the hardening of the tissue, it helps it to withstand the disruptive qualities of hypertonic and hypotonic solutions during the following steps of histological preparation. Fixation changes furthermore the refractive indices of cells and tissue constituents in varying degrees, so that features with the same or a very similar refractive index can be differenciated in the microscope. Generally, fixation facilitates the action of dyes; it may however, in some cases as well hinder the action of a certain dye.

III.4.2.2 Rules for optimal fixation of bone

To receive an optimal result from fixation, that means to be able to examine a tissue with the least artefactual change as possible, the following rules should be followed (outlined for bone) /38/:

- Heavy metals should be avoided. They cause pigmentary deposits.
- The fixative has to be adequate for the purpose as different fixatives fix different features differently well.
- The number of fixative solutions should be kept as low as possible.
- For bone especially, the penetrating capacity of fixatives is not higher than 0.5cm/24h. To reach penetration of all parts of a tissue block in a reasonable time, one of the dimensions of the block of bone should be smaller than 0.5cm.

- The volume of fixative should be at least twenty times the volume of the bone block.
- Fixation time should be 24 hours at 20°C or three days at 4°C.
- Evaporation of the fixative should be avoided.
- After fixation, the specimens should be stored in 70% ethyl alcohol in distilled water.

III.4.2.3 Composition of fixatives

A fixative is the fixing agent plus a suitable vehicle, usually a buffer containing various salts /37/.
Living cells contain a variety of solutes from small ion species to large molecules. They are surrounded by a semipermeable membrane. The cell as a whole is, therefore, subject to osmotic stress when the concentration of solutes in the external medium is changed. This stress leads to volume changes of the cells, due to the movement of water across the cell membrane to reach equilibrium.
The ideal fixative would, therefore, match the natural environment of the living tissue exactly with respect to pH, osmolarity and ionic constitution. Swelling, shrinkage and/or disruption of delicate tissue components and dissolution of certain components could so be prevented. The composition of the fixative is different from the natural extracellular tissue fluids. This mismatch leads to the extraction of cellular materials and/or the deposition of fixative components. This happens in the period before the tissue is adequately fixed, particularly when the fixing agent penetrates or reacts slowly /37/. The ionic constitution of the fixative has therefore to be adjusted by a suitable vehicle.
However, if a piece of tissue is immersed in a fixative, the strong buffering capacity of the tissue proteins tends to keep the pH within a narrow range /37/.

The choice of a suitable vehicle is governed by the required properties:

1) It has to be able to contain a constant pH during fixation.
2) It must have a suitable osmolarity when mixed with the fixing agent. This prevents swelling or shrinkage of the tissue during fixation.
3) The ionic constitution must be suitable. Otherwise, extraction or precipitation of materials can occur during fixation.

For perfusion fixation, the vehicle should additionally have the following properties:

4) lack of toxicity,
5) colloid osmotic pressure.

For electron microscopy, the fixatives are normally adjusted to a pH near neutrality. Commonly, in most cases pH values from 6.5 to 8.0 are acceptable /37/. For the examination of undecalcified bone, all acid solutions have to be avoided /39/. The fixative wil therefore be adjusted to a pH value of 7.0 to 7.2 in most cases.

The osmolarity of the fixative is mainly important for the examination of cells.
The correct osmolarity for a fixative however, cannot be calculated due to insufficient knowledge about the internal osmotic pressure of living cells. Furthermore, the permeability of membranes is altered during fixation.

The osmolarity of a fixative can be adjusted in three ways /37/:

- alteration of the concentration of the buffer (and not the concentration of the fixing agent),
- addition of sodium chloride,
- addition of non-ionic components such as for example sucrose or glucose.

III.4.2.4 Buffer solutions
(according to Glauert /37/ unless otherwise stated)

Phosphate buffers and cacodylate buffers are the most commonly used buffer solutions for the fixation of bone.
Phosphate buffers are the most physiological of all buffer solutions. They are, therefore, excellent for

slowly penetrating and reacting fixatives like osmium tetroxide. Disadvantages are that precipitates occur and that they get slowly contaminated with micro-organisms.
Due to the first reason, they are not recommended for electron microscopic examinations. Large atypical crystals that are thought to be artefacts are seen in TEM-sections after fixation in phosphate-buffered formaldehyde (and glutaraldehyde). The occurence of these crystals has not been reported if cacodylate buffers have been used. Calcified tissue should, therefore, only be preserved in phosphate-buffered fixatives if decalcification is intended. The crystallite artefacts are thought to be due to an increase in the concentration of phosphate ions in the fixative mixture reacting with calcium where the latters concentration is high /40/.
One of the most commonly used phosphate buffers is Sörensen's buffer. By use of various ratios of sodium-dihydroxy-phosphate (NaH_2PO_4) to disodium-hydroxy-phosphate (Na_2HPO_4) the pH of the buffer can be adjusted to a value suitable for the tissue examined and the desired purpose.

Cacodylate buffers were introduced by Sabatini et al. /41/ in 1963. They are based on sodium cacodylate ($Na(CH_3)_2AsO_2$). They are easy to prepare, stable and, unlike phosphate buffers, they do not support the growth of micro-organisms.
Because of their content of arsenic they are toxic and have to be handled with great care and safety precautions. A further disadvantage is that they may act as fixative.

III.4.2.5. Fixing agents

The most common fixing agents are osmium tetroxide, various aldehydes and ethanol. Their characteristics are shortly explained in the following.

1. Osmium tetroxide
Osmium tetroxide is an excellent fixative for the examination of cellular fine structure under the electron microscope. It preserves delicate cytoplasmic processes and does not cause any alterations in mitochondria and fat droplets /37/.
It penetrates and reacts very slowly however, so that the specimens should be very small to ensure good and equal fixation over the whole cross-section of the specimen.
Osmium tetroxide destroys the osmotic properties of the cells; the osmolarity of the fixative is therefore irrelevant /42/.
The ionic constitution of the buffer however, is important, if osmium tetroxide is used as a primary fixative, due to its slow penetration rate, and has to be chosen very carefully (/37/, /43/).
Straight osmium fixation of calcified tissue does not give satisfactory results for transmission electron microscopy, unlike the observations made with soft tissues. Immersion fixation in 1% osmium tetroxide in 0.1M cacodylate buffer (pH 7.2 - 7.4) frequently results in the washing out of matrix components in bone and cartilage, especially of the pericellular matrix around osteocytes and chondrocytes. Therefore, osmium is predominantly used as a post-fixative. A popular primary fixative, that contains osmium tetroxide, is Millonig's constant osmolarity phosphate buffered osmium tetroxide /40/. The use of phosphate buffers, however, is not recommended for the fixation of calcified tissues (compare 4.2.4).
For scanning electron microscopy purposes though, osmium tetroxide plays an important role as primary fixative, despite its poor penetrating properties /44/.
In this application, the main advantage is that osmium tetroxide-fixed cells are not fixed to each other as it happens with glutaraldehyde due to its cross-linking ability. The cells may therefore be removed by dissection, even in dry condition. This is an important aid in determining cell-cell and cell-matrix relationships /44/.

As mentioned above, osmium tetroxide plays an important role as second fixative after primary aldehyde fixation. It reacts with components that are not fixed by aldehydes and it acts as stain /37/.

Osmium tetroxide vapours are harmful; solutions have to be handled with great care in a closed fume-cupboard.

2. Aldehydes
Aldehydes are used as fixatives for light and electron microscopic purposes.
Glauert /37/ states that aldehydes are not suitable as general fixatives for electron microscopy when

they are used alone. Sabatini et al. /45/ found that fixation with an aldehyde, especially glutaraldehyde, followed by secondary fixation with osmium tetroxide, gave results comparable to those with osmium tetroxide alone.
Even though, double fixation with glutaraldehyde and osmium tetroxide has many advantages over fixation with osmium tetroxide alone /37/. The size of the tissue block is less critical; dimensions of several millimetres are acceptable. Furthermore, the specimens can be stored in buffer in the cold for periods up to several months before post-fixation, without any obvious alterations in their fine structure.

Glutaraldehyde reacts very rapidly with proteins. It stabilizes structures by cross-linking before there is any opportunity for extraction by the buffer to occur /37/.
As the cells remain osmotically active during glutaraldehyde fixation, the osmolarity of these fixatives must be chosen carefully (/37/, /46/). Tissues are very sensitive to variations in the buffer osmolarity /47/. One has to bear in mind that the osmolarity of the fixative vehicle and not of the fixing agent has to be adjusted. This can be done by either alteration of the buffer osmolarity or by addition of sucrose, glucose or sodium chloride /37/. The buffer concentration should be modified to approximately iso-osmotic conditions. The tissue should be neither exposed to hypo-osmotic solutions, resulting in specimen swelling, nor to hyper-osmotic solutions, resulting in specimen shrinkage. Hyper-osmotic solutions however, are the less damaging. Most fixatives are therefore hyper-osmotic (rather than hypo-osmotic) /47/.
Changes in glutaraldehyde concentration are less critical than variations in buffer osmolarity /47/.
Variations of pH in the range between 6.8 and 7.5 seem to have no effect on fixation of fine structure. A pH under 7.5 should be chosen to prevent polymerization and the loss of reactive groups /37/. In the case of the examination of undecalcified bone, the pH has to be neutral or higher, otherwise decalcification occurs.
Generally, phosphate buffers and the addition of a small amount of calcium or magnesium are recommended /37/. Fixatives should always be prepared freshly.
Glutaraldehyde is the most commonly used and the best of the usual fixatives for electron microscopy /44/. It penetrates more slowly than formaldehyde and its use is therefore not advised for large tissue blocks fixed by immersion. Perfusion fixation should be used where possible.
The use of 0.15M sodium cacodylate-buffered 2 - 3% glutaraldehyde is advisable as fixative and storage medium for calcified tissue. For scanning electron microscopic purposes, long-term storage of specimens in glutaraldehyde is well possible. The cross-linking process combined with a partial polymerization may even lead to the retention of cells in fractured matrix preparations which are otherwise lost. Long-term storage can lead to an orange-brown colour of the samples. This does not however, seem to affect the scanning electron microscopic examination /44/.

Another aldehyde commonly used in fixative solutions is formaldehyde or paraformaldehyde.
Paraformaldehyde and formaldehyde are recommended for light microscopy, transmission electron microscopy and combined light and electron microscopy. Though glutaraldehyde is superior for cellular preservation, paraformaldehyde is a good compromise if the solutions are freshly prepared. After prolonged storage however, the preservation of cells is no longer sufficient neither for light microscopy nor for electron microscopy. Paraformaldehyde has no advantages over formaldehyde /48/.
For TEM-applications, formaldehyde freshly prepared from paraformaldehyde powder compares favourably with glutaraldehyde. The fresh preparation of formaldehyde avoids the use of commercial formaldehyde solutions which often contain the harmful contaminant methanol (/37/, /40/). The paraformaldehyde powder should be not too old as the eventually occuring polymerization affects its solubility.
For light microscopy however, Burkhardt /49/ recommended formaldehyde in a mixture with methanol.
Formaldehyde, like glutaraldehyde, reacts with proteins. As it is a monoaldehyde (i.e. mono-functional) in contrast to the difunctional glutaraldehyde, less cross-linking occurs with formaldehyde than with glutaraldehyde. Formaldehyde reacts slowly and the reaction is reversible. It reacts with lipids /37/.
The main advantage of formaldehyde is its high rate of penetration, as compared with glutaraldehyde or osmium tetroxide. Relatively large blocks of tissue are therefore well fixed /37/.
The permeability properties of the membranes are partly preserved during formaldehyde fixation. The osmolarity of the fixative has therefore to be adjusted carefully /37/.

Formaldehyde fixatives can be prepared with phosphate or cacodylate buffers. The addition of a small amount of calcium chloride is recommended /37/.
Phosphate buffers however, are not recommended for electron microscopy for the reasons outlined in chapter 4.2.4.

After fixation, the specimens should be washed. For prolonged storage, they should be transferred to 70% alcohol to prevent autolytic processes from resuming /48/. A slight disadvantage of formaldehyde fixative is that it changes the colour of fuchsin used as block stain or for unembedded ground sections towards violet. The stain appears therefore less brilliant.
Neutral formaldehyde causes shrinkage of specimens and may therefore modify the histological aspects of the interface between soft and hard tissue /50/.
Furthermore, formaldehyde undergoes progressive acidification even when buffered (/48/, /50/, /51/). This is due to contamination with formic acid. Demineralization, formation of acidic minerals, and fading of tetracycline and other fluorescent dyes may occur. Formol should, therefore, be neutralized (with $CaCO_3$) and buffered /48/.
Formaldehyde as fixative for specimens intended for scanning electron microscopy is possible, but not recommended /44/ due to the following disadvantages:

- It leads to the vesiculation of cell surfaces during fixation and is, therefore, possibly the worst of the common fixatives for scanning electron microscopy examinations in which the cell surface is an important feature.
- The value of anorganic specimens is spoiled due to a partial dissolution of the mineral component by the decomposition of formaldehyde to formic acid.
- The use of formaldehyde prevents the enzymatic removal of cell and/or matrix components.

Wide use as primary fixatives have solutions that contain formaldehyde as well as glutaraldehyde /37/. The addition of calcium chloride is advisable, and, for electron microscopy, secondary fixation with osmium tetroxide is necessary.
The rational behind this combined use of formaldehyde and glutaraldehyde is the following: formaldehyde penetrates tissues much more rapidly than glutaraldehyde. It was supposed, therefore, that formaldehyde would temporarily stabilize the structures, before glutaraldehyde subsequently gives a more permanent fixation /37/.

3. Ethanol
Alcohol in concentrations of 40 to 50% was originally recommended by Frost /52/ in 1958 for the preparation of unembedded, fuchsin-stained, ground sections.
It is neutral and removes lipids to a certain extent. It penetrates however only slowly. This drawback can be overcome by cooling to 4-7°C. In this temperature range, autolytic enzymes are almost completely inactivated. A rather homogenous fixation throughout the specimen is achieved /48/.
The main advantage of alcohol as fixative is the good preservation of fluorochromes and the good stainability if fuchsin is added to the dehydrating alcohol steps /48/. Recker /53/ sees as further advantage that dehydration and fixation are carried out at the same time. He states further that the overnight washing in water, which is necessary with formalin and leads to the swelling of tissues, is avoided. Alcohol as fixative causes less shrinkage during dehydration and less retraction at the bone-bone marrow interface than formalin /53/.
Alcohol preserves the mineral matrix very well, but soft tissues and cells in particular are very badly fixed. Disintegration of cell membranes occurs, leading, for example, to the lysis of erythrocytes (/48/, /50/). Nevertheless, alcohol in concentrations of 40% to 100% is a standard fixative for light microscopy in many laboratories /48/. Unless good cell morphology is required for examinations with the scanning electron microscope, specimens which are intended to be stored at ambient temperatures, should be kept in 70% ethanol /44/. This strength ethanol does not cause any shrinkage or expansion of cells which can at least be recognized if necessary.
Ethanol has the advantage that it can be washed out to permit the utilization of proteases and other enzymes which may be employed to digest cells and/or matrix components. Furthermore, it is a step in the right direction towards defatting the bone sample which is desirable in most cases /48/.

III.4.2.6 Recommended fixative solutions for bone

Many different fixatives have been recommended for different purposes and different tissues. A summary of the common fixatives for the examination of bone, classified depending on their usefulness for light microscopy, scanning and transmission electron microscopy, is given in the following.

1) Fixatives for light microscopy:

- 70% ethanol in distilled water /38/
- 40% ethanol /53/
- alcohol in concentrations of 40% to 100% (/39/, /48/, /54/)
- neutral buffered formaldehyde solution (/38/, /39/, /54/)
- 10% formalin and alcohol 1:1 /55/
- Zenker's fixing solution /38/
- Carnoy's solution /48/

2) Fixatives for scanning electron microscopy:

- buffered 4% formaldehyde /38/
- 0.15M sodium cacodylate buffered 2-3% glutaraldehyde /44/
- Karnovsky's solution /38/
- 70% ethanol /44/
- osmium tetroxide /44/
- osmium tetroxide/boric acid/borate /44/
- thiocarbohydrazide and osmium tetroxide /44/
- cetylpyridinium chloride /44/

3) Fixatives for transmission electron microscopy:

- Stefani, DeMartino & Zamboni /38/
- Karnovsky's solution /38/
- 3% glutaraldehyde solution /38/
- osmium tetroxide (Caulfield's fixing solution) /38/
- osmium tetroxide /40/
- "suitable" aldehyde,
  buffer wash in 0.1M cacodylate buffer,
  post-fixation in 1% osmium tetroxide in 0.1M cacodylate buffer /40/

4) Fixatives for combined light and electron microscopy:

- glutaraldehyde /48/
- paraformaldehyde /48/

III.4.3 Dehydration

III.4.3.1 Aims and reasons of dehydration

Dehydration of biological specimens is the second step in most histological preparation schemes. The water in the specimen is substituted by a solvent that is miscible with the embedding medium if the specimens are to be embedded or that has more convenient values of the temperature and the pressure at the critical point for critical point drying.
Most embedding media are not soluble in water. The specimens are therefore dehydrated by passing them through a sequence of solutions the last of which is miscible with the embedding medium /37/.

III.4.3.2 Common dehydration agents

The most commonly used dehydration agents for all sorts of specimens and all applications are acetone and ethanol (/37/, /40/). They achieve very similar results /33/. Acetone however, readily takes up water so that incomplete dehydration is possible to occur. Mostly, therefore, ethanol is preferred /37/.
Dehydration of undecalcified bone is not different from "normal" histology /48/. Only the hardening of the tissue by clearing solutions such as xylene and chloroform can be ignored for plastic embedding of this special tissue.

III.4.3.3 Chemical and morphological effects of dehydration /37/

General morphological studies have shown that the effects produced during dehydration are mild against the changes occuring during fixation.
The common dehydrating agents are excellent lipid solvents. As major chemical effect of dehydration lipids are extracted, therefore, from the specimen by the dehydrating agent. A loss of lipids up to 95% can occur, the majority of which takes place at concentrations of ethanol or acetone higher than 70%.
Further, the extraction of proteins is possible. The losses are, though, only up to 4%. The majority of protein losses occur however in lower concentrations of ethanol in water.
These chemical changes are accompanied by shrinkage of tissues, cells and subcellular components.

III.4.3.4 Dehydration schedules

For dehydration, the fixed specimen is passed through a graded series of solutions of increasing concentrations of the dehydrating agent in water, ending with pure or absolute dehydrating agent. The volume of the solution has to be at least ten times the volume of the specimen /37/.
The dehydration can be done in the cold or at room temperature. If it is started in the cold, the temperature is risen to room temperature when the specimens are in a 90% or 95% solution of the dehydrating agent in water /37/. For transmission electron microscopy, Dickson /40/ recommends acetone dehydration at room temperature and alcohol dehydration at 4°C until absolute alcohol is reached /40/.

For bone, as for every tissue, thorough infiltration and substitution of the media is essential as the quality of the final blocks is greatly improved by the removal of fat and lipids from the specimen. Cutting problems are so reduced /48/. Defective dehydration results in poor resin penetration of tissue blocks. Often holes appear on sectioning in these cases /40/. In the case of critical point drying, several observations indicate that an obvious retention of water or ethanol in a sample intended for critical point drying causes shrinkage after drying /44/.
Enough time should be allowed, therefore, for each dehydration step, and the solutions should be replaced regularly /48/. Usually, several changes of 100% ethanol or acetone are employed to minimize the contamination with water /44/. Further, it is important that moisture absorption in absolute ethanol or acetone is prevented. This can be achieved by storing the pure solvents over anhydrous copper sulphate in a glass stoppered bottle. The solvent bottle has then to be kept topped up and a pipette is used to remove the liquids /40/.

Some dehydration schedules begin at concentrations of ethanol or acetone of 30%. At concentrations lower than 60% however, both, ethanol and acetone, can cause disruptive swelling of cells in the absence of divalent cations. Although this is very unlikely in intact calcified tissue and has never been demonstrated, it is recommended to begin the dehydration schedule in 70% ethanol or acetone in water, just to be on the safe side. No significant volume changes will occur then /44/.

Approved dehydration schemes for bones of various sizes are for example:

ethanol 70%	duration of treatment	by standard iliac crest biopsy	2 hours
	duration of treatment	by proximal end of rat tibia	2 days
	duration of treatment	by transverse slice of human femur	3 days

ethanol 95%	duration of treatment	by standard iliac crest biopsy	2 hours
	duration of treatment	by proximal end of rat tibia	2 days
	duration of treatment	by transverse slice of human femur	3 days
ethanol 100%	duration of treatment	by standard iliac crest biopsy	2 hours (2x)
	duration of treatment	by proximal end of rat tibia	1 day (2x)
	duration of treatment	by transverse slice of human femur	2 days (2x)
ethanol 100%	duration of treatment	by standard iliac crest biopsy	12 hours
	duration of treatment	by proximal end of rat tibia	1 day
	duration of treatment	by transverse slice of human femur	4 days

In urgent cases, the dehydration times can be decreased by the following means:

- agitating the specimens throughout dehydration,
- carrying out the dehydration at room temperature and not in the cold,
- using more changes of each solution,
- using smaller blocks of tissue /37/.

As mentioned above, most losses of lipids occur at concentrations of ethanol higher than 70% in water. Furthermore, most shrinkage of specimens or specimen components occurs at the 80% concentration step /44/. The partial dehydration by Idelman /37/ avoids therefore the use of these higher concentrations of ethanol. The dehydration schedule is however, dependent on the fact that the used epoxy resin (Epon 812) is soluble in 70% ethanol in water.

Continual dehydration series and small concentration jumps did not prevent or slow down shrinkage. There is, therefore, no logic in a 95% step /44/. This is understandable if one considers that the chemical changes that occur during dehydration result in shrinkage also of tissue, as outlined in 4.3.3.

III.4.4 Embedding

Embedding of a biological specimen consists of the permeation of the fixed tissue block with a substance which will, when the tissue is properly impregnated, present as uniformly a consistency as possible to the cutting edge of the knife /38/.

III.4.4.1 Goals and applications of embedding

Embedding is essential to facilitate the sectioning of specimens thin enough for transmission light and electron microscopy (/38/, /39/). The embedding medium has to provide support for the specimen and it has to have the suitable consistency for microtomy.

The goals of embedding undecalcified bone are, therefore, the following (/37/, /48/, /53/):

- The embedding medium must be miscible with water or with the dehydrating agent.

- The bone specimen must be thoroughly penetrated by the embedding medium and not only be surrounded. For good results, the infiltration procedure has therfore to be as slow as possible and the embedding medium should have a low viscosity before hardening (i.e. as a monomer for synthetic resins).

- During sectioning, the tissue should be little deformed and remain free of cracks and fissures. Ideally, the embedding medium has therefore to be as hard as calcified bone itself. This avoids vibration and fracture during the sectioning process. The blocks should further harden homogenously.
  The embedding material has, on the other hand, to be supple enough to facilitate the

sectioning and the handling of the sections. The sections should be easy to stretch and attach firmly to gelatine-coated slides. This is the reason for adding a softener to, for example, methyl methacrylate.

- The embedding medium should polymerize/harden uniformly and show little volume change on polymerization in case of synthetic resins.
- The consistency of the blocks should not change, even after prolonged storage.
- The blocks have to be easy to stain, or the embedding medium must be easy to dissolve after sectioning so that any desired staining technique can be applied.
- During polymerization, the temperature should not rise over 45°C to 50°C to avoid damaging the tissue.
- The infiltration and polymerization/hardening time should be short.
- Finally, the embedding medium should be not too expensive, easily available, and uniform from one batch to another.
- For electron microscopy, the embedding medium has further to be stable under the electron beam.

Mostly, there has to be a compromise between these requirements as none of the presently available embedding media fulfills all these expectations. For transmission electron microscopy, it is especially important to find a satisfactory compromise between the resin viscosity and the background granularity of the polymerized block /40/.

For scanning electron microscopy of calcified tissue, embedding is an unusual requirement /44/. Embedded specimens are therefore only used for scanning electron microscopy if it is imperative for a correlative method to be employed or if the primary purpose is the production of an ultraflat surface upon which stereological investigations can be conducted using BSE-imaging (BSE = backscattered electrons). Topography-free specimens of resin embedded material give only density-dependent information using the BSE-mode in the scanning electron microscope (compare 5.4).

