AN INVESTIGATION INTO THE EFFECTS OF AN ANTIMETABOLITE (METHOTREXATE) ON BONE HEALING

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Thesis submitted in part fulfilment for
the Degree of Master of Dental Surgery

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TABLE OF CONTENTS

TABLE OF CONTENTS (cont'd)

TABLE OF CONTENTS (cont'd)

TABLE OF CONTENTS (cont'd)

TABLE OF CONTENTS (cont'd)

PRECIS

The use of cytotoxic agents is now an accepted adjunct to the management of malignant disease. It has been suggested that these agents also cause retarded wound healing. However, a search of the literature has not revealed any experimental study on the effects of these drugs on healing in bone.

The present investigation is to determine the effects of the cytotoxic agent Methotrexate, on the healing of a bone wound.

A drill hole was made in the mid-shaft of the femur in the Sprague Dawley rat and the subsequent healing that took place was studied by using routine histological methods, augmented by some histochemical observations.

Three experimental series were established:

1. A control group with no drug administration.
2. An experimental group with Methotrexate administered after the operation (OM series).
3. An experimental group with Methotrexate administered prior to operation (MO series).

The result of the control group indicated that the defects healed in a manner similar to that described by previous investigators - by the formation of 'periosteal', 'endosteal' and 'internal' calluses.

The effects of intraperitoneal administration of Methotrexate with the dosage used in the present investigation did not appear to have any significant effect on bone healing. There were some minor alterations in the pattern of healing in the initial stages in the OM series, but recovery soon took place following the cessation of drug administration.

DECLARATION

This thesis is submitted in part fulfilment of the requirements for the Degree of Master of Dental Surgery in the University of Adelaide. Candidature for the degree was satisfied by obtaining the Degree of Bachelor of Science in Dentistry in 1974.

This thesis contains no material which, except where due mention is made, has been accepted for the award of any other degree or diploma in any University. To the best of my knowledge, this thesis contains no material previously published or written by another person, except where due reference has been made in the text.

R.H.B. JONES

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INTRODUCTION

Radiotherapy is an established method for the treatment of malignancies. It is also well known that it causes prolonged and deleterious effects on tissues leading to retarded wound healing. This is especially so in bone, where 'osteoradionecrosis' may develop as a result of treatment, and in these patients, oral surgery in the post-irradiated phase carries a grave risk of chronic osteomyelitis of the jaws.

In addition to radiotherapy and surgery, anti-cancer chemotherapy is now an accepted adjunct to the management of malignant disease. Chemotherapy of cancer can be used alone or in conjunction with radiation or surgery, either for palliation or for cure. The results of its use against inaccessible or inoperable neoplams have been promising.

The effects of anti-cancer chemotherapeutic agents on normal body tissues are not well documented. It has been suggested that these agents also cause retarded wound healing in soft tissues. Studies on this subject have been inadequate and superficial. Further, a search of the literature has not revealed any experimental study on the effects of these agents on the healing of bone wounds. Knowledge of this is of importance in the management of head and neck malignancies because patients receiving anti-cancer chemotherapy often have to undergo oral surgery, particularly the extraction of teeth. Thus, the dentist who treats these patients must ask the question - does anti-cancer chemotherapy carry the same risks as radiotherapy?

The present investigation attempts to answer this question by studying the effects of METHOTREXATE on the healing of a cortical defect placed in the mid-shaft of the rat femur.

Methotrexate is one of the most commonly used cytotoxic agents in the management of head and neck malignancies. It inhibits cell division by blocking an essential step in the conversion of folic acid to tetra-hydrofolic acid during DNA synthesis.

In recent years, the use of a cortical defect has been accepted as a model for the study of bone healing. Its use offers many advantages over the traditional 'long-bone fracture' as an experimental model.

Routine histological methods are used to compare the healing of the cortical bone wound in control and experimental animals. Histochemical methods are used to identify the various biochemical components present in the healing wound and provide a subsidiary approach to the present study of osseous repair.

The first part of this thesis reviews the literature relevant to the subject of this investigation in the following areas:

1. The management of cancer and anti-cancer chemotherapy.
2. The biology and histology of bone repair.
3. The effects of radiation and anti-cancer chemotherapy on wound healing.

The second part of the thesis discusses the methodology of the present investigation and presents its findings.

DEFINITION OF TERMS

Throughout this Thesis, certain terms have been used and to clarify their use in the present text they have been defined in the following way:

Neoplasm : A neoplasm is an abnormal mass of tissue, the growth of which exceeds and is uncoordinated with that of normal tissues and proceeds in the same extensive manner after cessation of the stimuli which evoked the change.

Tumour : A tumour is defined as an abnormal growth of tissue, and in this thesis has been used synonomously with the term neoplasm.

Cancer : The term cancer is a generic term encompassing all malignant tumours or neoplasms.

Anti-Cancer Chemotherapy : The use of chemical agents in the treatment of cancer. The term chemotherapy literally means 'curing by chemicals'. However, the term is restricted to the use of a chemical compound in a patient to kill an infective organism or neoplastic cell.

Anti-Cancer Chemotherapeutic Agent : A chemical agent used in the treatment of cancer.

Cytotoxic Agent : A cytotoxic agent is any chemical agent which kills cells.
It is recognised that not all cytotoxic agents are used in the chemotherapy of cancer, many are too toxic for clinical use. However, the terms cytotoxic agent, anti-neoplastic agent and cancer chemotherapeutic agent have been used synonomously in this thesis.

Radiation : Radiation applies to two different forms of energy:
(1) Electromagnetic radiation, e.g. X-rays and Gamma rays.
(2) Particulate radiation,e.g. Alpha and Beta particles.

Irradiation The exposure to radiation.

DEFINITION OF TERMS (cont'd)

Repair : The replacement of lost tissue by granulation tissue which matures to form scar tissue.

Healing : Healing refers to the body's replacement of destroyed tissue by living tissue.
In the present Thesis the terms 'repair' and 'healing' have been used synonomously.

CHAPTER 1

THE TREATMENT OF CANCER

A. METHODS OF CANCER THERAPY

(1) Surgery

(2) Radiotherapy

(3) Chemotherapy

B. THE CHEMOTHERAPEUTIC AGENTS

(1) The Alkylating Agents

(2) The Anti-metabolites

(i) Anti-purine agents

(ii) Anti-pyrimidine agents

(iii) Folic Acid inhibitors

(3) The Antibiotics

(4) Hormones

(5) Plant Extracts and Others

C. THE ACTION OF CYTOTOXIC AGENTS ON THE CELL CYCLE

D. GENERAL PRINCIPLES OF CANCER CHEMOTHERAPY

E. THE ADMINISTRATION OF CYTOTOXIC AGENTS

CHAPTER 1

THE TREATMENT OF CANCER

The earliest known account of the treatment of cancer by a chemical substance is found in the Ebers Papyrus written about 1500 B.C. It describes the treatment of ulcerative lesions of the skin with arsenical compounds. HIPPOCRATES recognised the use of caustics and cautery in the treatment of the above type of lesions, but warned that only the superficial ones could be treated successfully. CELSUS in A.D. 30 described the topical use of certain substances in the treatment of cancer and also advocated the excision of cancer of the breast, face and lip. GALEN viewed cancer as a systemic disease caused by 'black bile', and used surgery, diet and purgatives.

Little is known of what happened during the Dark Ages. The Renaissance brought an enlightened attitude to many things, including medicine. By the 17th century, advances in the basic sciences were gathering momentum. HARVEY in 1628 had demonstrated the anatomy of the circulation and JANSSEN about this time invented the microscope. HUNTER, the foremost surgeon/anatomist of the time differentiated between "consequent cancers in opposition to the original" and also recognised the importance of lymphatic spread.

BERNARD PEYRILHE was the first to attempt tumour transplantation. He recognised the difficulty in finding a suitable chemical agent for the treatment of cancer. This resulted from recognition of the similarity between normal and malignant tissues. He reasoned that any agent which could kill one type of cell could kill the other.

Until the latter part of the 19th century, the surgeon reigned supreme. The discovery of X-rays by ROENTGEN (1895) opened up a new era

of diagnosis. The discovery of radium by the CURIES added a further weapon to the rapidly increasing armamentarium of the therapist, but one which was in the nature of a double-edged sword.

During the last decade of the nineteenth century foundations were laid down for two lines of attack on cancer:

1. BEATSON (1896) described the hormonal effect of ovariectomy on two patients with breast cancer.
2. EHRLICH (1898) described the necrotising effects of an alkylating agent, ethylinamine, on animal epithelial tissues. Some fifty years later, this substance was examined for its cancericidal properties.

LOEB (1901) successfully transplanted tumours in rats. This discovery was an important development in the understanding of cancer and the assessment of cytotoxic agents.

A. METHODS OF CANCER THERAPY

From the above historical review it can be seen that various forms of treatment have been used in the management of 'cancer'. These form the basis for the treatment of cancer today, and can be classified into:

(1) Surgery

The role of surgery in 'cancer' therapy is well known and needs no repetition. The principles of cancer surgery are defined by the potential of the cancer for local infiltration and for metastatic spread. The knife must therefore always pass through healthy tissues both on the surface and in depth. Despite the efficacy of block removal in operable malignancies, recurrence and/or metastases are observed.

Surgical manoeuvres may push neoplastic cells into deeper tissues and into lymph vessels, or there may be a direct contamination of the wound by malignant cells. Various 'follow-up' regimens have been instituted to overcome these problems. Postoperative radiotherapy has been used in an attempt to destroy any neoplastic cells which might not

have been removed. Cytotoxic agents have been prophylactically used in order to prevent the so-called "seeding". The use of cytotoxic agents in this way has led to the question of whether these agents might be detrimental to healing.

(2) Radiotherapy

Radiotherapy can be defined as the therapeutic use of ionising radiation. The dosage of ionising radiation required to destroy virtually all the cells of certain neoplasms is less than that which will destroy normal tissues. On this ratio of sensitivity (the therapeutic ratio), the clinical usefulness of radiotherapy depends. Radiation is thought to act biologically through its ionising action, but the precise changes which result in cell death are not well understood.

(3) Chemotherapy

Although many substances have been used to treat cancer in the past, modern chemotherapy commenced with the use of nitrogen mustard as a chemotherapeutic agent against leukaemia in 1946.

With the exception of L-asparaginase, an enzyme that specifically inhibits the growth of some strains of leukaemic cells, all drugs used in cancer chemotherapy are non-specific in their action and are cytotoxic to both neoplastic and normal cells. As a result, the clinical use of these drugs is accompanied by a degree of toxicity. The more intense the mitotic activity of a tissue, the greater is its sensitivity to a cytotoxic agent. Neoplastic cells are the most active mitotically, and are therefore the most affected. However, normal cells undergoing rapid proliferation are also severely affected. These cells include those of the haemopoietic system and of the intestinal mucosa.

B. THE CHEMOTHERAPEUTIC AGENTS

The criteria for success from chemotherapy in the treatment of bacterial disease cannot be applied to the "battle" against tumour cells. The penicillins for example, block the synthesis of the bacterial cell

wall but do not affect the cell membrane of animal cells in this way. However, despite some differences, the tumour cell and normal cell have the same origin. For this reason, it was considered that the possibility of finding substances which would selectively kill tumour cells was unlikely. In 1946, some cases of Hodgkin's disease were also successfully treated with nitrogen mustard (GILMAN and PHILIPS 1946).

Nitrogen mustard is a highly toxic substance and hence great care in dosage is required. Following this success other substances were synthesised and it was hoped that a substance would be found which would kill tumour cells with minimal toxic side effects on other cells.

Up to the present, over one quarter of a million chemical compounds have been synthesised and tested for their effectiveness against tumours, not one of which has been found to be a "wonder drug". However, many have earned their place alongside the surgeon's knife and the high energy beams used by the radiotherapist.

For a long time the elevated rate of cell division in a tumour has been considered to be the aspect of the tumour which makes it most vulnerable in treatment. The best known chemotherapeutic agents are, therefore, substances which attack D.N.A. synthesis.

The cytotoxic agents used in cancer chemotherapy can be summarised by the following table:

Guanine and adenine are alkylated, chain breakage follows and hence a defective D.N.A. is produced. These agents represent a heavy handed approach to the problem, and there is a more subtle indirect approach by the use of antimetabolites.

The alkylating agent, mustard gas was used in World War 1 as a weapon. Besides severe burning of the skin and fatal lung damage due to the formation of hydrochloric acid, other side effects were noticed. Primarily severe damage was noted in proliferating tissues, such as the intestinal mucosa and bone marrow. In 1931, ADAIR and BAGG studied the action of mustard gas on the skin and on neoplasms of the skin, and treated squamous cell carcinomas, malignant melanomas and senile warts with favourable results.

During the Second World War, research in gas warfare led to the synthesis of nitrogen mustard and sulphide mustard. These substances were found to have cytotoxic effects on proliferating cells. By 1946, the value of nitrogen mustard as an anti-neoplastic agent was recognised (GILLMAN and PHILIPS 1946). This was quickly followed by triethylenemelamine (PHILIPS and THIERSCH 1950).

Nitrogen mustard was found to be a highly reactive substance, but did not effectively enter the tumour cells because it was taken up by other cells and serum components. For this reason, other agents were developed in order to circumvent this problem. The best known of these is Cyclophosphamide (Endoxan).

Others include Busulphan which is 1:4 dimethane-sulphonoxybutane and Alkeran which is p-di(2-chloroethyl)-amino-L-phenylalanine.

(2) The Anti-metabolites

One of the most interesting phases of modern cancer chemotherapy arose from the concept of competitive inhibition by WOODS(1940) who worked on the effects of sulphonamides on bacterial growth. He suggested that the inhibition of bacterial growth was brought about by the blockage of an

essential metabolic pathway by competitive inhibition.

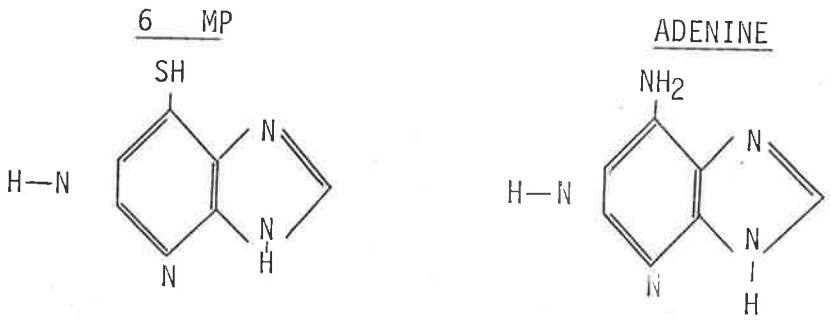
A variety of metabolites are produced during cell metabolism (protein intermediates amino acids and the nucleotides). Chemical substances which closely resemble these metabolites may, by competitive inhibition, block the pathways of normal cell metabolism.

ANGIER et al. (1946) found that folic acid stimulated the production of leukaemic cells in leukaemia, and from this postulated that an anti-folic or folic acid inhibitor would decrease the production of leukaemic cells. FARBER (1948) observed the first drug induced remission of leukaemia using aminopterin. Investigations into the mode of action of aminopterin found that the conversion of folic acid to tetrahydrofolic acid was inhibited. Many other drugs were tried, but the only successful ones were related to aminopterin, of which amethopterin (from here on will be referred to as 'Methotrexate') is a derivative.

Three classes of anti-metabolites are important in cancer chemotherapy:

(i) Anti-purine agents

The first planned experiments to find an anti-neoplastic drug which had a specific effect were carried out by ELION et al. (1953) who produced 6-mercaptopurine (6-MP). This drug has been successfully used in the treatment of leukaemia. Structurally, 6-MP resembles adenine.



If instead of adenine, 6-MP was incorporated into D.N.A., there would have to be some effect on D.N.A. synthesis. 6-MP can in fact be incorporated into both R.N.A. and D.N.A., but its principle effect appears to be indirect. The 6-MP is converted to 6-MP nucleotide and as such inhibits the conversion of inosinic acid to adenylic acid, an important step

in nucleic acid synthesis.

In addition, 6-MP blocks early steps in purine synthesis by a false negative feed back of biosynthesis. 6-MP can normally be catabolized into 6-thiouric acid by the action of xanthine oxidase in the mammalian cell. In general, tumour cells contain a low level of xanthine oxidase and as a result 6-MP has a greater effect on tumour cells than on normal cells.

(ii) Anti-pyrimidine agents

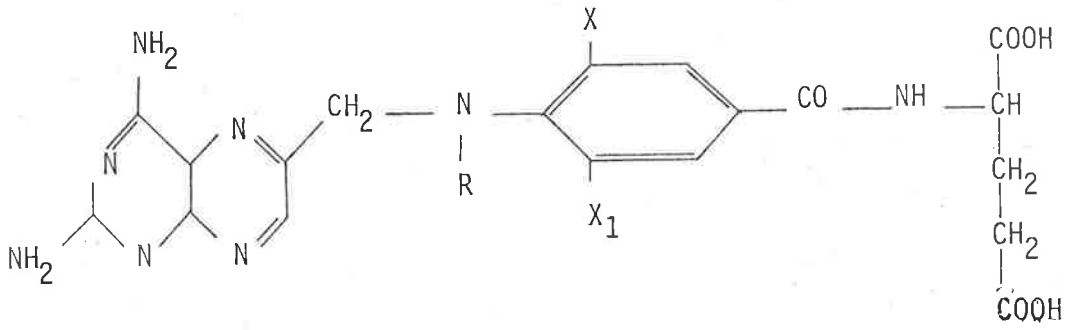
5-fluorouracil is an example of an anti-metabolite which inhibits pyrimidine synthesis.

(iii) Folic Acid inhibitors

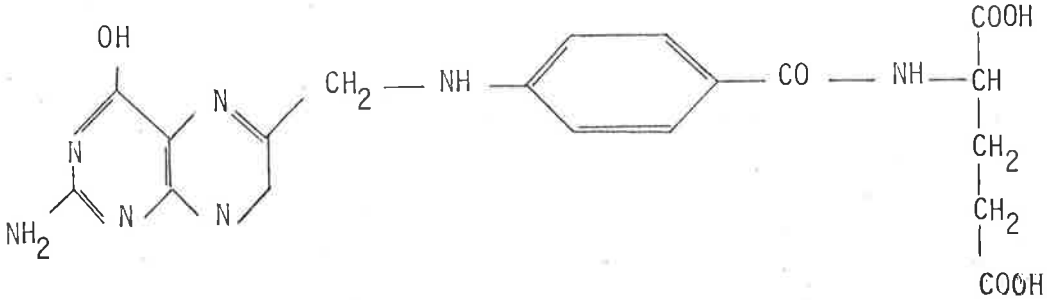
A third group of anti-metabolite, the Folic Acid inhibitors, act by inhibiting folic acid synthesis, which is important in both purine and pyrimidine synthesis.

These agents are structurally very similar to Folic Acid and are classic examples of competitive inhibition.

The Folic Acid inhibitors can be divided into two groups. Group I Inhibitors which have the following general formula



Their resemblance to Folic Acid (below is evident



The Group I Inhibitors include:

Aminopterin, where the R, X and X_1 groups are replaced by H.

Amethopterin (Methotrexate), where the R group is replaced by CH_3 and the X and X_1 group replaced by H.

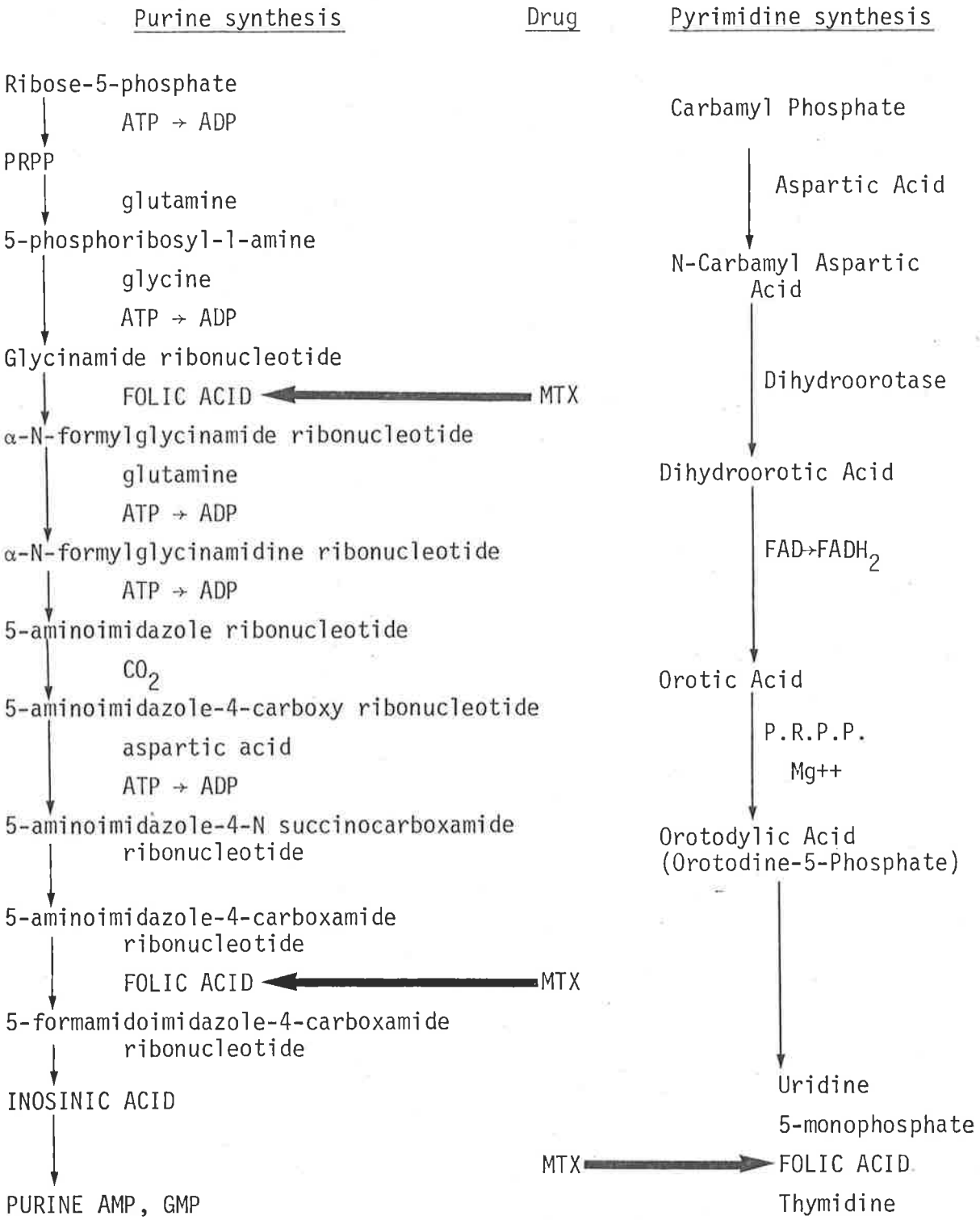
Dichlor Methotrexate, where the R group is replaced by CH_3 and the X and X_1 are replaced by Cl.

The Group I inhibitors act by inhibiting the conversion of Folic Acid to tetra-hydrofolic acid by an "irreversible" combination with the enzymes folic reductase and dihydrofolic reductase (HALL 1962, DELMONTE and JUKES 1962).

The Group II inhibitors combine "reversibly" with the above enzymes and are ineffective against neoplastic disease (DELMONTE and JUKES 1962).

The following diagram shows the sites of action of methotrexate in relation to purine and pyrimidine synthesis.

In summary methotrexate inhibits the following reactions:



MTX indicates methotrexate

Areas of inhibition by methotrexate are shown by ⟶

(3) The Antibiotics

BROCKMAN introduced actinomycin C in 1949 which was found to be useful in the treatment of Hodgkin's disease, but it also had some bad side effects. WAKSMAN (1940) later developed a similar but less toxic antibiotic actinomycin D.

A number of other antibiotics have since been found to be effective against neoplastic cells. These include Rubidomycin, Bleomycin and Mitomycin.

The actinomycins interfere with the synthesis of certain forms of R.N.A., but the mode of action is not fully understood.

(4) Hormones

Steroids were found to play an important role in the management of cancer. In 1940 HUGGINS et al. reported the use of stilboestrol in the treatment of prostatic cancer in dogs. HUGGINS and MOULDER (1944) followed BEATSON's work (1896) and studied the effects of ovariectomy and adrenalectomy in dogs. Early results were disappointing but with the isolation of adrenocorticotrophic hormone (ACTH), success in the treatment of chronic leukaemia (PEARSON et al. 1949), and of acute leukaemia (FARBER et al. 1950) was achieved. However, it has been superseded by the use of gluco-corticoids such as prednisolone. At present, steroids play an important role in the management of acute leukaemia and breast cancer with the oestrogens being most effective in the treatment of prostatic carcinoma.

(5) Plant Extracts and Others

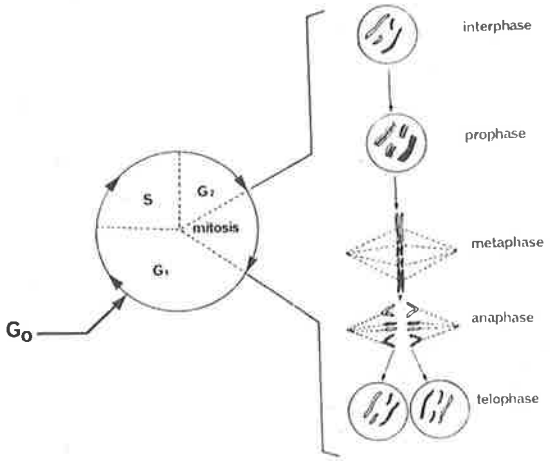
Plant extracts have been known to have cytotoxic effects. Demecolcin was isolated by SANTAVY and REICHSTEIN (1950) and has been used in the treatment of myeloid leukaemia. Vincaleukoblastine was found to be beneficial in the treatment of myeloid leukaemia (CUTTS, BEER and NOBLE, 1957, 1960).

The mode of action of these agents and others such as hydrox-

yurea and natulan is not understood. Hydroxyurea is specifically active against dividing cells. It would appear however, that these agents act in a similar way to the other types by interfering with cell division (CONNORS 1969).

C. THE ACTION OF CYTOTOXIC AGENTS ON THE CELL CYCLE

Fig. 1.1 The Cell Cycle



The classification just discussed may be misleading, as not all of the drugs under the same heading have the same effect on the cell cycle, as is the case with the alkylating agents. Nitrogen mustard has a toxic action on cells in all phases of the cell cycle, including those at rest (G_0), whilst cyclophosphamide and phenylalanine mustard spare cells in G_0.

Hence the chemotherapeutic agents may also be grouped according to their action on the cell cycle (refer to Fig. 1.1, 1.1a).

(a) Drugs which act on all phases of the cell cycle, including G_0 are nitrogen mustard and ionising radiation.

(b) Drugs which are active in all phases of the cell cycle, but spare G_0 include cyclophosphamide, phenylalanine mustard, actinomycin D and 5-fluorouracil.

(c) Drugs which are active only in one phase of the cell cycle, sparing cells in other phases and G_0 are the vinca alkyloids (vincristine and vinblastine), cytosine arabinoside, bleomycin, hydroxyurea and methotrexate.

Fig. 1.1a

CLASS OF AGENT	AGENT	SUGGESTED ACTION
A	Radiation Nitrogen Mustard	Kill cells in all portions of generation cycle. Sensitivity not marked to proliferative phase.
B	5 Fluorouracil Actinomycin D Cyclophosphamide	Kill cells in all or most portions of generation cycle. Sensitivity depends on cells in proliferative state.
C	H^3 Thymidine Vinblastine Methotrexate	Kill cells in one portion of generation cycle

BRUCE (1966) studied the effects of a variety of drugs on transplantable lymphoma in mice. He also studied the effects of these drugs on the haemopoietic system. Using the spleen colony method of TILL and McCULLOCH (1961) they were able to calculate the fraction of normal haemopoietic colony-forming cells and lymphoma cells surviving after the administration of the drugs. According to the type of curves obtained, they were able to classify the drugs into broad classes in a similar way to the above (see Figs. 1.2, 1.3, 1.4).

It can be seen from these facts that the action of drugs on the cell cycle is important in the treatment of cancer. This ensures the use of the correct combination of drugs and also the method of administra-

tion. Those drugs which do not spare cells in G_0 should be given continuously to a tolerated toxicity and treatment repeated when the peripheral blood has returned to normal. Drugs sparing cells in G_0 should be given in massive doses, intermittently, to ensure maximum destruction of the tumour cell population.