III.4.4.2 Embedding media
(after Carleton /39/ if not otherwise stated)

Many embedding media have been used in histology. The most common ones are listed below with a short description of their characteristics.

1. Paraffin wax
Tissue has to be dehydrated prior to infiltration with molten paraffin wax. After infiltration is completed the wax is allowed to harden by cooling.

2. Ester wax
Ester wax has a lower melting point than paraffin wax, but it is harder when solidified. It is advantageous for the cutting of thin sections with minimal shrinkage of the tissue.

3. Water soluble waxes (polyethylene glycol waxes)
These embedding media allow the direct transfer of tissue from aequeous fixative solutions to the wax for infiltration. Dehydration and clearing are avoided and shrinkage of the tissue is therefore reduced. The cutting and manipulation of the sections however, is more difficult than with paraffin wax. Furthermore, the blocks must be kept in dry atmosphere.

4. Cellulose nitrate (celloidine and low viscosity nitrocellulose)
Again, specimens have to be dehydrated before they are infiltrated with a solution of cellulose nitrate

in a suitable solvent (usually alcohol or ether). After infiltration, the solvent is allowed to evaporate. This method has the advantage that no heat is used.
It is an especially suitable method for large pieces of bone and brain.

5. Double-embedding
Double-embedding combines the paraffin and cellulose nitrate method to use the advantages of both.

6. Synthetic resins
These are especially used for the embedding of specimens of undecalcified bone, for the preparation of sections for the electron microscope, and for 1µm to 2µm sections for combined light and electron microscopic examinations.
There are three main types of synthetic resins in general use /37/:

- epoxy resins,
- polyester resins,

and

- methacrylates.

The epoxy resins are the most widely used resins. They have most of the required properties listed above (compare 4.4.1). They polymerize uniformly with very little volume change and are relatively stable under the electron beam. Their main disadvantage is their high viscosity which leads to a longer infiltration time as compared with the methacrylates.
Chemically, they are polyaryl ethers of glycerol which bear terminal epoxy groups. The small shrinkage is due to the fact that the polymerization is an addition process and that the resin is highly associated in the uncured state. The uniformity of polymerization is caused by the hardening being an addition reaction and therefore not dependent on an initiator. The reaction is therefore not affected by the presence of impurities /37/.

Polyester resins have similar excellent properties as the epoxy resins. Some of their components are however not stable during storage /37/.

The methacrylates are excellent embedding media for light microscopy; for transmission electron microscopy however, they have been largley abandoned due to uneven distortion artefacts that can be caused during polymerization /40/. They polymerize unevenly and with considerable shrinkage and they are unstable under the electron beam. They are, therefore, no longer used as routine embedding media /37/. Improved formulations are however anticipated /40/.
Pure methacrylates are hard and brittle. For most applications in light microscopy, methyl methacrylate is used. Other methacrylates are used as well, though not as often, for example butyl methacrylate, glycol methacrylate and ethyl methacrylate. Softeners are added to adjust the consistency of the blocks /48/.
In common use are also mixtures of n-butyl and methyl methacrylates with an added catalyst. The hardness of the final block is adjusted by varying the ratio of n-butyl to methyl methacrylate, the hardnees increasing with the proportion of methyl methacrylate. Impurities in the embedding medium or even the specimen itself can initiate polymerization. Polymerization may start, therefore, in one region before others. The addition of a small amount of initiator effects a more uniform polymerization. The large shrinkage may damage the specimen /37/. The use of partially polymerized methacrylate is, therefore, recommended so that a part of the shrinkage will have occurred before the specimen is placed in the embedding medium (/37/, /50/).
One common failure in the preparation of methacrylates is bubble formation. It is due to overheating of the resin at the onset of polymerization and sometimes difficult to control. As liquid methacrylates are poor heat conductors, the overheating is mostly confined to the centre of the sample. The boiling starts generally at the tissue-medium interface or in the tissue itself. One should, therefore, generally wait at room temperature until polymerization starts spontaneously /48/.
Whether the inhibitor should be removed or not depends on the brand and even on the batch. It should be tested, therefore, whether polymerization starts easily or difficult /48/.
Vibration of the overheated mass at the onset of polymerization should be carefully avoided. It can trigger rapid bubble formation and even produce an "explosion" so that the embedding medium is changed into a solid foam within a couple of minutes. Extreme care should be taken also at opening the oven or displacing specimens in the unpolymerized state /48/.

7. Gelatine and other aequeous media:
These embedding media provide support, for example, for tissues to be cut on the freezing microtome.

III.4.4.3 Chemical and morphological effects of synthetic resins
(after Glauert /37/ if not otherwise stated)

The changes occuring in the tissue during embedding are slight compared to those occuring during fixation and dehydration.
Only little dimensional change is observed during infiltration of the embedding medium into most specimens. Little shrinkage occurs also during the polymerization of epoxy and polyester resins. The dimensional changes from embedding in methacrylates, however, are large.
Epoxy resin monomers extract small amounts of lipid, the exact amount depending on the preceding fixation and dehydration schedule.
The methacrylates are powerful lipid solvents, even at low temperatures.

III.4.4.4 Standard embedding methods for synthetic resins

Usually, complete embedding mixtures are applied for infiltration, containing the accelerator or activator. The mixtures are freshly prepared during the later stages of dehydration /37/. Infiltration is effected by combining a linking agent with the resin mixture /40/.
The specimens are infiltrated by passing them through a sequence of solutions of increasing concentrations of resin mixture in the dehydrating agent until this dehydrating agent has been completely replaced by the final embedding mixture /37/.
The infiltration with viscous embedding media is accelerated by placing the vials that contain the specimens on a slow rotary shaker to keep the specimens in a constant motion (/37/, /40/) or by using a continuous stream of gas bubbles to keep the embedding medium circulating around the specimens /37/.
The resin mixtures have to be blent thoroughly. Air bubbles can be removed by applying a vacuum (700 to 750mbar) at room temperature.

III.4.5 Preparation of thin sections

Thin sections of calcified tissue are produced either by sectioning blocks of tissue on a microtome or ultramicrotome, depending on the section thickness, or by a combination of sawing and grinding on abrasive substrates.

III.4.5.1 Microtomy of calcified tissue

Microtomy is used to achieve sections for transmission light microscopy.
Important features of microtomes are their stability and precision. They should have a motor drive and an adjustable speed control. Further, for the cutting of bone embedded in synthetic resin, they should be provided with special knives for the cutting of plastic embedded objects.
The variables that can be controlled on the microtome at the sectioning of hard objects are the cutting speed, the sectioning angle and the section thickness.

The cutting speed has to be low for hard material.

The sectioning angle is explained in figure III.9. Simplified, it is the sum of the clearance angel and the knife angle. With an increasing sectioning angle, the cutting process changes from a peeling to a planing and finally to a scraping process. To avoid cracks, fissures and fragmentation of the specimen a small sectioning angle seems preferable. Such knives however, have the disadvantage of

deformation and fast abrasion of their edge. The stability and durability of the knife is therefore increased by a higher knife angle, and most commercially available knives have a knife angle between 35° and 50°. The clearance angle does not give much freedom for adjusting the sectioning angle. It should be kept between 5° and 10° /48/.

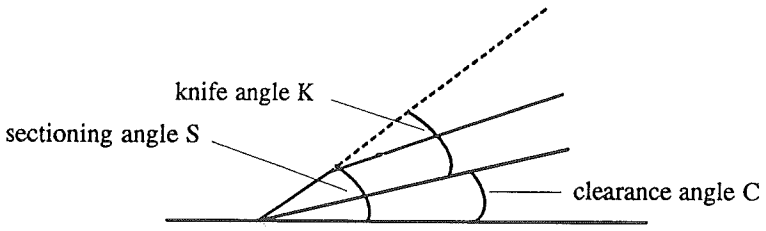


Fig. III.9: Sectioning angle, clearance angle and knife angle

With standard microtomes, section thicknesses between 2 and 10µm can be obtained of specimens embedded in hard synthetic resins /39/. The best results have been achieved with thicknesses between 4 and 7µm. Even though the sections are the less susceptible to the artefacts mentioned above, the compression of the section by metal knives for thicknesses below 3µm is too severe. For section thicknesses over 10 to 12µm the danger of introducing cracks increases /48/ and shattering and splintering of the embedding medium occurs /39/.

The sections should be cut with a slow, steady cutting stroke /39/. Often, tungsten-carbide tipped profile knives are used /53/. 70% alcohol, terpeneol or a mixture of equal parts alcohol and terpeneol can be used as lubricant /39/.
After cutting, the sections are mounted on glass slides. Many artefacts are introduced during the processing of the sections for mounting after the actual cutting process /48/.

III.4.5.2 Ultramicrotomy of calcified tissue
(after Dickson /40/)

Ultramicrotomy provides ultrathin sections of desirable areas of semithin sections for transmission electron microscopy. The areas are usually identified by light microscopy.
Ultramicrotomy deals with section thicknesses up to 1µm. Semithin sections are in the thickness range of 0.5 to 1.0µm, ultrathin sections have thicknesses below these values. Ultramicrotomy has to be performed in a dust and vibration free environment.
The cutting is performed with glass or diamond knives. Glass knives should always be freshly broken. Diamond knives are expensive, and it is important to choose a suitable diamond for the material to be cut. They have however many advantages over glass knives: the cutting edge holds its sharpness over a long period, a larger surface area of the section can be obtained, and serial sections can be easily collected from the large trough around the knife edge.
Diamond knives have to be treated with great care, as the thin cutting edge is hard and brittle. Any stress other than compressive one causes nicks in the delicate edge. Bending stresses on the knife and cutting perpendicular to the edge have therefore to be avoided.
With glass knives, sections up to a size of approximately 0.8mm x 1.0mm can be cut. A guide to the section thickness is obtained from the reflection colours produced by thin sections floating on the trough liquid.

The following problems can arise during ultramicrotomy:

- Scratches on the section vertical to the knife edge:
  These are due to a bad knife edge or to the dislodgement of mineral, possibly as a result of

poor resin impregnation.

- Holes in the section are also a result of mineral dislogdement.

- Section compression leads to wrinkles and folds that are visible as dark, wavy lines parallel to the knife edge. It is caused by improper embedding of the specimens or if the sections have dried on the knife edge.
- Chatter means closely spaced variations in the section thickness parallel to the knife edge. It is a result of high frequency vibration. The cutting speed and/or the sectioning angle have to be changed. For hard blocks, a reduction of the sectioning angle is advised to try.

Sectioning problems may reflect the underlying direction of calcifying collagenous fibres which constitute a hard tissue.
Furthermore, mechanical artefacts and suboptimum fixation conditions are encountered very close to the tissue surface, particularly after immersion fixation. Sections from deeper in the specimen are therefore often of higher quality.

After cutting, ultrathin sections are lifted onto copper grids. These grids exist with support bars of varying width.

III.4.5.3 Sawing and grinding

This method produces thin sections by a combination of sawing and grinding. First, thin sections of well-fixed bone that may or may not be embedded are sawn with a fine saw. These sections are then either manually or mechanically ground.
Manual grinding is effected on abrasive paper or rough glass plates. 70% alcohol is used as lubricant. Of embedded specimens, sections as thin as 20µm can be obtained. The final sections are mounted stained or unstained /54/.
Mechanical grinding is effected on rough rotating glass plates. The method has been developed by Stürmer /56/. An English description can also be found in Anderson /38/.

III.4.6 Staining

(after Schenk et al. /48/ if not otherwise stated)

As staining of specimens is not a main aspect of the work presented here, only the basic idea is outlined and some common stains are listed. The given references on preparation methods for bone or basic textbooks on histology and histochemistry provide deeper information on the principles of staining and the composition of the named staining mixtures.

Staining for light microscopy is used to reveal specific information about calcifying and calcified matrices and the cells involved in their formation, maintenance and breakdown. Specific aims for staining calcifying tissue is firstly the reliable discrimination of mineralized and unmineralized matrix compartments. Further, cells and cellular details shall be well visualized and, to a certain extent, tissue components shall be identified qualitatively by simple histochemical means.
It has to be stressed again, as already outlined above (compare 4.4.1), that embedding media have a great influence on the stainability and have possibly to be removed.
Some common stains for different purposes in the field of light microscopy are the following:

- basic fuchsin,
- Giemsa stain,
- methylene blue,
- toluidine blue (for ultramicrotome sections),
- Paragon stain,

and the

- von Kossa reaction.

For transmission electron microscopy, the most common substances for the staining of calcified tissues are uranyl acetate, lead citrate, phosphotungstic acid (PTA), and osmium tetroxide. These high molecular weight stains impart electron density in varying degrees to proteins, nucleic acids, phospholipids, polysaccharides, fat and glycogen /40/.
Double staining with uranyl acetate and lead citrate is routinely used for calcified tissue /40/.

III.4.7 Drying methods

III.4.7.1 Introduction

The major cause of damage on drying of hydrated specimens is the surface tension of the liquid contained in these specimens (usually water if dehydration has not been completed). Water, the most common intra- and extracellular environmental medium, has the highest surface tension of all common liquids ($\gamma_{H2O} = 73 \cdot 10^{-3}$N/m). The evaporation of water through and around a soft hydrated specimen leads to crushing and tearing forces and therefore to distortions and disruptions in the specimen /57/.
There are several possibilities of decreasing surface tension or its damaging effects /57/:

- hardening and toughening of the specimen by fixation with aldehydes, osmium tetroxide or other fixatives;
- examination of fresh or fixed specimens in the wet state before the vacuum and the electron beam cause obvious damage: this method has the additional advantage that coating of the specimen with a conductive layer becomes unnecessary due to the high conductivity of moist specimens;
- examination of the specimen in the frozen state: problems arise from the need to prevent the deposition of an obscuring coating of frost, the limitation of examination time and the difficult storage of the specimens;
- evaporative drying from a solvent of lower surface tension: surface artefacts may however be produced which are dependent on the solvent molecular polarity;
- freeze drying;
- critical point drying (CPD).

The last three drying methods are shortly explained in the following chapters, with stress on critical point drying as this method has been used in the work presented here.
The aspects of fixation have been dealt with in chapter 4.2 above; examination of wet or frozen specimens has not been performed in this work. For further information on these subjects, the reader is referred therefore to the special literature (compare the literature cited by Cohen /57/).

The purpose of all drying methods is the preservation of the shapes and sizes of hydrated specimens after they are fully dried.

III.4.7.2 Evaporative drying (air-drying)

(after Boyde /44/ if not otherwise stated)

This drying method can be used in calcified tissue research if the dense matrix and/or the dense mineral only are of interest. If cells have to be examined as well, evaporative drying is not appropriate due to the resulting shrinkage.
Evaporative drying can be performed from water or from a solvent with a surface tension lower than water. The second possibility causes less shrinkage than the first one, due to the high surface free

energy of water. Recommended solvents are ethanol, acetone, ether, xylene, toluene, chloroform, or, best of all, 1,1,2-trichlorotrifluoroethane.
It has to be stressed again that only stable and strong specimens should be air-dried. Delicate structures cannot withstand the enormous forces of surface tension on drying.
The consequent shrinkage can be greatly reduced though by arranging the drying process in an environment that is saturated with the vapour of the solvent employed.

III.4.7.3 Freeze drying
(after Reimer/Pfefferkorn /58/ if not otherwise stated)

Freeze shock and subsequent freeze drying is one possibility to avoid drying artefacts.
Firstly, the specimens are shock-frozen. It is very important to cool down the material in a time in the order of milliseconds to under -140°C to minimize damaging the structure through the formation of ice crystals. At this cooling rate, it becomes more likely that the water solidifies in fine crystalline or glassy structure. Only this vitrification ensures an optimal conservation of the cells. At very low cooling rates, ice crystals form mainly between the cells from few seeds. Thereby, water is removed from the cells so that the salt concentration in the cells is rising. The freezing point is lowered in the inner of the cells. Shifting of tissue constituents, shrinkage and cracks in the tissue are the result. With increasing cooling rate, the resulting ice crystals become smaller and form within the cells. Therefore, cell structures are damaged.

After freezing, the temperature of the specimen may be risen to a value not above -80°C. Otherwise, the ice recrystallizes with the damaging effects described above. Freeze drying means the sublimation of the ice in high vacuum. The liquid phase is hereby avoided.
At relatively low temperatures, the vapour pressure is low too, so that a pressure better than 10^{-2} Pa is necessary.
The drying process can take up to several days, depending on the kind and size of specimen. This is the main disadvantage of the freeze drying method.

Besides freeze drying from water, there are many other possibilities. They are listed in the survey given by Boyde in 1984 /44/.

III.4.7.4 Critical point drying (CPD)

Critical point drying has several advantages in comparison with the drying methods explained above:

- "bulky" specimens in the range of a few mm^3 can be prepared without loss of preservation of internal structure at a microscopical level;
- after examination in the scanning electron microscope, the specimens can be impregnated with resin and sectioned for transmission electron microscopical examination;
- fractography in a most versatile variety is possible.

a) Idea of critical point drying:

Critical point drying avoids the liquid:vapour boundary line in the phase diagram /44/ (compare fig.III.10.). For all substances, this boundary line ends at the critical point (CP) /58/. The transition from liquid to vapour occurs in a pressure vessel with the pressure exceeding the critical value at the critical temperature. Surface tension forces are therefore circumvented by preventing evaporation /44/.

b) Theory of critical point drying: /57/

For critical point drying, a sturdy container with valved orifices for filling and venting is about half filled wiht a very volatile liquid (the transitional fluid), for example liquid CO_2 under pressure. On warming the container, the liquid expands and evaporates, and if the container is about half full, evaporation balances expansion so that the surface of the liquid remains approximately in the center.

The expanding, but nearly incompressible liquid becomes less dense whereas the compressible gas or vapor phase becomes increasingly dense. The total density (neglecting the slight volume increase of the container) remains approximately constant. During this process the interface or meniscus between the two phases becomes less distinct. As the densities and other properties of the two fluid phases approach each other, the interfacial or surface tension decreases. At a specific temperature, the critical temperature T_c, the densities and all other properties of the two phases are identical. In this so-called critical point (CP) the meniscus and the surface tension vanish. The pressure and density at the critical point are called the critical pressure p_c and critical density D_c. The critical surface tension is zero; at CP, the two phases are truly continuous. At temperatures above T_c, the fluid behaves more like a gas. If there is a specimen in the liquid and the container heated to a temperature above T_c the specimen is brought from a liquid to a dry vapor environment without being in contact with a surface. No distortion effects of surface tension can take place. While the temperature rises, the valve is slightly opened to let the gas stream out. When atmospheric pressure is reached the dry specimen may be removed. This is the essence of CPD.Most CPD is performed by going around the critical point. This means that the specimen is dried by passing the fluid through T_c at a pressure higher than p_c.

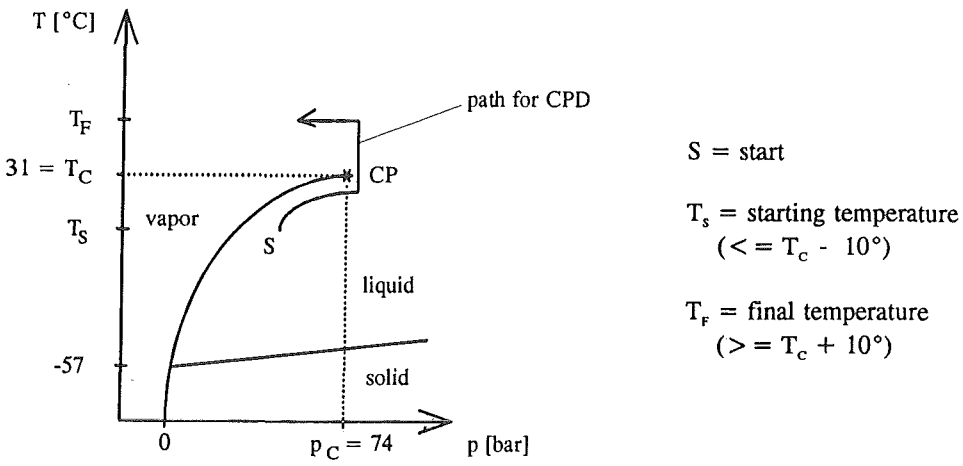


Fig. III.10. Phase diagram of CO_2 with path for CPD

c) Practical aspects of critical point drying

Critical point drying directly from water is not possible, as the values for the critical pressure and temperature are too high (p_c = 214.4 bar, T_c = 374°C) /58/. The tissue water has therefore to be replaced with a substance which has convenient values of the critical temperature and pressure, the so-called transitional fluid. In common use are carbon dioxide and monochlorotrifluoro-methane (Freon 13) (/44/, /57/).
None of the extant transitional fluids is miscible with water however /57/. The water in the specimen has therefore to be replaced with any of several dehydration fluids, usually ethanol or acetone. As not all dehydration fluids mix with the transitional fluids the dehydration solvent has to be substituted by the so-called intermediate fluid that mixes with the dehydration solvent as well as with the transitional fluid (/44/, /57/, /58/). In common use are ethanol, acetone, isoamyl acetate, Freon TF and 1,1,2-trichlorotrifluoro-ethane (Freon 113) (/44/, /57/).

d) Processing of specimens for critical point drying:
(/57/ and own experience after recommendations of Dr. A.H.L. Chamberlain, Department of Microbiology, University of Surrey, Guildford/Surrey, UK)

The following steps have to be performed for critical point drying of biological specimens, outlined here for ethanol as dehydrating agent, acetone as intermediate fluid and CO_2 as transitional fluid (T_c=31°C, p_c=74bar) (this scheme has been used for bone specimens in the work described in this report - compare chapter 6.2):

- sectioning and cleaning if necessary;
- fixation in any common fixative solution for scanning electron microscopy;
- dehydration in ascending ethanol series;
- substitution of dehydrating agent (ethanol) with intermediate fluid (acetone) in ascending acetone series;
- placement of the specimen in intermediate fluid (acetone) into the critical point drying container (bomb); the temperature has to be at least 10° to 15° below T_c;
- after sealing of the bomb liquid CO_2 is introduced through the slightly opened inlet valve until the specimens are covered by liquid;
- the temperature should be approximately 20°C which is about 10° below the T_c of CO_2; the transitional fluid is then less dense than the intermediate fluid so that the latter will drain the bottom;
- flush the bomb for 5min, leave the specimens in liquid CO_2 for 1h, flush for 5min (general guidelines are available for other intermediate and transitional fluids and different specimens);
- for the actual drying process, the chamber should be more than half full of liquid at a temperature of T_c-10°; the specimens have to remain covered by liquid;
- the temperature is then risen to about 10° above the critical temperature; if the pressure exceeds the safe level, the bomb is slightly vented;
- the temperature is kept high and the container slowly vented through the outlet valve.

The dried specimens should be examined by scanning electron microscopy as soon as possible after the drying process; until then they should be stored in a vacuum desiccator.

e) Artefacts caused by critical point drying: /57/

The main artefact encountered with critical point dried specimens is shrinkage. Mostly, however, this shrinkage is quite uniform and merely results in a reduction in volume without a change in proportions. There is no apparent distortion therefore in most cases.
Shrinkage may result in a cracking of cells, especially if they are stretched on a rigid surface as bone for example. Furthermore, wrinkling of the cell membrane and very small holes in cell membranes can occur.
Critical point drying can develop latent errors of previous processing. Previous steps in specimen preparation, as fixation, sectioning, dehydration, have therefore to be performed with great care to obtain an optimal result in critical point drying.

III.5. SCANNING ELECTRON MICROSCOPY

III.5.1 Scanning electron microscopy (SEM) in calcified tissue research

Scanning electron microscopy is a very adequate tool in calcified tissue research if surfaces are of interest. Vice versa, calcified tissue is especially suitable for examination with the SEM in comparison with soft tissues, due to its lower water content. Therefore, the first biological applications of the SEM were in the field of calcified tissue research in 1961 /44/.

Problems relate to the study of cells in a calcified matrix. Their examination is here even more difficult than for other soft tissue as the shrinkage of the cells and the surrounding mineralized matrices differ widely /44/.

The SEM is used in calcified tissue research in the following areas /44/:

- examination of cell shape, extent, and surface features on an interface (for example osteoblasts on bone),
- examination of the three-dimensional distribution of matrix surfaces, exposed by appropriate chemical and physical approaches,
- mapping of the three-dimensional distribution of the mineralized phases in anorganic samples.

III.5.2 Principal functioning of the scanning electron microscope

(after Hayes /59/ if not otherwise stated)

Image formation in the scanning electron microscope is different from the traditional spatial focusing method. Most images (for example in light microscopes, transmission electron microscopes, telescopes, eyeglasses, the human eye) are formed by focusing the radiation after it has left the specimen thus achieving the necessary one-to-one correspondence between points on the specimen and points on the image. This correspondence however can never be realized in existing microscopes due to imperfections in real lenses (CF). In contrast, the scanning electron microscope forms the image through a time sequence of points, like television imaging does. All focusing takes place prior to interaction with the specimen. The image points are adressed in time.

The SEM is composed of a probing system and a display system. In the probing system, the interaction between the specimen and the electrons of the primary beam takes place. After leaving the filament, the primary electrons are focused by means of electron lenses to a very fine point on the surface of the specimen. Interactions between the electrons and the material of the specimen at this point induce various forms of radiation (characteristic X-rays, infrared, visible light and streams of electrons). All these types of radiation leave the specimen in a variety of directions. The intensity of the chosen sort of radiation is used to modulate the brightness of the beam of the display cathode ray tube as it rests at the corresponding point on the image forming screen of the display tube (display system).

In a lens imaging system (light microscope or transmission electron microscope), the information signal leaving the specimen is a vector quantity containing directional (localization) and amplitude and phase (information) components.

In a scanning system, the information signal is a scalar quantity. All localization is a function of time, the video system only contains the amplitude component. Therefore, only the number of electrons or photons leaving a certain spot has to be measured, whereas the measurement of their direction is not necessary.

The two electron beams in the probing and display system are synchronized as they sweep over the same number of lines. The area of the array of lines (the raster) is of different size on the specimen and on the face of the cathode ray tube. Magnification is achieved, therefore, by having the display raster very much bigger than the synchronous specimen raster.

The useful range of magnification of 10 to 50000 times (and now even higher) is dictated by the resolving power of the instrument (upper limit) and by the lens design and placement of the specimen (lower limit).

III.5.3 Interaction between electrons and specimen
(after Reimer/Pfefferkorn /58/ if not otherwise stated)

As the primary electrons (PE) of the electron beam enter the specimen, elastic and inelastic scattering events occur. Due to these scattering processes the electrons loose some of their energy, so that their range is finite.
Inelastic scattering hereby causes energy losses primarily in a range of up to 50eV. It therefore produces mainly slow secondary electrons (SE) with energies below 50eV.(compare Fig. III.11). a thin surface layer with a thickness between 10 and 100A. These electrons are called group 1 electrons. Only electrons of this group allow high resolution in the SEM as their area of evasion is determined by the evasion depth and the diametre of the primary electron beam.
There is a smaller probability that faster secondary electrons are produced by electron-electron impacts. These cannot be distinguished from backscattered electrons (BSE) (group 2) the latter being electrons which are deflected in wide angles after elastic scattering at the atomic nuclei. Their energies range from the primary energy down to 50eV. On passing the surface, they as well may produce secondary electrons; these can evade if they are produced not deeper from the surface than 10 to 100Å (group 3).

The electrons of these last two groups originate from an area with a diametre of 0.1µm to several microns around the point in which the primary beam hits the sample. The value of the signal from these groups is therefore influenced by the whole structure of the specimen in the diffusion cloud up to the sampling depth of the BSE.
BSE which hit the walls of the specimen chamber originate secondary electrons from there. These are also collected by the positive potential of the collector.
The few backscattered electrons which are scattered at the walls a second time are also counted into group 2.

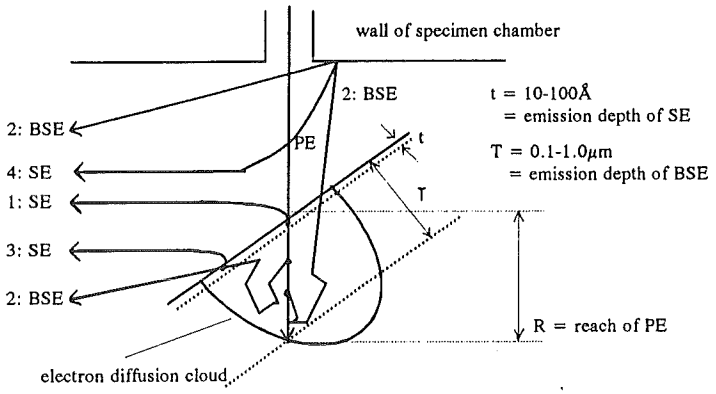


Fig. III.11. Interaction between electron beam and specimen

III.5.4 Scanning electron microscope modes
(after Boyde /44/ if not otherwise stated)

The operator of the scanning electron microscope can choose the following parameters according to the aim and subject of his work:

- beam voltage and current,
- specimen orientation,
- detector strategy

and

- signal mode(s).

The first signal mode is the secondary electron mode. The standard secondary electron detector in the SEM collects a high proportion of low energy (<50eV) secondary electrons (SE) and a small proportion of high energy backscattered electrons (BSE) (compare chapter 5.3). There is also a proportion of secondary electrons collected which arise from BSE leaving the sample. This proportion varies with the beam energy. The quality and information of the SEM-image varies therefore markedly with the accelerating voltage. Routine SEM of biological samples is best conducted at lower accelerating voltages because their densities are generally lower than those of non-biological materials. Furthermore, dried biological samples are non-conductors. Lower accelerating voltages are recommended therefore to decrease charging and damage of the specimen.
For routine work on calcified tissue, Boyde recommends an accelerating voltage of 10kV.

The enhanced secondary emission mode is achieved by floating the sample in a disk-shaped or cylindrical surround at a negative potential of operator choice. The enhanced emission of secondary electrons is useful for extracting information from deep holes, for example.

The backscattered electron mode requires accelerating voltages higher than 10kV for optimal imaging. The SEM is therefore usually operated in this mode at accelerating voltages of 20kV to 30kV. Modern solid state backscattered electron detectors are constructed in a ring configuration. The beam passes through the centre of the detector complex. A flat specimen may therefore be evenly illuminated at normal beam incidence.
Backscattered electrons give information about the density of the superficial layers of the specimen and about its topography if it is not totally flat.

Density-dependent, i.e. atomic number, contrast can be abstracted on very flat surfaces. With the appropriate technique, very low density differences can be detected and imaged. In contrast, samples of uniform density only show topographic contrast.

The BSE-mode has no advantages over the SE-mode concerning the complication of interpreting the origin of image contrasts. Only if it can be assumed that the sample is either flat or of uniform density, this is not a problem any more.

The main advantage of backscattered electrons is that they are not, due to their high energy, influenced by low strength fields induced by injecting the electron beam into non-conductive samples. Charging problems as they occur in imaging with secondary electrons can so be simply overcome.

For the converted backscattered electron mode, the sample is floated at a small positive potential in the range of +50V to +200V. The escape of secondary electrons is thus prevented (as outlined above (chapter 5.3), secondary electrons have energies in the range of 0eV to 50eV). Those electrons which escape the specimen can liberate secondary electrons where they strike other surfaces in the specimen chamber. These secondary electrons are collected by the usual Everhart-Thornley biassed scintillation detector and are called converted backscattered electrons (CBSE). The advantage is that much lower accelerating voltages are possible than with the solid state detector, which begins to work efficiently only from 10kV on upwards. The CBSE-mode can effectively eliminate charging problems. Furthermore, surface topographical contrast of etched, flat specimens is effectively enhanced.

III.6. MATERIALS AND METHODS

III.6.1 Bone

For light and scanning electron microscopy, specimens from the long bones (tibia) of sheep, goat, horse, dog and rabbit have been used. The sheep, goat and horse bones were used fresh (the goat bone frozen), the dog and rabbit bones were received embedded in PMMA from University Hospital Essen.
For transmission electron microscopy, fresh horse and PMMA-embedded dog bone have been used.

III.6.2 Specimen preparation for light and scanning electron microscopy

Metallurgical specimen preparation has been used to reveal the structure of bone. The following preparation schemes have been performed:

Preparation scheme 1 (sheep bone):

1. fixation in 40% ethanol
2. cleaning of the bones
3. sectioning with hack-saw and Accutom (Struers) (diamond blade)
4. air-drying
5. storage at -20°C
6. vacuum infiltration with a metallographical resin (Epofix - Struers, Epoxide - Bühler, Araldite - Ciba Geigy)
7. grinding on SiC-paper (320, 500, 1000) (compare fig. III. 12)
8. polishing (compare fig. III. 12)
9. etching (some specimens, compare fig. III. 12)

--> light microscopy

10. sputtering with gold or carbon

--> scanning electron microscopy

Preparation scheme 2 (goat bone):
(Bone was frozen when received.)

1. thawing at room temperature
2. fixation in 40% methanol
3. cleaning
4. sectioning with hack-saw and Accutom (Struers) (diamond blade)
5. embedding in metallographical resin (Acryfix - Struers)
6. grinding on SiC-paper (320, 500, 1000) (compare fig. III. 12)
7. polishing (compare fig. III. 12)
8. etching (some specimens, compare fig. III. 12)
9. de-embedding (mechanically or dissolution of the resin in acetone)

--> light microscopy

10. further sectioning of the specimens
11. dehydration in ascending ethanol series (70%, 90%, 95%, 100%)
12. substitution of ethanol by acetone (ascending acetone series: acetone : ethanol 1:2, 1:1, 2:1, 100% acetone)
13. critical point drying from acetone and CO_2 (compare fig. III. 12)

--> light microscopy

14. sputtering with gold

--> scanning electron microscopy

Grinding and polishing has been performed on an automatic grinding and polishing machine (Struers). The parameters can be seen from fig. III. 12. Good polishing results have also been achieved by omitting the 6µ-polishing step. The 1µ-step, however, has then to be prolonged to about 10 minutes.
It is important to polish on hard bases if no topography shall be produced and very flat surfaces are to be achieved. Softer bases effect bad border contrasts and reveal the structural components (lamellae) of bone.

	Grinding			Polishing	
Base	SiC	SiC	SiC	PAN-W 6µ (hard)	MOL 1µ (soft)
Grain	320	500	1000	--------	------
Lubricant	H_2O	H_2O	H_2O	red	red
Speed	300	300	300	150	150
Pressure	90 (3)	90 (3)	90 (3)	90 (3)	90 (3)
Time	until plane (2min)	0.5 min	0.5 min	4 min	3 min

Grinding and polishing scheme for bone

Number	Reagent	Time	Reagent	Time
1	5% HCl	2min		
2			5% NaOCl	7min
3	5% HCl	2min	5% NaOCl	1min
4	5% HCl	1min	5% NaOCl	5min

Etching recipes

Dehydration:	70% EtOH	90% EtOH	95% EtOH	100% EtOH
	20min (or storage)	20min	20min	20min
Intermediate Fluid	EtOH/acetone 2:1	EtOH/acetone 1:1	EtOH/acetone 1:2	100% acetone
	10min	10min	10min	10min
Transitional Fluid	CO_2	CO_2	CO_2	
	flushing 5min	equilibrate 1hour	flushing 5min	
Critical Transition	Temperature Rise to	Pressure Rise to		
	> 31°C	> 71.5bar		

Critical point drying scheme

resin	type	curing time	hardening time	viscosity
Epofix	cold mounting epoxy resin	30min at 25°C	8h at 25°C	550 cp at 25°C
Epoxide	epoxy resin	2-3h	2-3h at 30-35°C 6-8h at room temp.	950 cp
Araldite	epoxy resin	2.5h	24h	"very low"*

* Remark: Exact data for the viscosity were not available.

Characteristic data of the used resins

Fig. III. 12: Preparational data for bone

Etching is an - original metallographic - method*) which reveals different components of a material due to their different rates of removal. The etching pattern has to be interpreted in terms of what was the surface like before the etching procedure rather than in terms of what is the surface. Therefore, the differences in the rate of removal of surface layers which are no longer there have to be kept in mind. For etching of bone, the following reagents can be used: diluted acids (for example, hydrochloric acid - HCl) or powerful organic solvents (for example, sodium hypochloride - NaOCl). Acids demineralize the bone, so that after drying the distribution of the lamellae which consist basically of collagen is visible. Organic solvents dissolve the cells and the organic components of the bone matrix. The gross patterns of the mineralization of bone and the matrix surrounding cells, cell prospects and spaces in the hard tissue can be studied. Combined etching with an acid and an organic solvent (first HCl, then NaOCl) reveals the depth to which the acid has penetrated in the first instance. Differences between less and more mineralized osteons can be seen because denser osteons develop a rougher texture than recently formed less well mineralized ones /60/.

III.6.3 Embedding experiments

The bone specimens were vacuum infiltrated with three types of synthetic resins used in metallography. Some specimens were infiltrated with ink-stained resin to show the success or failure of the infiltration procedure. Further, curing of the resins under vacuum and environmental pressure were compared.

The following resins were used:

- Epofix (Struers: resin and hardener),
- Epoxide (Buehler: resin and hardener) and
- Araldite (Ciba Geigy: resin CY 1301 and hardener XY 1300).

The characteristic data of the resins can be seen from fig. III. 12.

III.6.4 Specimen preparation for transmission electron microscopy

Usually in biology, specimens for transmission electron microscopy are prepared by ultramicrotomy. Ultramicrotomy requires skilled personnel. Hard specimens are very difficult to section without causing artefacts (compare chapter IV) and the difficulties become more severe if compounds of hard tissue and an implant material are to be sectioned, due to the different properties of the two materials. In materials science, for example for the preparation of semiconductors for transmission electron microscopy, specimens are prepared by polishing the central area of a circular specimen to a certain thickness and subsequent ion beam thinning if necessary. This preparation method is relatively easy to perform. The disc-shaped specimens have to be handled carefully, however, due to brittleness of the thin area. Areas of special interest of an implant-bone interface can be selected and specifically prepared for transmission electron microscopy.
The preparation scheme for examination in a JEM 200 CX Jeol electron microscope is given in the following survey. figure III. 13 elucidates the preparation scheme.

Preparation scheme 3 (horse bone):

1. fixation in 40% methanol
2. sectioning with hack-saw, half-automatic and automatic (Accutom) diamond saws (thickness of cross-sections: 0.5mm)
3. drilling of small discs with a special drill (diametre of discs 3mm according to TEM-specimen holder)
4. polishing of these discs on fine-grained SiC-paper to a thickness of 150µm
5. dimpling to a thickness of 70µm
6. ion-etching

*) in modern microstructural analysis the term "metallographic" is substituted by "microstructural" and metallographic methods for preparation are microstructural preparation methods

A later experiment was performed without ion beam etching. The specimen was dimpled instead until it seemed "thin enough" to be examined by transmission electron microscopy. As this experiment was only a preliminary one, the exact thickness of the specimen has not been determined during the thinning process.

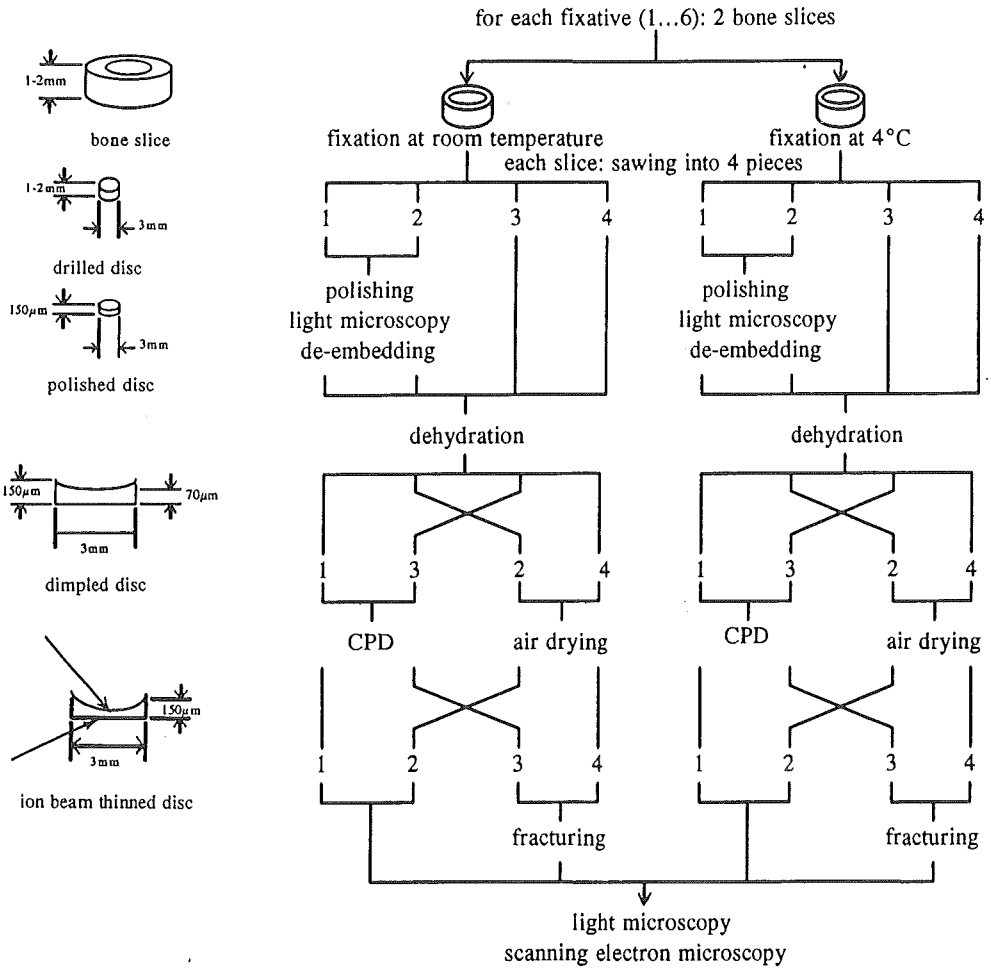


Fig.III.13. Preparation scheme for TEM-specimens (left) and for fixation experiments (right)

III.6.5 Fixation experiments

There are many fixative solutions which have been used to fix bone. Each of them has its special field of application. Most frequently, the following solutions are recommended for light microscopy and scanning and transmission electron microscopy:

1. 10% formalin in phosphate buffer,
2. 2.5% glutaraldehyde in phosphate buffer,
3. paraformaldehyde / glutaraldehyde (1:1) in phosphate buffer,
4. 40% ethanol,
5. 70% ethanol,
6. 10% formalin / 100% ethanol (1:1).

Phosphate buffer is known to produce artefacts in mineralized tissue due to calcium phosphate formation. It has been used here in spite of that disadvantage due to its harmlessness as compared with cacodylate buffer which is highly toxic. The formation of calcium phosphates does not alter the results of these experiments as only comparative data were to be achieved.

The fixative solutions listed above have been compared in the work described here. For each fixative solution, the following experiments have been performed:

- fixation at room temperature versus fixation at 4°C,
- air-drying versus critical point drying,
- analysis of polished surfaces,
- examination of fracture surfaces in the SEM.

For these experiments, a long bone of a horse has been obtained from a casualty animal slaughterer. The animal was slaughtered because of old age. The exact age and health state of the animal, however, is not known.

Fixation was performed within two hours after the death of the animal.
The preparation of the specimens is described in the following scheme and shown schematically in figure III. 13:

1. preparation of twelve cross-sections, thickness about 1cm, from the fresh bone,
2. fixation in the solutions listed above, two sections per fixative, fixation time: 2 days; one specimen has been fixed at room temperature, the other one at 4°C,

3. washing of specimens 1 - 3, 6 (aldehyde fixed) in double distilled water for one hour.

The following steps are performed respectively for each fixative solution:

4. sawing of the cross-sections into four small blocks,
5. two of these blocks: - embedding in metallographical resin (Acryfix)
   - grinding and polishing according to the method described above

--> light microscopy

6. de-embedding of the specimens
7. dehydration in ascending ethanol series (70%, 90%, 95%, 100%)
   (compare fig. III. 12 - dehydration)

8. respectively one set of specimens (one polished and one non-polished specimen)
   --> air-drying
   --> critical point drying from CO_2 (intermediate solvent: acetone) (compare table 6.3)

--> light and scanning electron microscopy of the polished specimens

9. fracturing of the non-polished specimens in liquid nitrogen

--> light and scanning electron microscopy of the fracture surfaces

III.7. RESULTS AND DISCUSSION

III.7.1 Specimen preparation for light and scanning electron microscopy

The sheep bone specimens were air-dried at elevated temperatures. After embedding in a metallographic fast hardening resin (Epofix - Struers), they were examined by light and electron microscopy.
Reflective light microscopy proved to be an effective tool in showing the lamellar structure of bone.Fig.III.14. shows a cross-section of bone, prepared as described above. The structure of bone is well visible, with lamellar bone at the outer circumference and Haversian bone towards the middle of the section. Unetched specimens show some topography due to the varying hardness of their components. The cement line, being devoid of collagen and therefore the hardest structural component, stands out of the otherwise smooth surface. Contrast is enhanced by using polarized light.

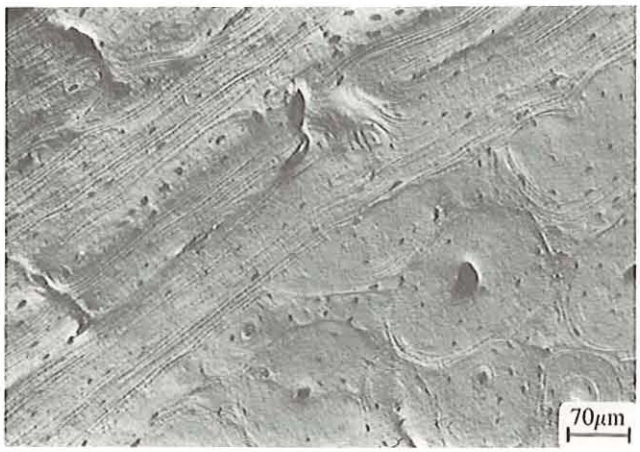


Fig.III.14. Reflected light micrograph of bone

The specimens showed extensive cracking due to the drying method. This could be concluded from later experiments with more careful drying procedures (air-drying at room temperature, critical point drying). Cracks never developed again to the extent seen in these sheep bone specimens.fig.III.15 a) (light microscopy) and b) (scanning electron microscopy) show the cracks in a bone cross-section. Bigger cracks nearly go through the whole width of the section with their path partially following the orientation of the lamellae, partially neglecting the structure of the bone.

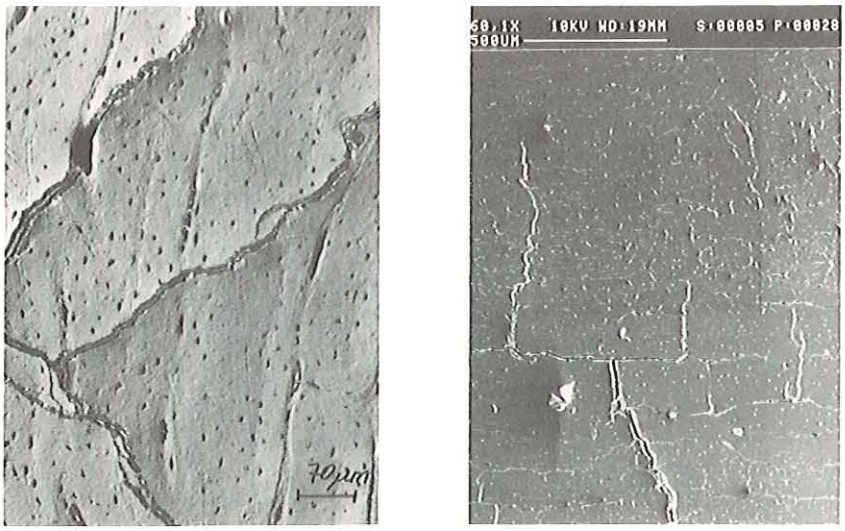
With scanning electron microscopy, fibrils protruding from the surfaces and partially bridging the crack can be seen in some of the cracks (fig.III.16.). They may be collagen fibres disrupted during crack propagation. The crack has proceeded parallel to the lamellae, separating them at an interlamellar cement line. This can be concluded from fig.III.16.b), showing the lamellar organization of collagen fibres on one crack surface. The cracks might develop due to the sudden expansion of water during drying at elevated temperatures. This assumption is based on later light microscopic observations: specimens which showed hardly any cracks after air-drying at room temperature developed many cracks when they were evacuated in a coating unit. The sudden expansion of water or alcohol possibly remained in dehydrated specimens causes an explosive like cracking of the bone structure. There are the less cracks the better the specimens are dried before electron microscopic examination.