FIG. 1.2

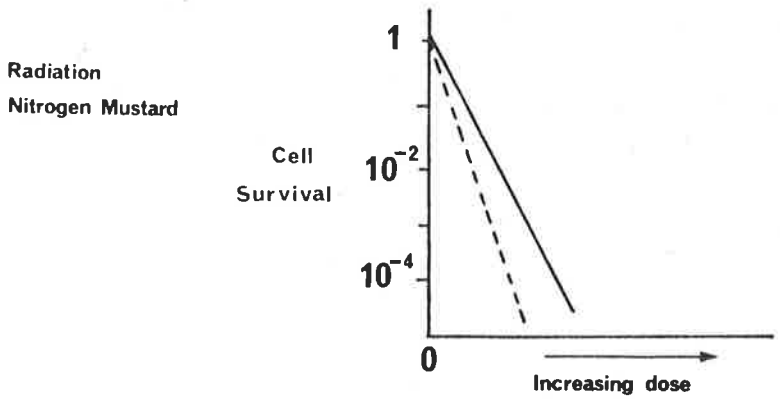


FIG. 1.3

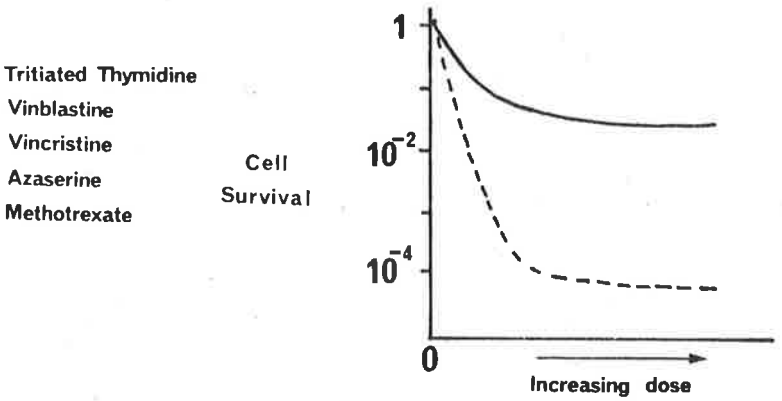
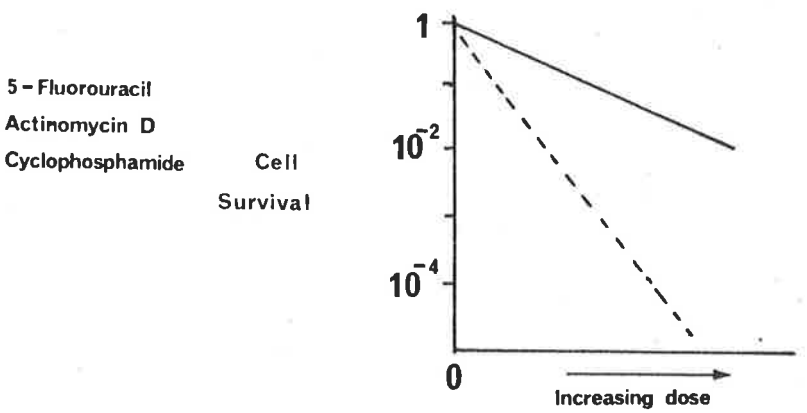


FIG. 1.4



D. GENERAL PRINCIPLES OF CANCER CHEMOTHERAPY

One of the fundamental considerations in modern cancer chemotherapy is the timing in the use of drugs in relation to the cell cycle to ensure maximum therapeutic response with minimal toxicity.

No single treatment, whether with one drug or a combination of several drugs will eliminate all of the cells in any neoplastic population, as it only reduces a constant percentage, not a constant number of cancer cells. This is in accordance with first order kinetics. In view of this principle, it is no easier to reduce one hundred neoplastic cells to one cell than it is to reduce one million cells to ten thousand. In each case there is a 99% reduction. The ultimate goal is to kill all of the cells in a population. Theoretically this is possible by constant chemotherapy, but general toxicity and host factors make this impracticable, in spite of recent advances in drug administration.

The immediate response to a single treatment is directly proportional to the rate of growth of the tumour and inversely proportional to the number of neoplastic cells present. This is valid not only because of the large tumour cell population to be destroyed, but also for immunological reasons. The prior use of surgery and/or radiotherapy to reduce the tumour mass, is therefore, a rational procedure and favours the action of drugs.

Since the reduction in cell population produced by these drugs follows the principle of first order kinetics, treatment should continue as long as there is evidence of good control. The ability of a drug to control a tumour depends upon:

1. The sensitivity of a tumour to the drug, and
2. the development of resistance.

If the disease progresses unchecked despite treatment, then some alteration in treatment plan should be undertaken.

E. THE ADMINISTRATION OF CYTOTOXIC AGENTS

The administration of these agents can be carried out by several methods, the most often used are:

1. Systemic administration by the following routes:
   - intravenous
   - intra-muscular
   - intra-peritoneal
   - oral
2. Regional administration by arterial
   - infusion
   - perfusion

In order to obtain a therapeutic response, a drug should be given in divided daily doses or by continuous intravenous drip. The biological effects are increased by continued administration in comparison with those produced with single daily doses. For example, with methotrexate the effects are increased five-fold via continuous administration (SULLIVAN 1967).

Head and neck tumours are often in a location supplied by one or more readily accessible arteries. Techniques therefore have been devised for the administration of anti-neoplastic agents to a regional area in order to enhance the effects of the drug. These methods involve regional perfusion and arterial infusion. Regional perfusion (SULLIVAN 1967) involves the temporary exclusion of the cancer involved area from the general circulation for up to one hour, during which time large doses of chemotherapeutic agents are circulated in the extra corporal circuit.

In arterial infusion (SULLIVAN et al. 1959), a continuous twenty four hour infusion of drug is pumped through a small catheter placed into a known site in the arterial supply of the tumour. The rationale for this method of administration is based on the following factors:

1. The use of the arterial route results in a higher concentration of drug in the area of the tumour.

2. The continuous administration of an anti-cancer agent over a protracted period of time will result in a continuous anti-cancer effect and hence all cells of a given tumour population will be exposed to the anti-cancer agent as they sequentially enter the active vulnerable phase.
3. The use of anti-metabolic/antidote combination (methotrexate/citrovorum factor) will allow the administration of massive doses of drug by minimising the systemic side effects.

CHAPTER 2

THE BIOLOGY OF BONE HEALING

A. GENERAL ASPECTS OF WOUND HEALING

B. OSTEOGENESIS IN BONE REPAIR: AN HISTORICAL PERSPECTIVE

C. THE ROLE OF THE SKELETAL TISSUES IN OSTEOGENESIS

(1) The Origin of the Osteoblast

(2) The Role of the Periosteum in Bone Healing

D. THE ROLE OF 'INDUCTION' IN OSTEOGENESIS

(1) The Development of the 'Induction Hypothesis'

(2) The Stimulus for Osteogenic Induction

E. THE BI-PHASIC THEORY OF OSTEOGENESIS

F. FUNCTIONAL ASPECTS OF THE OSTEOBLAST

(1) The Pleuri-potential of the 'Progenitor Cell'

(2) Osteoblasts and Ground Substance

(3) Osteoblasts and Bone Matrix Formation

(4) Osteoblasts and Calcification

CHAPTER 2

THE BIOLOGY OF BONE HEALING

The remarkable capacity of the body for structural and functional reconstruction following injury is well known. In the repair of soft tissue wounds, necrotic tissue and blood clot are removed and replaced by invading granulation tissue. The new tissue matures into a fibrous 'scar' which is subsequently remodelled to harmonise anatomically and functionally with the normal connective tissue of the region.

Bone repair follows a similar sequence. The wound is organised by osteogenic cells as well as fibroblasts, with the resultant 'callus' containing new bone, fibrous tissue and often cartilage.

As this project deals with the effects of the antimetabolite Methotrexate on bone healing it is important to discuss osteogenesis and bone healing in some detail.

A. GENERAL ASPECTS OF WOUND HEALING

Although wound healing has been the subject of medical research for a long time, it is only recently that attempts have been made to correlate the various aspects (EDWARDS and DUNPHY 1958, FLOREY and JENNINGS 1962, CHEN and POSTELTHWAIT 1964, DUNPHY and VAN WINKLE 1969, PEACOCK and VAN WINKLE 1970, FORRESTER 1973).

DUNPHY and his associates (DUNPHY and UDUPTA 1955, DUNPHY and EDWARDS 1956, EDWARDS and DUNPHY 1958) studied the chemical and histochemical sequences in wound healing and correlated these findings with histological and tensile strength studies of the healing wound. They postulated two phases in wound healing.

As acute inflammation subsides, the fibroblast rapidly becomes the most dominant cell. The first evidence of repair occurs at some distance from the wound. During the first hours after wounding, the accumulation

of mucopolysaccharides has been identified by histochemical means (DUNPHY and UDUPTA 1955). This occurs well before collagen formation is apparent in the wound. A similar type of reaction is seen in the periosteum and endosteum in bone healing as will be described in the next chapter.

The first phase of healing begins shortly after injury, lasts for about 5 days and has been called the substrate phase. Previously this histologically quiescent period was labelled the "lag phase" (WHIPPLE 1940). In contrast, DUNPHY et al. stressed that the "lag phase" was a productive phase, during which the "building blocks" of repair were manufactured and accumulated in the healing zone.

The second phase begins about the 5th postoperative day and lasts until healing is complete. During this collagen phase, collagen is laid down and is responsible for the increase in tensile strength, along with the re-establishment of tissue continuity in the injured part.

In osseous repair, the "collagen phase" is followed by a third phase, the osteogenic phase (UDUPTA and PRASAD 1963) where granulation tissue is replaced by bone. This phase starts about 3-4 weeks postoperatively and is responsible for the strength in the healing wound.

Many reports have indicated the importance of the ground substance during collagen formation. Fibroplasia may be divided into three functional phases:

1. The proliferation of fibroblasts
2. The formation of ground substance
3. The formation of collagen.

Both collagen and the mucopolysaccharides account for a major portion of the organic matrix in bone. There is general agreement that these components are elaborated by osteoblasts during osteogenesis (PRITCHARD 1956, 1972, GERSH 1960, FITTON-JACKSON 1960). Discussion on the importance of the ground substance in wound healing and collagen synthesis are included in Appendices I and II for reference.

B. OSTEOGENESIS IN BONE REPAIR: AN HISTORICAL PERSPECTIVE

The controversy over which tissue component plays the major role in healing of a fracture has been going on for centuries. HIPPOCRATES maintained that only bone marrow was responsible for callus formation. GALEN on the other hand considered the fracture to be joined by some undefined cement-like substance which, however, was never transformed into bone.

These views were unopposed until the eighteenth century when DUHAMEL (1742-1743) repeated the madder experiments of BELCHIER, and found that the newly formed bone was coloured red. DUHAMEL (1742) traced the origin of the red callus to the periosteum. However, the conclusion that the periosteum was the source of the new bone created a great controversy. JOHN HUNTER (1770) strongly opposed this view and claimed that the periosteum had no osteogenic capabilities.

Following these early investigations many of the experiments on bone formation and repair were designed to clarify the role of the periosteum in osteogenesis.

GEGENBAUER (1864) thought that there was a specific cell in the cambial layer of the periosteum, the marrow cavity and the lamellar systems of bone which was responsible for bone formation and he called this cell an 'osteoblast'.

As early as 1867 OLLIER maintained that transplants with intact periosteum could survive and grow in response to functional stress. He transplanted autogenous bone into intra-muscular and intra-osseous sites and found that osteoblasts were responsible for new bone formation on the surface of the transplant.

BARTH (1895) noted that transplants of calvarium devoided of periosteum, became necrotic and were replaced by new bone arising from the tissues of the graft bed.

On the other hand, MacEWEN (1912) stated that "the periosteum is of

great use in limiting....the osteoblasts...." and implied that bone forming cells resided within bone and not the periosteum.

G. AXHAUSEN (1908, 1909) confirmed OLLIER's work by showing that transplants could remain alive and were the source of new bone. He found that new bone was formed by the proliferation of periosteal cells and also by cells of the endosteum and bone marrow. The osteogenic potential of these tissues has since been repeatedly demonstrated (MAYER and WEHNER (1914), McGRAW and HARBIN (1934), MAYER (1919), NATHAN (1921), KEARNS (1934), URIST and McLEAN (1941), BERTELSEN (1944), HAM and HARRIS (1956, 1972), RAY and HOLLOWAY (1957).

LEXER (1924, 1929) stated that these cells were not histologically recognisable as "osteoblasts" in the latent state but became so with adequate stimulation. LEXER defined the stimulus as being inflammation, whereas necrosis of bone (AXHAUSEN 1920, 1951, 1956), CUSHING (1969) and other conditions in which bone nutrition was interrupted were thought to be the responsible factors (AXHAUSEN 1920, 1951).

Bone marrow also contributes to osteogenesis. RICHTER, SUGG and BOYNE (1968), BURWELL (1964), PETRAKOVA, TOLMACHEVA and FRIEDENSTEIN (1963), CUSHING (1969), OWEN (1970), FRIEDENSTEIN et al. (1968) have reviewed the literature on autogenous red marrow grafts and believe that the potential of this tissue to form new bone was due to the availability of the cells lining the sinusoids of the marrow (endosteal cells) which can differentiate into osteoblasts. This view is shared by others (ZAMBONI and PEASE (1961), WEISS (1961), TRUETA (1962, 1963), FORNES and BARKER (1963). Other osteogenic cells from the marrow also contribute to osteogenesis in bone repair as will be discussed in the next chapter.

Apart from skeletal tissues, other tissues have been thought to take part in bone formation. MARCHAND (1901) postulated that osteoblasts were formed from mesenchyme cells. BASCHKIRZEW and PETROW (1912) supported this hypothesis, saying that undifferentiated connective tissue cells of mesenchye origin were pluri-potent and gave rise to bone forming cells. LERICHE and POLICARD (1928) also supported this view.

Scientific discussion about the problem of osteogenesis has been dominated by the conflict between the osteoblast and the induction hypothesis. Are the cells of bone and periosteum the only cells responsible for new bone formation? Can the connective tissues, if properly stimulated, be transformed into osteogenic cells and produce new bone?

C. THE ROLE OF SKELETAL TISSUES IN OSTEOGENESIS

(1) The Origin of the Osteoblast

In adult life, when growth has ceased, osteoblasts virtually disappear from the periosteum to become osteocytes. Since the osteoblast does not divide, the cells needed for new bone formation, repair and development, must be recruited from some cells other than the differentiated osteoblast. The identification of cells with osteoblastic potential and an understanding of the stimulus responsible for the release of this potential is important.

When an adult bone is injured, exposed to infection, irritations, vascular disturbance or some abnormal situation, proliferating cells appear in the periosteum (PRITCHARD and RUZICKA 1950). From this it can be seen that cells with potential for proliferation and differentiation into osteoblasts are present throughout life, and that this ability can be readily and rapidly evoked by a variety of stimuli. These cells have been called cambial cells, osteogenic cells, preosteoblasts and osteoprogenitor cells (YOUNG 1964, PRITCHARD 1972).

So far the discussion has centred around the periosteum, but osteoblasts are found elsewhere.

In developing membrane bone, osteoblasts are first found in the centre of masses of condensed mesenchyme, where they differentiate into a mass of cells inter-meshed with a wide plexus of capillaries (PRITCHARD 1972).

In cartilage bone the osteoblasts first appear in the depths of the perichondrium; at about the same time the local cartilage cells

hypertrophy and their matrix calcifies. As the bone grows in width, new osteoblasts are produced peripherally from a layer below the fibrous periosteum (PRITCHARD 1972). Evidence for this has been found by HAM (1930), PRITCHARD (1952), TONNA & CRONKITE (1963), OWEN (1963).

Various ideas on the origin of these cells have been put forward. They could be derived from vascular endothelium, blood borne cells or from cartilage cells.

In the maintenance of cortical bone, osteoblasts and osteoclasts appear and disappear in the vascular haversian and Volkman canals of bone. These cells are thought to be derived from the mesenchymal cells which are present in these canals (PRITCHARD 1972).

Pritchard summarises as follows "The essence of the theory is the postulation of a dynamic pool of cells in bone which can assume the morphology and functional status of progenitor cells, osteoblasts, osteoclasts and chondroblasts as conditions dictate. It is probable that they also modulate to fibroblasts in certain circumstances, and it is not inconceivable that the haemopoietic cells of the bone marrow, or at least the haemopoietic stem cells may belong to the same interconvertible modulating system".

(2) The Role of the Periosteum in Bone Healing

The periosteum consists of two layers, an outer layer and an inner layer. The outer layer consists of dense fibrous tissue, a small number of cells and numerous blood vessels. The inner layer contains a greater number of cells, is less vascular and more elastic. In addition to these features, the periosteum has two main functions. It provides support for the blood vessels and nerves entering the bone and also provides anchorage for tendons. Its second function relates to the osteogenic nature of the cambial cells, which differentiate into osteoblasts and produce bone.

Despite the varying emphasis placed by different authors on the

importance of the different bone elements in osteogenesis, an overwhelming majority of the investigations carried out during recent years indicate that the periosteum is of primary importance for bone regeneration in fracture healing.

Experimental studies in rats, have shown that if the periosteum is removed with bone, fibrous union occurs when the fracture gap is more than 2.0 mm. in width (PRITCHARD 1946, MULHOLLAND 1959). However, in the presence of an intact periosteum, rapid bony union results in fractures where a gap of up to 17 mm. was present (MULHOLLAND, 1959, McCLEMENTS, TEMPLETON and PRITCHARD 1961).

D. THE ROLE OF 'INDUCTION' IN OSTEOGENESIS

In early stages of repair, it is clear that collars of callus which form around the two fragments of a fracture have their origin from the osteogenic layer of the periosteum. As these collars continue to grow and approach one another, the gap between them is invaded by a large number of cells with obscure origins. Thus any of the invading cells could be responsible for production of the callus. Are they osteogenic cells which have emigrated from the periosteal collars; or are they fibroblasts from the surrounding connective tissues? (HAM and HARRIS 1956, 1972). Since callus later appears in this site, it must be questioned whether or not fibroblasts can participate in osteogenesis.

Some authors see no fundamental distinction between the inherent abilities of the fibroblasts and osteoblasts to form bone, and stress the pluri-potential of mesenchymal tissues in response to functional requirements. Thus, they believe that the environment, rather than the cell, is the important factor in determing bone formation. Others consider that osteogenic cells have an inherent tendency to form bone and that fibroblasts can be induced to do so by certain poorly understood factors.

(1) The Development of the 'Induction Hypothesis'

The idea that mesenchymal cells other than osteoblasts may form bone is not new (MARCHAND 1901, BASCHKIRZEW & PETROW 1912, MARTIN 1927, LERICHE & POLICARD 1928.)

SACCERDOTTI & FRATTIN (1902), HUGGINS (1931), were able to produce new bone by metaplasia of connective tissue under the influence of epithelium from the urinary tract. It has been recognised that other implants have similar properties and include gall bladder epithelium and (HUGGINS & SAMMETT 1933), human amnion tissue culture cells (ANDERSON et al. 1964, WLODARSKI et al. 1970).

Heterotopic bone formation is known to occur in a number of clinical and experimental situations. For example, bone formation was found to occur in sclerotic aortas, and in kidney repair following partial removal in dogs.

BASCHKIRZEW and PETROW (1912), WERECHINSKI (1925), found that new bone formation occurred after transplantation of bone devoid of periosteum and marrow. They introduced the concept of fibrous tissue metaplasia to explain the origin of this new bone. This concept was further supported by ORELL (1934), who found that bone formed around transplants which had been pre-treated by boiling and freezing (ENGSTRÖM & ORELL 1943). This then led to the "INDUCTIVE HYPOTHESIS" which proposes that recipient host connective tissue cells were capable of being stimulated by an inductor substance in the transplant.

Living skeletal tissues, including bone (GOLDHABER 1961, RAY & SABIT 1963), cartilage (URIST & McLEAN 1952) and bone marrow (CUSHING 1969) also possess the property of bone induction. Although, with these tissues it is difficult to differentiate between induction and osteogenesis due to transplanted osteoblasts.

URIST & McLEAN (1952) noted that when cortical bone, devoid of viable cells was transplanted into the anterior chamber of the eye, a small

deposit of bone was produced after a latent period of 4 weeks. They claimed that this new bone originated from the perivascular connective tissues of the host, during resorption of the implant. These results were substantiated later, by experiments of BRIDGES & PRITCHARD (1958) and by RAY & SABIT (1963). When devitalized implants are used, any bone formation can be confidently attributed to induction. The induction capacity of these implants can be enhanced by decalcification, either in weak acid solution, or by chelation (VAN de PUTTE & URIST 1966). How and why this occurs is not completely understood, however the problem has been extensively discussed by URIST, DOWELL, HAY & STRATES (1968).

McLEAN and URIST (1968) have defined the term induction as being 'the influence that a tissue may have upon another in close contact with it, as a result of which the second tissue is induced to exhibit activities not previously in evidence'. It is apparent that no common factor can link such diverse inducing agents. The mechanism and nature of the induced cell remains speculative. However, it is generally accepted that 'induction' plays an important part in osteogenesis (MAXIMOW & BLOOM 1952, VAN PATTEN & WHITTICK 1955, BASSETT 1959, 1962, YOUNG 1962).

(2) The Stimulus for Osteogenic Induction

CHALMERS et al. (1975) have postulated 3 requirements for osteogenic induction:

1. An osteogenic precursor cell
2. An inducing stimulus
3. A tissue environment which is favourable for osteogenesis.

The existence of an osteogenic precursor cell has been established and is thought to be a primitive mesenchyme cell (OWEN 1970), which is widely distributed and possibly circulating in the body (OSTROWSKI & WLODARSKI 1971).

The existence of an inducing agent has been established but

there are divergent opinions as to the nature of the stimulus involved.

LERICHE and POLICARD (1928) proposed that embryonic cells elaborated a pre-osseous substance in response to a local calcific stimulus. However, in the case of myositis ossificans and in calcifying laparotomy wounds (WILLIS 1950), no such local over-supply of calcium salts has been demonstrated.

Trauma or infection has also been suggested as the stimulus responsible for metaplasia of connective tissues (NEUHOFF 1923, RHODE 1925).

Examination of various types of extracts of bone, bone marrow or periosteum (LEVANDER 1938, ANNERSTEN 1940, BERTELSEN 1944, LACROIX 1949, WILLSTAEDT, LEVANDER and HULT 1950) has indicated the existence of certain substances which possess specific osteogenetic qualities, the so-called osteogenins. Other authors (HELLSTRADIUS, 1947, HEINEN, DOBBS and MASON 1949, LINDAHL and ORELL 1951) attributed a non-specific effect to these substances.

In order to determine if an inductor is liberated from an area of bone repair, HURLEY et al. (1959) interposed a millipore filter between the area of healing and the overlying soft tissues. They observed no evidence of bone formation on the soft tissue side of the filter. On the other hand, GOLDHABER (1961) using both a millipore filter diffusion chamber as well as an immunization technique to discount the possibility of cells escaping from the chamber, was able to demonstrate bone formation on the host side of the chamber which contained a homograft. From these studies, Goldhaber concluded that the formation of new bone on the host side of the filter was the result of a "diffusable osteogenic inductor" coming from the new bone laid down inside the filter chamber.

When other tissue extracts (MOSS 1958, 1960, ANDERSON et al. 1960) were implanted into the anterior chamber of the eye or intra-cranially into rats, cartilage, osteoid-like tissue and at times bone were formed. Chondroitin sulphate has been shown to accelerate the rate of repair around

implants inserted into surgically prepared defects in the skulls of rats (BURGER, SHERMAN and SOBEL 1962).

Different agents have been found to induce bone formation. UPTON (1972) found that autogenous metaphyseal bone, fresh autogenous metaphyseal bone + bone marrow produced bone not only within his modified diffusion chambers but outside as well. Odontogenic tissues (e.g. decalcified dentine) have also been found to induce bone formation (YOEMANS and URIST 1967, MORRIS 1969, 1971).

Mechanical stress in bone, has also been found to be associated with osteogenic activity, for example, MURRAY (1936) reported bone formation in a tendon when excessive tensile stress was applied. Later work by BASSETT and BECKER (1961), SHAMOS, LAVINE and SHAMOS (1963) has shown that bone exhibits piezoelectric effects when deformed. BASSETT (1964) noted that weak currents in vitro stimulated osteogenesis and he postulated that the same principle might also apply in vivo (BASSETT 1966). This has since been confirmed in tooth movement studies (ZENGO, PAWLUK and BASSETT 1973).

Extra-cellular fluids from soft tissues may also play a part in encouraging osteogenesis by providing a rich nutrient source to effect an optimal osteogenic environment (HURLEY, STINCHFIELD, BASSETT and LYON 1959, BASSETT, CREIGHTON and STINCHFIELD 1961, BOYNE 1963).

The precise nature of this environment can only be postulated, but it is possible that there may be a spectrum of influence ranging from enhancement to inhibition. CHALMERS et al. (1975) have suggested that spleen, liver and kidney are inhibitors while muscle and fascia may enhance osteogenesis. If inhibition is incomplete then chondrogenesis rather than osteogenesis is induced (KOSKINEN et al. 1972, WLODARSKI et al. 1973). Other substances such as heparin (STINCHFIELD et al. 1956), ethane -I-hydroxyl -I- diphosphonic acid (EHDP), (CHALMERS et al. 1975) and cortisone

(THOMPSON & URIST 1970), have been found to suppress osteogenic induction.

E. THE BI-PHASIC THEORY OF OSTEOGENESIS

The situation regarding osteogenesis in the repair of bone may be summarised by W. AXHAUSEN's (1956) bi-phasic theory of osteogenesis:

> "Bone regeneration occurs in two osteogenic phases, the first, and physiologically the most important phase, originates in the pre-existing specific cells and begins after several days. The inception of the second phase, however, originating in the non-specific connective tissues, requires several weeks".

In this "theory", W. Axhausen combined the osteoblast "theory" and inductive "theory". URIST and McLEAN (1952) were in agreement with Axhausen, but in addition they tried to classify, in order of merit, the osteogenic activity and inductive capacities of various tissue transplants.

Osteogenic activity

tissue cultures

periosteum

bone marrow

endosteum

cancellous bone

fibro cartilagenous callus

cartilage, epiphyseal and articular

compact bone

devitalised bone

Induced bone formation

Taken from URIST and McLEAN (1952)

In line with W. Axhausen's bi-phasic theory, PRITCHARD (1964) introduced the terms 'osteogenic' and 'fibroblastic' blastemas. He defined a 'blastema' as a mass of proliferating, migrating, differentiating and matrix-producing cells.

Thus, in the meduallary cavity and beneath the cambium layer of the periosteum, an 'osteogenic blastema' develops; while from the fibrous periosteum and extra-periosteal tissues, a 'fibroblastic blastema' is formed.

The osteogenic blastema possesses immediate bone and cartilage-forming powers by virtue of its already highly differentiated state. The fibroblastic blastema normally confines its activity to collagen fibre production, but also seems to have latent osteogenetic powers by virtue of the pluri-potentiality of its mesenchymal tissues, which may be elicited to produce bone under special circumstances.

F. FUNCTIONAL ASPECTS OF THE OSTEOBLAST

The productivity of the osteoblast has been estimated in various ways. OWEN (1963) has calculated, from studies using tritiated thymidine and tritiated glycine, that the osteoblast actively secretes for three days and in this time each cell is responsible for three times its own volume of matrix.

TONNA (1966) showed that osteoblasts remain on the surface of bone for fifty four days. Tetracycline labelling can be used to locate and measure the amount of new bone formation (LACROIX 1951, PONLOT 1960, VANDERHOEFT, KELLY and PATTERSON 1962, LANDEROS and FROST 1964).

In recent years, many important advances in the basic mechanisms of bone formation and calcification have been made. These fundamental issues have been dealt with in detail in several monographs (SOGNNAES 1960, WEIDMANN 1963, RICHELLE and DELLEMAGNE 1965, DEISS 1966, McLEAN and URIST 1968, BLACKWOOD 1964, GLIMCHER 1968, BOURNE 1972) and are beyond the scope of this review.

The following sub-sections deal briefly with some of these issues where it is considered pertinent to the subject of this thesis.

(1) The Pleuri-potential of the 'Progenitor Cell'

Periosteal cells are capable of differentiating into osteoblasts, chondroblasts and osteoclasts which indicates their common origin from a 'progenitor cell' (HAM 1930, PRITCHARD 1961, 1963, 1964, 1972), HAM and HARRIS(1972).

External factors or stimuli which cause these stem cells to differentiate in their various directions have been much debated and according to PRITCHARD (1972) it is generally accepted that parathormone favours the formation of the osteoblast. The mechanism by which differentiation takes place is still uncertain and may involve many complex mechanical, electrical and chemical factors.

Poor vascularity has been suggested as a factor which favours the formation of cartilage rather than bone (HAM 1930, FELL 1932, GIRIS and PRITCHARD 1958, TEMPLETON 1960, BASSETT 1962, SHAW and BASSETT 1967), while HALL (1967) claimed that pressure rather than a low oxygen tension is by far the more important factor.

It is by no means clear which factors influence the subsequent differentiation of the 'Progenitor' cell and YOUNG (1964) stated "In the ultimate analysis these hormonal, vascular and mechanical factors must act through the genetic machinery of the stem cells, switching on and off the appropriate sets of genes; but much more work is needed before this can be put on a firm factual basis".