Similar fibres can be seen at the interface between the bone and the embedding material (Epofix) (fig.III.17). They are Sharpey fibres which attach the periosteum to the bone. Because the periosteum

was only loosely attached before embedding due to the drying procedure, it was pulled off the bone surface when the embedding material shrank during hardening.

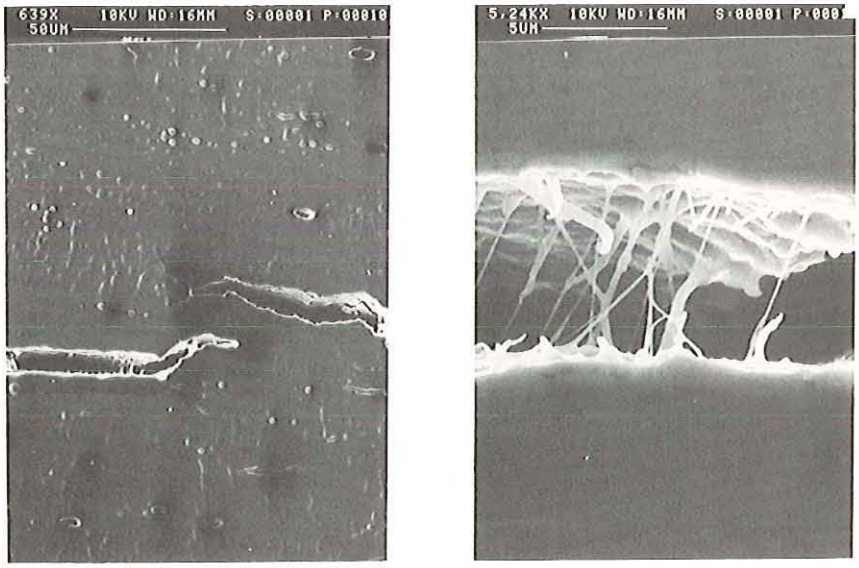
For the examination of flat surfaces by scanning electron microscopy BSE-mode proved to be superior to SE-mode due to the following reasons: Because secondary electrons mainly show topography contrast, structural features other than surface voids cannot be distinguished (fig.III.18). However, the interior surface structure of these voids is easily visible, for example the organization of collagen fibres on the inner surface of a Haversian canal (fig.III.19). In SE-mode, 5kV accelerating voltage were found to be optimal. Higher accelerating voltages lead to charging of the specimens to the point where further examination becomes impossible, especially for bulky specimens embedded in a metallographic non-conductive resin. Charging results in bright seams at crack walls and bony voids as can be seen in fig.III.20.
In BSE-mode, charging is not visible due to the higher energy levels of the collected electrons. Subsurface voids are revealed because backscattered electrons gather information from a greater depth than secondary electrons (fig.III.21). Furthermore, the lamellar organization of bone is more clearly visible. Specimens are, however, seriously damaged due to the high accelerating voltages necessary for achieving optimal images. 25kV accelerating voltage were found to be a reasonable compromise to enhance the the BSE-image by an increased accelerating voltage on one hand and to prevent severe damage of the specimen before examination is completed on the other hand.
Water retained in the specimen and, additionally, at higher magnifications, concentration of the energy of the incident beam with its subsequent heating and electron displacement effects are responsible for the damage by the electron beam. Due to ionization effects, in organic material bonds between carbon and hydrogen are disrupted and hydrogen atoms and smaller radical groups are released. The extent of damage in an organic (HCl-etched) specimen is shown by comparison of figs.III.22.a), b) and c): The area scanned during examination at a higher magnification (fig.III.22.b) appears rough as compared to its former state.

Etching is a suitable method for enhancing the contrast of the specimens as well as to minimize the damage caused by the electron beam. The optimum technique to achieve the first objective is etching with HCl (fig.III.23.a). As the mineral component is dissolved, the organization of the organic phase can be seen. The lamellae, whose structural components are the collagen fibres, are easily visible (fig.III.23.b).
The latter objective is best realized by combined etching with HCl and NaOCl (compare fig. III. 12). Anorganic (NaOCl-etched) specimens contain no structural water thus there is no shrinkage during drying and less damage by the electron beam because the component especially susceptible for damage is removed.
Fig.III.24 shows how the rate of damage is reduced by certain etching routines. Part a) shows a survey of the specimen shown in fig.III.22 after that series was taken, part b) shows the surface of a specimen etched with HCl and NaOCl after examination at higher magnifications. Areas of damage are visible but they are not as significant as in part a). Rougher areas have been more mineralized than smoother surface areas. The specimen etched with HCl only still contains its organic phase in the surface which is the phase likely to be damaged by the electron beam. The smooth structure of the specimen surface after combined etching with HCl and NaOCl indicates that all osteons nearly have the same level of mineralization.



a) b)

Fig.III.15. Cracking of bone sections
a) light micrograph, b) scanning electron micrograph



a) b)

Fig. III.16.Cracks with fibrils
a) survey, b) magnified view of upper crack shown in a)

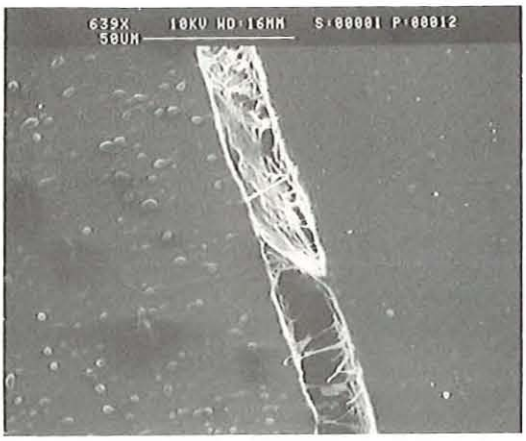


Fig.III.17. Interface bone - resin (Epofix)

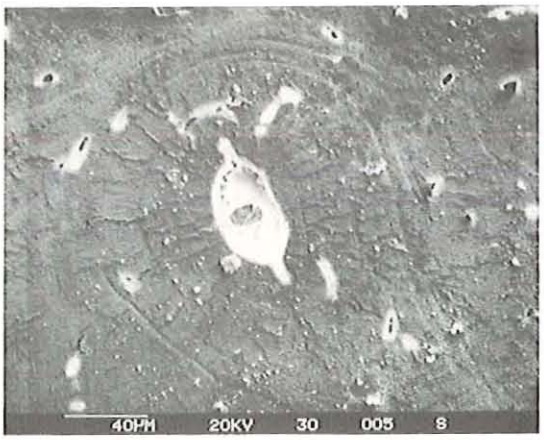


Fig.III.18. Structure of polished bone cross-section as seen in SE-mode

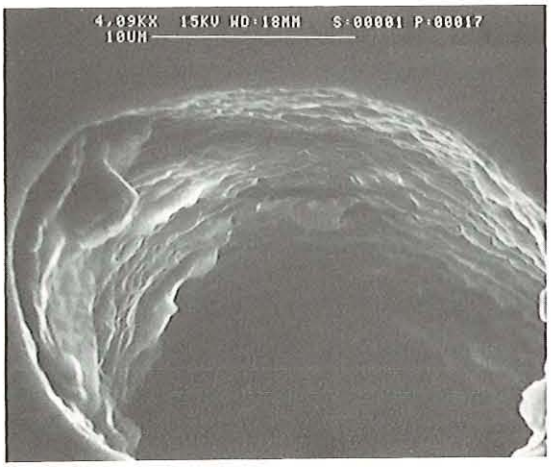


Fig.III.19. Haversian canal (SE-mode)

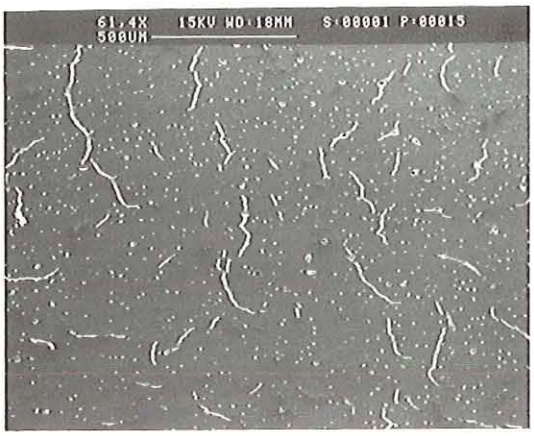


Fig.III.20. Charging in SE-mode

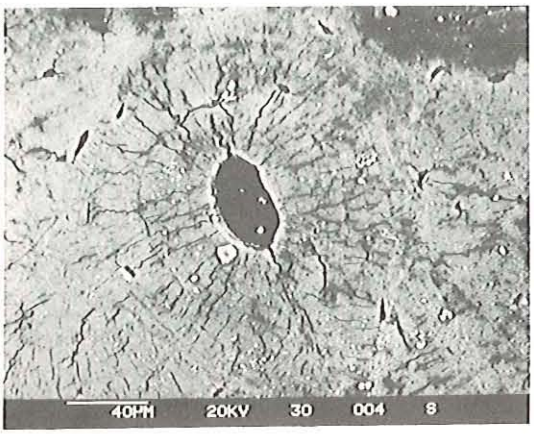
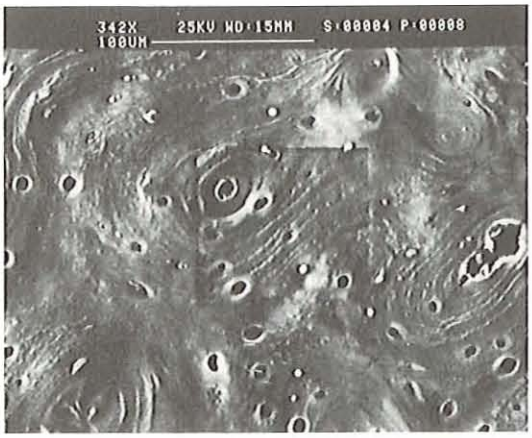
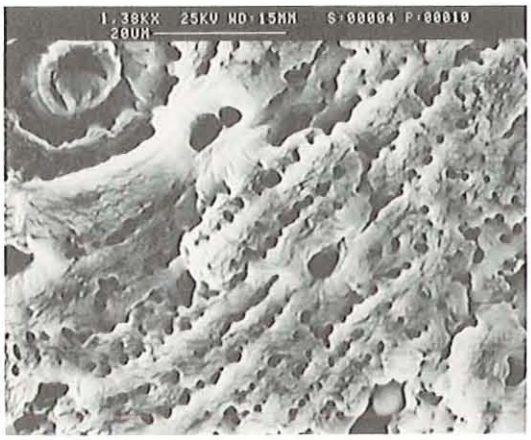


Fig.III.21. Structure of polished bone cross-section as seen in BSE-mode

a)



b)



c)

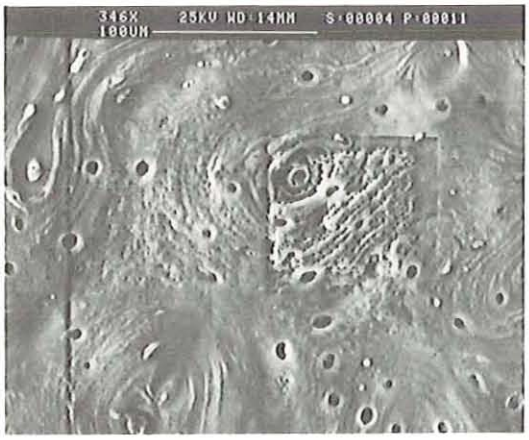


Fig.III.22. Damage by the electron beam
(etching: 2min 5% HCl)

a)

b)

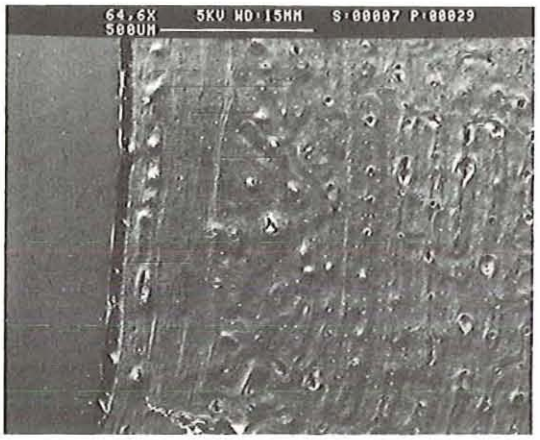
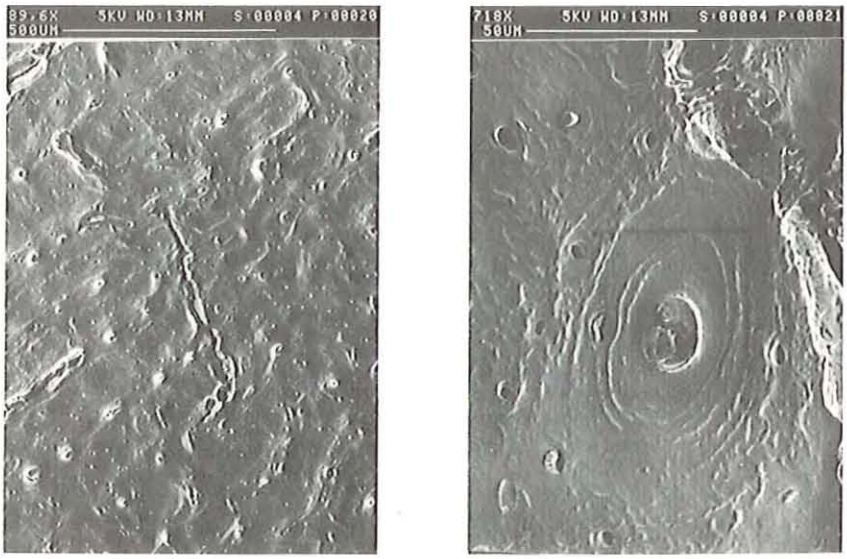


Fig.III. 23. Comparison of damage by the electron beam in two differently etched specimens
a) high rate of damage in HCl-etched specimen,
b) low rate of damage in HCl/NaOCl-etched specimen



a) b)

Fig.III.24. Structure of bone as visible after etching with HCl
a) survey, b) Haversian system

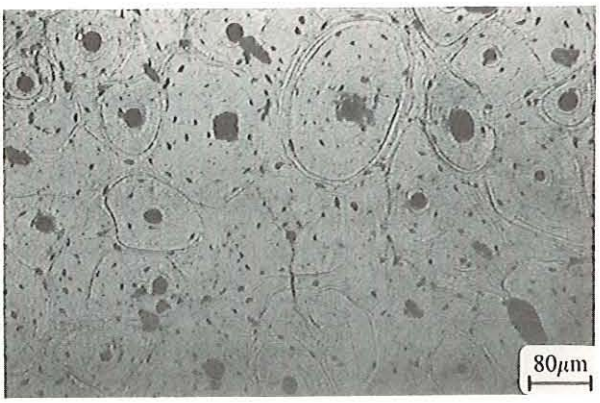
III.7.2 Critical point drying (CPD)

The goat bone specimens were critical point dried as described above (compare chapter IV) and not embedded when examined by scanning electron microscopy. They were machined considerably smaller than the sheep bone specimens because of size restrictions in the critical point drying apparatus. The structure and state of the specimens after polishing, but before drying can be seen from fig.III.25 There are small cracks radially through the Haversian systems (a) and others following the lamellae (b). They may be due to the fact that the bone was stored frozen before preparing it for examination. The cracks might also develop when the specimen surface unintendedly dries out in course of the preparation or during observation in the light microscope.

Cracking of the specimens due to water evaporating in the vacuum of the electron microscope is avoided. After critical point drying, in polished, unetched cross-sections similar cracks as before drying can be seen (fig. III.26). The cracks seem to be a little bit wider, possibly due to volume changes during drying.
After etching, some of the cracks show fibrils protruding from the crack walls, as can be seen in figure III.27a). The broken fibrils are seen in demineralized specimens in cracks parallel to the lamellae or in Volkmann's channels. They are therefore likely to be broken collagen fibres, exposed due to the attack of the etching reagent. Radial cracks do not show these fibrils (fig.III.27b), obviously due to the internal structure of the bone.
After demineralization with hydrochloric acid, the organization of the organic phase in the surface is revealed (fig.III.27). The osteons, which contain relatively more organic material than the ground substance, which is mainly composed of minerals, stand above this ground substance. The organization of the collagen fibres into lamellae can be clearly seen.

Furthermore, the specimens were not damaged any more by the electron beam since water is thoroughly removed. Fig.III.28 shows a series comparable to fig.III.22. The HCl-etched specimen is not damaged by the electron beam. Consequently, there are no square imprints on the specimen surface which show the area scanned by the electron beam at higher magnifications.

a)



b)

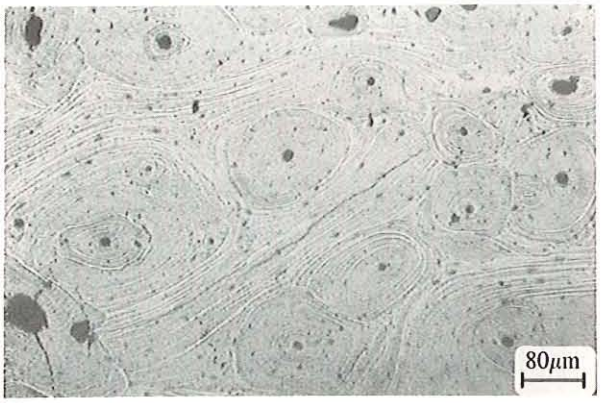


Fig. III.25. State of goat bone specimens after polishing and before drying
a) radial cracks through Haversian systems,
b) cracks following the lamellae

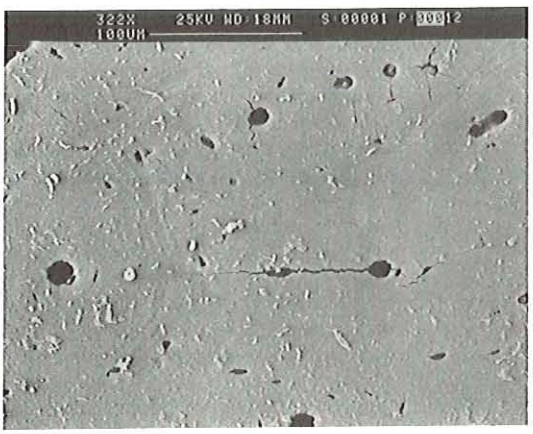
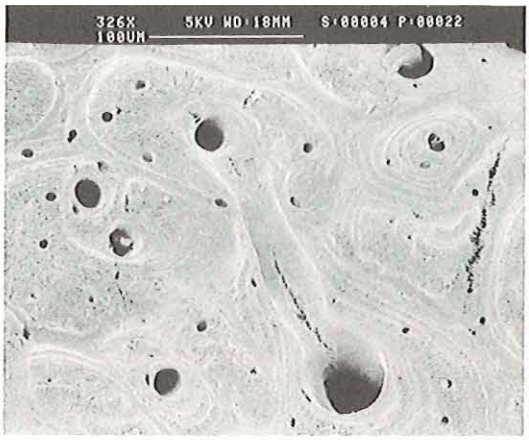


Fig. III.26. Cracks in unetched CPD-specimens (SEM: BSE-mode)

a)



b)

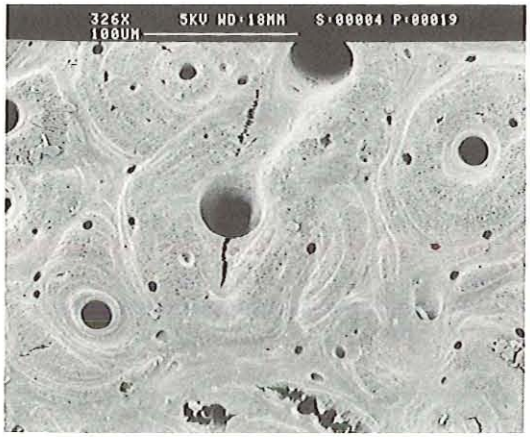
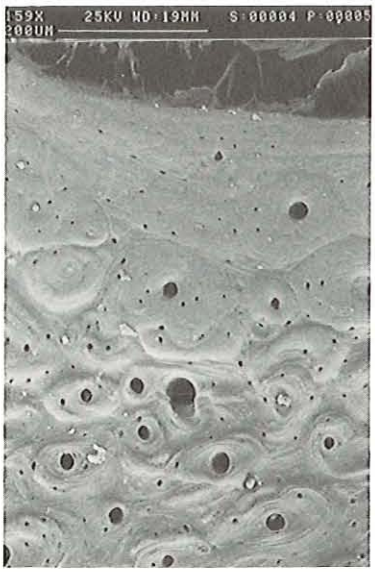
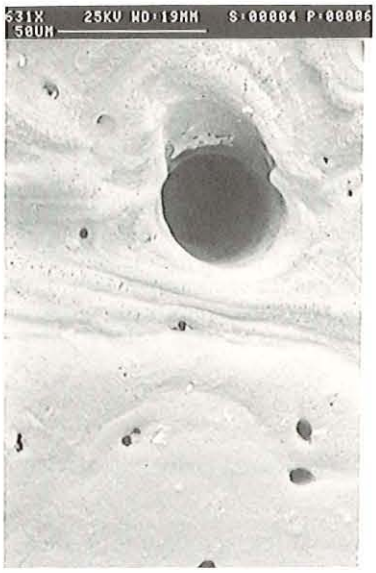


Fig. III.27. Cracks in HCl-etched CPD-specimens (SEM: BSE-mode)
a) cracks with fibrils, b) cracks without fibrils

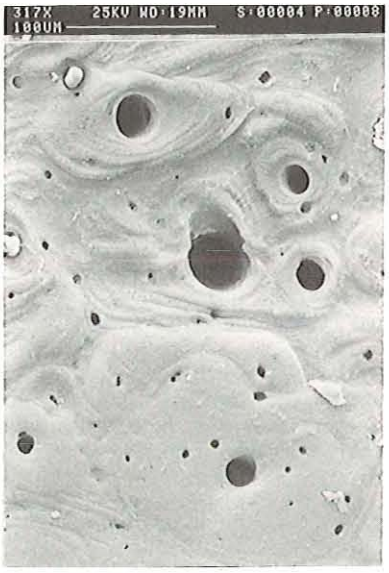


a)



b)

c)



d)

Fig. III.28. Documentation of lack of damage after CPD (etching: 2 min 5% HCl)
a) lowest magnification, taken first
b) higher magnification, taken second
c) highest magnification, taken third
d) lower magnification, taken after a, b, c

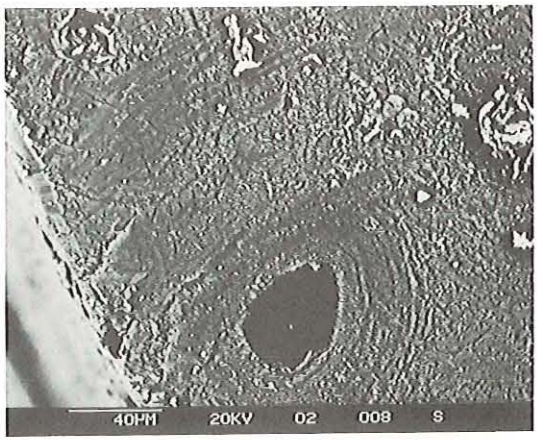
III.7.3 Embedding experiments

The metallographical resin "Epofix" did not infiltrate the bone as proved by staining the resin. The hardening time of several hours is too short and the viscosity of the resin too high to infiltrate the relatively small and narrow pores of bone thoroughly. Furthermore, due to the shrinkage during hardening the resin separated from the specimen surface. A gap between the bone and the resin can easily be seen even by light microscopy (compare figs.III.17. and III.18. a) above).
Due to the difficulties encountered with "Epofix" two other resins with lower viscosities and longer curing times were tested. They neither were suitable for infiltrating bone. There are gaps between the bone and these resins as well. After staining of the resin, no infiltration into the pores of the bone sections can be seen.

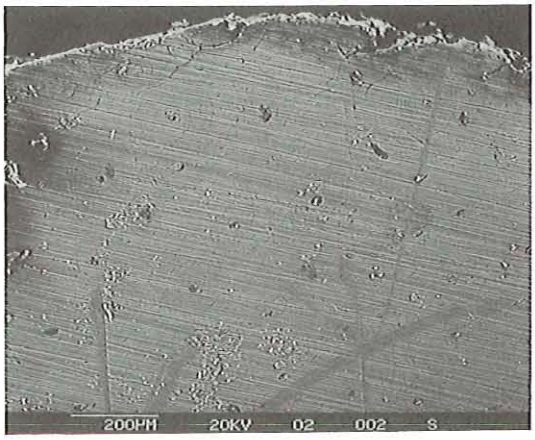
III.7.4 Specimen preparation for transmission electron microscopy

Dimpling may be an appropriate method for preparing specimens for transmission electron microscopy if certain problems are considered.
Specimens are, down to a thickness of about 80µm, hardly damaged by the preparation procedure even if they are not embedded. This can be seen from figs.III.29 a) to c), which show the surface of bone specimens prepared for transmission electron microscopy after various preparation steps. There are only a few cracks visible at the circumference of a bone disc drilled out of a slice of bone which was cut on an automatic saw with a diamond blade (fig.III.29 a). The structure of the bone is - though not distinctly - revealed, prooving the good quality of the cut. After grinding the specimen to a thickness of about 150µm, the structure of the bone is better visible and a smoother surface has developed (fig. III.29 b). Fig.III.29 c) finally shows the specimen surface after dimpling at the border between dimpled (right) and polished (left) area. To achieve an even surface for dimpling, the specimen was polished with a special dimpling tool prior to the actual dimpling procedure. As topography contrast is excluded density differences of the elements in the specimen surface are visualized (atomic number contrast). Less well mineralized osteons appear darker, therefore, than more mineralized ones due to their lower calcium content. The specimen surface shows many scratches after dimpling. It is advisable therefore to polish the thinned area with a polishing tool on the dimpler to remove the scratches shortly before the final thickness is reached.
Fig.III.30. shows the surface of a dimpled specimen after ion beam thinning and after ion beam thinning and TEM-examination. The ion beam thinned surface looks rather smooth, only the scratches from the grinding process are still visible. After TEM-examination, the surface of the specimen appears rough. The specimen was too thick for being transmitted by electrons and has been damaged by the temperature during the transmission process.
Further experiments have to be performed, however, to enable a final statement whether ion beam etching is appropriate for preparing bone specimens for the TEM or whether dimpling on its own is the more advantageous method.
Fig.III.31. shows a TEM-micrograph of a specimen prepared by dimpling. There are certain areas of the specimen thin enough to be transmitted by the electron beam. The existence of hydroxyapatite is shown by its characteristic diffraction pattern. Due to the size of the specimens, heating is a severe problem. It is essential, therefore, to cool the specimens during examination with liquid nitrogen and to lead the heat off by holding the specimen between two copper grids to prevent damage by the electron beam. Fig.III. 32. shows a SEM-micrograph of a dimpled specimen after TEM-examination. The dark area was burnt during examination. There are cracks in the specimen, probably developed during examination in the electron microscope.
It seems to be possible to prepare bone specimens for transmission electron microscopy just by dimpling. Further experiments have shown, however, that it may be advantageous to embed the specimens to avoid mechanical damage during dimpling. It is not easily possible otherwise to obtain a coherent area thin enough for TEM-examination.

a)



b)



c)

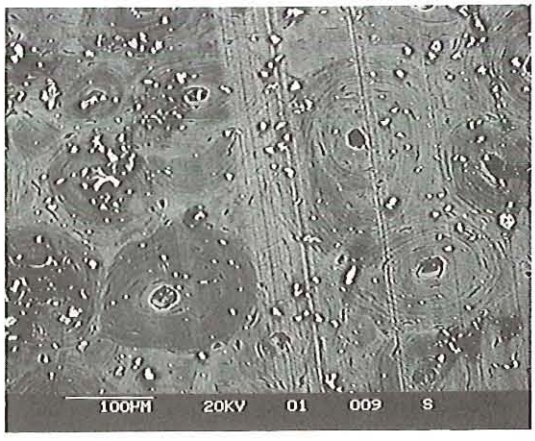
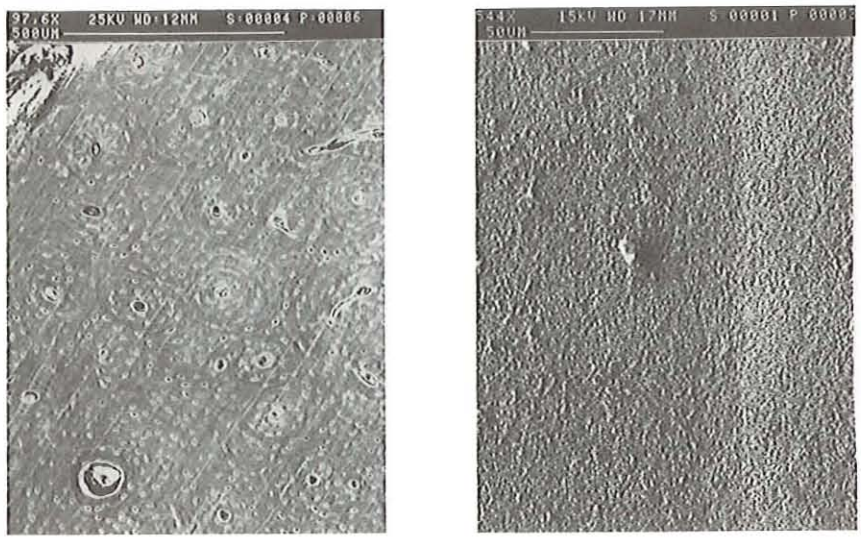
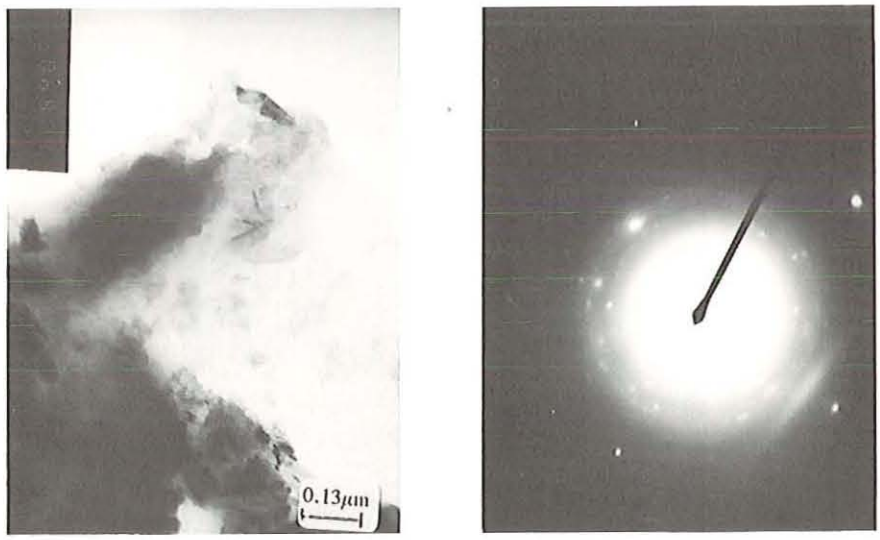


Fig.III.29 Surface of bone after certain TEM-preparation steps
a) after drilling, b) after grinding, c) after dimpling



a) b)

Fig. III.30. Surface of ion beam thinned specimen
a) before, b) after TEM-examination



a) b)

Fig.III.31. Dimpled bone specimen
a) TEM-micrograph,
b) selected area diffraction pattern (hydroxyapatite)

Fig.III.32. Damage in dimpled TEM-specimen

III.7.5 Fixation experiments

From the comparison of the polished specimens the following conclusions can be drawn:

- in average, fixation at room temperature leads to less cracks in the specimens than fixation at 4°C;

- if fixation is performed at room temperature, critical point dried specimens seem to have less cracks as compared with air-dried specimens. Further, during examination in the SEM, critical point dried specimens develop less cracks than air-dried specimens. A possible reason might be that critical point dried specimens are more thoroughly dried. This is confirmed by the results reported above. Air-dried specimens might still contain water or ethanol which will evaporate quite roughly in the vacuum of the SEM or the coating unit.

- Many specimens show dark rings at the circumference of the Haversian systems and/or colour differences within the osteons (dark to light inside-out). The specimens fixed in 40% ethanol are the only ones which do not show this feature.

No significant differences can be seen on the fracture surfaces of the differently fixed and dried specimens. Cells cannot be detected in the lacunae. The blood vessels in the Haversian canals are preserved but mostly they show shrinkage and more or less a destruction of their geometry due to rupturing along the Haversian canal even if this canal is vertical to the fracture surface.

It has to be born in mind that these results have been obtained with a very limited number of specimens. They therefore can only show trends but they are not adequate to derive final statements.

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IV.Kontrastierung von Knochen und Implantatwerkstoffen

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IV.1. Problemdefinition und Zielsetzung

Die quantitative Erfassung des Gefügeaufbaus stellt das Bindeglied zwischen der Herstellung und dem Einsatzverhalten eines Werkstoffes dar, mit dem das kostspielige Trial- und Error-Verfahren durch eine vom Verständnis geleitete Versuchsführung zur Entwicklung neuer Werkstoffe ersetzt werden kann.
Die quantitative Gefügeanalyse ist seit mehreren Jahrzehnten ein wichtiger Bestandteil der Kennzeichnung von Werkstoffen. Die Anwendung der Stereologie und der zur Datenerfassung für die Ermittlung stereologischer Kennwerte notwendigen Bildanalysen sind nicht auf die Werkstoffkunde und Werkstofftechnik beschränkt.
Mit denselben Verfahren werden z. B. in der Medizin und der Biologie die Morphologie von Geweben und anderen organischen Stoffen, in der Geologie und Mineralogie der Aufbau von Gesteinen und Mineralen analysiert.
Eine mikroskopische Betrachtung der Probenoberfläche nach dem Glätten im Auflicht-Hellfeld läßt gar keine oder nur sehr wenige Einzelheiten erkennen. Es sind entweder Präparationsartefakte oder solche Gefügeelemente, die sich durch ihre Eigenfarbe oder durch ihr unterschiedliches Reflexionsvermögen vom Umfeld unterscheiden. Analog einem latenten Bild in der Fotografie muß das unsichtbare Gefüge erst entwickelt und haltbar gemacht werden, bevor es ausgewertet werden kann.
Im Gegensatz zur Fotografie strebt man nicht unbedingt die Wiedergabe aller Gefügeelemente gleichzeitig an, sondern begnügt sich mit den jeweils wichtigsten, eigenschaftsrelevanten Bestandteilen. Das können Zweitphasen in einer Matrix, Korngrenzen, Versetzungen, Poren u.a. sein.
Da das menschliche Auge beleuchtete Gegenstände nur durch Helligkeitsunterschiede oder Farben wahrnimmt, müssen die Elemente eines Werkstoffgefüges zu ihrer Erkennbarkeit Helligkeits- oder Farbunterschiede aufweisen. Das Reflexionsvermögen und die Farbe der Phasen - sie stellen die wichtigsten Gefügeelemente dar - unterscheiden sich für eine Differenzierung nicht hinreichend voneinander; sie müssen konstrastiert werden [2,3].
Biologische Proben werden normalerweise vor den lichtmikroskopischen Untersuchungen nach unterschiedlichen speziellen Methoden eingefärbt. Bei anorganischem Material läßt sich durch geeignete Präparation der Probenoberfläche diese Wechselwirkung so beeinflussen, daß ein möglichst großer Kontrast zwischen den Phasen, Kristalliten oder Korngrenzen des Gefüges entsteht. Die Kontrastierungsverfahren beruhen entweder allein auf dem Einsatz optischer Hilfsmittel (Polarisation, Hell- und Dunkelfeldbeleuchtung, Filter, Phasenkontrast, Interferenzkontrast u.a.) ohne Änderung der Probenoberfläche [4] oder auf der Bildung bzw. Abtragung von Oberflächenschichten.

Abtragung erfolgt beispielweise über Materialzerstäubung durch positive Ionen beim kathodischen Ionenätzen [5,6,7,8], Schichtbildung dagegen beim Bedampfen [9] sowie in den meisten Fällen auch beim chemischen bzw. elektrochemischen Ätzen [3] und auch bei dem hier zu besprechenden Gaskontrastieren [10,11,12].
Für die quantitative Gefügeanalyse von Werkstoffen ist häufig eine Kontrastierung der angefertigten Schliffe erforderlich.
Als Verfahren bei optimaler Schonung der Probe wurde das Gaskontrastieren bekannt, das sich sowohl auf metallische wie auf nichtmetallische Proben anwenden läßt.
Im Prinzip werden dabei Gasionen an der Probenoberfläche angelagert, die bei verschiedenen Phasen unterschiedliche Schichtdicken ergeben, deren Interferenzfarben die gewünschten Kontraste liefern.
Zu klären ist die Anwendbarkeit dieses Verfahrens auf Knochenpräparate von Knochen mit implantierter Biokeramik.
Es ist eine Vakuumkammer herzustellen mit gekühlter Probenhalterung, geeigneter Gestaltung der Kathode und eventuell erforderlichen Blenden. Zum Betrieb gehört die Erzeugung, Messung und Konstanthaltung des erforderlichen Vakuums, sowie die Dosierung des Arbeitsgases.
Verschiedene Gase sind auf ihre Eignung zu prüfen, die geeigneten Betriebsspannungen sind zu ermitteln und Kontrastierungsversuche an vorliegenden Knochenpräparaten sind durchzuführen.

IV.2. Charakterisierung

IV.2.1. Knochen

Knochen können als Verbundwerkstoffe betrachtet werden. Ihr Gefüge, das aus anorganischem und organischem Material und Poren besteht, ist biologisch und mechanisch optimiert; ihr Wachstum ist u.a. mechanisch stimulierbar, wobei ein fibrillär-schuppiger Habitus bevorzugt wird. Etwa 30% des Feststoffanteils bestehen aus organischem Material, davon wiederum mehr als 90% aus Kollagen - einem fibrillären Skleroprotein.
Der Rest wird von einer amorphen Grundsubstanz, die verschiedene Verbindungen wie Polysaccharide u.a. enthält, gebildet.
Etwa 70% des festen Knochenmaterials sind anorganischer Natur und bestehen im wesentlichen aus Calciumphosphaten (Ca:P-Verhältnis=1,6....1,73), die in speziellen Knochenzellen und an Kollagenfibrillen gebildet werden. Hierfür wird eine Kristallkeimbildung durch Komplexe aus Kollagen und anorganischen Gruppen angenommen, die die Ausscheidung eines etwa zu 60% kristallinen Materials (Basis Apatitgitter) bewirkt. Dabei überwiegt im kindlichen Knochen Defektapatit, der allmählich durch Ionenaustausch ($2H^+ \longrightarrow Ca^{2+}$) zu Hydroxylapatit altert. Außerdem findet sich im Knochen auch Tricalciumphosphat (TCP) als Füllsubstanz [13].

IV.2.2. Implantatwerkstoffe

Unterschiedliche Ursachen können es erforderlich machen, Teile von Knochen temporär oder dauerhaft zu ersetzen bzw. Lücken auszufüllen.
Generell müssen Implantatmaterialien bestimmte Kriterien erfüllen (Abb. IV.1), um als biokompatible Werkstoffe eingesetzt zu werden. In Abbildung IV.2 sind die verschiedenen Typen dargestellt und erläutert.

- Werkstoff-Eigenschaften
  - mechanische Stabilität
  - thermisches Verhalten wie Knochen
  - chemische Stabilität gegenüber Körpermedien
  - biologische Stabilität gegenüber mikrobakteriellem Angriff
- Physiologisch verträgliches Verhalten
  - Biokompatibilität (-tolerant, - inert, - aktiv)
  - nichttoxisch etc. wirkend
- Herstellung
  - ökonomisch vertretbarer Aufwand
  - Anwendung üblicher Techniken
  - oberflächenvergütbar
- Anwendung
  - einfache Handhabung
  - mittels einfacher Methoden sterilisierbar

Abb.IV.1 Kriterien für Implantatmaterialien

1	-	resorbierbare Implantate - Aufzehrung
1 2	-	biotolerante Implantate - relativ verträgliche Korrosionsprodukte
2	-	bioinerte Implantate - kaum Reaktionsprodukte
3 4	-	bioaktive Implanate - Reaktionsgrenzschicht; Verwachsung

Abb.IV.2 Verhalten von Implantaten in biologischer Umgebung:
1=Korrosion; 2=Einscheidung; 3=Bildung einer dynamischen Grenzschicht; 4=Verwachsung

In Abbildung IV. 3 sind eine Reihe von Implantataufgaben an Beispielen und in Abbildung IV. 4 die konventionell genutzten Implantatmaterialien dargestellt.

Im Rahmen der Arbeit von Wihsmann und Berger [13] wurden Untersuchungen nach physikalischen und chemischen Eigenschaften bzw. biologischem Verhalten der Systeme

P_2O_5-CaO-Al_2O_3	(PCA)
P_2O_5-CaO-SiO_2	(PCS)
P_2O_5-CaO-MgO	(PCM)
P_2O_5-CaO-Al_2O_3-SiO_2	(PCAS)

durchgeführt.

Aufgabe	Beispiel	Mittel	Internationaler Stand	offene Fragen
a) temporäre Stützfunktion	Röhrenknochen-bruch	Titanlegierung Schiene und Schraube	im allgemeinen ausreichend	Unverträglichkeit (akute Toxizität)
b) temporäre Ausfüllung	Wurzelresektion, kleine Knochen-defekte	resorbierbares Material (TCP), Körnungs-reaktionen	im Tierversuch gelöst, erste klinische Erprobung	Korrelation zwischen Resorption und Wachstum ist nicht ausreichend kontrollierbar
c) dauerhafte nichttragende Funktion	Knochendefekte (z.B. Orbitalboden)	Bioglas oder Biovitrokeram	im Tierversuch gelöst, erste klinische Erprobung	weitere Optimierung
d) dauerhaft tragende Funktion	Oberschenkel-gelenkersatz	Korundprothese (Biokeramik); "Knochenzement" PMMA	z.Z. mit einer wahrscheinlichen Anwendungsdauer von 15 Jahren	keine echte Knochenverbindung, z.T. Alterung des PMMA bzw. Unverträglichkeit
e) dauerhaft tragende Funktion mit vollständigem Verwachsen mit dem Knochen bzw. Aktivierung des Knochenwachstums	Zahnwurzelimplan-tate, Unterkiefererersatz	Biovitrokeram, Bioglas	Erfolge im Tierversuch	bislang unzurei-chende Festigkeit (Verbunde?) evtl. kritisch für Weichgewebe
f) Verbundmaterial (Ultraschallver-schweißen), (Bioaktiver Knochenzement)	Knochenbrüche (Implantatverbund und Knochen-Implantat-Verbund)	Bioglas, Biovitrokeram (Körnung)	erste Tieranwen-dungen	Korrosion der nicht bioaktiven Kompo-nente; Oberflächenanteil der bioaktiven Kom-ponente sehr hoch

Abb.IV.3 Knochendefekte und Implantattypen

Werkstoff-gruppe	Metalle	organische Polymere	anorganische nicht-metallische Werkstoffe	Verbunde
Beispiele	Platin Platin-Iridium Gold Silber Titan Titan-Legierungen (mit Al, Mn, V) Co-Cr-Legierungen Co-Cr-Mo-Legierungen Co-Cr-Mo-Ni-Legierung Tantal Rostfreier Stahl (z.B. FeNiCrMn) etc.	Polymethylmethacrylate (PMMA) Teflon Polypropylen Polyurethane Kollagen, Lösungen Gelantine etc.	Knochenporzellan Al_2O_3-Keramik TiO_2-Keramik Hydroxylapatit (HA) Tricalciumphosphat (TCP) bioaktive Gläser Vitrokeramiken und Sinterglaskeramiken Calciumaluminate $MgAl_2O_3$-Spinell Glaskohlenstoff	Proplast PTFE (C-Fasern) Ti-Leg., pyrolyt. Kohlen-stoff Bioglas-beschichtete Al_2O_3-Keramik und Cr-Co-Mo-Legierung HEMA-Hydrogel-gefüllter, poröser Apatit
Vorteile	hohe Biegefestigkeit guter Röntgenkontrast	angepaßter E-Modul	bioinertes bis bioaktives Verhalten im Gewebe, hohe Verschleißfestig-keit, (Oxidkeramik)	Vorteile bzw. Nachteile können nicht verallgemei-nert werden
Nachteile	Korrosion (Metallose)	rasche Alterung, geringe Dauerfestigkeit, toxische Monomere	geringe Biegefestigkeit (hohe Sprödigkeit)	

Abb.IV.4 Konventionell genutzte Knochenimplantatmaterialien [13]

Es wurden werkstoffkundliche Untersuchungen zur Korrosion, histologische Fluoreszenzmessungen, die durch Einspritzen von Tetraverin in den noch lebenden Körper vorbereitet und mittels UV-Licht-Anregung zu kontrastreichen Bildern führen, Untersuchungen zur Bioaktivität mit Hilfe der Elektronenstrahlmikroanalyse bzw. Rasterelektronenmikroskopie sowie Ausstoß- oder Haftkraftmessungen in Abhängigkeit von der Verweilzeit in den entnommenen Knochen-Implantat-Verbundproben durchgeführt. [14].

IV.3 Kontrastierungsverfahren

IV.3.1. Ätzen

IV.3.1.1. Methodenübersicht

Nach dem Endpolieren (Fertigpolieren) ist der Anschliff nur dann zur makroskopischen bzw. mikroskopischen Gefügebeobachtung geeignet, wenn sich die Gefügebestandteile bereits zu erkennen geben. Das trifft jedoch zu, wenn die Gefügebestandteile das Licht mit verschiedener Intensität reflektieren, wobei die Unterschiede im Reflexionsvermögen mindestens 10% betragen müssen. Die ist gegeben, wenn die Gefügebestandteile eine ausreichende Eigenfarbe haben oder wenn sie durch die vorausgegangene Schleif- und Polierbehandlung genügend unterschiedlich abgetragen wurden (Reliefwirkung) oder wenn Risse, Poren, Lunker und dergleichen festgestellt werden sollen.
Im allgemeinen zeigt eine fertigpolierte Schliffffläche kein Gefüge, da das auffallende Licht nahezu gleichmäßig reflektiert wird; die geringen Reflexionsunterschiede zwischen den Gefügebestandteilen liegen unter der Wahrnehmungsgrenze des menschlichen Auges.
Somit muß das Gefüge in der Regel zur Sichtbarmachung kontrastiert werden. Für diese Kontrastierung hat sich in der gefügeanalytischen Präparationstechnik der Begriff "Ätzen" eingebürgert, obgleich es sich nicht immer um Ätzvorgänge im eigentlichen Sinne handelt (Lösungsvorgänge, bei dem Teile der Oberfläche stärker angegriffen werden als andere).

Die Möglichkeiten zur Gefügekontrastierung sind zahlreich und ganz verschiedenartig. Je nachdem, ob die Kontrastierung ohne oder mit einer Veränderung der polierten Schliffffläche vorgenommen wird (Abb. IV 5), unterscheidet man zwischen "optischen" oder "elektrochemischen (chemischen)" bzw. "physikalischen" Ätzmethoden.

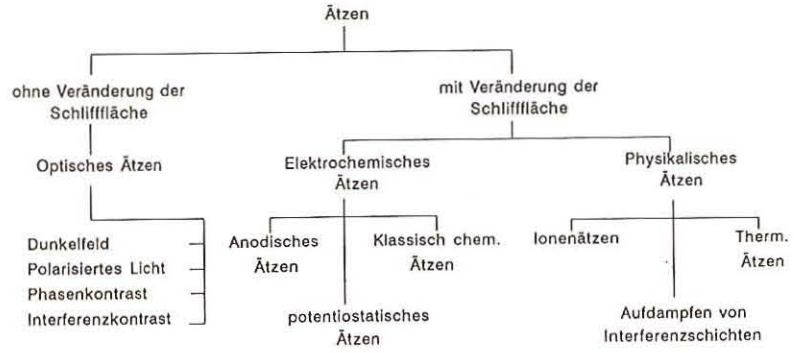


Abb.IV.5 Methoden zum gefügeanalytischen Ätzen

IV.3.1.2. Optisches Ätzen

Das "Optische Ätzen" beruht auf der Anwendung spezieller Beleuchtungsverfahren, die alle das Prinzip des Köhlerschen Strahlenganges beibehalten, welches auch der üblichen Hellfeldbeleuchtung zugrunde liegt.