(2) Osteoblasts and Ground Substance

The importance of ground substance in the repair of soft tissue wounds is well recognised (see APPENDIX I). SCHOENBERG & MOORE (1958), ANTONOPOLOUS et al. (1965), suggested that osteoblasts were able to synthesize mucopolysaccharides. Electron microscope studies indicate that sulphation of these mucopolysaccharides occurred in the golgi apparatus and were then secreted into the extracellular space (GODMAN & LANE (1964), FEWER et al. 1964).

Intra-cytoplasmic periodic acid Schiff (PAS) positive granules have been shown in tissue culture experiments during the early phases of bone matrix formation (JOHNSON 1960, BASSETT 1962). These granules were present just prior to the appearance of extra-cellular metachromasia. This sequence suggests that osteoblasts are in some way associated with mucopolysaccharide production.

While this data indicate that mucopolysaccharides are elaborated by osteoblasts, little is known about the function of these substances in bone formation. Different workers link them with fibrilogenesis (SHATTON & SCHUBERT 1954, FITTON-JACKSON & RANDALL 1956, MEYER 1960), initiation of calcification (SOBEL & BERGER 1954, THOMAS 1961) and inhibition of mineralization (GLIMCHER 1960).

(3) Osteoblasts and Bone Matrix Formation

The organic matrix of bone is composed of a mucopolysaccharide-rich ground substance and collagen.

The knowledge of the location, morphology and histochemistry of osteoblasts in conjunction with the results of labelled studies leave no doubt that these cells actively secrete bone matrix (PRITCHARD 1972). Evidence is overwhelming that the osteoblast takes in amino acids, glucose, sulphate and manufactures mucopolysaccharides and glycoproteins and collagen which form the matrix of the osteoid. The extensive dilated granular endoplasmic reticulum, the large golgi apparatus (FELL 1925, HILL 1936, PRITCHARD 1952), numerous mitochondria (SHELDON and ROBINSON 1957, SCOTT and PEASE 1956, ASCENZI and BENDETTI 1959), PAS + ve granules (DUDLEY and SPIRO 1961) and the conspicuous nucleoli (WEIDENRICH 1928) provide the necessary machinery to carry out this function.

Radioautographic studies using ^{35}S- sulphate have been carried out in order to try and ascertain the existence and function of the mucopolysaccharides in matrix formation (DZIEWIATKOWSKI 1951, 1952, BELANGER (1954). AMPRINO (1956) found ^{35}S- sulphate within the osteoblast and then later in the bone matrix. FITTON-JACKSON and RANDALL (1956) provided

further evidence that osteoblasts were directly involved in the deposition of sulphated mucopolysaccharides in bone tissues. There is limited information about the formation of glycoproteins in bone. However, studies using tritiated fructose have shown that glycoproteins are manufactured by the osteoblast and then secreted to the bone matrix (LEBROND and WEINSTOCK 1972).

The function of osteoblasts in synthesizing collagen has been clarified considerably by the use of radioactive labelled precursors (CARNARIO and LEBLOND (1959), DEISS (1962), TONNA et al. (1963), VAES and NICHOLS (1962), OWEN (1963), LEBLOND (1963), YOUNG (1964), TONNA (1966), OWEN (1970), LEBLOND and WEINSTOCK (1972). FITTON-JACKSON and SMITH (1957), reported that osteoblasts in tissue culture converted C^{14}-L-proline to C^{14} hydroxyproline which is a characteristic component of collagen (see APPENDIX II). LEBLOND et al. (1959), CARNARIO and LEBLOND (1959) and YOUNG (1962) observed tritiated glycine first in osteoblasts, then in the surrounding matrix. They concluded that the protein material was synthesised in the cells and was subsequently secreted to form the fibrils.

Collagenase (NICHOLS 1966, FULLMER and LAZARUS 1967, RODAN and ANBAR 1967) and acid phosphatase (WERGDAL and BAYLINK 1969, RADDEN and FULLMER 1969) are found to be present in the osteoblast. This would indicate that the osteoblast could break down the bone matrix as well as manufacture it.

(4) Osteoblasts and Calcification

The organic matrix is present before evidence of calcification can be detected (SCOTT and PEASE 1956, MOLNAR 1959, JOHNSON 1960, LEBLOND and WEINSTOCK 1972). This uncalcified material has been referred to as 'osteoid'.

Results have indicated that the osteoblast can elaborate components of the 'osteoid' including mucopolysaccharides, glycoproteins and collagen. The addition of collagen occurs at the free edge of the osteoblasts. This layer is in direct contact with a second layer where bone

salts are deposited in an amorphous form (MOLNAR 1959). According to LEBLOND and WEINSTOCK (1972) a 'frontier line' can be demonstrated between the osteoid and the calcified matrix which concentrates such substances as alizarin and tetracycline. These workers have also been able to demonstrate glycoproteins immediately beyond the 'frontier line' at the edge of the calcified matrix. In the next layer the smallest crystals of bone appear to bear a definite relation to an alignment with the 640 Å repeating pattern of the underlying collagen fibrils. This early stage of ossification can be detected with the electron microscope but not by standard histological techniques.

It has been hypothesized that mineralization of an osseous matrix is initiated by some interaction between calcium, phosphate and an, as yet, unidentified nucleation site or template on the collagen fibril (GLIMCHER 1960, GLIMCHER et al. 1968, ROBINSON and SHELDON 1960, BOURNE 1972). PAUTARD (1966) suggested that the osteoblast may take a part in calcification by the transportation of calcium from the blood stream to the calcifying matrix, and that the calcium diffused from the fine processes of the osteoblast (ARNOTT and PAUTARD 1967). In addition to this process, FROST (1967) thought that the final stages of mineral uptake also occurred by direct diffusion from the tissue fluids.

THE HISTOLOGY OF BONE REPAIR

A. HEALING OF FRACTURES IN LONG BONES

(1) The Periosteal Reaction

(2) The Osteocytes

(3) The Endosteum and Marrow Reaction

(4) Cartilage Formation within the Fracture

(5) Quantitative aspects of Callus Formation in Fracture Healing

B. THE HEALING OF CORTICAL BONE DEFECTS

(1) The Histology of Healing Cortical Defects

(2) The External vs the Internal Callus

(3) Cartilage Formation in the Healing of Cortical Defects.

CHAPTER 3

THE HISTOLOGY OF BONE REPAIR

A. HEALING OF FRACTURES IN LONG BONES

Healing of bone has mainly been studied by observing the healing of fractures in long bones, of which ROBBINS (1961) gives the following brief summary:

> "Immediately after a fracture, there is considerable haemorrhage into the fracture site from ruptured vessels within the bone as well as from the torn periosteum and surrounding soft tissues. A haematoma is formed that fills the fracture gap and surrounds the area of injured bone. During the twenty four to forty eight hours that follow, inflammation results in oedema, vascular congestion and infiltration by leukocytes, chiefly neutrophils.
>
> After two days, the neutrophils are accompanied by a large number of macrophages that begin phagocytosis of necrotic tissue and red cell debris. At the same time fibroblastic repair begins with invasion of the blood clot followed later by the formation of callus in and about the fracture site.
>
> After the first few days, newly formed bone and cartilage is present in the area of the fibro-vascular response. In the course of the succeeding days the bony spicules become sufficiently numerous and aggregated to create a temporary bony union of the fracture. By this time, the inflammatory reaction has largely subsided and repair is well under way.
>
> In an uncomplicated fracture, the bony callus usually attains its maximal size at about the end of the second or third week. This callus is increasingly strengthened by the precipitation of bone salts, and the widening of the newly formed delicate bony spicules, and is at

the same time remodelled by osteoclastic and osteoblastic activity. During this reconstruction, the internal callus which fills the marrow space is resorbed. If the fracture has been well aligned, virtually perfect reconstruction of the bone is accomplished".

WEINMANN and SICHER (1955) classified the histological sequence in healing bone as follows:

(a) Formation of haematoma;

(b) organisation of the haematoma;

(c) formation of fibro callus;

(d) formation of primary bony callus;

(e) formation of secondary bony callus;

(f) functional remodelling of the fractured bone.

HAM and HARRIS (1972) consider that the division of fracture healing into such compartments is misleading and that "a fracture is repaired, not by a series of calluses of different origins but by means of the proliferation and differentiation of the members of a special cell lineage, and that the one callus that they begin and continue to form is the only one that forms if union occurs, and that the concept of their being different calluses at different times is based on misinterpreting the different stages seen in the progressive remodelling of a single callus".

The following sections deal with some of the more important aspects of fracture healing.

(1) The Periosteal Reaction

The external callus (periosteal) of a healing fracture develops from the periosteum. The role of the periosteum in osteogenesis has already been dealt with in the previous chapter.

The contribution of the periosteum to the repair of bone is of major importance as an intact periosteal tube is able to regenerate a

large segment of rib or the entire diaphysis of a long bone. In the young this regeneration is complete. However, in the adult with less active periosteum the end result is imperfect and incomplete. The role of the periosteum can be demonstrated in the healing of a fracture when the marrow cavity is almost completely obliterated with a steel rod, as with the case of a "K" nail. Regeneration is in most cases rapid and efficient and is mainly by the periosteum, but in the neck of the femur where the periosteum is not present or is inactive, the repair of bone is slow (McLEAN and URIST 1967).

Twenty four hours after fracture, the most salient feature is that the periosteum, particularly near its torn edge, has become considerably thickened because of the proliferation of the cells in its osteogenic layer. As a result of this the fibrous periosteum on each side of the fracture is lifted away from the surface of the bone. Sections made on subsequent days show these two collars have become increasingly thicker, have grown in towards each other and eventually fused. Thus the external callus is formed by the growth of the cells within these collars. TONNA and CRONKITE (1961, 1962) found after twenty four hours many labelled cells not only in the osteogenic layer close to the fracture, but also along the length of the shaft that had been fractured.

(2) The Osteocytes

Following a fracture the osteocytes in the lacunae near the fracture line become necrotic or degenerate with pyknosis of the nuclei. This is because the source from which these cells obtain nourishment is lost as the result of fracture. According to HAM (1952) the osteocytes in compact bone are never more than 1/10th of a millimetre away from a functioning capillary. The result following a fracture is, therefore, that there is a short segment of cortical bone on each side of the fracture in which the osteocytes die. This dead bone is removed and replaced by new living bone (HAM and HARRIS 1972).

(3) The Endosteum and Marrow Reaction

The formation of external callus has been discussed in conjunction with the periosteal reaction, and now the internal callus will be discussed in relation to the endosteum and marrow cells.

The endosteum is a cellular membrane which lines the marrow cavity and haversian canals of a bone and covers the bony trabeculae present in the marrow cavity. The endosteum contributes to the formation of the internal callus in much the same manner as the periosteum does to the external callus.

However, the second source of osteogenic cells which constitutes the internal callus has to be considered. As mentioned in Chapter 2, there is much evidence to indicate that certain cells in the substance of the marrow can readily form bone. It is probable that part of the internal callus develops from this source. The origin of these osteogenic cells in the marrow substance is not clear. It was believed that both osteogenic cells and cells which became the reticular cells of marrow were derived from the periosteal bud of a bone-to-be. Early experiments with marrow transplants, based on the above assumption led to the belief that the reticulum of the marrow may be transformed into osteogenic cells during repair, as it was believed that these marrow cells retained great mesenchymal potentiality.

In the last decade these former views have been questioned as a result of knowledge derived from marrow transfusion studies in irradiated animals (TILL and McCULLOCH 1961, BECKER et al. 1963). These studies indicate that red bone marrow is derived from stem cells (colony forming units) which do not originate from the periosteal bud. In the mouse, these colony formers were first found to develop in the yolk sac which then enter the circulation to colonise various haemopoietic sites (MOORE and METCALFE 1970). Therefore it now seems hazardous to assume that the stem cells responsible for haemopoiesis in the marrow of a bone are derived from the mesenchyme cells of the periosteal bud. Hence, the concept of fixed re-

ticular cells developing from the periosteal bud and retaining great mesenchymal potentiality is open to question (HAM and HARRIS 1972).

Two alternative sources have been considered:

(i) That bone formation in the marrow close to a fracture arises from free stem cells of the marrow which develop into bone-forming cells by "induction".

(ii) That the marrow is normally permeated with osteogenic cells originating from the periosteal bud, which are normally in a resting state and cannot be distinguished from other marrow cells. These cells are stimulated into an active phase during repair. According to HAM and HARRIS (1972), this later alternative seems to be more likely.

(4) Cartilage Formation within the Fracture

The appearance of cartilage in the fracture most often occurs in the healing of fractures of long bones where considerable displacement of the fragments has occurred or where there is a large defect (McLEAN and URIST 1967).

The significance of cartilage formation in a fracture according to LACROIX (1953) revolves around its organising ability; it not only determines the form of the skeletal tissues but also organises them. The formation of cartilage in the fracture does not require any explanation on the grounds of metaplasia, for cartilage is one normal differentiation product of osteogenic cells (see CHAPTER 2). Cartilage formed within a fracture does not remain for long, as it is rapidly replaced by cancellous bone in the same way as cancellous bone replaces cartilage at the diaphysis of developing bone.

(5) Quantitative aspects of Callus Formation in Fracture Healing

Clinical studies have shown that the amount of callus formed during fracture healing is related to the extent of separation between the fragments. If the fragments are widely separated and displaced, con-

siderable callus is formed which takes a long time to undergo remodelling, months and sometimes even years. The remodelling of callus results in the formation of new trabeculae in planes adapted to withstand stress and strain. If the fragments unite at an angle to one another the new bone that forms in the remodelling process is added to the concave surface and bone is resorbed from the convex side.

ANDERSON (1965) examined the effects of accurate and rigid fixation with respect to primary healing and found that whenever fixation of a fracture is rigid, healing occurs by internal callus formation with minimal external callus. He considered the type of fixation to be unimportant so long as the two fragments were anatomically apposed, with minimal gap between the two fragments and they were completely immobilised. If the ends were not accurately apposed and if the immobilisation was not complete then healing occurred by means of abundant external callus and slow absorption of a large haematoma.

B. THE HEALING OF CORTICAL BONE DEFECTS

Healing following fracture has been well documented. However, there have been few descriptions of repair of a cortical defect in bone apart from those of ELY (1927), BOURNE (1944), PRITCHARD (1956), MURRAY, HOLDEN and ROSCHLAU (1957), MELCHER and DREYER (1961, 1962) MELCHER and IRVING (1962, 1963, 1964), RADDEN (1965), RADDEN and FULLMER (1969).

(1) The Histology of Healing Cortical Defects

MELCHER and IRVING (1964) described the healing of bone in a circumscribed defect in the rat femur. According to them the formation of the haematoma, its organisation and subsequent development of fibrous callus takes place as in the healing of a long bone, as described earlier.

RADDEN and FULLMER (1969) have described the early events in this form of defect more fully as follows:

(a) Two days after the production of the drill hole, a large blood clot was found to occupy the drill hole, extending into the adjacent medullary cavity and out into the

neighbouring soft tissues. At the edges of the clot, granulation tissue was found, accompanied by an inflammatory cellular infiltrate and exudate. This was more substantial in the soft tissues than in the medullary cavity. Subperiosteal bone formation occurred at sites distant from the hole.

(b) At three days there was a clear margin between the blood clot and the edges of the granulation tissue. The first evidence of collagen and internal callus was in the granulation tissue within the medullary cavity. At the same time there was a marked periosteal proliferation and external callus formation was noted.

(c) At four days no clot remained in the bony cavity, but residual clot was still found in the adjacent soft tissues. Extensive new bone was found in the medullary cavity. New bone had also been deposited on the cut edges of the bony defect. Some small bone spicules within the cavity appeared to be the nidus for new bone formation. The subperiosteal new bone was extensive and in many cases the new bone formation was greater at sites distant from the cavity than adjacent to the cavity. All of this new bone was found to occur on the surface of the old without any resorption and in many cases the osteocytes within the bone appeared to be dead or degenerative.

(d) After six days no residual blood clot was seen, the internal callus was extensive and filled the entire medullary cavity in many cases. In others there was considerable internal callus adjacent to the drill hole with further deposits on the opposite side of the medullary cavity. New bone was found at the cut edges of the defect with marked periosteal bone at sites distant from the edges of the defect. In a

few animals the formation of external callus was advanced and bridged the hole while in others at a less advanced stage organising callus still enclosed the hole.

(e) By seven days more new bone had formed in the region of the hole and the periosteal areas than in the region of the internal callus and endosteal areas. However, active remodelling was noted in the internal callus where resorption exceeded deposition.

(f) At nine days all of the drill holes were filled with callus, the periosteal reaction being pronounced, particularly in areas distant from the drill hole. In the deeper regions of the callus, active remodelling and resorption was evident. Maximum callus formation was evident at about eleven days and thereafter remodelling became more active with resorption of the callus by osteoclasts. Activity in the periosteal region became less obvious as time progressed and by twenty one days had almost ceased, however remodelling was still conspicuous.

(2) The External vs the Internal Callus

The internal callus is of major importance in the healing of cortical defects (MELCHER and IRVING 1962, 1964, BOURNE 1944, PALLASCH 1968, KAHNBERG 1974).

The internal callus appears to undergo four phases in this type of repair:

(i) It seals off the medullary cavity from the external environment of the bone, and

(ii) once this has been completed, it proliferates until most of the defect is filled with immature spongy bone.

(iii) Once the subperiosteal callus is well established, the internal callus is largely resorbed (MELCHER and IRVING 1962).

(iv) The internal callus adjoining the ends of the defect remains and is responsible for the attachment of the callus to the ends of the femur.

This is in marked contrast to the healing of a fractured long bone, where the external callus is primarily responsible for the maintenance of continuity between the two ends of the fracture, and to provide strength to the fracture area. Possibly the stimulus for the production of external callus is initiated by the rupture of the periosteum and intensified by the displacement of the fragments. Hence the size of the external callus is proportional to the amount of displacement between the fragments and the rigidity of fixation during the treatment (MELCHER and IRVING 1962, ANDERSON 1965).

The periosteal reaction is of secondary importance in the healing of cortical defects, as the need for rigidity and strength afforded by the external callus in the case of a fractured long bone is not needed.

The external callus can be artificially induced to attain proportions far in excess of those normally produced (PRITCHARD 1956, MURRAY, HOLDEN and ROSCHLAU 1957, MELCHER and DREYER 1962) but this callus only lasted as long as the artificially produced circumstance was maintained.

MELCHER and DREYER (1962) have also noted the formation of external callus on the surface of the femur opposite the defect. These authors believe that it was possibly stimulated by an alteration in the direction of the forces normally transmitted through that part of the bone.

KAHNBERG (1974) set out to determine whether the absence of the periosteum influences bone formation in circumscribed defects prepared without entering the medullary cavity. He found that bone regeneration started earlier and was more complete when the entire cavity was lined with periosteum than when all or part of the periosteum had been excised. The absence of osteogenic cells outside the defect was noticed when the periosteum had been excised and this absence was obvious for the first

two weeks, but the situation changed rapidly with the subsequent appearance of new osteogenic cells. The mechanism of this differentiation to osteogenic cells, according to KAHNBERG (1974) is still vague. According to the theory of induction, as described earlier (URIST and McLEAN 1952, ZACHALINI and URIST 1964), injury to the underlying bone promotes the differentiation of extra-skeletal connective tissue cells into osteogenic cells and new bone. This might also be explained by the activation of the preosteoblasts of TONNA and CRONKITE (1963) which were left in the fibrous connective tissue after resection.

(3) Cartilage Formation in the Healing of Cortical Defects

In the experiments of MELCHER and IRVING (1964), no cartilage was found in the endosteal callus but they did find cartilage in the periosteal callus. This has been reported, and is a normal occurrence in fracture healing particularly if the two fragments are widely separated. Healing from here follows the pattern of normal endochondral ossification, as has been described in the section on fracture healing. Cartilage formation has also been reported by BOURNE (1944) in the healing of a cortical defect in the rat, but he states that cartilage formation does not occur in the guinea pig. RADDEN and FULLMER (1969) however, state that they found no evidence of cartilage formation in the rat in their series.

THE EFFECTS OF CYTOTOXIC AGENTS AND RADIOTHERAPY ON WOUND HEALING

A. THE EFFECTS OF IRRADIATION

(1) The Effects of Irradiation on 'Fibroplasia'

(2) The Effects of Irradiation on Vascular Tissues

(i) Moderate doses

(ii) Large doses

(iii) Late effects

(3) The Effects of Irradiation on Bone

(i) Disturbance of bone growth

(ii) Degenerative changes

(iii) Disturbance of bone repair

B. THE EFFECTS OF CYTOTOXIC AGENTS ON WOUND HEALING

(1) The Effects of Cytotoxic Agents on 'Fibroplasia'

(2) The Effects of Cytotoxic Agents on Blood Vessels

(3) The Effects of Cytotoxic Agents on Bone

(4) Studies on Individual Cytotoxic Agents

(i) The Alkylating Agents

(ii) The Anti-metabolites

a. 5-Fluorouracil

b. Methotrexate

C. A COMPARISON BETWEEN THE EFFECTS OF IRRADIATION AND CYTOLOGICAL AGENTS ON WOUND HEALING

CHAPTER 4

THE EFFECTS OF CYTOTOXIC AGENTS AND RADIOTHERAPY ON WOUND HEALING

Although this thesis primarily deals with the effects of Methotrexate on bone healing, the aim of the project is to ascertain the effects of this drug in the light of the effects of irradiation on wound healing. It is therefore necessary not only to review the effects of cytotoxic agents on wound healing, but that of irradiation as well, in order that valid comparisons between the two may be made.

A. THE EFFECTS OF IRRADIATION

Radiation affects all phases of wound healing, the final changes depending upon:

1. the time of exposure;
2. the dosage given;
3. the physical properties of the ionising radiation.

(1) The effects of Irradiation on 'Fibroplasia'

RANTANEN (1973) has carried out an excellent study on the effects of irradiation on connective tissue repair. His work, and those of earlier investigators may be summarised as follows:

(i) Exposure to radiation at the 'substrate phase' of wound healing caused a delay in the proliferation of cells responsible for the production of ground substance and collagen (GRILLO and POTSAID 1961, RANTANEN 1973).

(ii) However, if exposure was carried out during the phase of collagen synthesis, increased amounts of insoluble collagen were formed without change in the number of cells (RANTANEN 1973).

The rate of gain in tensile strength is delayed, and this was thought to be due to a decrease in the rate of hydroxylation of collagen (DOBBS 1939, LAWRENCE et al. 1953, BLAIR et al. 1961, BRYANT et al. 1968, WEEKS 1968, ZELMAN et al. 1969, ARCHER et al. 1970, THOMPSON and BENNETT 1967, STAJIC and MILOVANOVIC 1970).

Despite the increased amount of insoluble collagen formed, the final tensile strength of the wound is decreased, and is attributed to the altered functional orientation of the collagen (WEEKS 1968, 1971, BRYANT et al. 1968, RANTANEN 1973).

(iii) If, however, the exposure was carried out after the fibres were fully orientated, then an increase in tensile strength was noted with an associated accumulation of insoluble collagen.

(2) The effects of Irradiation on Vascular Tissues

Changes in the vascular system are the most important changes and are responsible for the long-term problems associated with radiation.

(i) Moderate doses of irradiation cause a vasomotor disturbance of the small blood vessels in granulation tissue and the depression of vascular growth during the early phases of healing (VAN DEN BRENK 1956, BLAIR et al. 1961, GRILLO and POTSAID 1961, FOX 1969).

DOTTO et al. (1970) found that irradiation produced an initial depression of vascularisation, which they related to fibroblastic depression. Following this initial delay, vascularisation increased beyond normal values. This hypervascularisation did not occur with higher doses (DOTTO et al. 1970).

(ii) Large doses

Following the administration of large doses, severe vas-

cular changes were observed. MERVIN and HILL (1955) observed a reduced capacity of the endothelium to form new capillaries, thereby inhibiting the re-vascularisation of healing wounds. VAN DEN BRENK (1959) maintained that the inability to regenerate vascular tissues after irradiation was due to secondary hyalinisation of collagen and the formation of a fibrous barrier.

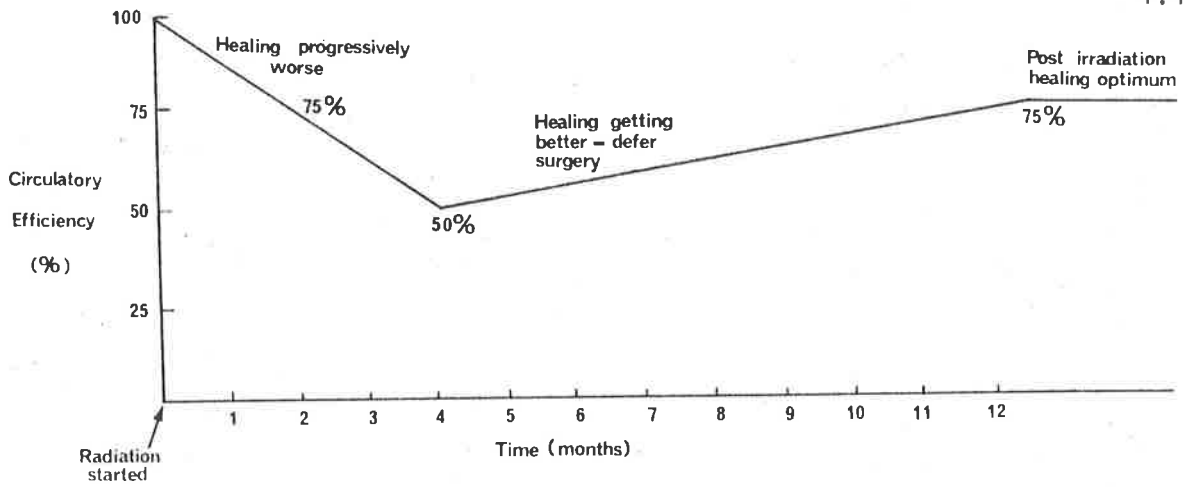
(iii) Late effects

BERDJIS (1960) examined the late effects of irradiation on vascular tissue and noted atherosclerotic changes in the arteries, particularly in the arterioles. There was atrophy of the muscular coat, fibrosis and thickening of the vessel walls (GILLIES and McINDOE 1933, McINDOE 1947, and CADE 1948). The end result was an obliterative endarteritis.

These changes have been considered to be in the blood vessels themselves, although there is evidence to suggest that they may be secondary to changes in the surrounding connective tissues (RHOADES 1948).

The changes in the blood vessels are progressive (CADE 1948, HOFFMEISTER et al. 1969). Their effects are to reduce the regenerative powers of the tissues, rendering them susceptible to infection and impairing their healing capacity (McINDOE 1948).

HOFFMEISTER et al. (1969) investigated the circulatory efficiency of blood vessels in irradiated bone over a 12-month period and arrived at the following graph.



The important point to note with this graph is that the circulatory efficiency decreases rapidly over the first 4 months, thereafter slowly increasing over the next 8 months to reach a plateau. The final circulatory efficiency is approximately 75% of that prior to irradiation. Along with this graph, they correlated the incidence of major complications following dental extractions in irradiated jaws. It was found that the frequency of major complications, e.g. chronic osteomyelitis, occurred most frequently at a time when the blood supply was at its lowest level.

(3) The Effects of Irradiation on Bone

Several reviews of the effects of irradiation on bone have been presented (CLEMEDSON and NELSON 1960, VAUGHAN 1956, 1972). Three main types of change have been noted:

1. Disturbance of bone growth;
2. degenerative changes;
3. disturbance of bone repair.

(i) Disturbance of bone growth

Histological evidence of the inhibitory effects on bone growth has been studied by BARR et al. (1943), MELANOTTI et al. (1961), LEVY and RUGH (1952), ADKINS 1968a, 1968b), and has consistently revealed disturbances of endochondral ossification.

Premature closure of the epiphyseal plates occurs, manifested by the presence of a bony plate on the epiphyseal

side. In the marrow there is a decreased number of osteoblasts, devitalisation of the bony trabeculae and an increase in the number of multinucleated cells.

(ii) Degenerative changes

As well as its influence on endochondral ossification and growth, ionising radiation has an effect on mature bone tissue.