Diese Beleuchtungsverfahren - im einzelnen handelt es sich um Dunkelfeld, Phasenkontrast, Interferenzkontrast und polarisiertes Licht - sind in vielen der handelsüblichen Mikroskope zu realisieren und meistens ohne merkliche Beeinträchtigung der Bildgüte leicht mit einigen Handgriffen gegeneinander auszutauschen.

IV.3.1.3. Elektrochemisches (chemisches und physikalisches Ätzen)

Beim elektrochemisches Ätzen metallischer Werkstoffe - oft auch als "chemisches" Ätzen bezeichnet - laufen elektrochemische Vorgänge ab, Reduktions-Oxidationsprozesse (Redoxprozeß).
Physikalische Grundvorgänge werden oft zur Kontrastierung von Gefügen ausgenutzt. Sie haben den Vorteil, daß sie saubere und von chemischen Reststoffen rückstandfreie Oberflächen ergeben. Sie bieten auch Vorteile, wo das elektrochemische Ätzen oft mit Schwierigkeiten verbunden ist, z. B. bei hohen Unterschieden in den elektrochemischen Potentialen der Gefügebestandteile, wie sie in Plattierungen vorkommen, bei porösen Materialien und bei der Untersuchung von Keramiken.

Bei der kathodischen Zerstäubung, auch Ionenätzen genannt, wird der Kontrast durch Austritt von Atomen aus der Schliffoberfläche erreicht. Zur Ablösung aus dem umgebenden Verband muß den einzelnen Atomen eine hierfür ausreichende Energie (Ablöseenergie) zugeführt werden. Diese Bedingungen treffen bei der kathodischen Zerstäubung zu. Hierbei wird die Probenoberfläche im Vakuum mit energiereichen Ionen (Betriebsspannungen von 1 bis 10 kV) beschossen, die eine, das Gefüge entwickelnde Materialzerstäubung bewirken.

Auch das thermische Ätzen, das in der Hochtemperaturmikroskopie ausgenutzt wird, beruht teilweise auf dem Austritt von Atomen aus der Oberfläche infolge thermischer Energiezufuhr. Allerdings ist die dominierende Triebkraft beim thermischen Ätzen die Bildung von leicht gewölbten Gleichgewichtsflächen der einzelnen Körner mit minimaler Oberflächenspannung [15].
Die Gefügesichtbarmachung durch aufgedampfte Interferenzschichten wird zwar oft zu den optischen Ätzmethoden gezählt; da sie jedoch keinen Eingriff in den Mikroskopstrahlengang, sondern eine Behandlung der fertigpolierten Schlifffläche erfordert, unterscheidet sie sich von den eigentlichen optischen Ätzmethoden und wird besser hier erwähnt.

Die Schlifffläche wird im Vakuum mit einer interferenzfähigen Schicht bedampft. Als Schichtwerkstoffe werden Substanzen mit hohem Brechungsindex wie z.B. ZnSe, TiO_2 usw. eingesetzt. Die Wirkung einer solchen Interferenzschicht besteht darin, daß die durch Interferenz geschwächte Wellenlänge an der Grenzfläche Objekt/Aufdampfschicht Mehrfachreflexionen erleidet, wodurch eine Kontraststeigerung zwischen den Gefügebestandteilen erfolgt. Neuerdings stehen sogenannte Gasreaktionskammern zur Verfügung, in denen das Aufdampfen sehr präzise durchgeführt werden kann [15].

IV.3.1.4. Gaskontrastieren

Beim Gaskontrastieren befindet sich die Probe in einer Vakuumkammer, in der eine Glimmentladung stattfindet. Abb. IV.6 zeigt den schematischen Aufbau einer Kontrastierkammer. An der Kathode (a) aus einem zunächst beliebigen leitenden Material liegt eine negative Gleichspannung von 1000 bis 2000 Volt. Das Gehäuse mit den Probenhaltern (c) ist geerdet, so daß zwischen der Kathode und der anodisch geschalteten Probe eine Potentialdifferenz entsteht.

Zu Beginn einer Beschichtung wird die Kammer (e) durch eine Vakuumpumpe auf einen Druck von ca. 10 Pa evakuiert. Mit Hilfe des Nadelventils (d) läßt sich der Partialdruck des jeweils gewählten Arbeitsgases einstellen.

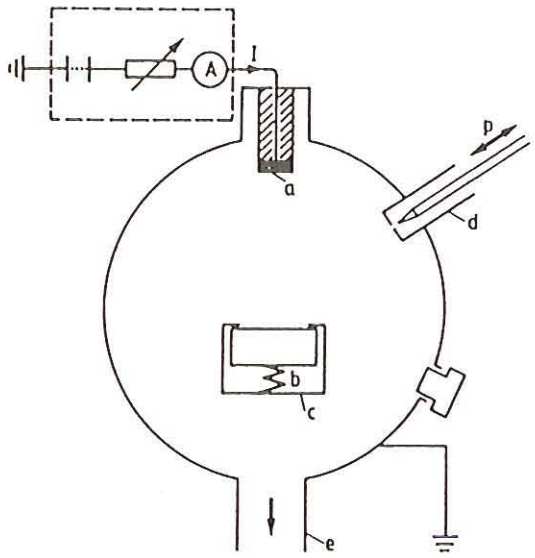


Abb.IV.6 Schematische Darstellung der Gasionenkammer

Nach Anlegen der Hochspannung zündet die Glimmentladung, die zur Abscheidung einer interferierenden Schicht auf der Probenoberfläche führt [16].
Daß solche Schichten Farbkontraste zwischen verschiedenen Gefügebestandteilen hervorrufen können, ist bekannt [17,18].
Grundsätzlich ist solche Schichtbildung nicht auf eine bestimmte Werkstoffgruppe beschränkt, sie findet also

- sowohl auf metallischen Werkstoffen, einschließlich intermetallischer Phasen
- auf Verbundwerkstoffen, einschließlich Hartmetallen
- wie auch auf keramischen Werkstoffen

statt.

Die Reaktionstendenz dieser verschiedenen Werkstoffe mit dem Kontrastiergas unter Betriebsbedingungen ist unterschiedlich. Demzufolge kann Schichtbildung

- entweder durch Wechselwirkung (Reaktion, Löslichkeit) zwischen dem Kontrastiergas und den Bestandteilen der Probenoberfläche ("Reaktionsschichten")
- oder durch Adsorption bzw. Sublimation der Gasatome bzw. -ionen oder -moleküle ohne Wechselwirkung an der Probenoberfläche ("Adsorptionsschichten")

erfolgen.

Im ersten Fall bestimmen die Reaktions- bzw. Lösungsenthalpien und deren Temperaturabhängigkeit die Schichtbildungskinetik. Da diese Enthalpien für die verschiedenen Gefügebestandteile insbesondere bei Verbundwerkstoffen, aber auch generell bei mehrphasigen Werkstoffen unterschiedlich sind, muß mit

- Unterschieden in der stofflichen Zusammensetzung und Struktur der Schichten und
- unterschiedlichen Wachstumsgeschwindigkeiten, d.h. unterschiedlichen Schichtdicken

auf verschiedenen Gefügebestandteilen gerechnet werden.

Temperaturerhöhungen, die die Reaktionsfreudigkeit stegern, können beim Gaskontrastieren durchaus auftreten.
Sublimation wäre dann begünstigt, wenn die Probe nicht mit der Anode verbunden und daher kälter als die Umgebung wäre, weil Erwärmung entfällt [19]. Im Gegensatz zu Reaktionsschichten sollten Adsorptionsschichten annähernd gleiche stoffliche Zusammensetzung, Struktur und gleiche Dicke bzw. Wachstumsgeschwindigkeit auf allen Gefügebestandteilen haben.

Die im Rahmen der Arbeit von Nold und Ondracek [20] durchgeführten Auger-spektroskopischen Untersuchungen zum Schichtaufbau bei gaskontrastierten Hartmetallen ergaben, daß

- von der ursprünglichen, unbeschichteten Oberfläche ausgehend eine Schicht wächst, in der die Elemente des Kontrastiergases und des Kathodenmaterials dominieren (vgl. Abb. IV. 7, Zone x_0-x_1).
- Elemente des Kontrastiergases während des Kontrastiervorganges in das Material eindringen und damit die ursprüngliche Oberfläche in ihrer stofflichen Zusammensetzung verändert wird (vgl. Abb.IV. 7, Zone x_1-x_1+dx)
- Elemente des Grundmaterials in der aufwachsenden Schicht vorliegen können, daß dies aber von der Zusammensetzung der betreffenden Phase des Grundmaterials abhängt (vgl. Abb. IV. 7, oben: HfC-Phase/kein HfC in der Schicht; Abb. IV. 7,
  unten: Co-Phase/Co in der Schicht).

Die beim Kontrastieren entstehende Schicht besteht demnach aus zwei Zonen, einer auf dem Material gewachsenen Zone und einer in das Material hineinreichenden Diffusionszone und ist insgesamt, wie auch hinsichtlich dieser Zonen, inhomogen bzw. kann aus verschiedenen Phasen oder Gefügebestandteilen stofflich unterschiedlich zusammengesetzt sein.

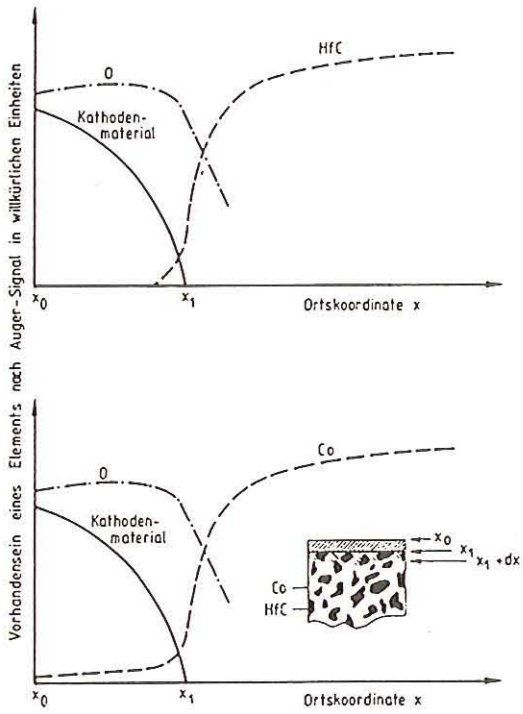


Abb.IV.7 Schichtaufbau bei gaskontrastiertem Hartmetall

Der Effekt der Gaskontrastierung läßt sich durch Reflexionsbetrachtungen erklären, was in der Literatur ausführlich behandelt wurde [24].

IV.3.2. Färben

Die histologische Beurteilung wird an 3 bis 8 µm starken unentkalkten Dünnschliffen vorgenommen.
Die Präparate entstehen nach vorausgegangener Einbettung der getrimmten Explanate in Polymethylmetacrylat durch Schneiden mit einer Diamantsäge und anschließendes fraktioniertes Schleifen.
Vor der Eindeckung der Dünnschliffe erfolgen Färbungen.

Die anfangs bei einigen Präparaten vorgenommenen Lichtgrün- und Methylblau-Färbungen wurden zugunsten einer einzigen Standardfärbung mit Toluidinblau aufgegeben, denn das Toluidinblau macht durch einen Metachromasieeffekt die färberische Differenzierung der Hartgewebsanteile in Abhängigkeit von ihrem Alter möglich.

Die aktuelle Farbe des Toluidinblaus hängt vom Polymerisationsgrad des Farbstoffes ab, d.h. in niedriger Konzentration zeigt er die orthochromatische Farbe Blau, in höherer Konzentration die metachromatische Farbe Rot. Toluidinblau wird im fixierten Gewebe an die Glykosaminoglyklame (frühere Bezeichnung: saure Mukopolysaccharide), gebunden

Da im Osteoid der Gehalt an Glykosaminoglykanen insgesant anteilmäßig gering ist, wird wenig chromotrope Substanz gebunden, so daß das Osteoid im histologischen Präparat blau erscheint. Mit zunehmendem Reifegrad des Knochens erhöht sich sein Gehalt an Glykosaminoglykanen. Folglich stellt sich ein junger Lamellenknochen im Stadium der Muration violett, ausgereiftes Knochengewebe in Regionen mit geringer Umbauaktivität dagegen ausgeprägt metachromatisch, d.h. rot, dar.

Die gute Abgrenzbarkeit des Bindegewebes - es zeigt ein tiefes Blau - ist ein weiterer Vorteil dieser Färbemethode [26].

IV.3.3. Vergleich der beiden Kontrastierungsverfahren

Verschiedene Autoren haben eindrucksvoll dargestellt, daß die Ultrastruktur des Knochenminerals und ebenso die Ultramorphologie des Keramik-Knochen-Verbundes maßgeblich von der Technik der vorausgegangenen Präparation abhängt. Diese erfolgt in der Regel in wäßriger Lösung, wodurch unvermeidbar Kristallwachstum und Rekristallisationsvorgänge in Gang gesetzt werden. Auf diese Weise wird die Realstruktur von Mineralphasen im ultramikroskopischen Befund nachhaltig verfälscht.

Derartige methodisch bedingte Artefaktbildungen können nur durch Ionen-Ätzung bzw. Gaskontrastierung vermieden werden - ein Verfahren, bei dem die Präparation der Probenoberflächen als trockener Prozeß und überdies ohne mechanische Kraftwirkung (Schneiden) erfolgt, so daß auch Mikrorisse und Kleinfragmentierungen vermieden werden [27].
Der Beschreibung dieses Verfahrens und seiner Anwendung auf Knochen, Biowerkstoffe und Knochen-Implantatwerkstoff-Grenzflächen gelten daher die folgenden Ausführungen.

IV.4. Die Gaskontrastierapparatur

Gasentladung, kurz Entladung, ist der Transport von Ladungsträgern (freie Elektronen und Ionen) durch Gase. Wegen ihrer größeren Beweglichkeit sind die Elektronen dabei mit etwa 99% beteiligt.

Der größte Teil des Raumes zwischen den Elektroden, die zur Schaffung eines den Transport bewirkenden elektrischen Feldes erforderlich sind, ist bei einer Gasentladung mit einem Plasma ausgefüllt.

Es gibt Ausnahmefälle mit induktiv oder kapazitiv angeregten HF-Entladungen (elektrodenlose Gasentladung).

Bei einer Gasentladung bilden sich in Elektrodennähe relativ dünne Raumentladungsschichten aus und zwar so, daß die Kontinuität des Stromes gewährt wird.
Wenn bei einer Gasentladung eine Leuchterscheinung auftritt, spricht man auch von einer Glimmentladung.

Gasentladungen können nach verschiedenen Gesichtspunkten eingeteilt werden (Abb. IV. 8).
Als den Ladungsträger- und Energiehaushalt bei Gasentladung bestimmende Elementarprozesse unterscheidet man:

- Generation von Ladungsträgern im Volumen, z.B. Ionisation von Molekülen durch Stoß von Elektronen, Photonen oder schnellen neutralen Teilchen

(summarisch beschrieben durch die Generationsrate) und an Grenzflächen (Elektronenemission);
- Umwandlung von Ladungsträgern durch Anlagerung von Elektronen an Moleküle (Bildung negativer Ionen), Umladung (Ladungaustausch zwischen positiven Ionen und Neutralteilchen);
- Vernichtung von Ladungsträgern im Volumen (summarisch beschrieben durch die Rekombinationsrate) und an Grenzflächen durch Rekombination an Oberflächen oder Neutralisation an Elektroden;
- Stoßprozesse bei der Ladunsträgerbewegung, beschrieben durch die Driftgeschwindigkeit, die Feldstromdichte und die Diffusionsstromdichte;
- Emission von Photonen (Strahlungsemission) und Absorption von Photonen, verbunden mit Anregung [28, 29, 30, 31].

Gasentladung	Kennzeichen	Beispiele
Selbständige	Die zur Existenz nötigen Träger werden von der G. selbst gebildet	Kaltkathoden-entladung z.B. in Glimmlampen
Unselbständige	Eine äußere Energiequelle (z.B. Glühkatode, einfallendes Licht) trägt unmittelbar zur Trägererzeugung bei	G. in Leuchtstoff-lampen
Stationäre	Strom und Spannung zeitlich konstant	alle Gleichstrom-entladungen
Nichtstationäre	Strom und Spannung zeitlich unveränderlich	Funke, Korona-entladung
Niederdruck-entladung	Gasdruck < 100 Pa	G. in Leuchtstoff-lampen
Hochdruck-entladung	Gasdruck > 100 Pa	Hg- oder Xe-Hochdrucklampe

Abb.IV.8 Einteilung von Gasentladungen

Der vorliegenden Arbeit liegt eine selbständige stationäre Entladung zugrunde, die ohne zusätzliche äußere Energiequellen selbsttätig abläuft.
Der einfachste Fall ist bei einem Gas zwischen zwei parallelen, ebenen Metallplatten gegeben. Herrscht zwischen den Platten ein geringes Potential, so ist das Gas praktisch ein vollkommener Isolator, bei Potentialerhöhung bis zu sog. Durchschlagspanung wird das Gas plötzlich leitend. Dieser Übergang von dem Isolator- zu den Leitereigenschaften kannin verschiedener Weise erfolgen.
In gewöhnlicher Luft von 1 at (105 Pa) geht bei einem Plattenabstand von z.B. 1 cm bei Erreichen der Durchschlagspannung ein Funke - d.h. ein kurzzeitiger Strom von hoher Intensität - über, wobei der ursprünglich sehr hohe Widerstand auf einen kleinen Wert von 1 Ohm absinkt; Zwischenwerte lassen sich in stationärer Form nicht verwirklichen.
Bei niedrigem Gasdruck (ca. 10 Pa) können im Übergangsbereich Isolator/Leiter auch selbständige Ströme (< 1 mA) auftreten. Der Widerstand des Gases beträgt in diesem Fall viele

Megaohm. Die Entladungen bei derart kleinen Strömen werden nach dem englischen Physiker Towsend benannt [32], der diese Erscheinung eingehend studierte.

Die Vorionisation, d.h. die Erzeugung der Ladungsträger vor und während des Entladungsaufbaus, erfolgt bei Verwendung einer ungeheizten Kaltkathode.

In der Kaltkathodenanordnung fließt bei geringer Gleichspannung zwischen Kathode und Anode schon ein schwacher Strom. Die Entladung ist unselbständig und hört auf, sobald die Ionisierungsursache verschwindet. Wird die Entladungsspannung zwischen Kathode und Anode erhöht, erzeugen die freien Elektronen durch Stoßionisation des Füllgases weitere Ladungsträger.

Die positiven Ionen bilden vor der Kathode eine Raumladung, zwischen Kathode und Raumladung steigt das Potentials an, wodurch die positiven Ionen eine genügend hohe Geschwindigkeit erhalten. Die auf die Kathode aufprallenden Ionen lösen dort Elektronen heraus. Aus der Kathode ausgetretene Elektronen werden auf eine zur Ionisierung ausreichende Energie beschleunigt. Durch die lawinenartig wachsende Anzahl von Ladungsträgern zündet die Entladung, der Strom steigt (stark) an, und eine selbständige Entladung setzt ein [31].

IV.4.2. Der Kammeraufbau

Die Gaskontrastierungsapparatur ist eine Vorrichtung zur Oberflächenbehandlung einer metallographischen, keramischen oder biologischen Probe mittels kathodischem Ionenätzen, Gasätzung oder zur Bedampfung von Beschichtungsproben, wobei zwischen den Oberflächen der Probe und einer Elektronenquelle eine elektrische Spannung angelegt und Gas in den Zwischenraum von Probenoberfläche und Elektronenquelle eingeleitet wird. [33].

Beim Ionenätzen wird die Abtragung des Materials von der Probenoberfläche durch Zerstäubung mit Hilfe positiver Ionen, dem kathodischen Ionenätzen, durchgeführt [5].
Im Gegensatz zu diesem kathodischen Ionenätzen beruht die Gasätzung auf der Anlagerung von Gasionen an die polierte Probenoberfläche.

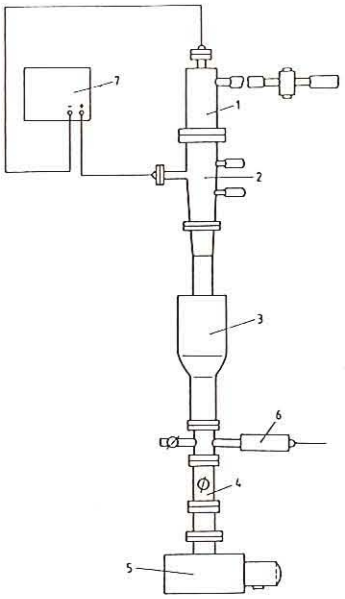
Die Gasionen kommen hauptsächlich durch Anlagerung von Elektronen an die neutralen Gasmoleküle zustande, also durch Oberflächenionisation, wobei ein relativ geringer Elektrodenabstand von ca. 10 mm, eine relativ geringe Spannung von 1 kV und ein relativ hoher Ätzgasdruck von mehr als 0,5 Torr (65 Pa) erforderlich ist. Die negativen Ionen wandern zur Anode. d.h. zur anodisch geschalteten Probe und führen dort zur Schichtbildung auf der Materialoberfläche.

Es wird im folgenden anhand eines Ausführungsbeispiels mittels der Abbildungen IV. 9 und IV. 10 näher erläutert.

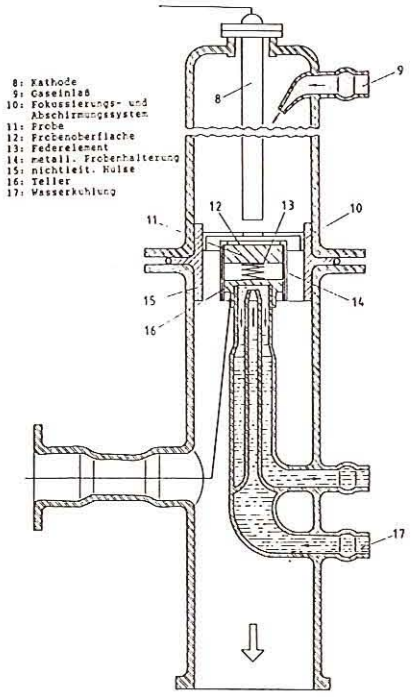
In Abb. IV. 9a ist eine Gesamtübersicht der erforderlichen Vorrichtung dargestellt. es handelt sich um eine aufrecht stehende, aus Glas gefertigte Anordnung mit den Gehäusen 1 und 2, einer an das Gehäuse 2 angeschlossenen Kühlfalle 3 sowie einem Teil 4 mit einem Entlüftungshahn.

Der Teil 5 ist eine Vakuumpumpe, eine Drehschieberpumpe der Firma Leybord-Heraeus. Außerdem wurde ein Meßgerät 6 mit Bereich 10^{-3} Torr (der Firma Leybord-Heraeus) zur Arbeitsdruckmessung benutzt.

Als Hochspannungsgerät wurde ein HCN-140-6500 der Firma Fug angeschafft. Das Hochspannungsgerät HCN-140-6500 (Hochspannungs-Chopper-Netzgerät) ist in der Lage, 6500 V



(a)



(b)

Abb.IV.9b Gesamtansicht (a) und Schnitt durch die Gehäuseteile 1 und 2 (b) der Gaskontrastiereinrichtung

mit 20 mA abzugeben. Es kann sowohl auf Konstant-Strom wie auf Konstant-Spannung geregelt werden.

Der Übergang von Konstant-Spannung auf Konstant-Strombetrieb und umgekehrt erfolgt automatisch mit scharfem Knick. Der Regelzustand wird durch LED`s bei den jeweiligen Einstellpotentiometern angezeigt.

Am oberen Teil 1 sind die Kathode und der Gaseinlaß mit Schlauch, am unteren Teil 2 die Anodenzuführung sowie die Zuführungen für die Probenkühlung angeordnet.

Der Schlauch für den Gaseinlaß zum oberen Teil 1 ist mit einem Nadelventil verbunden, mit welchem die Zufuhr des Arbeitsgases geregelt eingestellt werden kann.