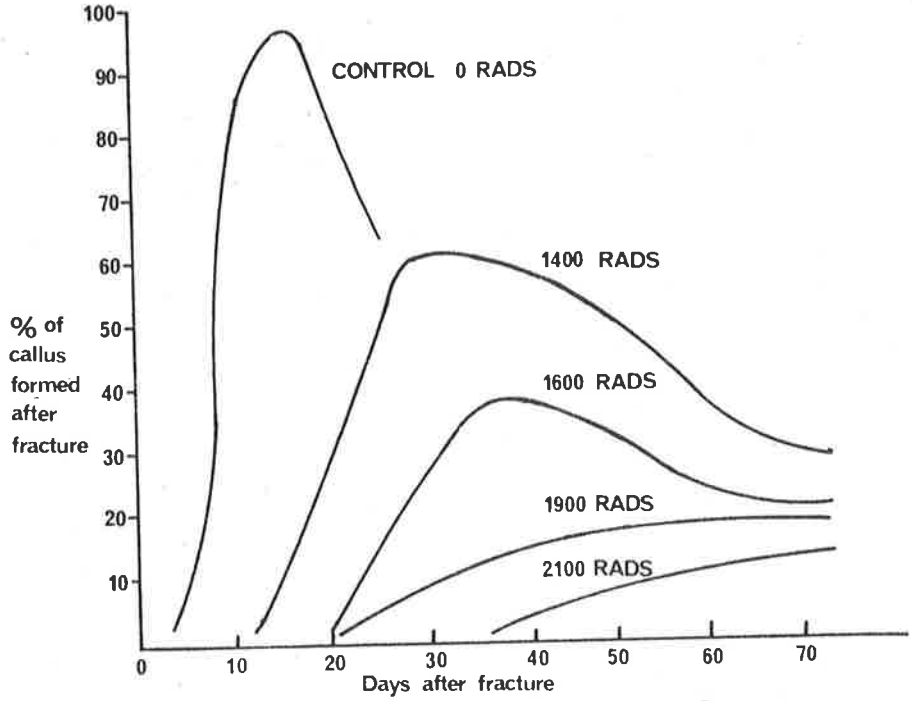
The histopathology of irradiated bone in humans has been studied and described by EWING (1926) and PHEMISTER (1926). HINKEL (1943a & b), HELLER (1948), COPELAND (1953), and BASERGIA, et al. (1961) have carried out similar experimental studies on animals. There is general agreement that the osteoblasts disappear along with the osteocytes. MELANOTTE and FOLLIS (1961) consider that the osteoblasts are over active in the early phases of radiation and then disappear later.

The fate of the osteoclast is disputed. COPELAND (1953) considered the osteoclast to be more radio sensitive than the osteoblast and considered that this explained the lack of bone resorption following radiation. GATES (1943) and HELLER (1948) found the bone marrow underwent hyaline, gelatinous or fibrous degeneration with a reduction in the number of and obliteration of the blood vessels with considerable increase in mineral deposit. Histologic examination of spontaneously fractured irradiated bone (BICKEL, CHILDS and PORRETTA 1961) has revealed similar changes. These changes in irradiated bone have been referred to as radiation osteitis (EWING 1926), radiation-osteodysplasia (VAUGHAN 1956) and osteoradionecrosis by REGAUD (1922a & b) a name which is most commonly used today.

(iii) Disturbance of bone repair

Few experiments have been carried out in order to ascertain the effects of irradiation on bone healing. Experiments in this area have studied the effects of irradiation on extraction socket healing (STEIN, BRADY and RAVENTOS 1957, FRANDSEN 1962, and SHEARER 1967). It is generally agreed that healing of the extraction wound is retarded by irradiation. Surface closure is retarded which allows a pathway for infection. Healing within the defect follows the same pattern, with a decrease in the amount of bone formation but as the interval between the time of extraction and irradiation increases, the impairment to healing of the extraction site is decreased.

In the case of normal fracture healing in mice, rapid proliferation of the periosteal cells starts soon after fracture and reaches a peak 32 hours later, with maximum callus formation occurring somewhere between 16 and 23 days (HAYASHI and SUIT 1971). In irradiated bone, it has been found that irradiation suppresses the amount of callus formed (STEIN et al. 1957, HAYASHI and SUIT 1971). The following graph taken from HAYASHI and SUIT's (1971) paper demonstrates the above facts.



Other important facts can be seen from this graph:

(1) As the dose of irradiation increases, not only does the amount of callus decrease, but the time at which the callus first appears increases from 1-2 days -- 35-36 days.

(2) The control graph (zero irradiation) shows a rapid increase in the amount of callus formation, which reaches a maximum by 16-23 days. However following this, a decrease in the amount of callus is noted which represents the remodelling phase. It can be seen that the amount of remodelling also decreases with increasing doses of irradiation. All phases of bone healing are effected by irradiation, from the substrate phase through to the remodelling phase, and as such behaves in much the same way as other connective tissues.

B. THE EFFECTS OF CYTOTOXIC AGENTS ON WOUND HEALING

In general the effect of anti-neoplastic agents depends upon the dose administered, the route of administration, and the time of administration in relation to the healing wound (CALNAN and DAVIES 1965, NEWCOMBE 1966).

The precise mechanisms leading to normal wound healing are still unknown. Histochemical studies have indicated that the deposition of ground substance and collagen are early events. Collagen deposition is vital to the increase in early tensile strength. Interference with the formation of collagen or any of its precursors at the site of injury may cause a decrease in tensile strength (STALEY et al. 1962).

It is thought that these agents generally act on the early phases of wound healing, by retarding activation, mobilisation, migration and proliferation of cells.

(1) The effects of Cytotoxic Agents on 'Fibroplasia'

It is generally agreed that the action of anti-neoplastic agents is chiefly against tissues which are actively proliferating. BARTON and

LAIRD (1957) found that regeneration of hepatic tissue was not affected by methotrexate when this regeneration followed fasting. However, where regeneration followed partial hepatectomy, and consequently there was an increase in liver cells, the nucleic acid metabolism was susceptible to inhibition by methotrexate.

DESPREZ and KIEHN (1960) carried out an investigation into the effects of cyclophosphamide on the healing of primary wounds, skin autografts and granulating wounds in mice. Initial experimentation showed that systemic effects of weight loss, leucopenia, and mortality were directly related to the dose of drug given. The tensile strength of the primary wound was found to decrease with increasing amounts of drug. However, the wounds healed in spite of relatively large administration of drug. Histologically they found a decreased inflammatory, cellular and fibrinous exudate in the early phases of wound healing. In the later stages they found a decrease in the deposition of collagen and maturation of the scar. The fibroblasts were found to be less mature than those in the controls. Cyclophosphamide had little effect on the "take" of skin grafts. However, they found definite retardation of the granulating wounds. Retardation of the inflammatory response, formation of granulation tissue and wound contraction was seen. Epithelial migration was retarded along with that of fibroplasia. The fibroblasts were immature with enlarged nuclei with fibrous strands in close proximity, but orderly deposition of collagen was not seen.

Many experiments have been carried out utilizing tensile and bursting strengths of wounds in order to quantitate the strength of the wound. The alkylating agents have been tested in this area. Triethylenethiophosphoramide (Thio-Tepa) was found to decrease the bursting strength of abdominal wounds (PISESKY et al. 1957). Triethylenemelamine was found to delay the healing of gastrotomy wounds (KREMENTZ et al. 1957), and FARHAT et al. (1958) found that systemically administered Mechlorethamine hydrochloride (nitrogen mustard) reduced the

tensile strength of laparotomy wounds. Inhibition of fibroplasia was thought to be responsible for this delay.

HARRIS and THOMAS (1961) using topically administered mechlorethamine hydrochloride, found a decrease of 52% in tensile strength of healing wounds in mice treated with 10 mg/100 ml of drug. No decrease of fibroplasia was evident, but abnormal healing of the wounds was found to be associated with a non-specific inflammatory reaction.

HARDESTY (1958) noted that the tensile strength of wounds treated with 9 mg/100 ml of mechlorethamine hydrochloride was decreased initially but returned to normal by the ninth day. Histologically a decrease in fibroplasia was observed.

STALEY et al. (1961, 1962) demonstrated a reduction in tensile strength of abdominal wounds in rats treated with 5-fluorouracil, mechlorethamine hydrochloride, streptovitacin A while thio-tepa and cyclophosphamide produced no effect on healing. KENNINGER (1962) observed poorly granulating wounds in three patients receiving mechlorethamine oxide hydrochloride (Nitpomin). This was confirmed histologically and functionally in gastrotomy wounds in guinea pigs.

(2) The Effects of Cytotoxic Agents on Blood Vessels

In a search of the literature, the author can find no reference to the effects of cytotoxic agents on proliferation of vascular tissues or on existing vascular tissue.

(3) The Effects of Cytotoxic Agents on Bone

As far as the author is aware, there has been no study carried out on the effects of cytotoxic agents on bone or bone healing.

(4) Studies on Individual Cytotoxic Agents

(i) The Alkylating Agents

Most of the publications on the effects of anti-neoplastic agents on wound healing are related to the alkylating agents. There is general agreement that these agents delay wound healing (KAIZER et al. 1961, HARDESTY 1958, FARHAT et al. 1958

PLUMMER et al. (1952) has shown that triethylenemelamine completely inhibits mitosis of chick fibroblast cultures for twenty four hours or longer. Although mitosis was stopped, migration of the cells still occurred. From this experiment they concluded that the rate and quality of wound healing may be effected.

CONN et al. (1957) stated "The fact that the drugs produce no demonstrable effect on wound healing seems a little surprising, as the basis for their therapeutic use is the cytotoxic and growth inhibiting effect they have on rapidly proliferating cells".

KAIZER et al. (1961) carried out experiments on wound healing in dogs with nitrogen mustard and methotrexate using histological and clinical methods of assessment. All animals perfused with nitrogen mustard developed a marked transient oedema. Of those animals wounded, a marked erythema surrounding the wound was observed, and in some cases a degree of infection and disruption of the wound occurred. About 25% of the wounds were found to break down and heal by secondary intention.

Histologically, abnormal fibroblasts were found which they described as being "similar to those in irradiated tissues, with bizarre shapes, sizes, and staining characteristics". There was a delay in active fibroplasia and proliferation of endothelial cells until the fourth to fifth postoperative day. The granulation tissue was in the nature of a filmy pale staining connective tissue which contained a small number of extracellular fibres.

GUPTA, SINGH and UDUPTA (1970) again studied the effects of cyclophosphamide on wound healing and found:

1. Epithelial union had not occurred by the twelfth postoperative day.

2. The gap between the epithelial edges was filled with a necrotic slough and the scab had not separated.
3. The healing of the underlying dermis was not complete, collagen fibres being present but no organisation of them evident.

(ii) The Anti-metabolites

The effects of the anti-metabolite group such as methotrexate (CALNAN and DAVIES 1965) and fluorouracil (STALEY 1961, GOLDMAN et al. 1969) have been tested by utilising tensile strength, bursting strength and histological assessment of healing wounds.

a. 5-Fluorouracil (5FU)

5FU has been found to retard healing (STALEY 1962, WALDORF et al. 1966, GOLDMAN et al. 1969).

DILLOHA et al. (1965) found that 5FU labelled with C^{14} had minimal systemic effect but had a strong local effect.

WALDORF et al. (1966) studied the effects of topical 5FU on the epithelialisation of the wound and postulated that interference with the synthesis of R.N.A. and its function produced a disturbance in the formation of epidermal proteins, including keratin. Application of the drug to normal skin had no deleterious effect, but when the skin was damaged, some effect could be demonstrated. They suggested that some damage was necessary for the drug to penetrate the epidermal barrier. However, it seemed more likely that the effect was due to the drug's interference on mitosis of the cells involved in the process of repair.

GOLDMAN et al. (1969) found that 5FU delayed the healing of intestinal anastomoses in rats and considered that this was due to a delay in collagen formation. STALEY et al. (1961) confirmed this when they carried out tensile strength experiments on abdominal wounds in rats.

Postoperative intra-peritoneal adhesions have remained a problem for the surgeon. Their formation involves all of the phases of wound healing. For this reason GOLDMAN et al. (1967) investigated the effects of 5-Fluorouracil (5FU) on the process of adhesion formation.

GOLDMAN et al. (1967) found that adhesions were prevented by using 50 mgs/kg per day dosage, but also found that marked general toxicity was found with this dose.

b. Methotrexate

KIEHN, DESPREZ and BENSON (1962) found that methotrexate did not affect a primary wound or the take of a skin graft or the formation of granulation tissue unless the dose exceeded the LD_{50} in mice. According to CALNAN and DAVIES (1965) methotrexate has a marked effect on the early stages of wound healing which is directly proportional to the dose administered.

C. A COMPARISON BETWEEN THE EFFECTS OF IRRADIATION AND CYTOTOXIC AGENTS ON WOUND HEALING

Although there is delayed healing when a wound is irradiated, clinically this is not as significant as the accumulative and long term effects of radiation on body tissues. Changes in the blood vessels, connective tissues and bone render the body susceptible to subsequent infection and delays the healing of wounds in tissues which have been irradiated a long time ago.

Thus, if devitalised bone (osteonecrosis) is traumatised as in the case of dental extractions, the wound does not heal, and infection will

lead to chronic osteomyelitis.

A review of the literature on the effects of cytotoxic drugs on wound healing has revealed inadequate and superficial investigations on this subject. Most of the studies were related to alkylating agents and referred to tensile strength studies. A small number have been of a histological nature and generally show a retardation of the early phases of wound healing by reducing fibroplasia. However, no study was found which relates to the effects of these drugs on the vascular system or on bone.

Cytotoxic drugs are effective against malignancy and are used alone, or in conjunction with radiotherapy and surgery. They may be used for palliation and for cure, their effectiveness against large inoperable tumours often being rewarding. These agents also retard wound healing during the period of administration.

At this stage it is not known if the cytotoxic drugs have an accumulative or lasting effect on body tissues in the same way as irradiation. Similarly it is not known if these drugs have any effect on the vascular tissues or on bone in the same way as radiation.

CHAPTER 5

OBJECTS AND METHODOLOGY OF THE PRESENT INVESTIGATION

A. OBJECTS

B. METHODOLOGY

(1) The Experimental Model

(2) Choice of the Experimental Animal

(3) Choice of the Anti-cancer Chemotherapeutic Agent

(4) Method of Administration

(5) Dosage

(6) Schedule of Drug Administration

(7) Establishment of a Control

C. THE HISTOLOGICAL INVESTIGATION

(1) The "lag phase" Mucopolysaccharide Production

(2) Cellular Proliferation

(3) Collagen Formation

(4) New Bone Formation

CHAPTER 5

OBJECT AND METHODOLOGY OF THE PRESENT INVESTIGATION

A. OBJECTS

Frequently, the mandible and maxillae are irradiated in the course of treatment of malignancies in the head and neck. The hazards of dental extractions in an irradiated jaw are now well known. Chronic osteomyelitis presents a problem for both patient and clinician alike, for this condition is often as troublesome as the original lesion.

As can be seen in the previous chapter, radiotherapy poses long term hazards in relation to healing wounds. Osteoradionecrosis leading to osteomyelitis has often been reported as a complication of radiotherapy to the head and neck (RANKOW & WEISSMAN 1971, GRANT et al. 1966, LAWRENCE 1946, MacCOMB 1962, MacDOUGAL et al. 1963, MARCHETTA et al. 1967, McLENNA 1955, MEYER 1958).

More and more patients with head and neck malignancies are now subjected to anti-cancer chemotherapy, either alone or in conjunction with other forms of therapy. However, little is known about the effects of these agents on healing of tissues. As far as the author is aware, no experimental studies have been carried out to determine the effects of these agents on bone healing.

From a clinical point of view, many questions must be posed by the dentist in the case of patients scheduled for anti-cancer chemotherapy. For example:

1. Does the state of the patient's oral condition need to be viewed in the same light as that of a patient receiving radiotherapy?
2. If dental extractions are needed, at what stage should they be carried out in relation to chemotherapy?

3. If the patient is already receiving or has received chemotherapy, what period of time should lapse between cessation of therapy and dental extraction?

Further, many patients are receiving combined radiotherapy and chemotherapy. In these patients, would the two forms of therapy have a synergistic effect on delaying wound healing?

At present, these questions have no ready answer, thus the OBJECT of this investigation is to study the effect of METHOTREXATE, one of the more commonly used anti-cancer chemotherapeutic agents, on bone healing. It is an attempt to provide some of the answers to the questions which have been posed.

B. METHODOLOGY

(1) The Experimental Model

Fracture of long bones has traditionally been used in bone healing studies. This experimental model has several disadvantages. For example the difficulty of maintaining fixation of the fracture in an experimental animal, leads to uncontrolled displacement of the fragments. It has been pointed out in Chapter 3 that the amount of external callus formed depends on this factor.

Dental extraction offers another possible method of producing a bone wound which has the advantages of being closely allied to the clinical situation faced by the dentist. However, the extraction wound is an "open" wound and is therefore complicated by contamination from saliva and micro-organisms and is subjected to trauma from coarse fibrous foods.

The use of a drill hole as an experimental model is now well established (BOURNE 1944, MELCHER & IRVING 1963, 1964, RADDEN & FULLMER 1969). It can be closed, is thus protected from external contamination and it needs no fixation. Another important advantage offered by this model is the accuracy with which the cortical defect can be repeatedly produced thereby standardising the experiment. PALLASCH (1968) stated that "....the healing response of the rat femur in which an osseous defect has been

created follows a predictable series of events. Therefore, these events could serve as an experimental model upon which to observe drug activity". This method of producing a bony defect was therefore used, the detailed technique of which will be discussed in the next chapter.

(2) Choice of the Experimental Animal

The rat was chosen as the experimental animal, as it is easily housed, cheap to buy and easy to handle. The large number of animals required in this project in order to obtain valid experimental results made the above factors very realistic. With such numbers it would have been impossible to house larger animals in the existing facilities of our laboratories. Young males weighing between 200-300 gms. were used exclusively. Males were chosen so as to eliminate any possible influence the oestrous cycle may have on the healing of cortical defects. Age is important for there is variation in healing with increasing age. Young animals of the same age were used in order to eliminate this problem and ensure the healing of the defect under optimal conditions.

(3) Choice of the Anti-cancer Chemotherapeutic Agent

In Chapter 1 a number of commonly used agents were discussed, of which the alkylating agents have been the most studied. For head and neck malignancies METHOTREXATE is one of the most commonly used agents. The effects of this drug on wound healing are not well understood as has been pointed out in Chapter 4.

Other agents such as Bleomycin and 5-fluorouracil are also commonly used. However these agents are not readily obtainable and the time available for the present investigation did not permit them to be included in this study.

(4) Method of Administration

Continuous arterial infusion in combination with the systemic administration of an antidote is the method of choice in the treatment of head and neck malignancies with anti-cancer chemotherapy in humans, (see Chapter 3). It would be reasonable to use the same method in the present

experimental study if it was technically possible. This method of administration would offer several advantages. It would be possible to infuse one leg and use the contralateral leg as a control in the same animal. It would also mean a sustained blood level and continual action of the drug on the tissues during the entire infusion period. Initially arterial infusion was attempted on several animals. Continuous infusion proved to be impracticable as it necessitated keeping the experimental animal restrained for long periods of time. However, it was thought possible for frequent regular arterial injections to be administered, for example on a daily basis. The larger size of the rabbit made this animal more suitable for this type of experiment and attempts were made to catheterise the external carotid artery on one side. This was achieved by passing a catheter backwards into the external carotid artery from the superficial ear artery, which as far as can be ascertained corresponds to the superficial temporal artery in man. Successful catheterisation was carried out, but problems were encountered:

1. It was almost impossible to maintain the catheter in position as the animal kept on dislodging the catheter from its intended position.
2. It was not possible to maintain the patency of the catheter due to its small diameter and the ease with which it became blocked with blood clot.

Various methods of burying the catheter subcutaneously were tried without satisfactory results. Similarly, attempts at maintaining the patency of the catheter by heparinisation also proved futile. Arterial infusion was therefore abandoned in favour of systemic administration via the intraperitoneal cavity in the same way as CALNAN and DAVIES (1965).

(5) Dosage

It was a difficult procedure to determine a suitable dose of METHOTREXATE for use in the present investigation. Previous experiments by CALNAN and DAVIES (1965) used various doses via the intraperitoneal

cavity and concluded that wound healing was depressed significantly (as demonstrated by tensile strength measurements) when a dose of 0.3 mgs/kgm for 5 days was used and that considerable depression was found with 0.9 mgs/kgm for 5 days. This later dose corresponded to the oral, systemic and intra-arterial infusion doses in man.

RALL (1963) stated that METHOTREXATE was cumulative and that the LD_{50} for the rat in a single dose was 100 mg/kg body weight. If the drug was given daily for 15 days, the LD_{50} was about 0.25 mg/kg/day. Thus, it seems that the LD_{50} varied widely depending on the schedule of drug administration (in this case a 30-fold difference).

MATHE' (1969) stated that the LD_{50} was also related to the method of administration, an aspect which RALL did not consider in his paper.

An estimation based on therapeutic doses in humans could be used, but the species difference and the different mode and schedule of administration in this study compared to the clinical situation, would render such an estimation invalid. For this reason, it was decided to determine the LD_{50} in rats using the intraperitoneal method of administration and from this to estimate the dosage to be given to the animals in the present investigation (see following section).

The rate of absorption of methotrexate from the intraperitoneal cavity, the blood levels reached, the rate of drug excretion and detoxification are not known in the rat. No attempts have been made in this investigation to determine the above factors.

(6) Schedule of Drug Administration

Various time schedules have been used for drug administration. CALNAN and DAVIES (1965) administered the drug (by daily intraperitoneal injection) for a period of 5 days, while RALL (1963) used a 15 day period in his calculation of the LD_{50} for the rat. RADDEN and FULLMER (1969) have found that a cortical defect in the femur is mostly filled with internal callus by the 6th day and had reached maximum callus formation by the 11th

day (see Chapter 3). Thus, active proliferation of cells, production of granulation tissue and callus, have mostly been completed by the 6th day. It therefore seemed reasonable to administer the drug for this time period.

However, in order to determine a therapeutic dose for the purpose of this experiment it is necessary to estimate the LD_{50} for the administration of intra-peritoneal METHOTREXATE in the rat. An arbitrary dose of 1 mg/day was given to 10 animals for a period of 6 days. This is equivalent to 5 mg/kg body weight for 6 days and comes to a total dose of 30 mgs/kg. All of the animals died, following the exhibition of the following signs:

(1) alopecia;

(2) profuse diarrhoea;

(3) petechial haemorrhages around the feet and nose of the animals.

Following this, the dose was halved to 0.5 mg of METHOTREXATE per day for 6 days. Once again, most of the animals died with similar clinical signs. Once again, the dosage was halved to 0.25 mgs/day and this time 100% survival was obtained.

It was therefore considered, that for intraperitoneal administration, the LD_{50} over a 6 day course of drug is somewhere between 0.25 mg/day - 0.5 mg/day (1 mg/kg-2 mg/kg) in male rats weighing between 200-300 gms. The dosage of 0.25 mg/day (1 mg/kg) was subsequently used in this experiment as the therapeutic dose. The total dose given was 6.0-7.5 mg/kg body weight depending on the exact weight of the animal. This dose was double that of RALL (1963) who gave an LD_{50} at a total dose of 3.75 mg/kg spread over 15 days and is in the upper limits of the doses used by CALNAN and DAVIES in their experiments.

There are two aspects in this investigation that are of particular interest. The first is: what are the effects of methotrexate on the healing tissues if administered during the period of wound healing? The

second aspect is: if a drug has a deleterious affect on healing, for how long would this effect last following the cessation of administration of the drug?

Two series of experiments were therefore planned. In the first series, methotrexate was administered simultaneously with wounding and continued daily for 6 days. This series of experiments was designated the O-M (Operation-Methotrexate) series. In the second series of experiments, methotrexate was administered daily for 6 days prior to wounding. This series was designated the M-O (Methotrexate-Operation) series.

(7) Establishment of a Control

As mentioned above, the ideal method of administration is via arterial infusion, and with systemic administration of Citrovorum factor would allow the contralateral leg to be used as a control in the same animal. Technically this proved to be difficult and so intraperitoneal administration was used. However, this necessitated the use of separate animals as a control. Thus, a separate series of animals matched for age, weight and sex were used on which the standard wound was produced and studied over the same time intervals (details in Chapter 6).

C. THE HISTOLOGICAL INVESTIGATION

In Chapters 2 and 3 it was pointed out that there are a number of phases that are of great importance in wound healing. These are:

1. The "lag phase" in which mucopolysaccharides are produced.
2. Cell proliferation.
3. Collagen formation.
4. Bone formation.

Attempts have been made in this investigation to compare the four stages of repair in the various series of experiments with the control wounds.

1. The "lag phase" Mucopolysaccharide production

The role of ground substance in the repair of soft tissue and bone wounds has been discussed (see Chapter 2 and Appendices I and II).

The presence of mucopolysaccharides in the ground substance can be identified by histochemical methods as will be discussed later. No attempt was made to assay the quantitative aspects of these substances. The connective tissue mucopolysaccharides appear to be more difficult to retain in tissue specimens than the mucopolysaccharides of epithelium (CURRAN 1961). Many fixatives have been tried but none is completely satisfactory. Some of the fixatives clearly cannot be used in conjunction with various stains, e.g. Zenker's solution abolishes metachromasia (HALMI and DAVIES 1953), interferes with the colloidal iron stains (FISHER and HAZARD 1954) and depresses the alcian blue method (WAGNER and SHAPIRO 1957). Fixation is unpredictable in general and in particular, fixation of the mucopolysaccharides is unsatisfactory. CURRAN (1961) found that formalin is an adequate fixative and has therefore been used in the present investigation.

Certain mucin stains have been available for a long time and have been used to stain the mucopolysaccharides. These include mucicarmine (SOUTHGATE 1927) and mucihaematin (LASKEY 1950). These substances stain the highly sulphated mucopolysaccharides, present in many epithelial mucins, strongly, but are of doubtful use in staining the mucopolysaccharides of connective tissue.

The following histochemical methods were used for the demonstration of ground substance in the present investigation:

(i) Periodic Acid Schiff reaction, with prior digestion with diastase (Appendix V)

(ii) The Alcian Blue method (Appendix V).

The periodic Acid Schiff (P.A.S.) reaction

The P.A.S. reaction was first introduced by McMANUS (1946) and HOTCHKIS (1947) for the demonstration of mucin; however this method has been adopted as an acceptable histochemical procedure. Periodic acid is a selective oxidant which attacks the 1,2 glycol, 1-hydroxy-2-amino, 1-hydroxy-2-alkyloamino and 1-hydroxy-2-keto groupings (BARKER and ANDERSON 1963). As a result of this oxidation, at least one aldehyde group is

formed which is subsequently demonstrated colorimetrically by the Schiff's reagent.

If glycogen is first removed by treating the tissues with diastase then a positive P.A.S. reaction strongly indicates the presence of glycoproteins, mucoproteins and glycolipids (BARKER and ANDERSON 1963). The neutral mucopolysaccharides also stain well with the P.A.S. reaction: however, these do not occur commonly in higher animals. Acid mucopolysaccharides show no distinct positive reaction in P.A.S. staining being either P.A.S. negative or weakly staining (BARKER and ANDERSON 1963).

The Alcian Blue method

Various histochemical stains have been used to demonstrate the presence of acid mucopolysaccharides in tissues, with varying degrees of specificity. These include Alcian Blue method, Astra Blue, Toluidine Blue and the colloidal iron method. In the present investigation the Alcian Blue method was used.

Alcian Blue is a copper phthalocyanin dye, which will stain acid (including sulphated) mucopolysaccharides by salt linkage with the acidic groups, (DRURY and WALLINGTON 1967). According to LYNCH et al. (1969), Alcian Blue is an excellent stain which is easy to use and has a resistance to a variety of counter stains. Its use in acid solution makes it more specific for acid mucopolysaccharides (PEARSE 1960). At a pH 2.5, both carboxyl and sulphate groups take up the stain, but if the PH is lowered to 1.0 only the sulphated groups will stain (LYNCH et al. 1969).

According to CURRAN (1961) positive results at a pH below 2.8 are suggestive of the presence of acid mucopolysaccharides. LEV and SPICER (1964) stated "Staining with Alcian Blue at pH 1.0 can be made selective for sulphated mucopolysaccharides by the simple expedient of blotting the sections dry after their exposure to the staining solution prior to dehydration and mounting". In the present investigation, P.A.S. was used to demonstrate the presence of glycoproteins or mucoproteins while Alcian Blue stain was used in order to try and demonstrate the acid muco-

Metachromatic dyes have also been used to study the mucopolysaccharides. The property of metachromasia was first discovered by HESCHL (1875) and the dyes so named by EHRLICH in 1877. However, the basis of this phenomenon (the staining of certain tissue components a different colour to the original dye) is still in dispute, but according to CURRAN (1961), it appears to depend upon the availability of consecutive, regularly spaced, anionic groups along the carbohydrate chain.

There are a number of dyes which produce metachromasia, but Toluidine Blue is the one most often used, while Celestine Blue provides a useful alternative (LENDRUM 1947).

HALE (1946) described a method of staining the sulphate groups of the mucopolysaccharides or the uronic acid groups of hyaluronic acid with dialysed iron and subsequent colouring with prussian blue. According to CURRAN (1961) the colloidal iron method discriminates between the acid and neutral mucopolysaccharides. However, BRADEN (1954) checked the specificity of the mucopolysaccharide stains and came to the conclusion that Hale's colloidal iron stain was non-specific for the mucopolysaccharides. PEARSE (1951), DAVIES (1952) and THONARD and SCHERP (1962) have all made the same observation. In view of this uncertainty about the specificity for the Hale's staining the Alcian Blue and periodic acid-Schiff reactions have been used to stain the mucopolysaccharides of ground substance in this project.

(2) Cellular Proliferation

By far the most reliable and accurate method of identifying dividing cells and to trace their fate is by the use of radioisotopes (e.g. tritiated thymidine) and their subsequent demonstration using the technique of autoradiography. However these methods are expensive and time consuming and are not permitted by the circumstances of the present investigation.

The use of colchicine to arrest mitosis has been used as a

relatively crude method of studying mitotic rates (BRUES 1936, SENTEIN 1942a, b and 1943a, b, LEBLOND and STEVENS 1948, BULLOUGH 1950). It has been used with success in the study of epithelial tissues (EIGSTI et al. 1949, EIGSTI, 1955). However, its use in the study of connective tissues is limited and the results are hard to identify (CHAU 1968). It was therefore decided to identify cell proliferation by routine microscopy.

(3) Collagen Formation

Collagen fibres are the most important functional element of granulation tissue. These fibres can be well demonstrated by the Van Gieson and Mallory's aniline blue stains (Appendix V).

Compared with collagen, reticulin fibres are much finer, of irregular course and widely anastomosed. These fibres are not stained by the methods used to stain collagen, or general stains such as combination of haematoxylin and eosin.

Special stains, of which silver impregnation is the most widely used, give reticulin fibres a uniform black colour. AMLER et al. (1964) have summarised the differences between collagen and reticulin fibres.

With additional techniques at least three varieties of reticulin fibres have beeen demonstrated by them.

1. Pre-collagenic fibres which are seen in embryonic tissues and wound healing (ROBB-SMITH 1957).
2. Fibres which are apparently prolongations of reticulin cells in the spleen and lymphoid organs (LILLIE 1952).
3. Argyrophilic fibres, which are neither related to reticulin cells, nor do they mature into collagen fibres, such as those fibres found in the stroma of the kidney and liver (KRAMER and LITTLE 1953).

In this project the silver impregnation technique of LILLIE (1946) has been used (Appendix V) to demonstrate pre-collagenic fibres.

(4) New Bone Formation

Various methods have been used to demonstrate new bone formation, some of which will be discussed here. The agent Chlorozolfast-pink is incorporated into the osteogenic matrix of bone, if given during the period of bone formation. It has been used to demonstrate the sites of new bone formation (WEATHERALL and HOBBS 1960, STOREY 1968). However, new bone is readily identified under the light microscope in routine H & E sections. The additional use of chlorozol-fast-pink was therefore not used.

The tetracycline antibiotics are also incorporated into the inorganic crystals of calcified bone tissues if administered during the period of calcification. Their subsequent demonstration requires the use of undecalcified sections and ultra-violet light microscopy. The time available for the present investigation precluded the use of this additional method of identifying sites of calcification in newly formed bone.

CHAPTER 6

MATERIALS AND METHODS

A. WOUND PRODUCTION

B. EXPERIMENTAL DETAILS

(1) Group I - Control

(2) Group II - Operation and then Methotrexate Administration (OM)

(3) Group III - Methotrexate Administration and then Operation (MO)

CHAPTER 6

MATERIALS AND METHODS

A. WOUND PRODUCTION

The model used for this experiment is based on that used by BOURNE (1944), MELCHER and DREYER (1961), MELCHER and DREYER (1962), MELCHER and IRVING (1963, 1964).

Under intra-peritoneal barbiturate (Nembutal*) anaesthesia an incision was made through the skin on the inner aspect of the leg of the rat. Further exposure was carried out by blunt dissection to expose the muscles attached to the femur, the femoral artery, vein and the sciatic nerve. These major structures were retracted from the operative site. An incision was made down to bone through muscle and in a plane parallel with the muscle fibres. The periosteum was reflected from bone and a drill hole was made in the mid shaft of the femur with a No. 3 inverted cone bur using a dental handpiece with an adequate water coolant. The drill hole was made through the entire thickness of the cortical bone.

The drill hole was irrigated with water, the periosteum replaced and the wound debrided. Following this a 3/0 black silk suture was placed through the muscle to provide accurate adaptation over the drill hole. In this way the muscle was approximated and the suture retained to act as a land-mark for orientation of the specimen when it was blocked. After the skin wound was closed with 3/0 black silk sutures, the animal was resuscitated and returned to the animal house. For details of the instrumentation and operative technique see Appendix III.

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*Nembutal: (Abbot's Veterinary Nembutal).
See Appendix III for details of anaesthesia.

B. EXPERIMENTAL DETAILS

Three groups of animals were used:

(1) Group I - Control

The animals in this group had a standard drill hole placed in the mid shaft of the femur of both legs. This group is designated C.

(2) Group II - Operation and then Methotrexate Administration.

The animals were operated on and then immediately after the operation, were given 0.25 mg of methotrexate daily via the peritoneal cavity for a total of six days. In this way the animals received the same total dose as those in the MO group. This group is designated OM.

(3) Group III - Methotrexate Adminstration and then Operation.

These animals had a standard wound and drill hole placed in the mid shaft of both femurs. Prior to operation they received a six day course of methotrexate via the intraperitoneal cavity. 0.25 mg of methotrexate was administered daily giving a total dose of 1.5 mgs. This group is designated MO.

These groups can therefore be summarised as follows:

Group I - Control = C

Group II - Operation and then methotrexate = OM

Group III - Methotrexate and then operation = MO

Histological and histochemical studies were carried out on the healing defects. To study the sequence of events in the healing of the defect, animals were sacrificed at the following intervals:

12, 24, 48, 72 hours

5, 7, 14, days

3, 4, 8, 12 weeks.

The experimental details are summarised in the following tables:

TABLE I. CONTROL C

GROUP I

TIME	NO. OF ANIMALS	BLOCKS
12 hours	3 animals	6 blocks
24 "	3 "	6 "
48 "	3 "	6 "
72 "	3 "	6 "
5 days	3 "	6 "
7 "	3 "	6 "
14 "	3 "	6 "
3 weeks	3 "	6 "
4 "	3 "	6 "
8 "	2 "	4 "
12 "	2 "	4 "

TABLE II. OPERATION - METHOTREXATE - OM SERIES

GROUP II

TIME	NO. OF ANIMALS	BLOCKS
12 hours	3 animals	6 blocks
24 "	3 "	6 "
48 "	3 "	6 "
72 "	3 "	6 "
5 days	3 "	6 "
7 "	3 "	6 "
14 "	3 "	6 "
3 weeks	3 "	6 "
4 "	3 "	6 "
8 "	2 "	4 "
12 "	2 "	4 "

TABLE III. METHOTREXATE OPERATED - MO SERIES

GROUP III

TIME	NO. OF ANIMALS	BLOCKS
12 hours	3 animals	6 blocks
24 "	3 "	6 "
48 "	3 "	6 "
72 "	3 "	6 "
5 days	3 "	6 "
7 "	3 "	6 "
14 "	3 "	6 "
3 weeks	3 "	6 "
4 "	3 "	6 "
8 "	2 "	4 "
12 "	2 "	4 "

Total number of animals used in this investigation (excluding the 30 animals used in the estimation of the therapeutic dose of methotrexate = 93

Total histological blocks = 186

total number of histological slides examined = 2,300

Animals were sacrificed at specified intervals as shown in Tables I, II and III. The femurs were then disarticulated from the hip and placed in 10% formolsaline until they were adequately fixed (forty eight hours). Following fixation they were reduced to the experimental area and decalcified with formic acid and then processed ready for paraffin embedding and sectioning with a rotary microtome, (Appendix V). Serial sections of 7 u were taken through the blocks in a longitudinal plane in four blocks through to twelve weeks, and transverse plane in two blocks through to four weeks. In this way a three dimensional picture could be built up over the most important time interval.

Serial sectioning of the blocks was made, keeping every fifth section, and of these, every fifth one was stained with haematoxylin and eosin. The remaining sections were stained with other specific stains in order to establish the sequential appearance of certain substances:

1. Ground substance
2. Reticulin fibres
3. Collagen fibres
4. New bone.

CHAPTER 7

RESULTS

A. INTRODUCTION

B. THE CONTROL SERIES

C. EXPERIMENTAL SERIES - OM

(1) Blood Clot Formation

(2) The Inflammatory Reaction

(3) Granulation Tissue Formation

(i) Fibroblasts

(ii) Ground substance

(iii) Extracellular fibre formation

(4) Bone Formation

(i) Endosteal callus

(ii) Subperiosteal callus

(iii) Internal callus

(5) Remodelling

D. EXPERIMENTAL SERIES - MO

RESULTS

A. INTRODUCTION

Figs. 7.1 and 7.2 show the low power, overall picture of the longitudinal and transverse sections of the femur of the rat with the experimental drill hole used in the present investigation. Subsequent photomicrographs, (except high magnification) will be designated as either Insert I or Insert II, to denote the areas shown.

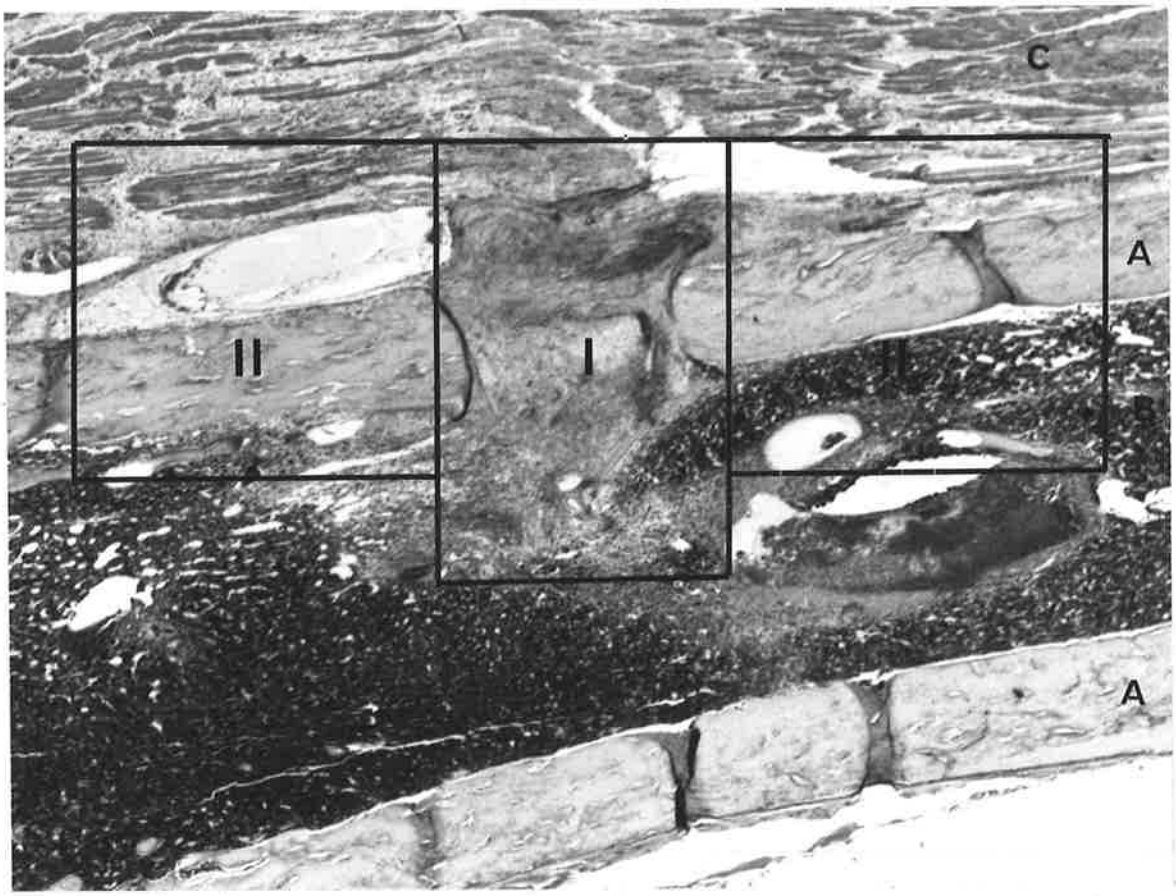


Fig. 7.1

Control 48 hours H & E x 5. Photograph of the rat femur in longitudinal section.

Insert I The experimental defect and surrounding soft tissues.

Insert II Cortical bone adjacent to the defect overlying soft tissues and marrow space.

A. Cortical bone

B. Marrow space

C. Muscles

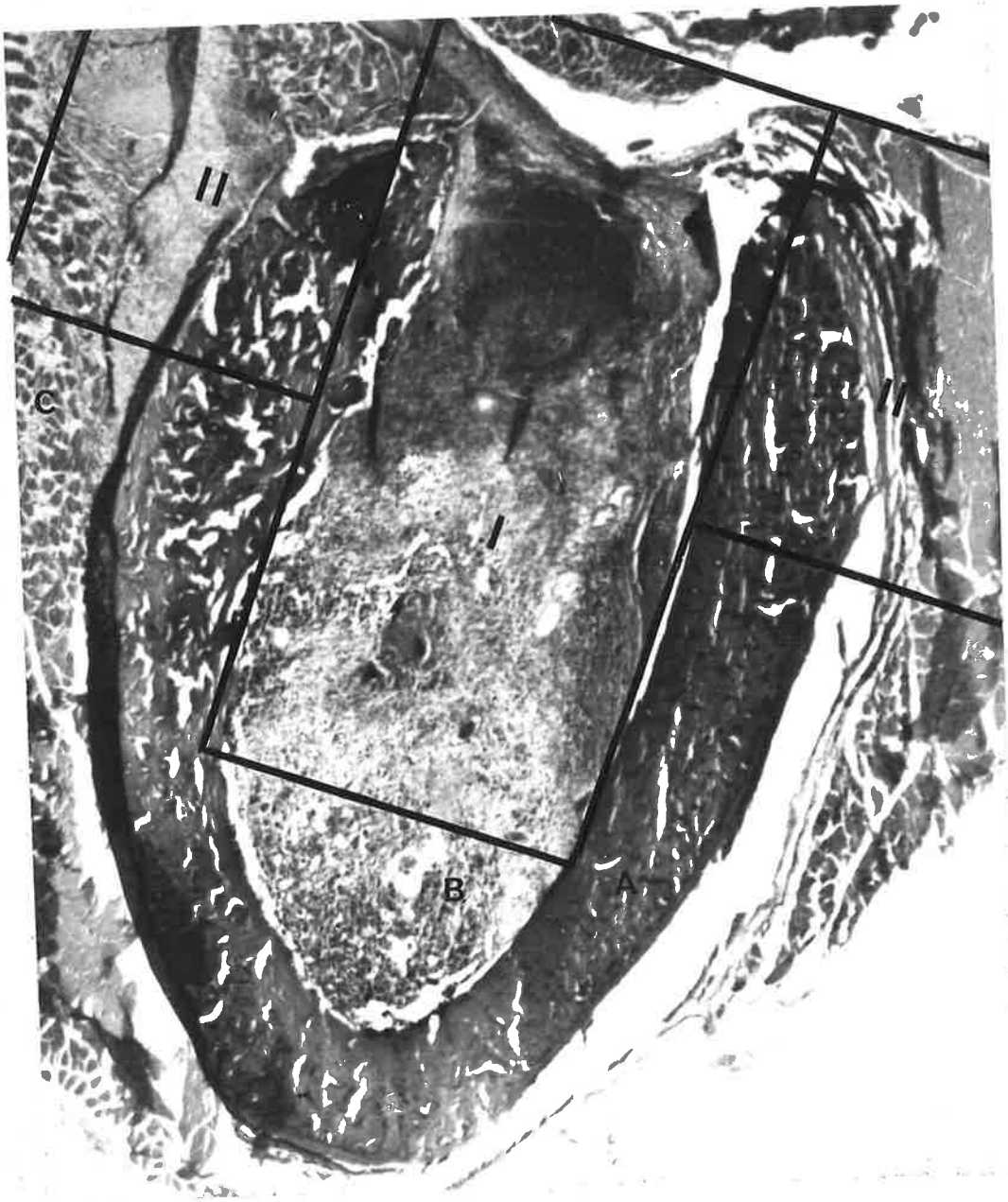


Fig. 7.2

Control 48 hours H & E x 5. Photograph of the rat femur in transverse section.

Insert I. The experimental defect and surrounding soft tissue.

Insert II. Cortical bone adjacent to the defect, surrounding soft tissue and marrow space.

A. Cortical bone (shaft of the femur)

B. Marrow

C. Muscles

In this investigation, the following terms have been used and are defined as follows:

(I) The defect area refers to the defect in the cortical plate and its extension into the marrow cavity.

(II) New bone formation is observed in three locations in relation to the healing cortical defect. These bone formations will be referred to as follows:

(1) Subperiosteal (or external) callus refers to the new bone formed subperiosteally over the external surface of the cortical bone.

(2) Endosteal callus refers to the new bone formed sub-endosteally over the internal surface of the cortical bone.

(3) Internal callus refers to the new bone formed within the defect itself.

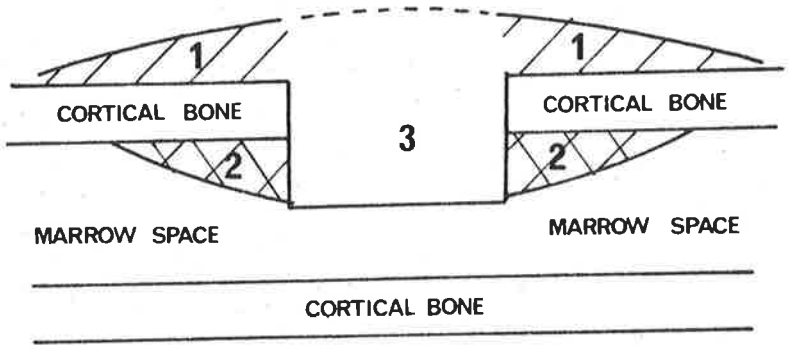


Fig. 7.3

Diagrammatic representation of the defect showing the various areas of new bone formation referred to above. Subperiosteal or external callus (1), Endosteal callus (2) and Internal callus (3).

In order to compare the healing processes in the control series with that of the experimental series, certain steps were taken to quantitate and/or qualitate the various histological observations. The results of these assessments have been tabulated and are presented by the use of histograms.

The following tissue components and substances have been assessed:

(1) Granulation tissue
    (a) Fibroblasts
    (b) Ground substances
    (c) Extra-cellular fibres
(2) New bone
    (a) Endosteal callus
    (b) Subperiosteal callus
    (c) Internal callus.

Fibroblasts have been assessed both quantitatively and qualitatively. Where possible, two sites have been chosen for observation. The first site was near the advancing front of the forming granulation, next to the lysing blood clot. The second site is in the most matured portion of the granulation. The number of fibroblasts per standard area (a rectangular area delineated on a special photomicrographic eye-piece) under high-power magnification (x 400) was recorded for each of the two areas. The morphology of the young fibroblasts was graded as follows:

Normal fibroblasts - these cells were plump or stellate in shape with large nuclei and loose staining chromatin material.

Abnormal fibroblasts - these cells were thin, spindly in shape with small pyknotic nuclei and darkly staining chromatin material.

Ground substances as demonstrated by the PAS stain (after diastase) were assessed for their extent of distribution. Both assessments were assigned arbitrary grades of +, ++, and +++.

The colour intensity of PAS staining is a variable feature even when a highly standardised staining procedure is used. The colour intensity of the PAS stain is therefore not a good guide of concentration and has not been used as a criteria for assessment.

The Alcian Blue stain for acid mucopolysaccharides failed to demonstrate any significant positive staining and was not assessed.

Three chief sites of deposition of PAS positive materials in the healing granulation have been observed. These can be summarised in the following way:

(a) The fibrin network of the blood clot.
This is of no significance in relation to ground substance production and was not measured.

(b) The perivascular accumulation of PAS positive material.
This is related to the glycoprotein-rich inflammatory exudate. It is uncertain as to the extent this contributes to production of ground substance.

(c) The PAS positive material which is associated with the cytoplasm of the young fibroblasts, and is also extra-cellularly in close association with these cells.

It has been assumed in this investigation that the last mentioned site of PAS positive material is an indication of the production of ground substances by the fibroblasts. It has been recognised that the PAS stain (after diastase) does not distinguish between mucopolysaccharides, muco-proteins and glycoproteins (see Chapter 5). The extent of distribution of this PAS positive material is taken to be an indication of mucopolysaccharide content.

Extra-cellular fibre formation has been demonstrated by the use of the silver impregnation (reticulin fibres - early collagen) and van Gieson

(collagen) stains:

These stains were assessed for their extent of distribution as being either +, ++ or +++.

An accurate assessment of the volume of callus formed entails three dimensional measurement in the vertical transverse, and horizontal plane of serial sections through the entire specimen. Such an undertaking is neither necessary or possible in the present project. The amount of endosteal callus formed is therefore assessed in one dimension only, using a scaled eye-piece in the microscope. The longitudinal sections have been used for this measurement where the horizontal dimension (d') of the callus is being measured (Fig. 7.4).

Measurements (d") of the sub-periosteal callus have been made in a similar way to that of the endosteal callus (Fig. 7.5).

The amount of bone formed in the internal callus has been assessed in relation to the size of the defect and assigned as being one-third (+) two-thirds (++) or all (+++) of the defect.

In all specimens, no alteration in the quality of the new bone formed has been noted. No attempt is therefore made to document this aspect of the results.

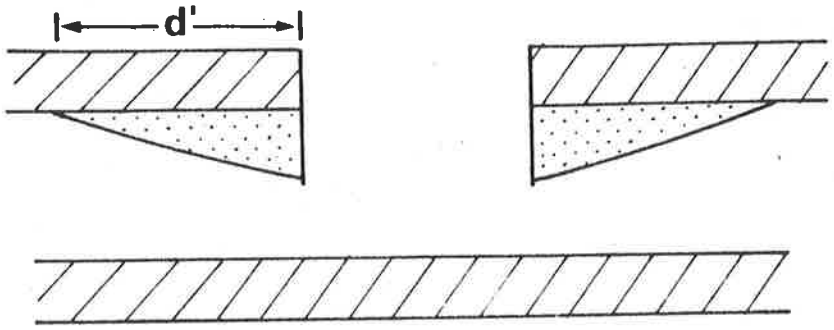


Fig. 7.4

Diagrammatic representation of the defect in longitudinal section showing endosteal callus measured (d').

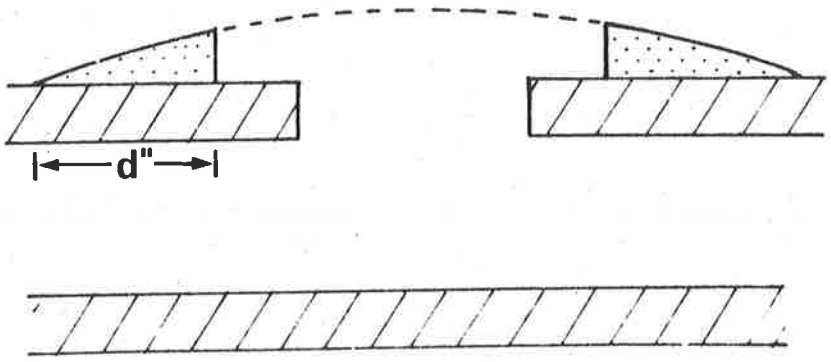


Fig. 7.5

Diagrammatic representation of the defect in longitudinal section showing the subperiosteal callus measured (d").

B. THE CONTROL SERIES

Healing of the defect in the control series can be summarised as follows:

(1) The formation of a blood clot.

(2) An inflammatory reaction, consisting of an exudate and cellular infiltrate follows the formation of the blood clot. The intensity of this reaction reaches a peak at 48 hours and rapidly subsides thereafter.

(3) Lysis of the blood clot is evident by 24 hours with the clot being completely replaced with granulation tissue by the 5th post-operative day.

(4) Granulation tissue formation can first be seen with the appearance of:

(a) Fibroblasts and young capillaries at the periphery of the defect by 48 hours.

(b) Ground substance formation can be demonstrated with the PAS stain at 48 hours, at the same time as the fibroblasts are seen.

(c) Fibre formation first appears at 48 hours with the demonstration of reticulin by the silver stain. Maturation of reticulin to collagen follows and can be demonstrated with the Van Gieson stain by 72 hours.

(5) Bone formation follows that of granulation tissue formation and can first be seen -

(a) Endosteally on the inner aspect of the cortical plate immediately adjacent to the edge of the defect by 48 hours with early evidence of endosteal activity by 24 hours.

(b) External callus formation appears at about the same time as that of endosteal callus formation, at about 48 hours with a thickening of the cambial layer and new bone formation at 72 hours; once again early evidence of subperiosteal activity being evident by 24 hours.

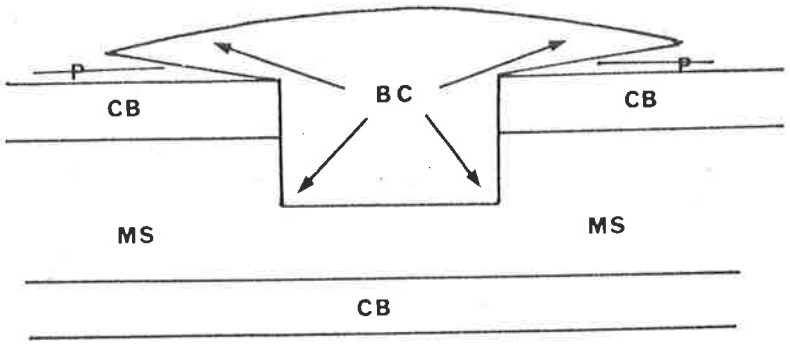
(c) Internal callus formation within the defect can be demonstrated at about 5 days and has filled the defect by 7 days.

At 7 days, the internal and endosteal callus has merged within the defect and by 14 days the external callus has merged with the internal callus over the top of the defect.

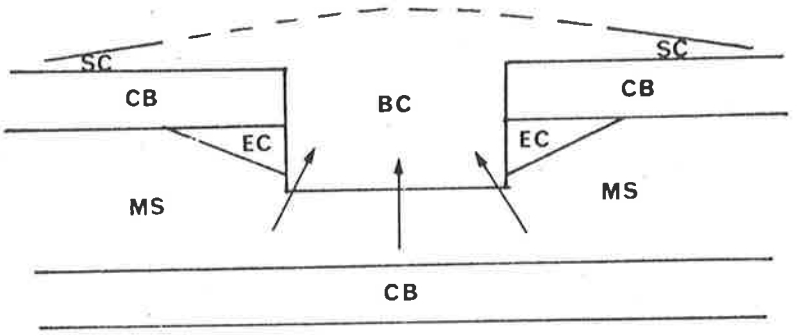
From this time onwards, remodelling is the most important and prominent feature seen, a feature which continues beyond the limits of this investigation.

The following diagrams summarise the healing sequence.

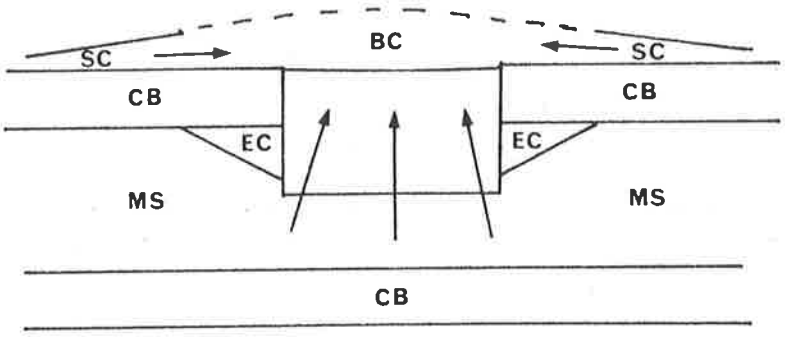
12 hours



48 hours



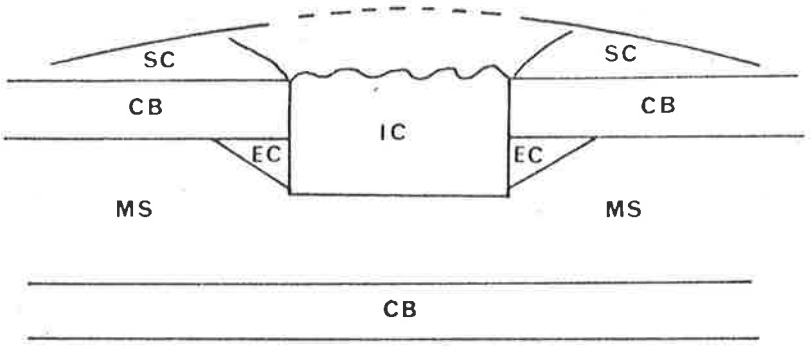
5 days



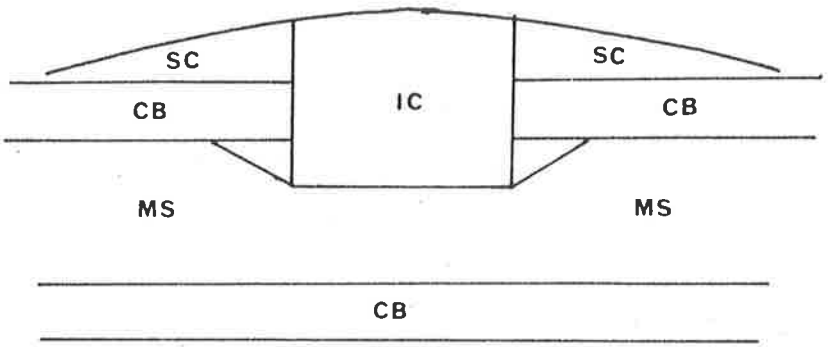
CB	CORTICAL BONE	MS	MARROW SPACE
BC	BLOOD CLOT	SC	SUB-PERIOSTEAL CALLUS
P	PERIOSTEUM	EC	ENDOSTEAL CALLUS

FIG. 7.6a

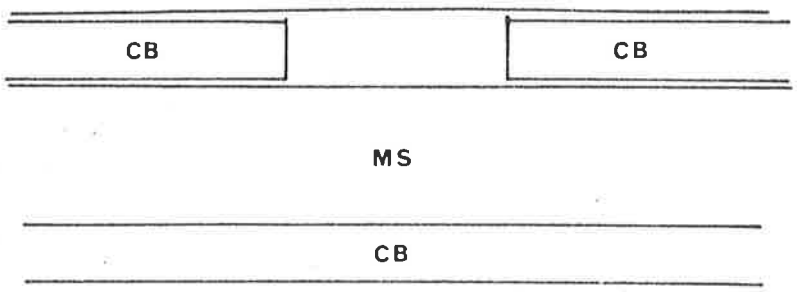
7 days



14 days



3 months



CB CORTICAL BONE
BC BLOOD CLOT
MS MARROW SPACE
SC SUB-PERIOSTEAL CALLUS
EC ENDOSTEAL CALLUS
IC INTERNAL CALLUS

FIG. 7.6b

12 hours

The defect is filled with a blood clot which extends into the adjacent medullary space and out into the overlying soft tissues (Fig. 7.7). An inflammatory reaction with a PAS positive exudate can be seen in and around the defect (Fig. 7.7) and in some sections outside amongst the overlying soft tissues.