Die Stromzuführungen sind mit dem positiven bzw. negativen Pol des geregelten Netzhochspannungsgerätes 7 verbunden.
Abb. IV. 9b zeigt einen Schnitt durch die Gehäuseteile 1 und 2, die aus Glas hergestellt sind.

Es wurden 3 Kathoden angefertigt (vgl. Abb. IV. 10):
eine Eisenhohlkathode, eine Hohlkathode aus Platin-Folie und eine Platindraht-Kathode

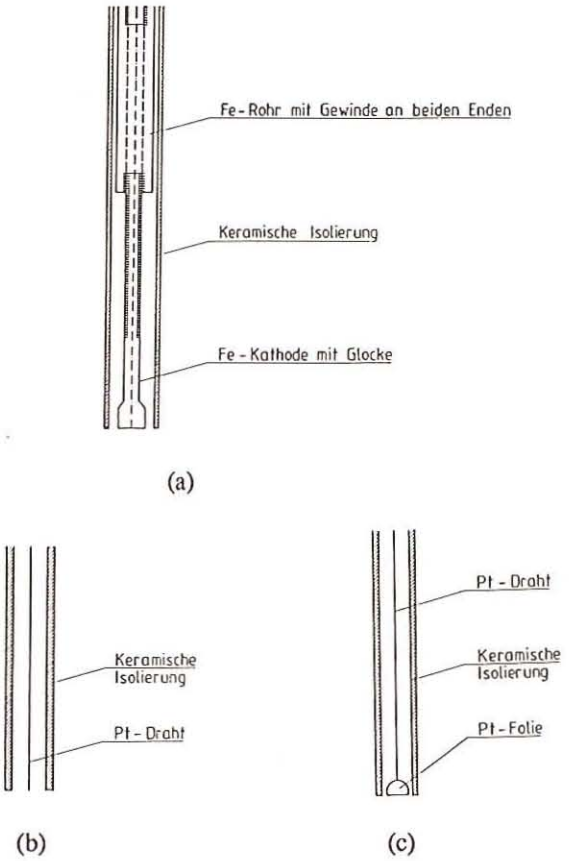


Abb.IV.10 Fe - (a), Pt-Folien - (b) und Pt-Draht-Kathode (c)

IV.5. Probenpräparation

IV.5.1. Präparationsfolge

Eine mikroskopierfähige Untersuchungsfläche an einer gefügeanalytischen Probe entsteht durch Anwendung einer Folge von Bearbeitungs- und Behandlungsverfahren in einem mehrstufigen Präparationsprozeß (Probeneinfassen, Grobschleifen, Feinschleifen, Läppen). Jede Anwendung eines solchen Verfahrens an einer Werkstoffprobe bedeutet einen "inneren Eingriff" mit beabsichtigten Gefügeveränderungen. Art und Ausmaß dieser Schädigungen sind im allgemeinen bekannt und begünstigen Fehtinterpretationen des mikroskopischen Untersuchungsbefundes. Die gefügeanalytischen Bearbeitungsverfahren sind sehr zahlreich und ihre Mechanismen grundverschieden, so daß hinsichtlich der unvermeidlichen Gefügeveränderungen in den oberflächennahen Randzonen qualitative und quantitative Unterschiede zu erwarten sind. Die Kenntnis der bei der Präparation ablaufenden Vorgänge und Wechselwirkungen kann Mißerfolge durch falsche Präparation stark einschränken und damit die Ausschußquote senken.
Das Herangehen an eine Untersuchungsaufgabe erfolgt weitgehend empirisch und ist stark von den Fertigkeiten und dem Geschick des Präparators abhängig. Auch gegenwärtig erfordern "schwierige" Proben Kunstfertigkeit, wenn sie exzellent präpariert werden sollen [34,35]. Es gilt der Grundsatz: Präpariere so gut wie nötig und nicht so gut wie möglich [36].
Abb. IV. 11 veranschaulicht den in sieben Stufen gegliederten Gesamtprozeß. Nicht in jedem Fall müssen alle Stufen in der angegebenen Reihenfolge durchlaufen werden. So ist z.B. das Reinigen der Proben, das hier als Schlußreinigung aufzufassen ist, auch schon nach solchen Behandlungen durchzuführen, bei denen die Gefahr des Verschleppens von Abrasivstoffen, Abrieb oder Chemikalienresten in den nächsten Präparationsstufen besteht.

Präparationsstufe		Wirkprinzip	Bearbeitungs- bzw. Behandlungsverfahren
Probenvorbereitung	Probenentnahme	Schmelzen	Brennschneiden, Reibtrennen, Elektronen-Plasmastrahltrennen, Elektroerosionstrennen
		Brechen	Abschlagen
		Scheren	Ab-, Ausschneiden
		Spanen	Abstechen, Sägen, Trennschleifen, Pulverstrahl-, Ultraschalltrennen
		chem. Lösen	Fadentrennen
		elektrochem. Lösen	Elysiertrennen
	Kennzeichnen	Druckumformen	Schlagstempeln
		Schmelzen, Verdampfen	Elektrogravieren
		Spanen	Einritzen, Vibrationsgravieren
		Adsorbieren	Beschriften
	Einfassen	Klemmen	Klammern
		Vernetzen, Binden	Umgießen, Imprägnieren, Formpressen
		chem. Abscheiden	Metallisieren
		elektrochem. Abscheiden	Galvanisieren
Probenbearbeitung	Ebnen	Spanen	Flachschleifen, Läppen, Mikrotomieren, Feinfräsen, Feindrehen
	Glätten	Spanen, Druckumformen	mechanisches Polieren
		chem. Lösen	chem. Polieren
		elektrochem. Lösen	elektrolytisches Polieren
		chem. Lösen	chem.-mechan. Polieren
		Spanen	Ätzpolieren
		elektrochem. Lösen	elektrolyt.- mechan. Polieren
		Spanen	
Probennachbehandlung	Reinigen	Desorbieren	Abbürsten, -putzen, -reiben
		Emulgieren	Waschen, US-Waschen
		chem. Lösen	Entfetten, Spülen, Dekapieren
		Verdunsten	Trocknen
	Kontrastieren	chem. Lösen	Lösungsätzen
		elektrochem. Lösen	anod. Ätzen, potentiost. Ätzen
		Oxydieren	Anlaßätzen, Reaktionsätzen
		Erodieren	Ionenätzen
		Adsorbieren	Aufdampfen, Sputtern

Abb.IV.11 System der gefügeanalytischen Probenvorbereitung für die Lichtmikroskopie

Ein Schema der gefügeanalytischen Probenpräparation ist in der Abb. IV. 12 dargestellt.

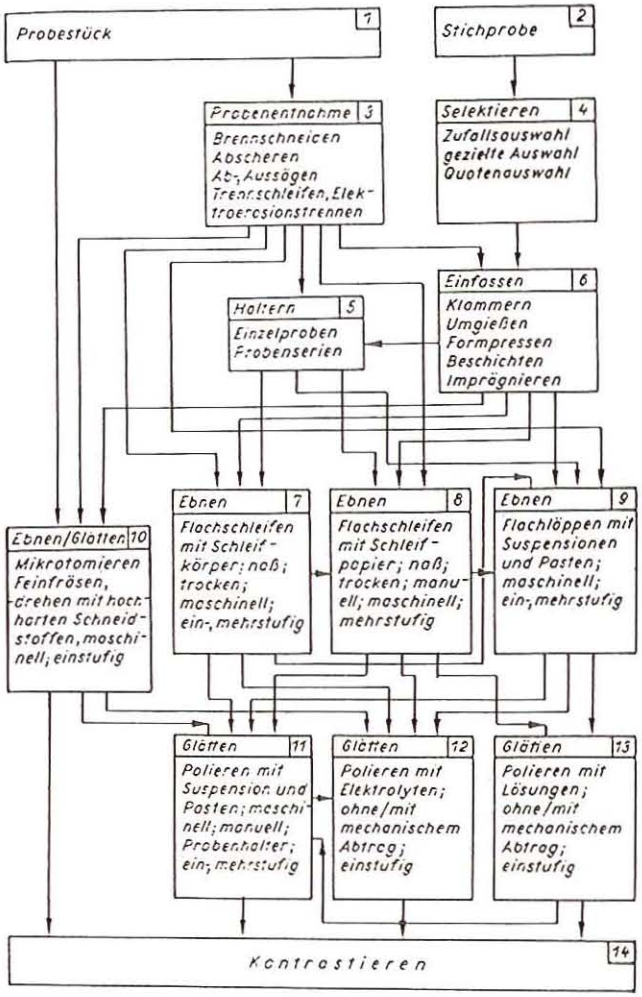


Abb.IV.12 Ablaufschema der gefügeanalytischen Probenpräparation

IV.5.2. Präparation poröser Werkstoffe

Poren entstehen bei der Werkstoffherstellung und liegen in aller Regel in Sinterwerkstoffen vor. Sie könnten bei Implantatwerkstoffen zur Aufnahme von Gewebe dienen, sind beim Knochen immer vorhanden und daher präparativ zu erfassen.
Es ist zweckmäßig, poröse Proben kalt und im Vakuum einzubetten.

Dadurch wird erreicht, daß die vorhandenen Hohlräume mit dem Harz infiltriert bzw. imprägniert werden. Da bei porösen Proben diesem Präparationsschritt besondere Bedeutung zukommt, sollen Vakuum-Imprägniergerät und Verfahren beschrieben werden.
Zu empfehlen ist ein kleines, handliches Imprägniergerät mit geringem Evakuiervolumen und mit einem Manometer, das kurze Evakuierzeiten bei Einsatz einer Wasserstrahlpumpe ermöglicht. Der Glaszylinder (Evakuierraum) steht über einen Dreiwegehahn mit der Vakuumpumpe oder mit der Atmosphäre in Verbindung. Über ein weiteres, verschließbares Kunststoffrohr kann das Imprägniermittel in den evakuierten Glaszylinder mit der Probe hineingesaugt werden.
Die zum Infiltrieren verwendbaren Harze sollen dünnflüssig sein, einen niedrigen Dampfdruck besitzen und geringen Schwund aufweisen. Polyester- oder Epoxidharze, z.B. EPOFIX, sind hierfür gut geeignet.
Die poröse Probe wird mit Alkohol entfettet und gründlich im Warmluftstrom getrocknet. Noch warm wird sie in die Einbettform gelegt und beides in den Glaszylinder eingesetzt. Dann wird evakuiert.
In Abhängigkeit von der Porosität kann die Pumpzeit zwischen 5 und 30 Minuten betragen.
Anschließend wird die benötigte Menge Harz mit Härter ca. 2 Minuten gut durchmischt. Um zu erreichen, daß das Harz dünnflüssiger wird, kann es vor dem Mischen vorsichtig auf ca. 40°C erwärmt werden.
Vor dem Beschicken mit der Einbettmasse wird der Druck am Manometer abgelesen und durch vorsichtiges Belüften auf 78 bis 130 hPA eingestellt. Bei geringem Druck beginnt nämlich die Einbettmasse langsam zu fließen, bis die Einbettform gefüllt und belüftet ist.
Der zunehmende Druck preßt das Harz in die offenen Hohlräume.
Die Aushärtzeit von ca. 8 Stunden bei Raumtemperatur kann durch Erwärmen auf ca. 60°C um etwa 3 Stunden verkürzt werden [37].
Die sorgfältig geschliffene, evtl. nachimprägnierte Probe wird vor dem Polieren mikroskopisch auf größere Schädigungen kontrolliert.
Um plane Oberflächen zu erhalten, soll mit möglichst wenig Polierstufen und auf relativ harten Tüchern gearbeitet werden. Zu lange Polierzeiten führen zu Reliefbildung und Abrundung der Porenränder.

Präparationsdaten für keramische Werkstoffe sowie β-Al_2O_3 sind in den Abbildungen IV. 13 und IV. 14 zusammengestellt [37, 38, 39, 40, 41].

	Schleifen			Polieren	
	1	2	3	1	2
Unterlage	Diamantschleifscheibe		Petrodisc-M	Pan-W	AP-CHEM
Korngröße	220	600	6 -Spray	3 -Spray	OP-5
Schmiermittel	Wasser	Wasser	blau	blau	--
Geschwindigkeit U/min	300	300	300	150	150
Druck N	150	150	120	150	90
Zeit min	bis plan	ca. 3	ca. 8	10	2

Abb.IV.13 Präparationsdaten für poröse Keramik bei Verwendung der Gerätekombination der Firma ABRAMIN

	Scheibe/ Tuch	Diamant-körnung	Umdrehungs-geschw.	Belastung	Zeit (min)
Schleifen	Diamant-geb. Schleif-scheibe	64 m	150/min	60 N*	5
Feinschleifen	Kunststoff Metallscheibe	6 m	150/min	60 N*	15
Polieren	hartes Chemie-fasertuch	3 m	150/min	60 N*	15
Feinpolieren	eiches Chemie fasertuch	1 m	150/min	60 N*	15

* Belastung bei 6 eingebauten Proben

Abb.IV.14 Präparationsdaten für β-Al_2O_3

IV.5.3. Präparation von Verbundwerkstoffen (Metall-Keramik)

Über Metall-Keramik-Verbundwerkstoffe sind Präparationsverfahren aus der Literatur bekannt, die auch im vorliegenden Fall interessieren, da es sich in beiden Fällen um die Verbindung von harten und weichen Materialien handelt.

Die heutige Anwendung von Metall/Keramik-Verbindungen im Raum- und Hochtemperaturbereich stellt ständig steigende Anforderungen sowohl an die Herstellungsverfahren als auch an die Haftfestigkeit dieser Verbindung.

In der Dentaltechnik werden durch Auftrocknen und Aufbrennen von Dentalkeramiken auf Goldlegierungeen mit haftvermittelnden Elementen komplizierte Formkörper hergestellt, deren Einsatz als Zahnersatz eine hohe Haftfestigkeit zwischen beiden Materialien bedingt.

Durch gezieltes Aufdampfen von Metallschichten auf Keramikgrundkörper entstehen Leiterbahnen, die in der Halbleiter- und Computertechnik eine große Rolle spielen. Plasmagespritzte Keramiken dienen als Wärmedämm- und Korrosionsschutzschichten auf Motorteilen, die hohen Temperaturen und aggressiven Medien ausgesetzt sind.

Im Hinblick auf die auflichtmikroskopische Untersuchung wird an einem Beispiel die gefügeanalytische Präparation von Verbundmaterialien beschrieben [42].

Bei der Präparation ist insbesondere zu berücksichtigen:

- hohe Härte der Keramik
- hohe Härteunterschiede
- Sprödigkeit der Keramik
- Porosität der Keramik
- unterschiedliches elektrochemisches Verhalten von Lötnaht, Keramik und Metall.

Aufgrund des stark unterschiedlichen elektrochemischen Verhaltens von Keramik, Metall und Lötnaht scheiden chemische Ätzverfahren zur Kontrastierung weitgehend aus.

Die vorab geschilderten Schwierigkeiten führten zur Erarbeitung der im folgenden beschriebenen Präparationen.

Beim Trennen von schichtförmig zusammengesetzten Proben aus weichen und harten Werkstoffen sollte zur Erzielung optimaler Schnittqualität senkrecht zur Schichtenfolge getrennt werden. Dies erfolgte mit einer bakelitgebundenen Diamanttrennscheibe, da bei metallgebundenen Scheiben an SiC-Proben ein Verschmieren der Trennfläche beobachtet wurde.

Als Einbettmittel wurde ein schnell aushärtender Kunststoff auf Basis von modifiziertem Polyesterharz mit äußerst geringer Schrumpfung verwendet.

Das Schleifen und Läppen von Verbundwerkstoffen ist in besonderem Maße von der Härte abhängig. Als Möglichkeit wäre hier das Schleifen auf bakelitgebundenen Diamantscheiben oder auf Stahlgewebstüchern unter Zugabe von Diamantphase zu nennen. (Abb. IV. 15).

Material: Keramik-Metall-Verbindung
Al_2O_3, SiC und Si_3N_4/Fe28Ni23Co

Einbettmittel: Technovit 4000

Trägerscheibe	Diamant-schleif-scheibe	Petrodisc-M	Petrodisc-M	Petrodisc-M
Unterlage	-	-	-	-
Korngröße	220/660	25 m	15 m	6 m
Schmiermittel	Wasser	blau	blau	blau
Drehzahl U/min	300	150	150	150
Last N	120	90	90	90
Zeit min	5-15/10	5	5	5

Abb.IV.15 Präparationsfolge beim Schleifen gelöteter Keramik-Metall-Verbindung auf einem halbautomatischen Schleif- und Poliergerät (Planopol/Pedemax) [43]

Das Polieren der Keramik-Metall-Verbindungen erfolgte auf harten Kunstfasertüchern, auf die Diamant der Korngrößen 6 µm und 3 µm aufgetragen wurde. Die Last betrug 90 N und die Polierzeit bei Aluminiumoxidkeramik 10 Minuten

IV.5.4. Präparation einer Implantatprobe

Präpariert wurde ein Kaninchenknochen mit intraossärem Implantat Bioverit II (Kalziumphosphatglas), P_2O_5-CaO-Al_2O_3-SiO_2-Na_2O (PCASN), welches 17 Wochen in einer Kaninchen-Tibia lag. Nach Entnahme erfolgte eine Formalfixierung und Einbettung in PMMA.
Geschliffen wurde mit SiC-180, SiC-320 auf Diamantschleifscheibe mit Alkohol.

Man schleift erst mit Druck für das SiO_2, dann mit geringem Druck, um die Zahl der Kratzer in dem weichen Knochen zu verringern.
Geläppt wurde mit Minimat in folgenden vier Arbeitsstufen:

1. mit einer Läppscheibe Metlap 10 mit 6 µm Diamantsuspension ohne Wasser (rot) 20 Minuten bei vollem Druck und dann mit schrittweise reduziertem Druck (3 min)

2. mit perforierter Scheibe Termet mit 6 µm Diamantsuspension (gelb) 10 Minuten bei vollem Druck und dann mit schrittweise reduziertem Druck (3 min)

3. mit Texmet weiß mit 3 µm Diamantsuspension 3 Minuten bei vollem Druck.
(Der Biowerkstoff hätte noch etwas klarer kommen können, und im Knochen waren noch Kratzer, aber man sollte vorsichtig sein mit einer Verlängerung der Polierzeit, da die Behandlung sonst zur Reliefbildung führt.)
Dan 2 Minuten mit schrittweise reduziertem Druck (von 9 auf 3).

4. mit der Scheibe Texmet weiß mit 1 µm Diamantsuspension 1 Minute bei vollem Druck.

IV.6. Kontrastierungsversuche und Meßergebnisse

Die im folgenden dokumentierten Meßergebnisse wurden für alle Materialien mit der gleichen Arbeitsweise erhalten:

Nach dem Einsetzen der Proben in den Probenhalter und dem Einführen der Kathode (unter Einstellung des Elektronenabstandes), wurden die Nadelventile geschlossen.
Durch die Vakuumpunpe wurde ein Vakuum kleiner als 1,3 Pa (Feinvakuum) erreicht. Dabei wurde gleichzeitig die Dichtigkeit der Apparatur geprüft. Es dauert etwa 2 Minuten, bis dieser Druck erreicht war.
Das angeschlossene DC-Hochspannungsgerät hat negative Polarität, die Anode lag auf erdnahem Potential und der Negativ-Pol lag hoch. Das ist wichtig, da die Anode mit Leitungswasser gekühlt wurde.
Mit Hilfe des Vakuummeßgerätes wurde der gewünschte Arbeitsdruck bei maximaler Saugleistung der Vakuumpumpe über ein Nadelventil in der Arbeitsgasleitung einreguliert.
Nachdem sich der Druck eingestellt hatte, wurde etwa 1 Minute geprüft, ob der Arbeitsdruck konstant bleibt. Dann wurde die Kühlwasserzufuhr geöffnet. Nun konnte das DC-Hochspannungsgerät eingeschaltet werden. Es brauchte etwa 1 Minute bis der Betriebszustand erreicht war und der gewünschte Sollwert für die Maximalspannung mittels Potentiometer eingegeben werden konnte. Außerdem wird ein Maximalwert für den Strom vorgegeben. Mit Einsetzen der Gasentladung muß man prüfen, ob die vorgegebene Soll-Spannung erreicht und gehalten wird. Sollte das nicht der Fall sein, dann bedeutet das, daß der vorgegebene Maximalstrom schon bei niedrigerer Spannung erreicht wurde. In diesem Fall war zu entscheiden, ob die Soll-Spannung zu erniedrigen war oder ein höherer Maximalstrom zugelassen werden sollte.
Nach Beendigung der Kontrastierdauer wurde die Spannung zurückgenommen. Das eine Nadelventil wurde geschlossen, das andere zur Entlüftung der Anordnung geöffnet. Der Druck steigt (>130 Pa), die Pumpe kann ausgeschaltet werden. Sie wurde unter dem Mikroskop betrachtet und fotographiert.
Dieser Arbeitsablauf wurde für jede weitere Kontraststufe wiederholt.
Das Gehäuse (Abb.IV. 9) ist nach jedem Versuch mit HCl zu reinigen, dann mit Leitungswasser und schließlich mit Aceton nachzuspülen, um die Oberflächenleitfähigkeit klein zu halten.

Abb. IV. 16 zeigt die Farbkontrastierung von konventionellem Stahl, Abb. IV. 17 diejenige von porösem Aluminiumoxid.