Except for directly over the defect, the periosteum is intact. At this stage, no periosteal or endosteal reaction can be seen. In most sections bony debris and bone filings can be seen within the defect (Fig. 7.7). This debris is presumably produced at the time of surgery and not washed away by irrigation.

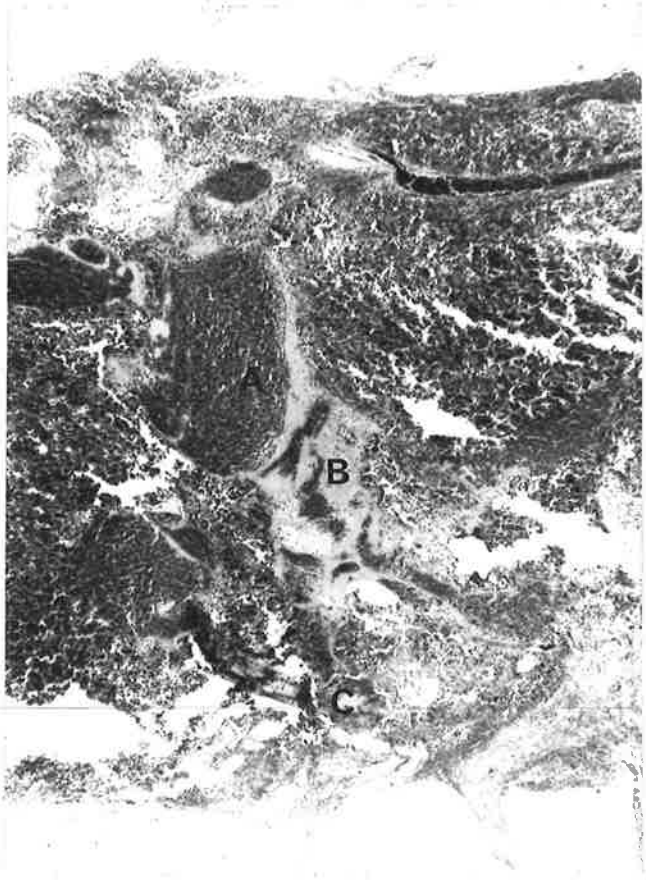


Fig. 7.7 (Insert I)

Control 12 hours H & E x 40. Photomicrograph showing a blood clot (A), inflammatory exudate (B) and bony debris (C).

24 hours

The defect still contains a large blood clot which shows evidence of lysis, leaving a PAS positive fibrin network (7.8).

A more extensive acute inflammatory reaction with an associated PAS positive exudate can be seen both within and outside the defect (Fig. 7.8).

An early endosteal reaction can be seen on the inner aspect of the cortical plate immediately adjacent to the margin of the defect (Fig. 7.9). The nuclei of cells in this area are plump and the cells contain PAS positive material in their cytoplasm. In the periosteum, a slight thickening of the cambial layer can be seen at some distance from the margins of the defect (Fig. 7.10). No new bone has been laid down at this stage.

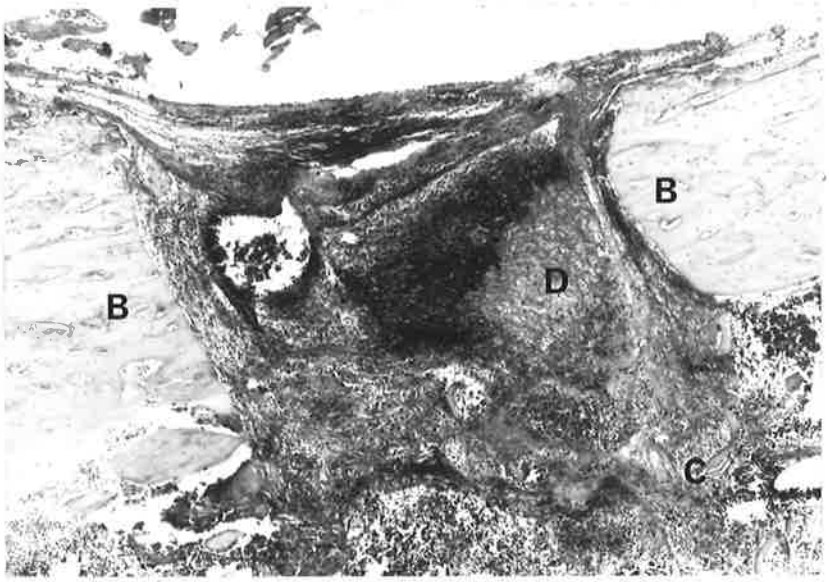


Fig. 7.8 (Insert I)

Control 24 hours H x E x 40. Photomicrograph showing the blood clot within the defect (A), cortical bone either side of the defect (B), bone spicules within the defect (C), and partially lysed blood clot (D).

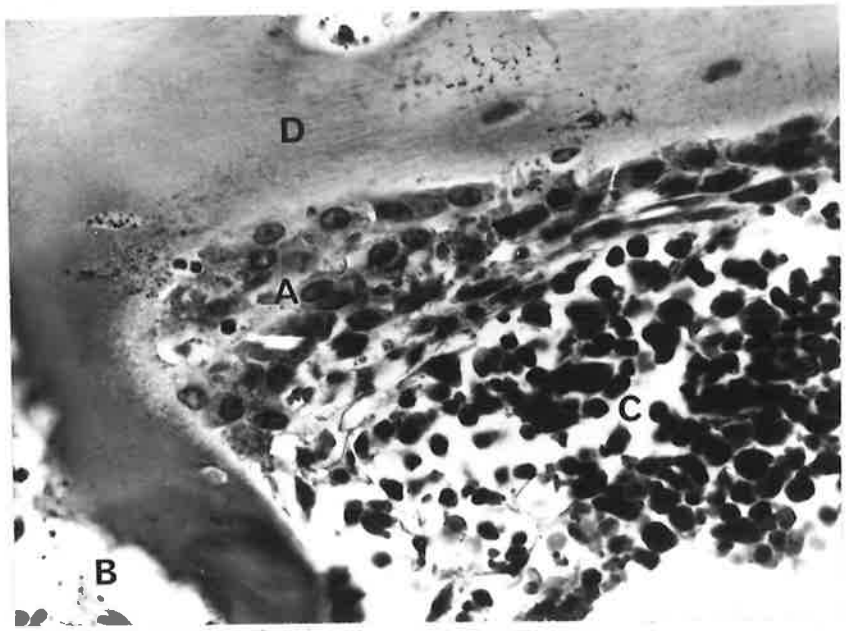


Fig. 7.9

Control 24 hours H & E x 400. Photomicrograph of the endosteal area immediately adjacent to the defect margin. Endosteal cells (A), defect (B), marrow cells (C) and cortical bone (D).

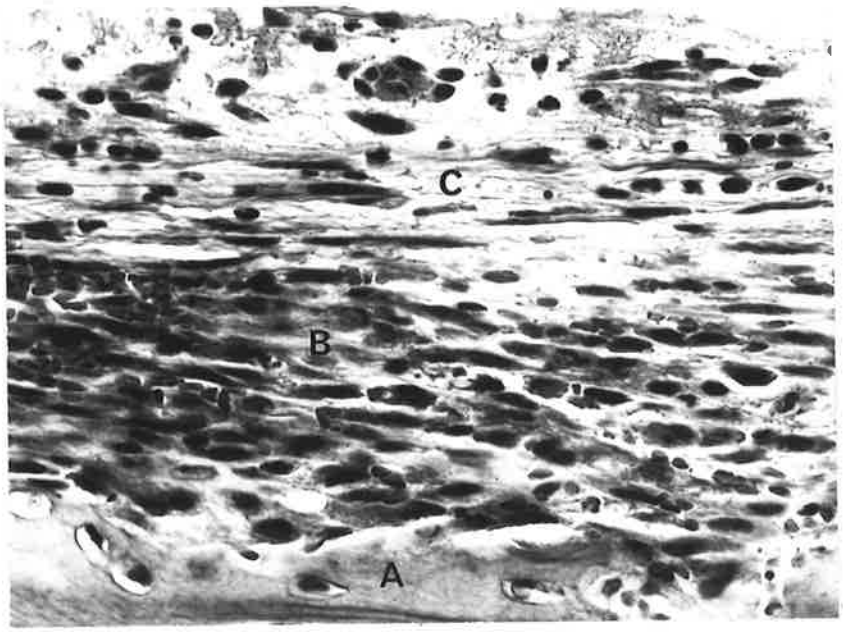


Fig. 7.10

Control 24 hours H & E x 400. Photomicrograph, showing the periosteal reaction away from the margins of the defect. Cortical bone (A), cambial cells (B) and fibrous periosteum (C).

Control 48 hours

A marked inflammatory reaction is evident which extends into the surrounding soft tissues.

There is early evidence of granulation tissue formation around the edges of the clot, which is progressively being lysed (Fig. 7.11 & 7.12). However, at this stage no collagen production can be seen. A faint PAS positive reaction can be seen between the cells of the granulation tissue thus indicating the production of ground substance.

Reticulin fibre formation is demonstrated by the silver stain within the defect. These reticulin fibres are laid down by the fibroblasts of the granulation tissue and appear to increase with a corresponding increase in the amount of granulation tissue (Fig. 7.15).

A more marked endosteal reaction is found on the inner aspect of the cortical plate immediately adjacent to the defect margins (Fig. 7.13).

There is a thickening of the cambial layer of the periosteum at some distance from the edge of the defect and in some sections there is evidence of subperiosteal bone formation (Fig. 7.14).

Fig. 7.11 (Insert I)
Control 48 hours H & E x 40. Photomicrograph of the defect showing blood clot (A), external blood clot (B), granulation tissue (C) and bone spicules (D).

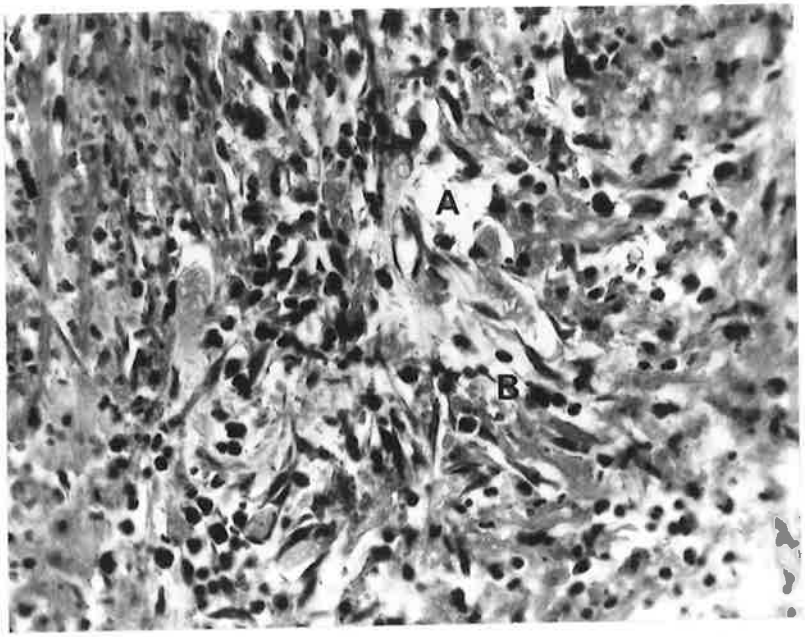


Fig. 7.12

Control 48 hours H & E x 100. Photomicrograph of the defect area in transverse section showing granulation tissue within the defect. Granulation tissue (A), fibroblasts (B).

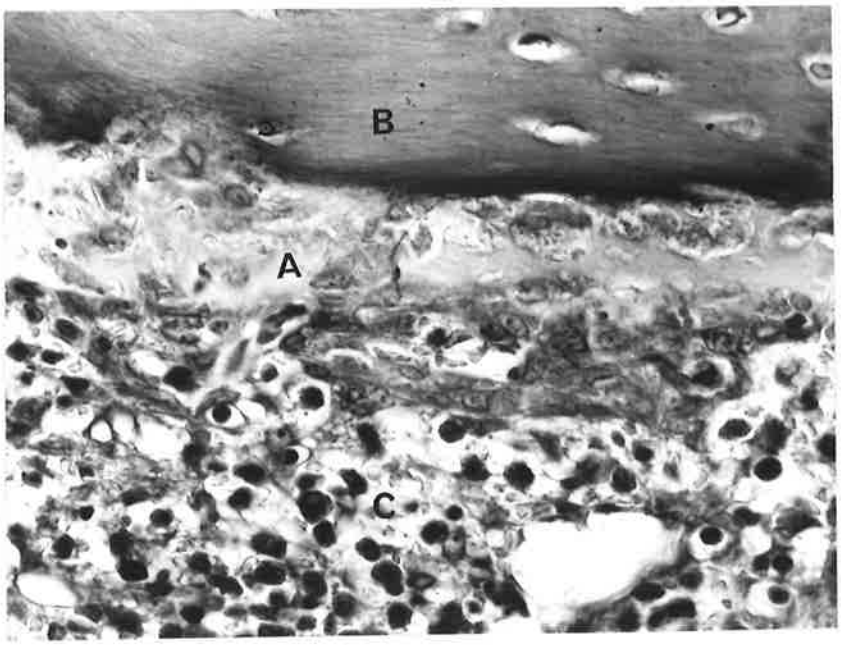


Fig. 7.13

Control 48 hours PAS x 400. Photomicrograph showing the endosteal reaction immediately adjacent to the defect margins (A), cortical bone (B) and bone marrow (C).

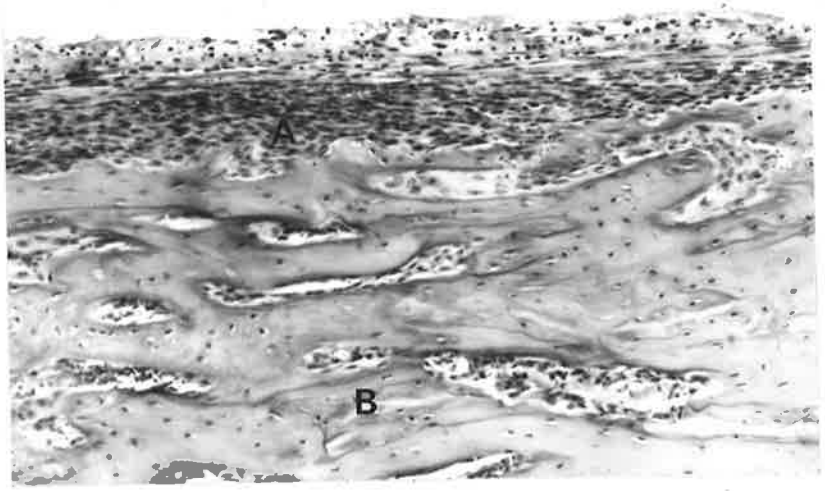


Fig. 7.14

Control 48 hours H & E x 100. Photomicrograph showing the periosteal response away from the margins of the defect. Periosteum (A) and cortical bone (B).

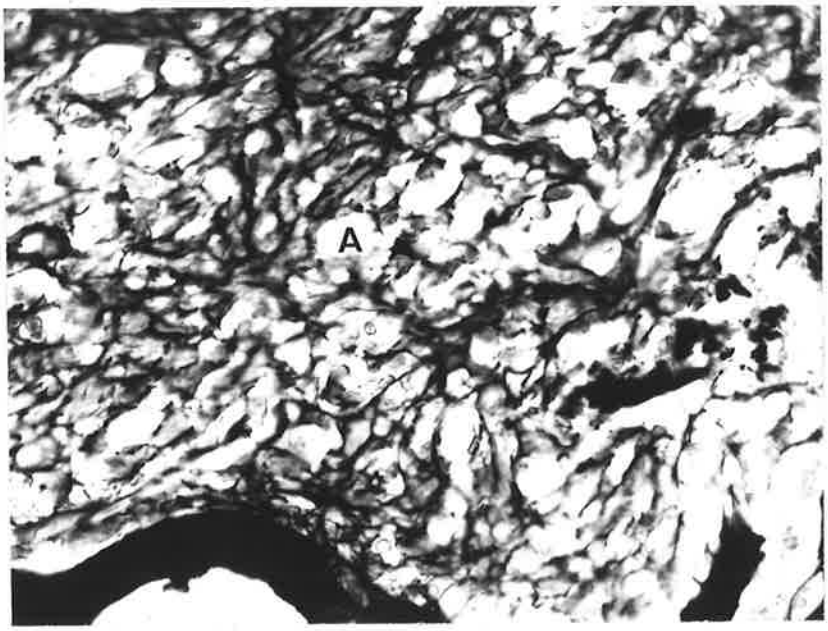


Fig. 7.15

Control 48 hours silver x 400. Photomicrograph showing reticulin fibre formation (A) in the young granulation tissue.

72 hours

At 72 hours postoperatively, healing is quite advanced in some specimens, with the defect almost completely filled with granulation tissue (Fig. 7.16) and only the remains of a fibrinous clot is present on the surface (Fig. 7.16).

The granulation tissue within the defect shows a distinct pattern of maturation. The centre is composed of young fibroblasts and relatively few reticulin (early collagen) and collagen fibres. Towards the periphery, there is a progressive increase in fibroplasia and fibre formation, as can be demonstrated by the silver and Van Gieson stains (Figs. 7.18, 7.19).

An intense PAS positive reaction could be seen in the granulation tissue at the periphery of the defect, indicating an increase in ground substance production (Fig. 7.17).

At this stage there is no new bone formation in the granulation tissue of the defect. However, endosteally and subperiosteally new bone formation is evident (Figs. 7.20, 7.21). This new bone formation appeared to be laid down directly on the old bone without any prior resorption. New subperiosteal bone formation can be seen occurring at a distance away from the margins of the defect (Fig. 7.21).

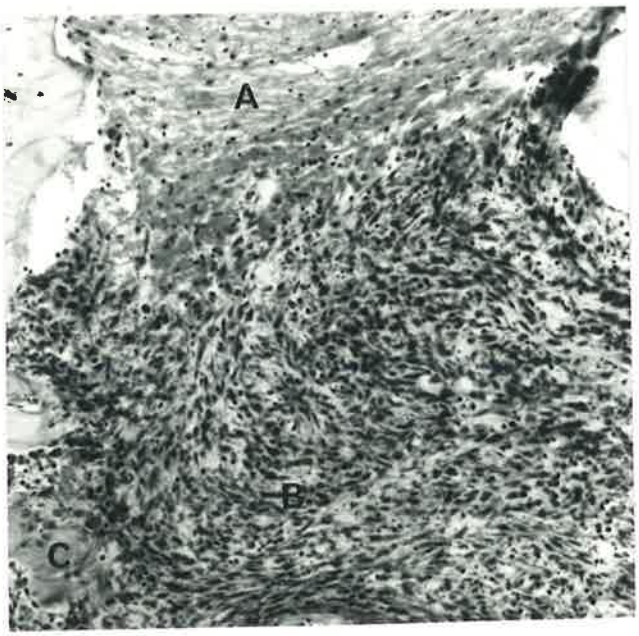


Fig. 7.16

Control 72 hours H & E x 100. Photomicrograph of the defect area showing a superficial clot (A), granulation tissue (B) and bone spicules (C).

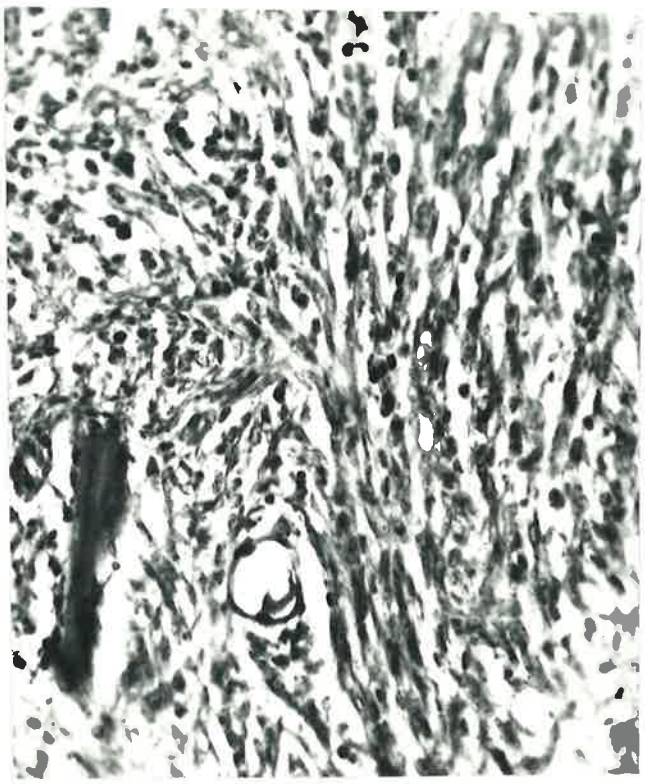


Fig. 7.17

Control 72 hours PAS x 250. Photomicrograph of the defect area showing PAS positive material within the granulation tissue of the defect.

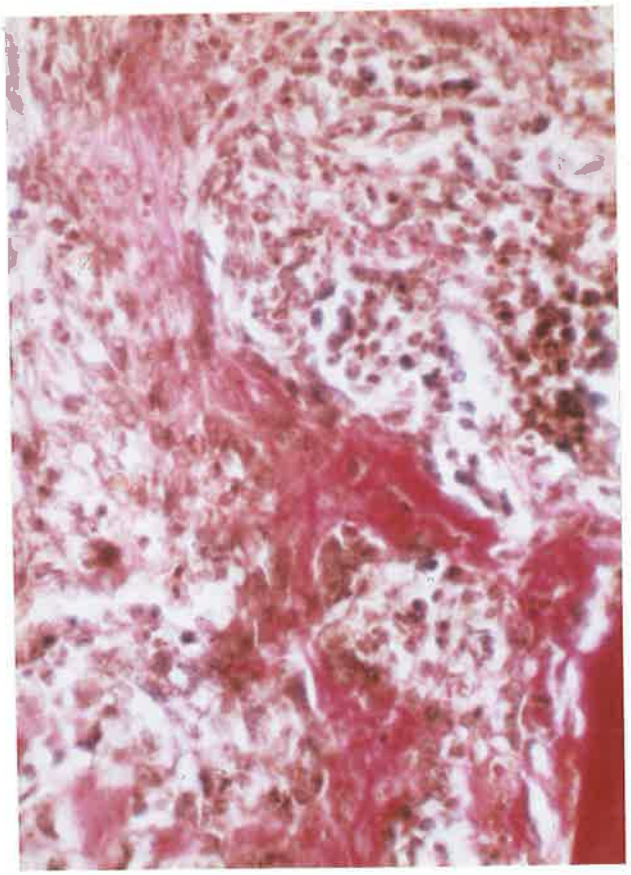


Fig. 7.18

Control 72 hours Van Gieson x 250. Photomicrograph of the defect area showing collagen formation at the edge of the defect.

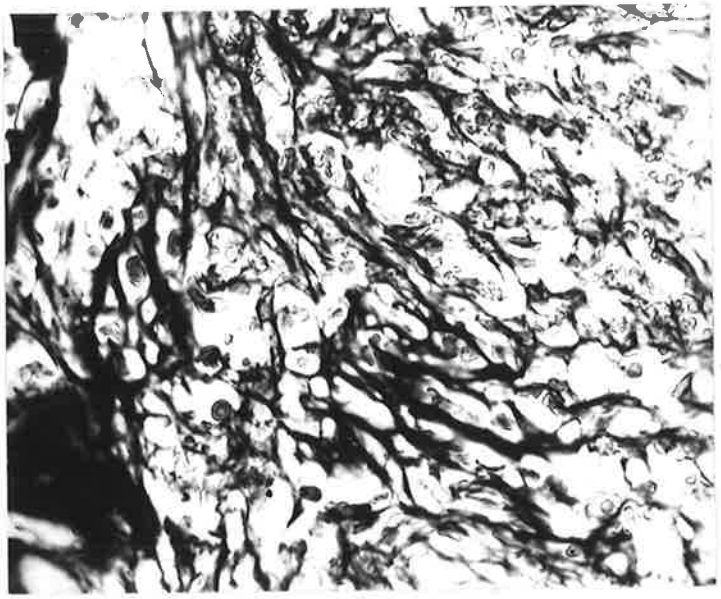


Fig. 7.19
Control 72 hours silver x 250. Photomicrograph of the defect area showing reticulin fibre formation in conjunction with collagen formation.

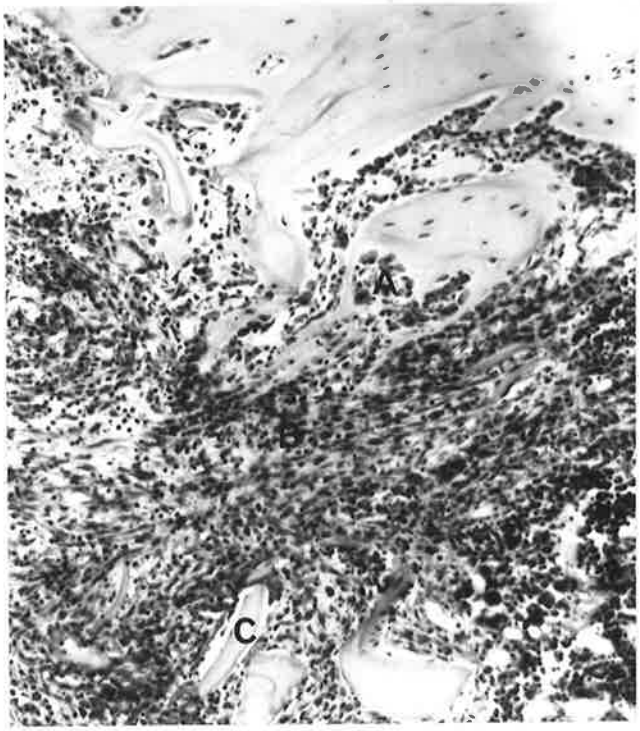


Fig. 7.20
Control 72 hours H & E x 100. Photomicrograph of the defect area showing the endosteal reaction (A) granulation tissue (B) and bone spicules (C) at the edge of the defect.

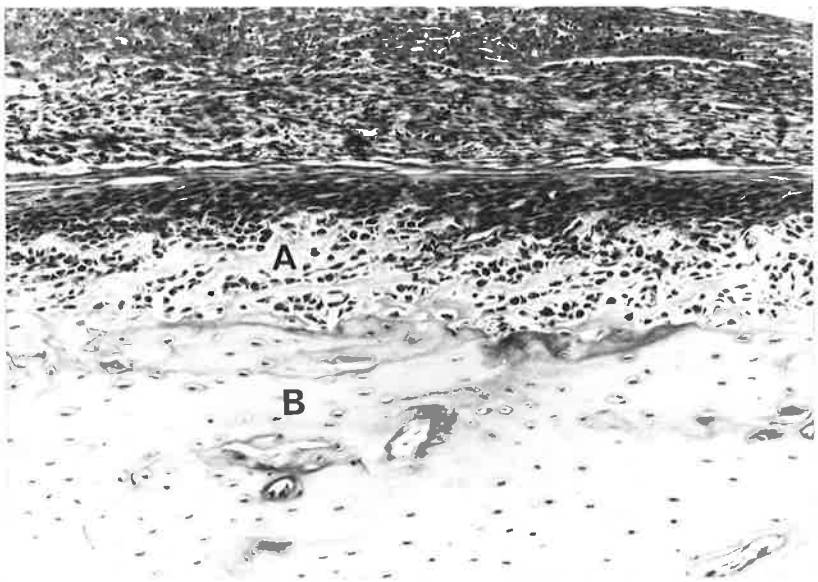


Fig. 7.21

Control 72 hours H & E x 100. Photomicrograph of the defect area showing the periosteal reaction, subperiosteal new bone (A), cortical bone (B).

5 days

The defect is completely organised with granulation tissue (Fig. 7.22). There is further maturation of the granulation tissue with further laying down of collagen. This is demonstrated by the gradual decrease in the number of reticulin fibres (as shown with the silver stain) as they are transformed into collagen (as shown by the Van Gieson stain) (Figs. 7.25, 7.24).

The first sign of new bone formation within the defect is shown at this stage and occurs at the base of the granulation tissue mass (Figs. 7.22, 7.23). The endosteal area at the edge of the defect is active with new bone formation (Figs. 7.22, 7.23).

New bone formation can also be seen in the subperiosteal areas (external callus) but has not reached the edges of the defect (Fig. 7.26).

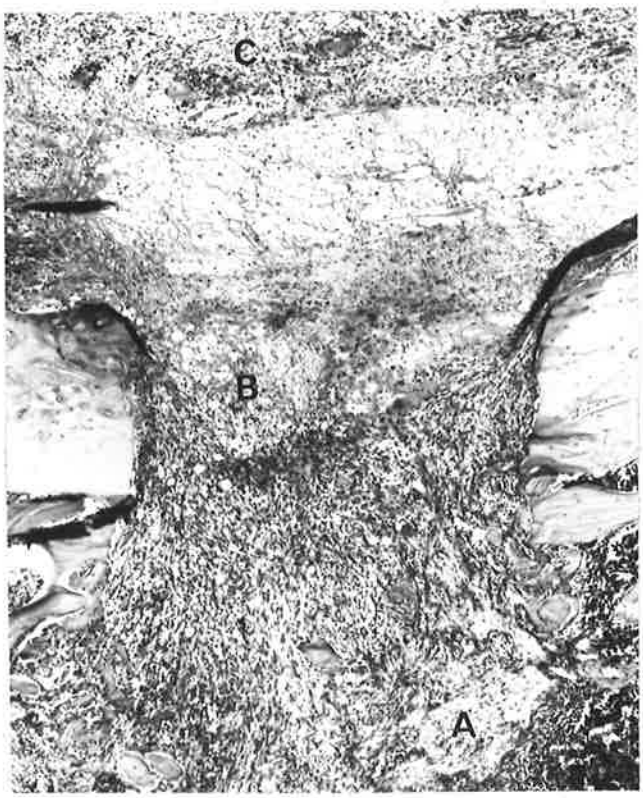


Fig. 7.22 Insert I

Control 5 days H & E x 40. Photomicrograph of the defect showing the internal callus (A), superficial clot (B) and overlying granulation tissue (C).

Fig. 7.23

Control 5 days H & E x 100. Photomicrograph showing new bone (A) in the internal callus at the periphery of the defect.

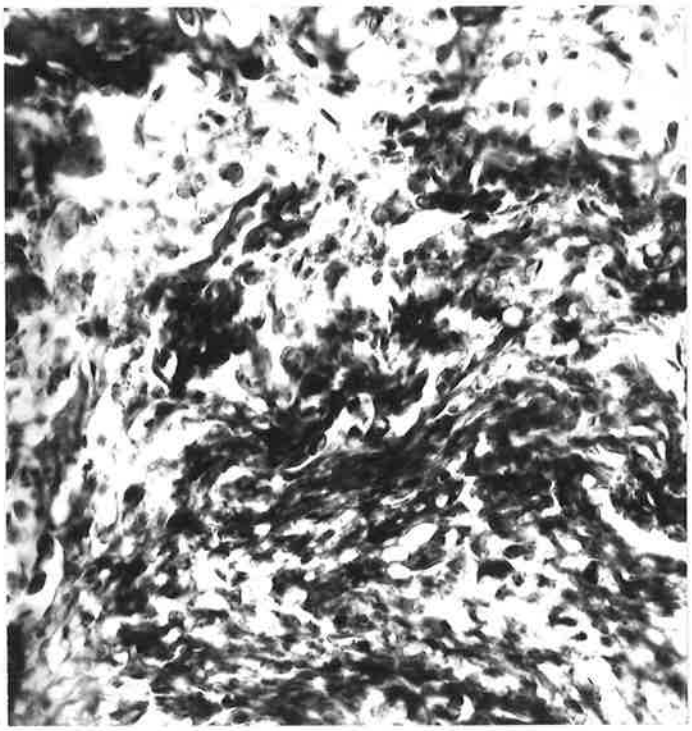


Fig. 7.24

Control 5 days Van Gieson x 250. Photomicrograph showing collagen formation within the defect in the internal callus.

Fig. 7.25 Insert I

Transverse section.

Control 5 days silver x 100

composite photomicrograph

showing dense reticulin fibre

network formation in the

centre (A). A less dense

reticulin fibre network (B)

indicating transformation of

collagen towards the periphery,

and new bone formation at the

periphery (C).

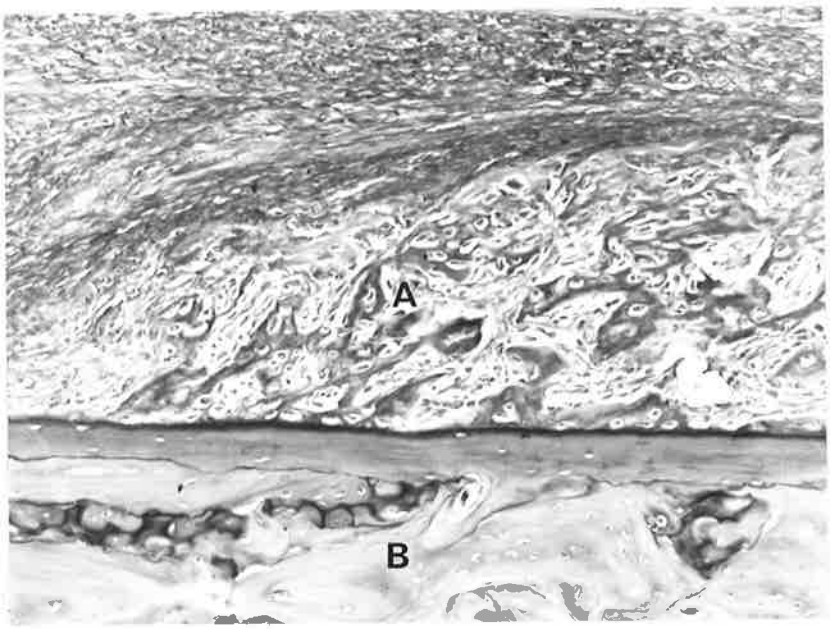


Fig. 7.26 Insert II

Control 5 days PAS x 100. Photomicrograph of the periosteal area showing external callus formation (A) and old cortical bone (B).

7 days

At 7 days the defect is completely filled with new bone which has reached the surface of the defect (Fig. 7.27). Beyond this, in the soft tissue, there is still a great deal of granulation tissue (Fig. 7.28).

The new bone in the defect (internal callus) fuses with the new bone formed from the endosteum (endosteal callus) which extends 1-2mm along the inner aspect of the cortical plate (Fig. 7.27) beyond the edges of the defect.

The internal callus is well organised with the trabeculations running at right angles to the long axis of the femur (Figs. 7.27, 7.29).

The external callus has reached the edges of the defect in some sections (Fig. 7.28).

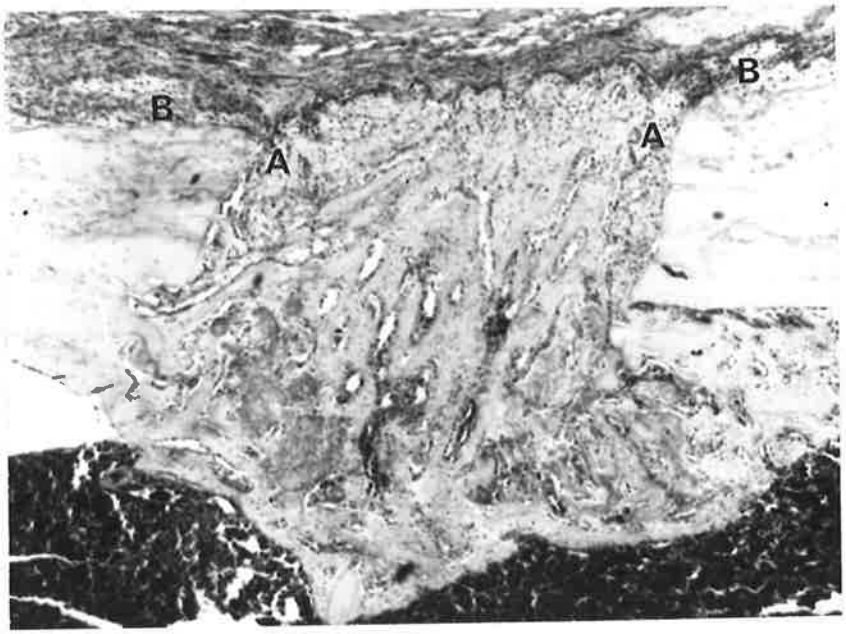


Fig. 7.27 Insert I

Control 7 days H & E x 40. Photomicrograph of the defect area showing the internal callus at the margins of the defect (A) with the external callus close to the edge of the defect (B).

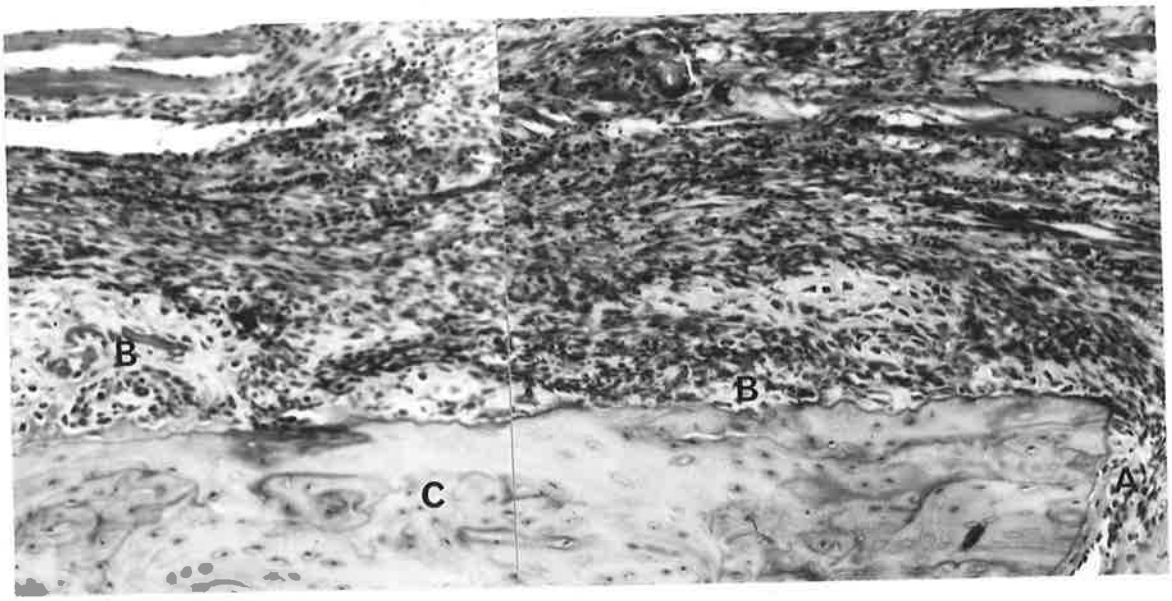


Fig. 7.28 Insert II.

Control 7 days H & E x 100. Composite photomicrograph of the defect area showing the internal callus (A) at the defect margin and the external callus (B) approaching the edge of the defect (C).

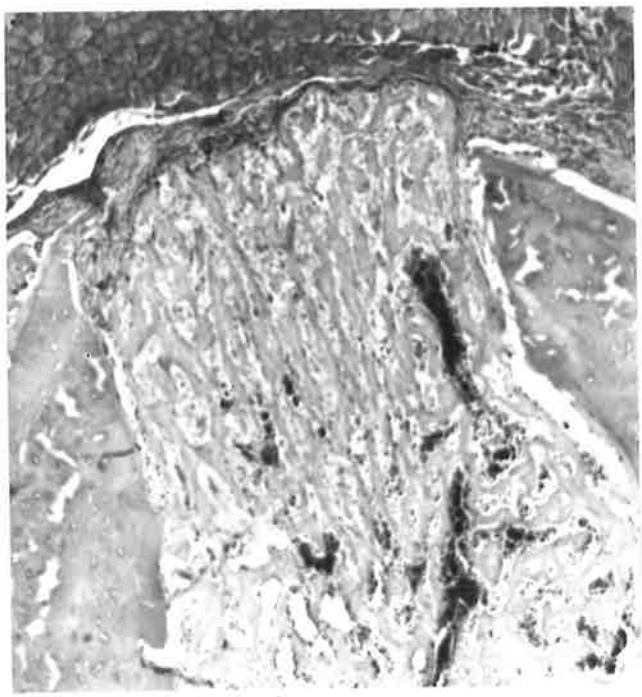


Fig. 7.29 Insert I.

Control 7 days H & E x 40. Photomicrograph of the defect in transverse section showing the internal callus at the defect margin, the external callus has not quite reached the margins.

14 days

By 14 days the defect is completely filled with callus (Fig. 7.30). It is quite evident that the formation of external callus starts at some distance away from the edges and then gradually extends towards the defect to merge with the internal callus (Figs. 7.30, 7.31) as healing progresses.

At this stage remodelling is evident with resorption of the internal callus in the medullary half to two-thirds of the defect. The trabeculae in this area becoming more sparse and the marrow space between the trabeculae becoming greater. At this stage the external callus still appears to be intact with no evidence of any active remodelling.

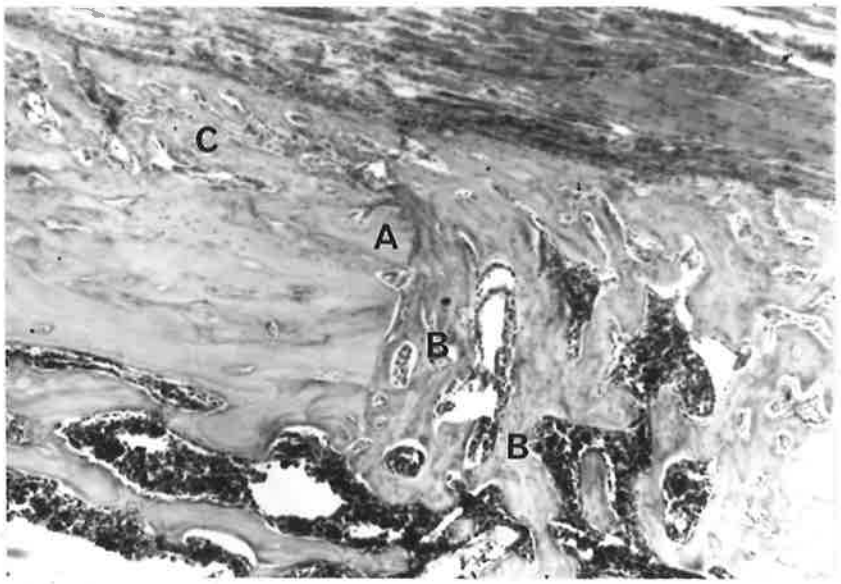


Fig. 7.30

Control 14 days H & E x 100. Photomicrograph of the defect area showing the defect margin (A), the internal callus (B), external callus (C) which has grown across the internal callus.

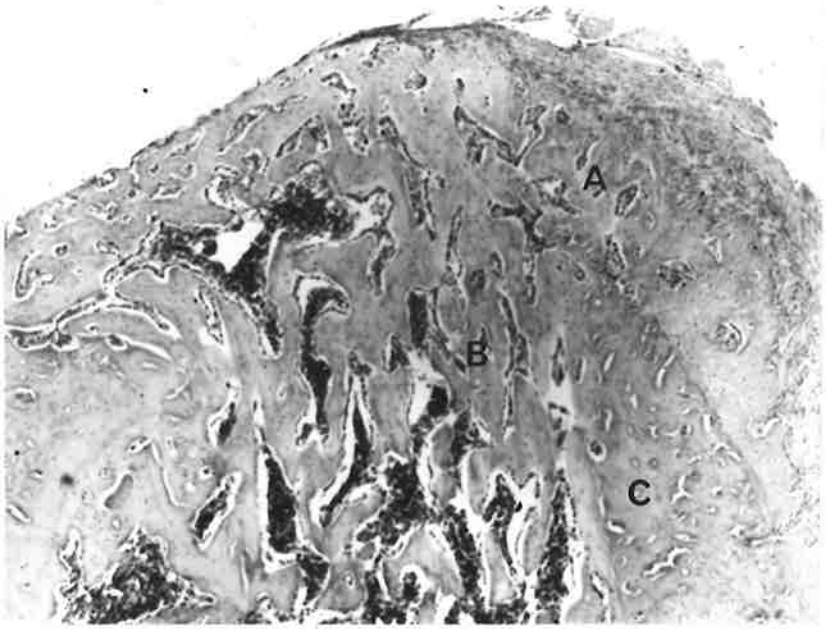


Fig. 7.31 Insert I.

Control 14 days H & E x 40. Photomicrograph in transverse section of the defect area showing the external callus (A), internal callus (B) and cortical bone (C).

3 weeks

At this stage remodelling of the defect is the most prominent feature seen (Figs. 7.32, 7.33). Remodelling can be seen in the medullary half to two-thirds of the defect with an increase in the medullary space and a decrease in the amount of internal callus (Figs. 7.32, 7.33). Remodelling of the external callus is not as marked as that of the internal callus.

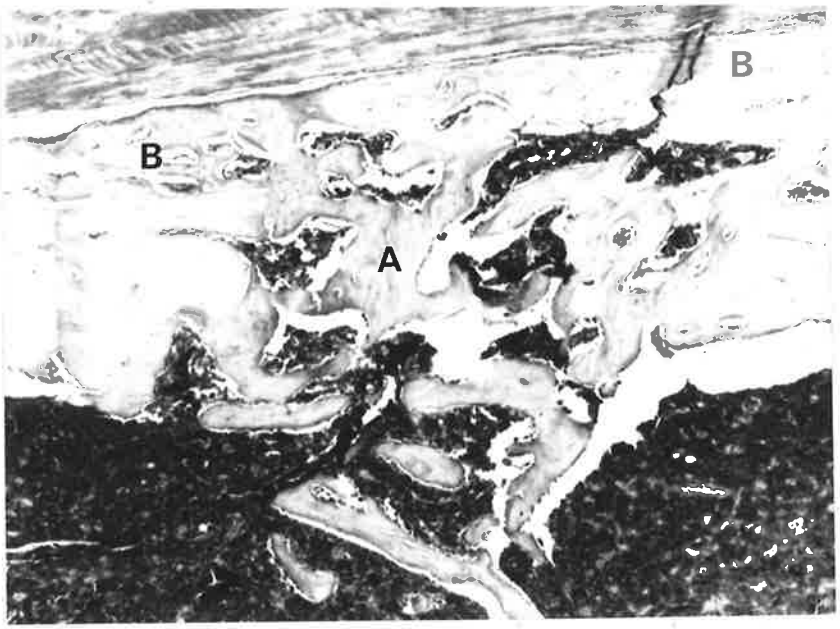


Fig. 7.32 Insert I

Control 3 weeks H & E x 40. Photomicrograph of the defect area showing the amount of remodelling of the internal callus (A), external callus (B).

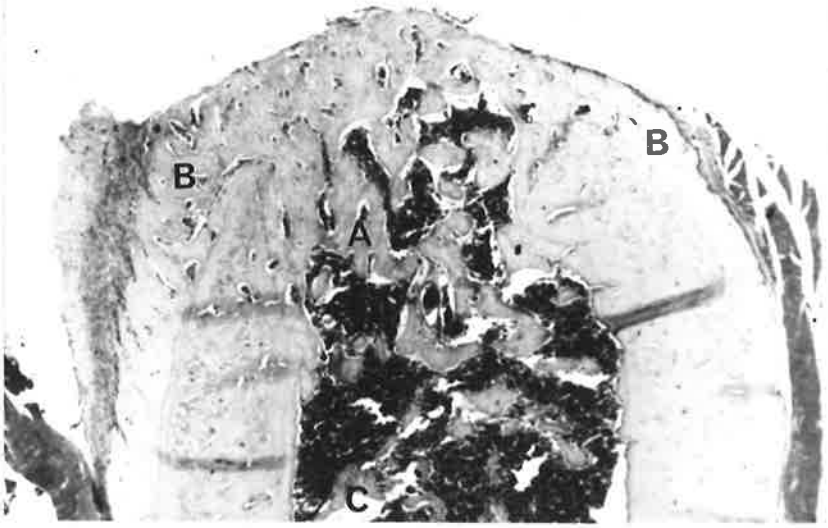


Fig. 7.33 Insert I and II

Control 3 weeks H & E x 40. Photomicrograph of the defect in transverse section showing the internal callus (A), external callus (B) which have merged. Remodelling of the internal callus (C).

4 weeks - 3 months

There is continuing remodelling of the external and internal callus (Figs. 7.34, 7.35, 7.36, 7.37). Much of the former bulk of the internal callus has been reduced (Figs. 7.36, 7.37). The direction of the trabeculae are still at right angles to the long axis of the femur, but over the 2-3 month time intervals, the callus gradually remodelled to lamellar bone (Figs. 7.34, 7.35, 7.36, 7.37).

This investigation terminated at the 3 month stage, but according to MELCHER & IRVING (1965), BOURNE (1944) and others, the remodelling continues for up to 12 months or more.

Fig. 7.34 Insert I.

Control 4 weeks H & E x 25. Photomicrograph of the defect area showing the defect to be almost remodelled.

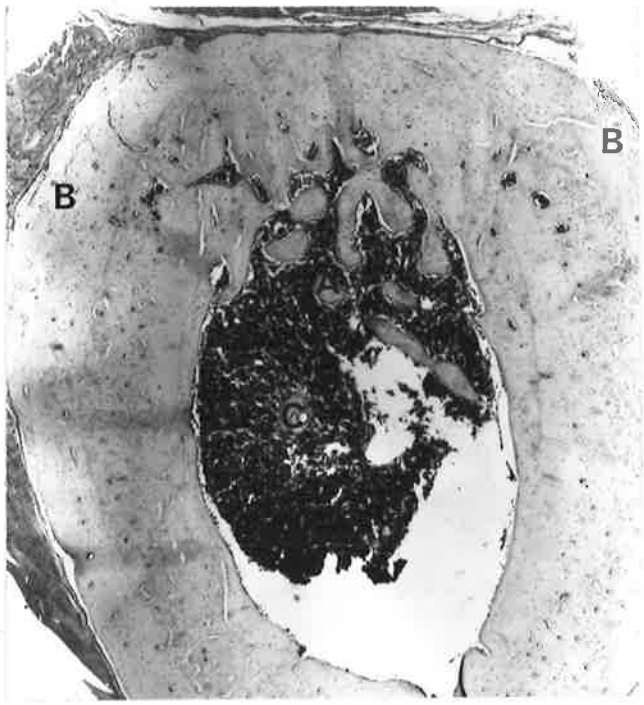


Fig. 7.35. Insert I.

Control 4 weeks H & E x 25. Photomicrograph of the defect in transverse section showing an almost remodelled defect. Internal callus (A), external callus (B) have merged with remodelling of the internal callus (C).

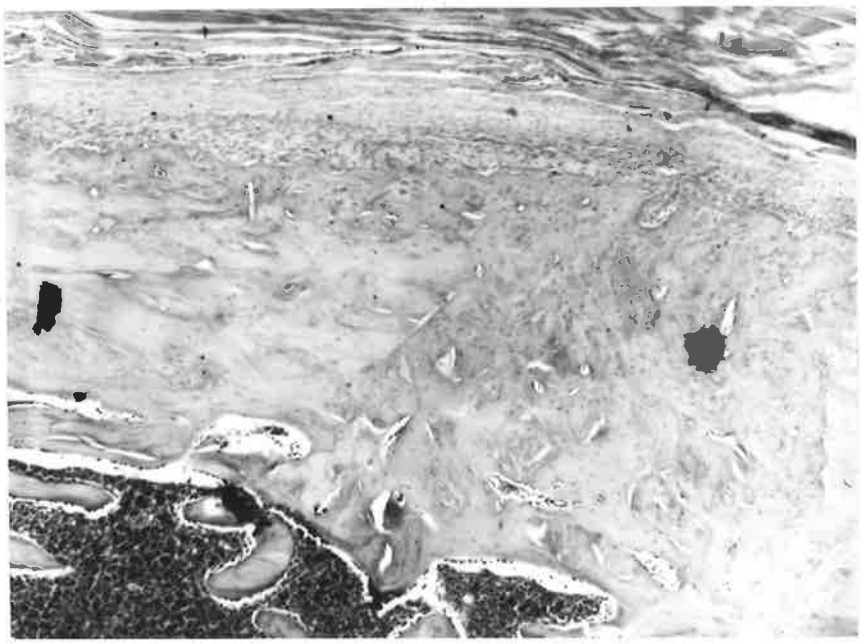


Fig. 7.36 Insert I and II.

Control 2 months H & E x 40. Photomicrograph showing the defect to be almost remodelled. However note that the trabeculae are still at right angles to the long axis of the femur. The internal callus has almost been completely resorbed.

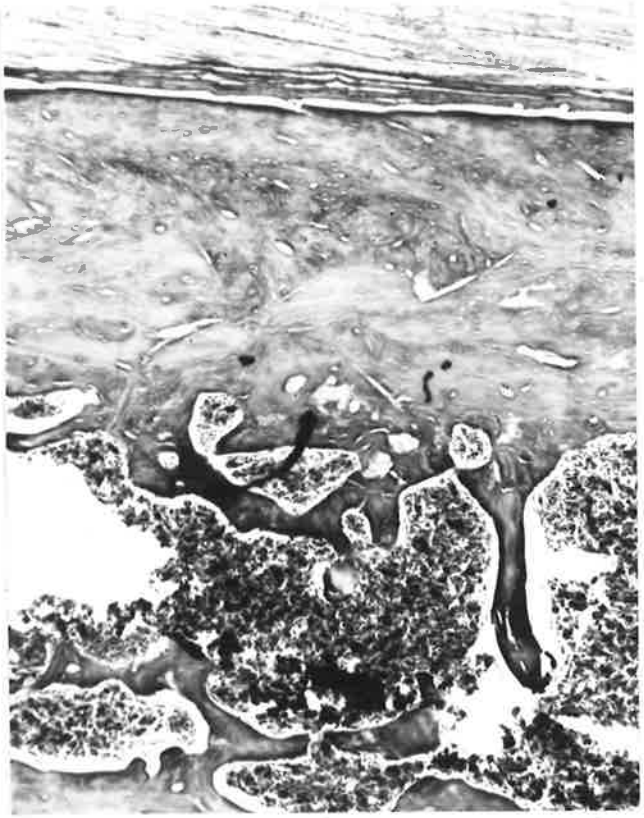


Fig. 7.37 Insert I.

Control 3 months H & E x 40. Photomicrograph of the defect showing an almost completely remodelled defect.

C. EXPERIMENTAL SERIES - OM

In general, the sequence of repair in the OM series follows that of the control series. However, some minor differences were observed. These differences will be discussed in the following section.

(1) Blood Clot Formation

The formation of blood clot and its subsequent lysis follows that of the control series. In some specimens the blood clot appears extensive and can be seen to extend into the adjacent medullary space and out into the overlying soft tissues.

(2) The Inflammatory Reaction

Generally the inflammatory reaction was greater than that of the control series. Evidence of an inflammatory response could be seen up to the 7 day specimens of the OM series, whereas the inflammatory response in the control series faded away soon after 48 hours. In over half of the specimens, this inflammatory response was quite marked.

(3) Granulation Tissue Formation

(i) Fibroblasts

Fibroblasts first appear at the periphery of the defect at 48 hours (Fig. 7.38) and continue to increase in number and distribution as the granulation tissue matures. Fibroblasts can also be seen in the more deeper areas and often in relation to the extensive blood clot seen in some specimens. The general arrangement of the granulation tissue appears haphazard in all specimens of the OM series, with the individual cells having a spindly shape (Figs. 7.39, 7.40). Other features were also noted which included poor organisation of the granulation tissue, increased oedema and poor staining.