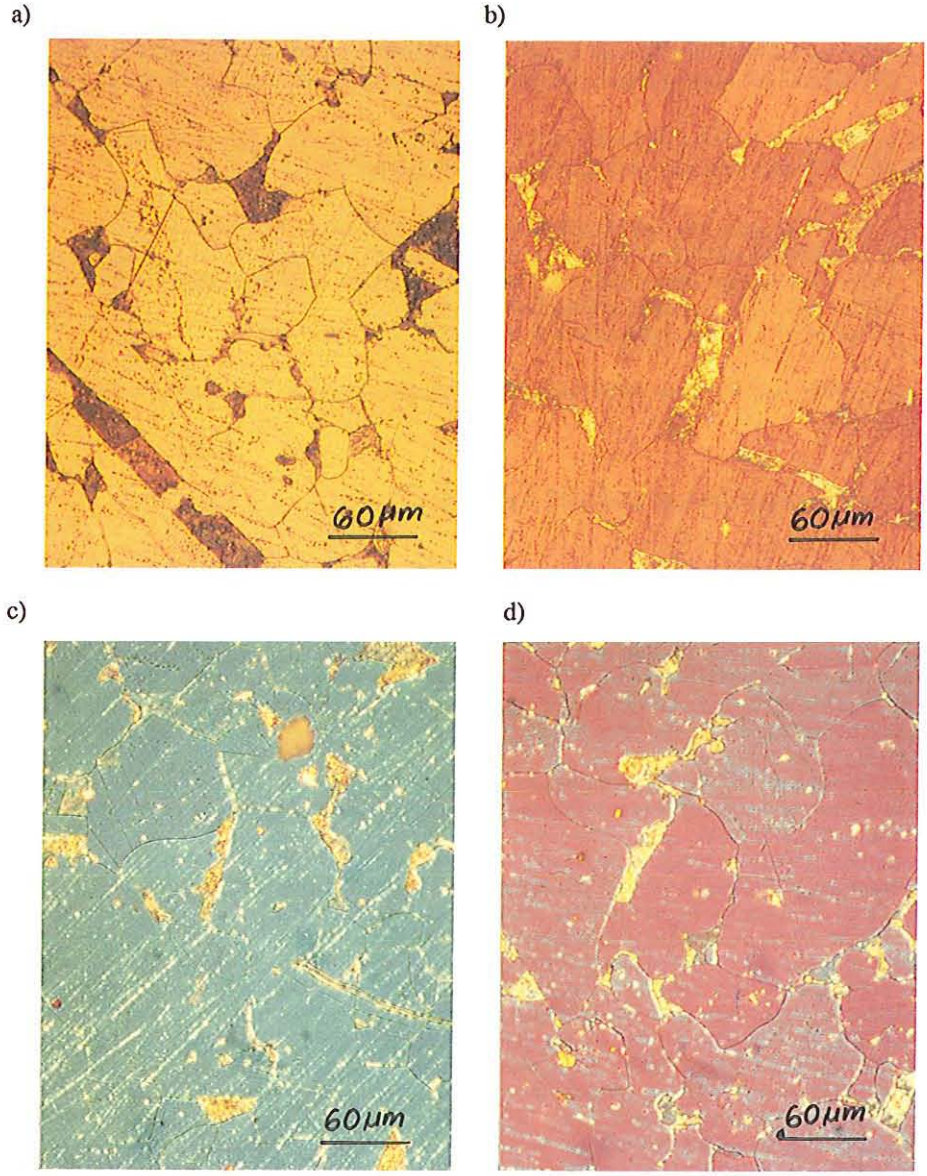


Abb. IV.16 Gaskontrastiertes Stahlgefüge
(C15; Sauerstoff, 26 Pa, 13 mm Elektrodenabstand, Pt-Kathode)
a=unkontrastiert
b= 5 min, 0.97 kV, 1.82 mA
c=10 min, 0.97 kV, 1.80 mA
d=20 min, 1 kV, 1.91 mA

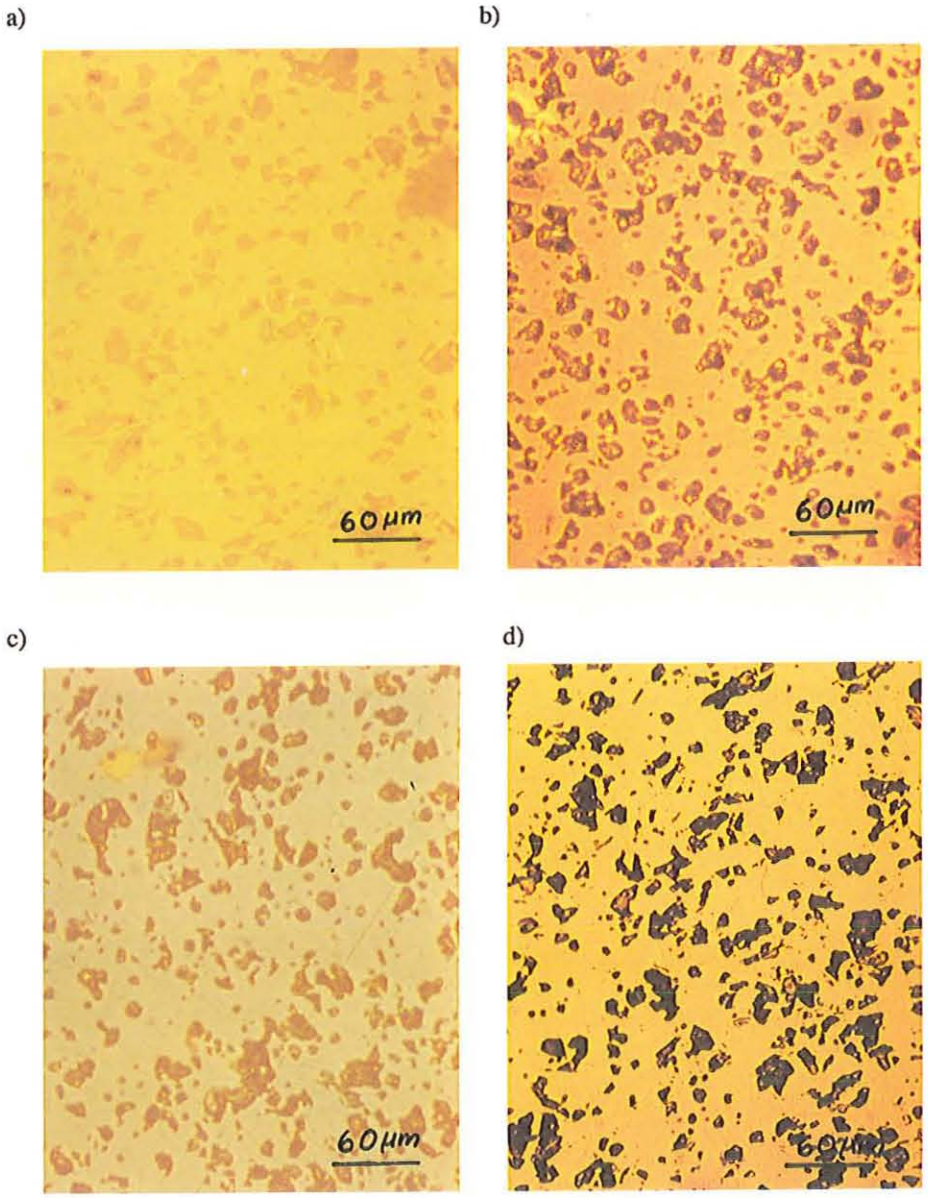
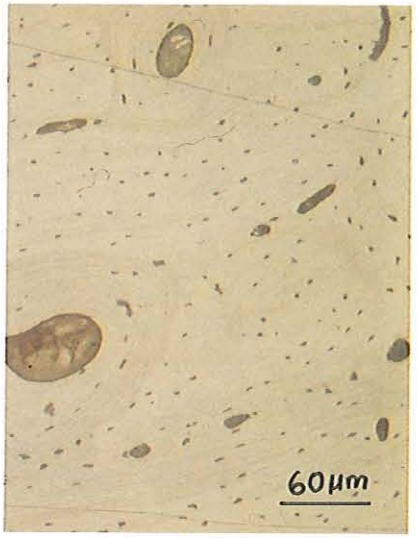


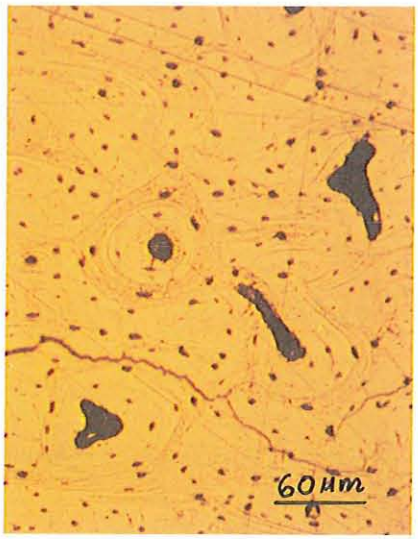
Abb. IV.17 Gaskontrastiertes Al_2O_3-Gefüge
(CO_2, 13 Pa, Elektrodenabstand 20 mm, Pt-Kathode, mit Poren)
a=unkontrastiert
b=15 min, 1.25 kV, 1.23-1.52 mA
c=25 min, 1.40 kV, 1.93 - 2.2 mA
d=35 min, 1.61 kV, 3 - 3.54 mA

In Abb. IV. 18 sind kontrastierte Gefüge von Kaninchenknochen mit Havers`schen Kanälen wiedergegeben und in Abb. IV. 19 diejenigen von Bioglas (Bioverit), dessen Verwachsung mit Kaninchenknochen die in Abb. IV. 20 gezeigte Grenzfläche bildet.

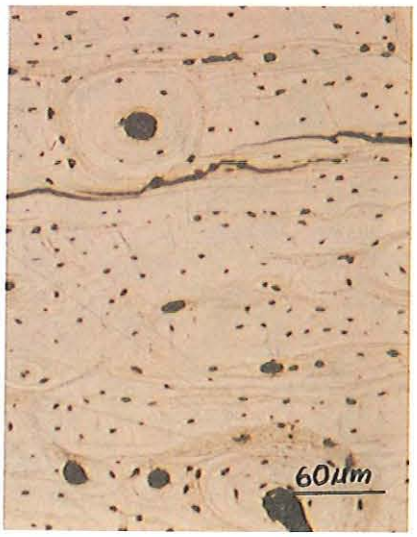
a)



b)



c)



d)

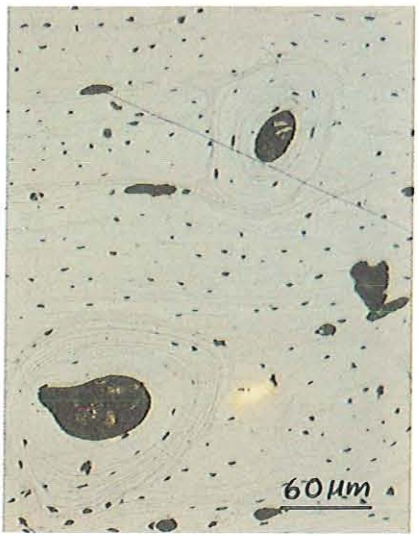


Abb. IV.18 Gaskontrastiertes Knochengefüge
(Kaninchen, Sauerstoff, 13 Pa, Elektrodenabstand 11 mm, Pt-Kathode)
a=unkontrastiert
b=10 min, 1.2.kV, 3.3 - 4.3 mA
c=20 min, 1.49 kV, 2.36 mA
d=25 min, 1.33 kV, 2.06 - 2.58 mA

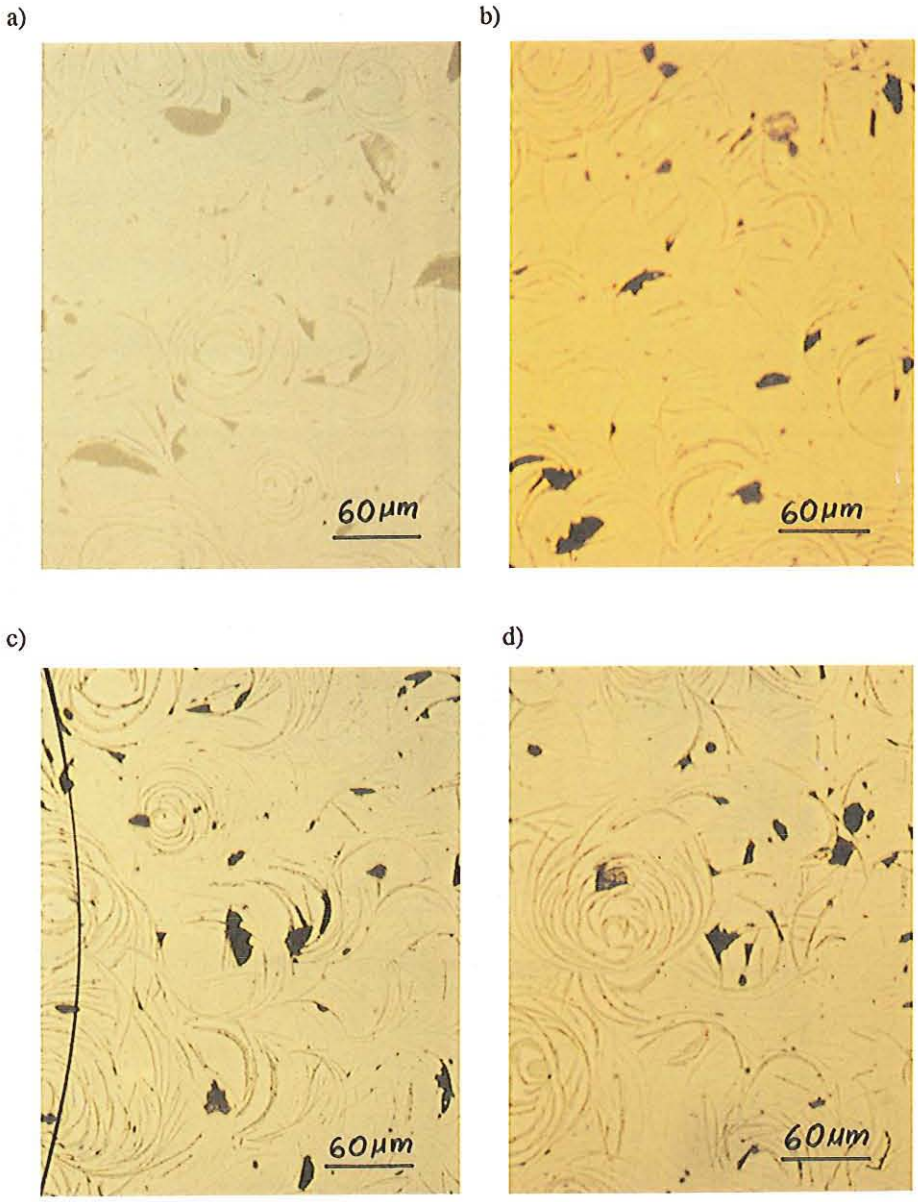
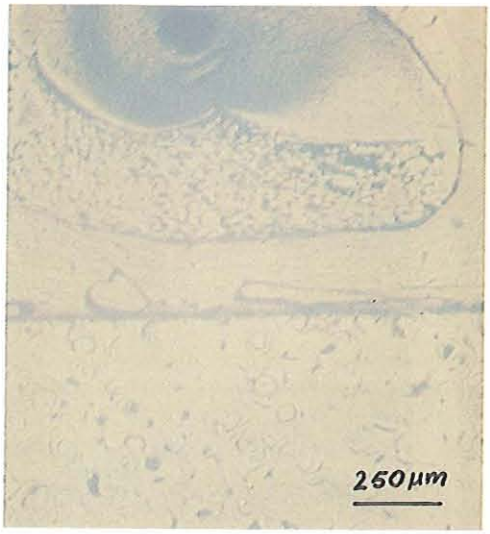
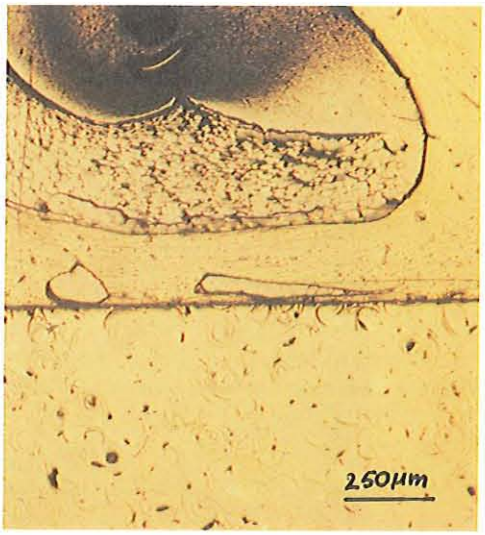


Abb. IV.19 Gaskontrastiertes Bioveritgefüge
(Glaskeramik, Sauerstoff, 13 Pa, Elektrodenabstand 11 mm, Pt-Kathode)
a=unkontrastiert
b=15 min, 1 kV, 2.49 - 2.68 mA
c=25 min, 1 kV, 2.95 - 3.21 mA
d=35 min, 1 kV, 2.30 - 2.81 mA

a)



b)



c)

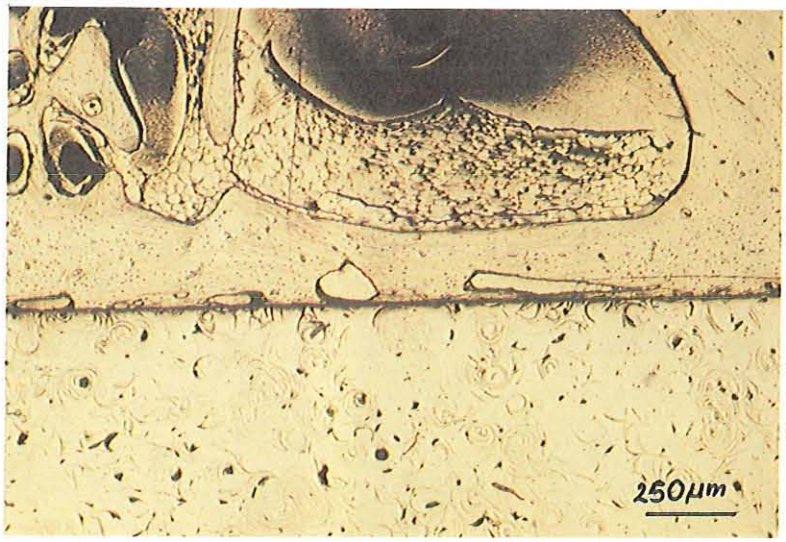


Abb. IV.20 Grenzflächengefüge Knochen-Bioverit
(Sauerstoff, 13 Pa, Elektrodenabstand 11 mm, Pt-Kathode)
a=unkontrastiert
b=15 min, 1 kV, 2.49 - 2.68 mA
c=25 min, 1 kV, 2.95 - 3.21 mA

Schließlich wurde noch die Grenzfläche zwischen Bioglas und Polyethylen gaskontrastiert (Abb. IV. 21) sowie eine Probe mit beiden Grenzflächen - Bioglas/Knochen und Bioglas/Polyethylen (Abb. IV. 22).

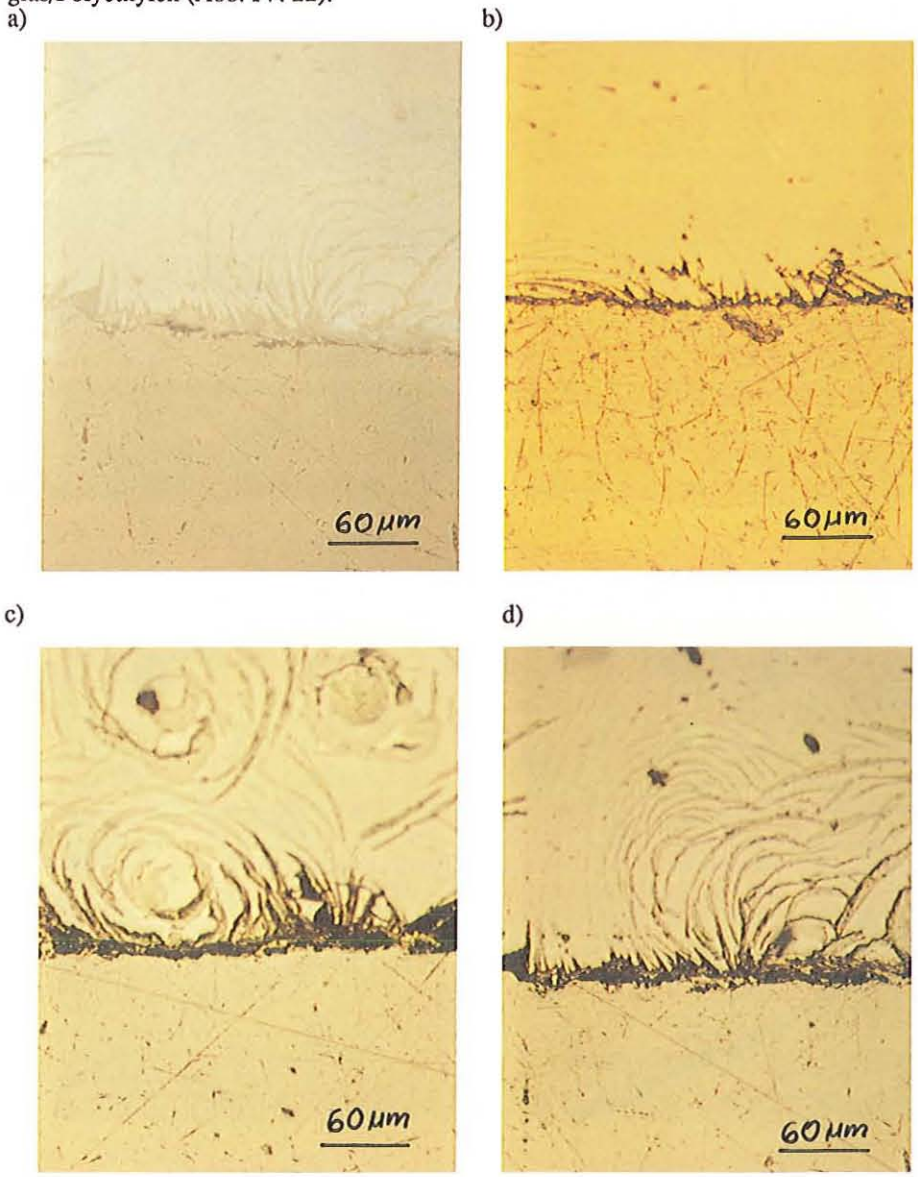


Abb.IV.21 Grenzflächengefüge Bioverit-Kunststoff
(Polyethylen, Sauerstoff, 13 Pa, Elektrodenabstand 11 mm, Pt-Kathode)
a=unkontrastiert
b=15 min, 1 kV, 2.49 - 2.68 mA
c=25 min, 1 kV, 2.95 - 3.21 mA
d=35 min, 1 kV, 2.30 - 2.81 mA

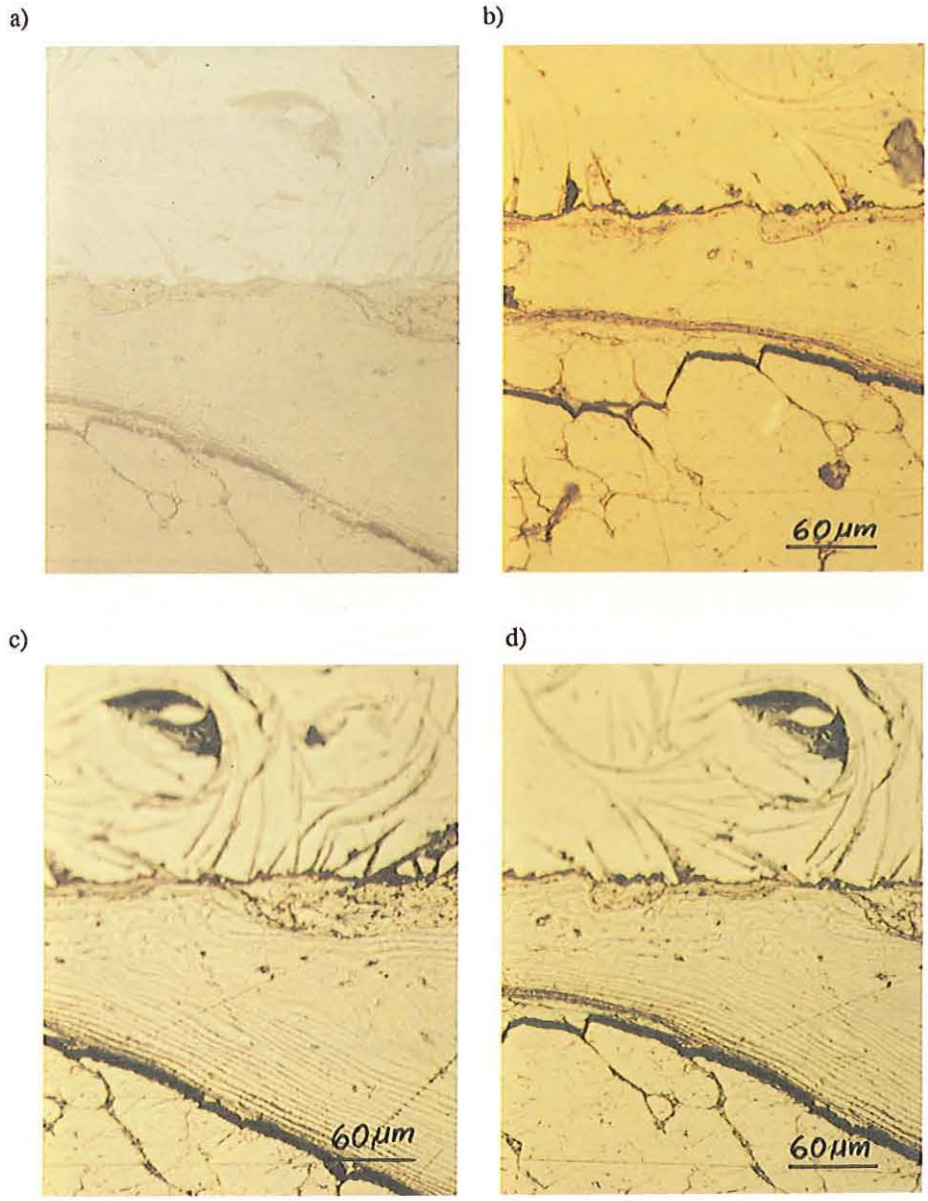


Abb. IV.22 Grenzflächengefüge Bioverit-Knochen-Kunststoff
(Sauerstoff, 13 Pa, Elektrodenabstand 11 mm, Pt-Kathode)
a=unkontrastiert
b=15 min, 1 kV, 2.49 - 2.68 mA
c=25 min, 1 kV, 2.95 - 3.21 mA
d=35 min, 1 kV, 2.30 - 2.81 mA

Die gewählten Bedingungen (Kontrastiergas, Kathodenmaterial, Elektrodenabstand, Spannung und Stromstärke) gewährleisten in allen Fällen Kontraststeigerung, ohne das eine - für organische Proben nicht tolerierbare - Erwärmung eintritt.

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