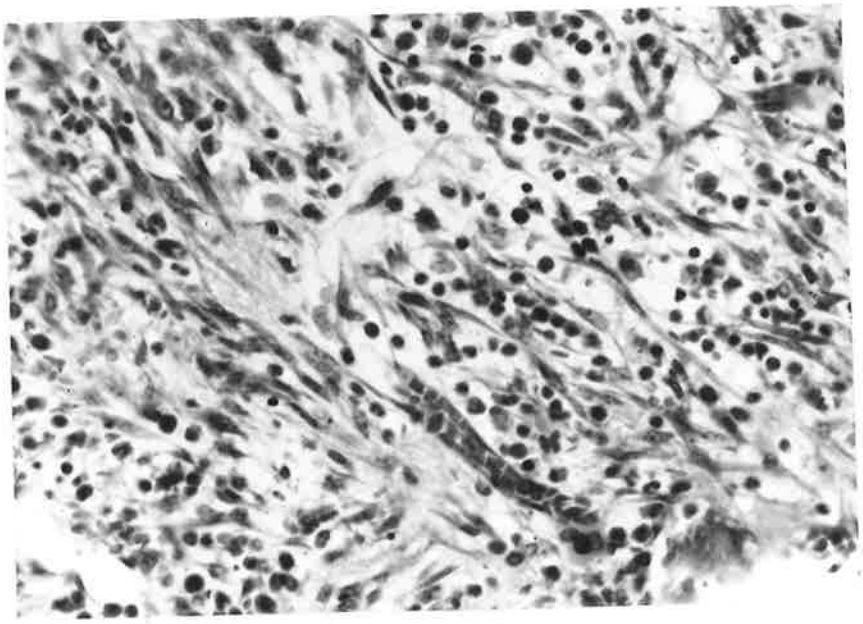


Fig. 7.38

OM 48 hours H & E x 250. Photomicrograph of the defect showing granulation tissue at the periphery of the defect.

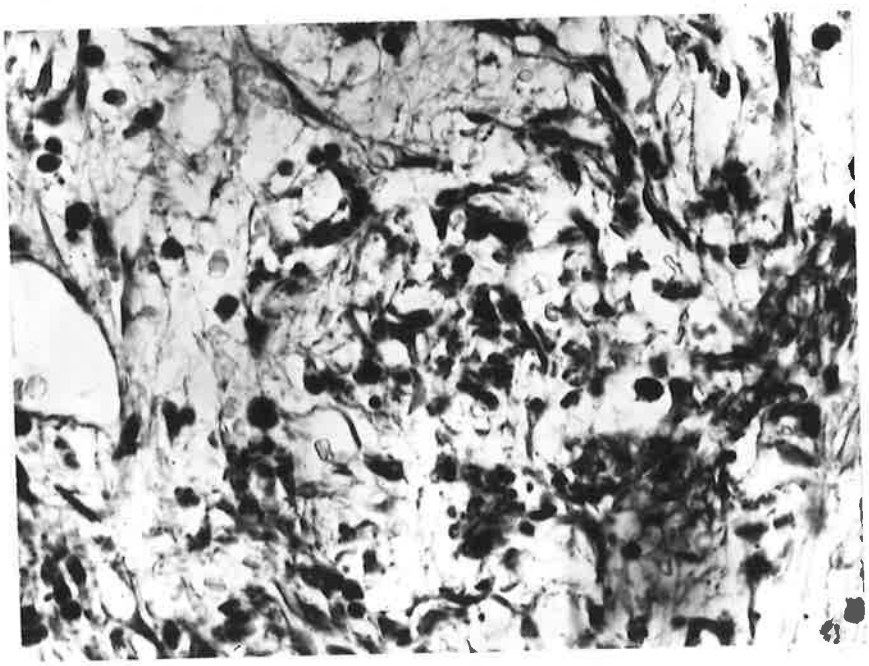


Fig. 7.39

OM 72 hours PAS x 400. Photomicrograph of the defect showing granulation tissue and ground substance. The organisation of the tissue is not orderly. Fig. 7.17 of control series for comparison.

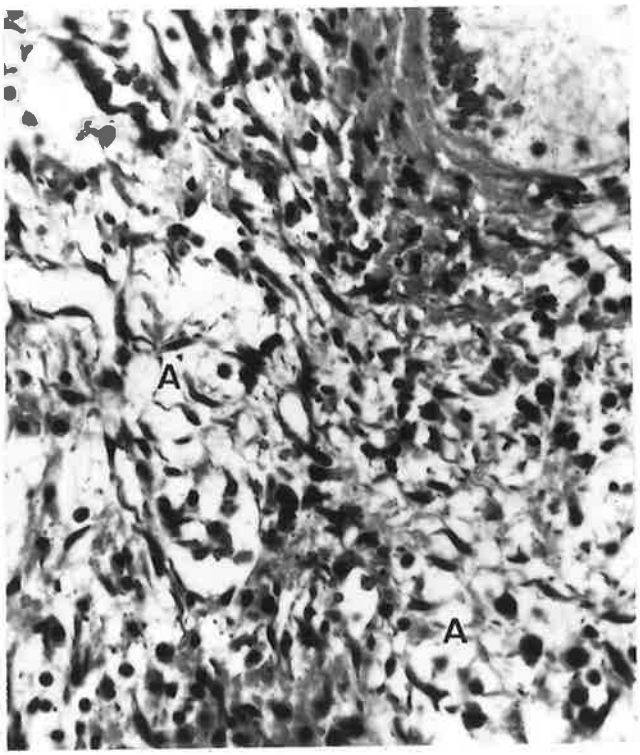


Fig. 7.40

OM 48 hours H & E x 250. Photomicrograph of the defect showing granulation tissue within the defect (A) showing a haphazard arrangement, spindly looking cells with pyknotic nuclei. See Fig. 7.12 of control series for comparison.

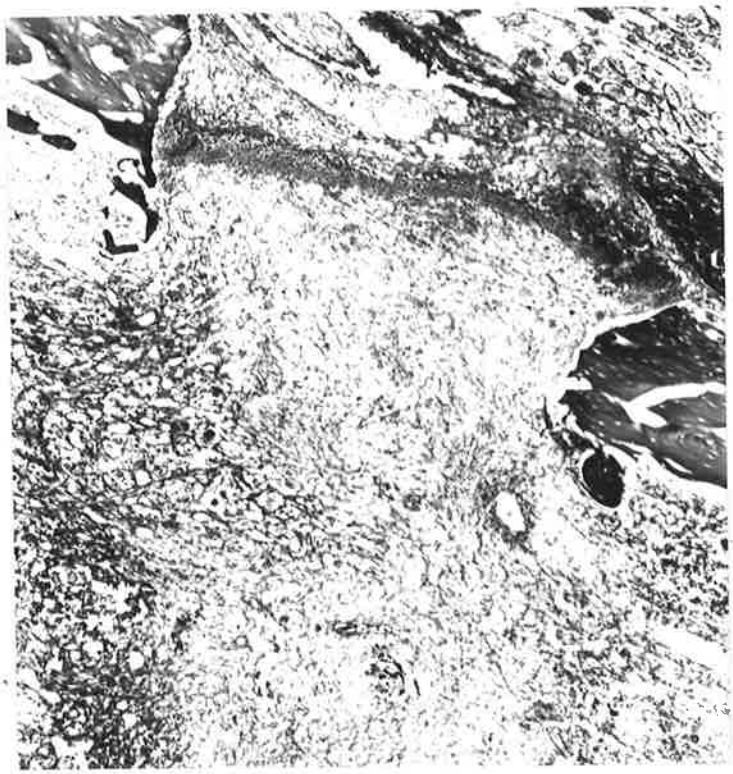


Fig. 7.41

OM 48 hours silver x 40. Photomicrograph of the defect in longitudinal section showing reticulin fibre formation mainly occurring at the periphery of the defect.

It can be seen from Table T.1 that the relative number of fibroblasts in the OM series is much less than that of the control series.

(ii) Ground substance

Ground substance, as demonstrated by the PAS stain, first appeared at 48 hours (Fig. 7.39) around the fibroblasts of the young granulation tissue. This ground substance increased in intensity of staining and in distribution as the granulation tissue matures. Table T.2 and hist ogram H.1 help to explain the observations made.

It can be seen from the hist ograms that the production and extent of the ground substance (indicated by the extent and intensity of PAS positive staining) is depressed in the OM series and follows the depressed number of fibroblasts seen in the granulation tissue in Table T.1.

(iii) Extracellular fibre formation

Reticulin fibre formation can be demonstrated by the silver stain in the granulation tissue at the periphery of the defect within 48 hours (Fig. 7.41). These fibres (early collagen) gradually increase in number and orientation as the granulation tissue matures. As these fibres are turned into collagen, the intensity of the silver stain regresses. Collagen (as demonstrated by the Van Gieson stain) (Fig. 7.42) fibres can first be seen at 72 hours and gradually increase in amount, distribution and orientation as the granulation tissue matures.

Table T.3 and hist ogram H.2 show the amount and extent of the reticulin fibres (silver stain) and Table T.4 and hist ogram H.3 show a gradual increase in the amount of collagen produced as the tissue matures. The amount of collagen is much less than that produced in the controls series. In addition to the

intensity and distribution of staining, it is evident from these results that collagen first appears in the controls by 48 hours and not until 5 days in the OM series.

Fig. 7.42

OM 72 hours Van Gieson x 250. Photomicrograph of the defect area showing collagen formation starting at the base of the defect.

(4) Bone Formation

(i) Endosteal callus

An endosteal response can be seen on the inner aspect of the cortical plate immediately adjacent to the defect by 24 hours (Fig. 7.43). New bone formation could be seen in this area by 48-72 hours and extends for some distance along the cortical plate (Fig. 7.44). A further endosteal reaction could be seen immediately opposite the defect (Fig. 7.46) and in more remote areas on the same side as the defect (Fig. 7.45).

The measurements made on the endosteal callus are presented in Table T.5 and have been visually presented in the hist ogram H.4. From the table and the hist ogram, it can be seen that the difference in the amounts of endosteal callus between the experimental and control series is minimal.

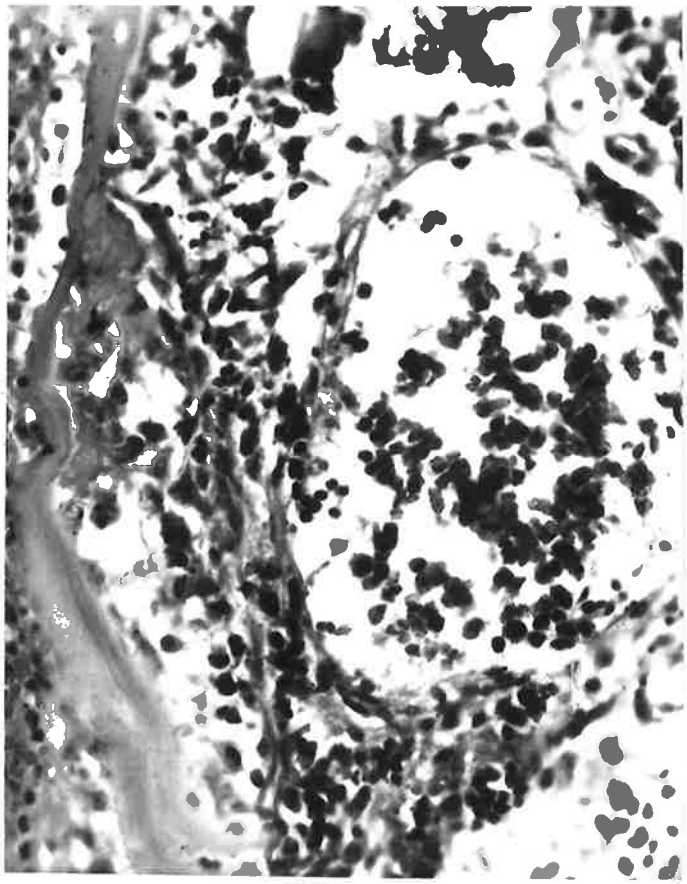


Fig. 7.43

OM 24 hours H & E x 250. Photomicrograph of the defect showing early endosteal callus formation in relation to a bone spicule at the margins of the defect.

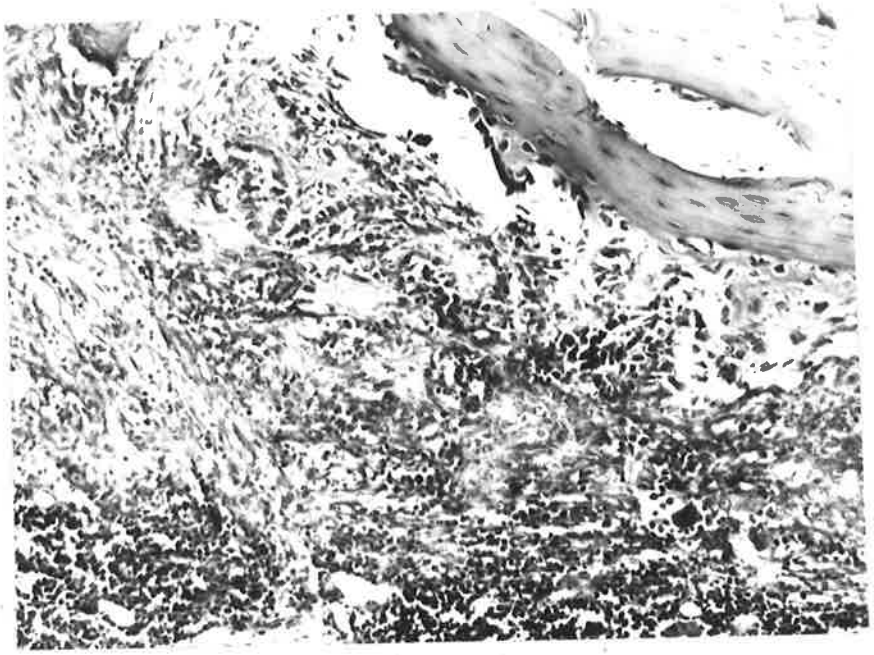


Fig. 7.44

OM 72 hours H & E x 250. Photomicrograph of the defect showing the endosteal response adjacent to the defect margin.

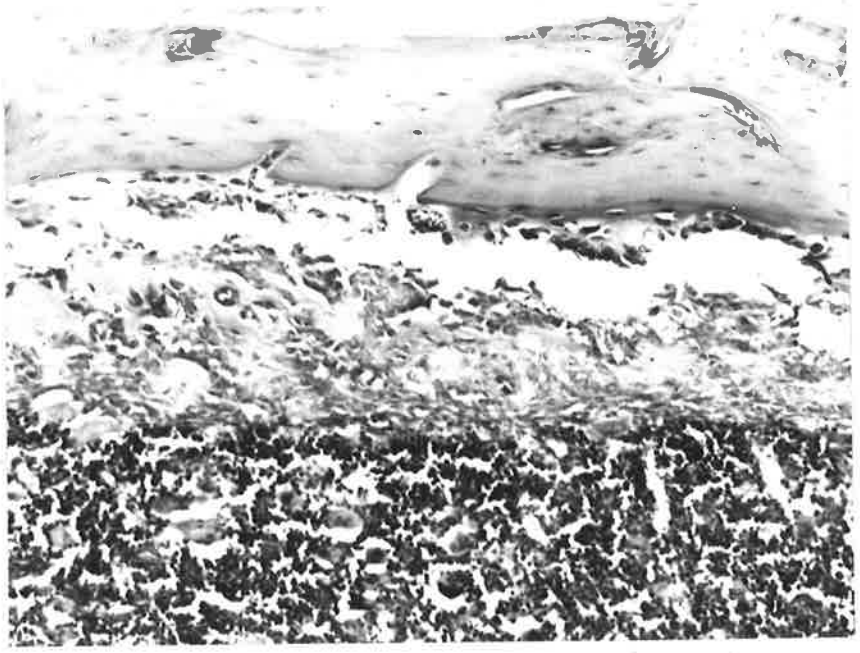


Fig. 7.45

OM 72 hours H & E x 250. Photomicrograph of the defect showing the endosteal response at a distance away from the margins of the defect on the same side.

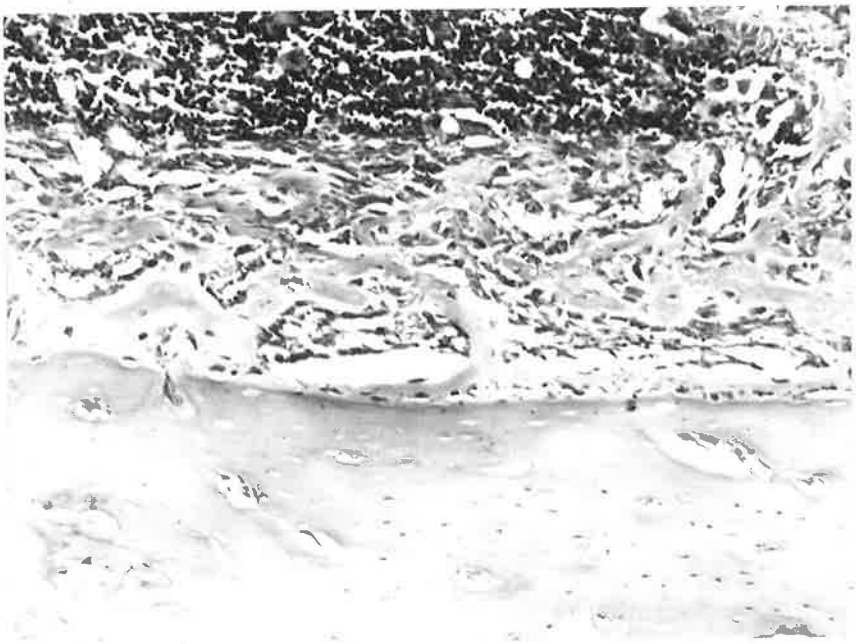


Fig. 7.46

OM 72 hours H & E x 250. Photomicrograph of the defect showing the endosteal reaction opposite the defect.

(ii) Subperiosteal callus

A periosteal reaction can be seen at 48 hours with a thickening of the cambial layer at some distance from the margins of the defect (Fig. 7.47). New external callus could be demonstrated by 72 hours (Fig. 7.48) and continued to be produced along the outer table of the cortical plate until it merged with the internal callus by the 14th day (Fig. 7.49). However the amount of external callus produced by the OM series was much less than that produced by the control and MO series. This difference was only seen in the early stages of healing and by 14 days there was no demonstrable difference between the experimental (OM) and control series.

Measurements (d") of the external callus are presented in Table T.6 and hist ogram H.5.

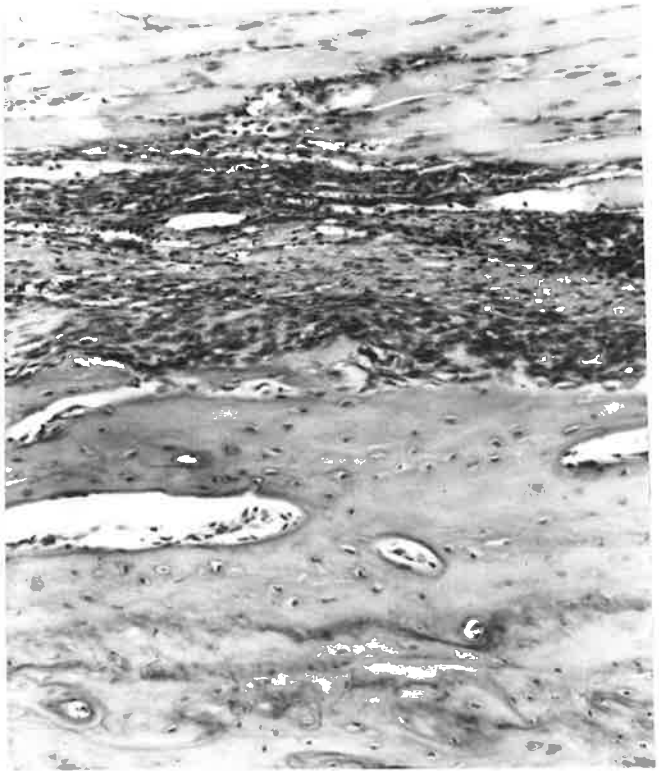


Fig. 7.47

OM 48 hours H & E x 100. Photomicrograph of the defect showing the periosteal reaction.

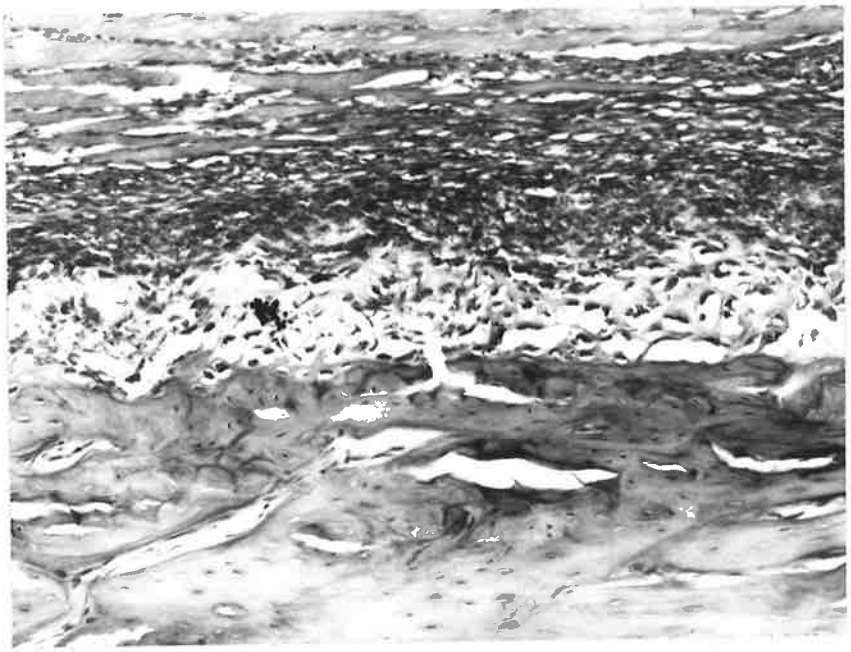


Fig. 7.48

OM 72 hours H & E x 250. Photomicrograph of the defect showing the periosteal (external callus) reaction.

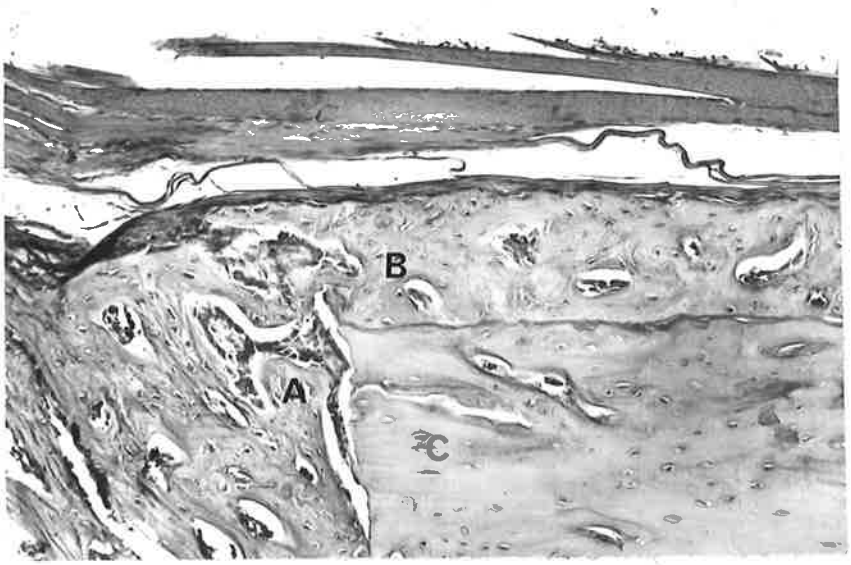


Fig. 7.49

OM 14 days H & E x 100. Photomicrograph of the defect showing the bridging internal callus (A) external callus (B) and cortical bone (C).

(iii) Internal callus

Bone formation within the defect could be seen by 5 days (Fig. 7.50) and had filled the defect between 7-14 days (Figs. 7.51, 7.52, 7.49). By 14 days the external and internal callus had merged over the top of the defect. No difference in the quality of the internal callus could be seen between the experimental and the control series. However, formation was retarded as demonstrated in Table T.7 and hist ogram H.6.

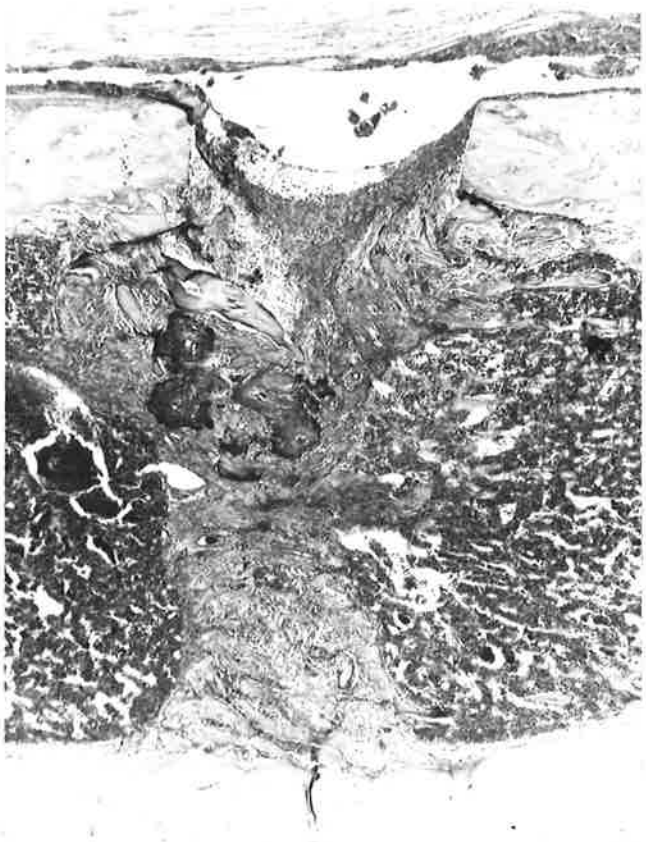


Fig. 7.50 Insert I

OM 5 days H & E x 25. Photomicrograph of the defect area showing granulation tissue formation and internal callus.

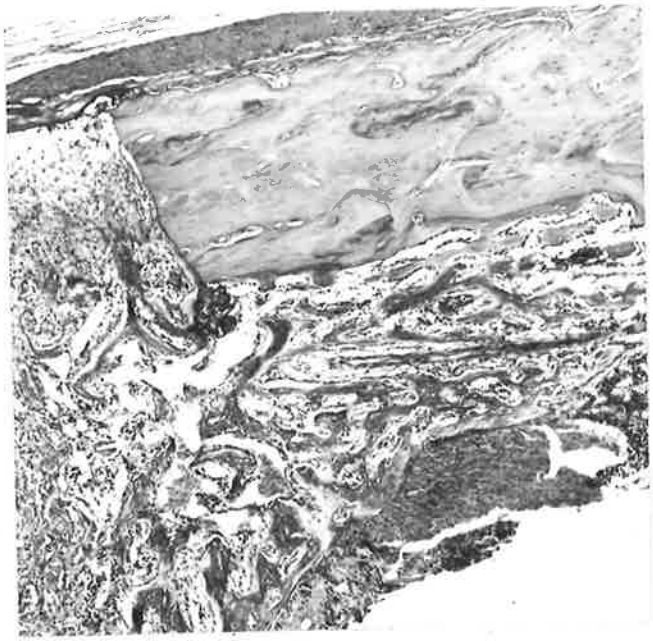


Fig. 7.51

OM 7 days PAS x 40. Photomicrograph of the defect showing the internal callus extending along the inner aspect of the cortical plate immediately adjacent to the defect.

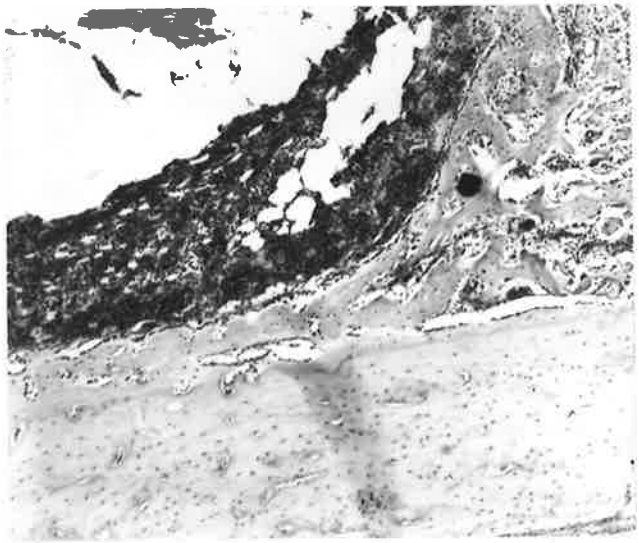


Fig. 7.52

OM 7 days H & E x 40. Photomicrograph of the defect showing internal callus formation extending along the inner aspect of the cortical plate opposite the defect.

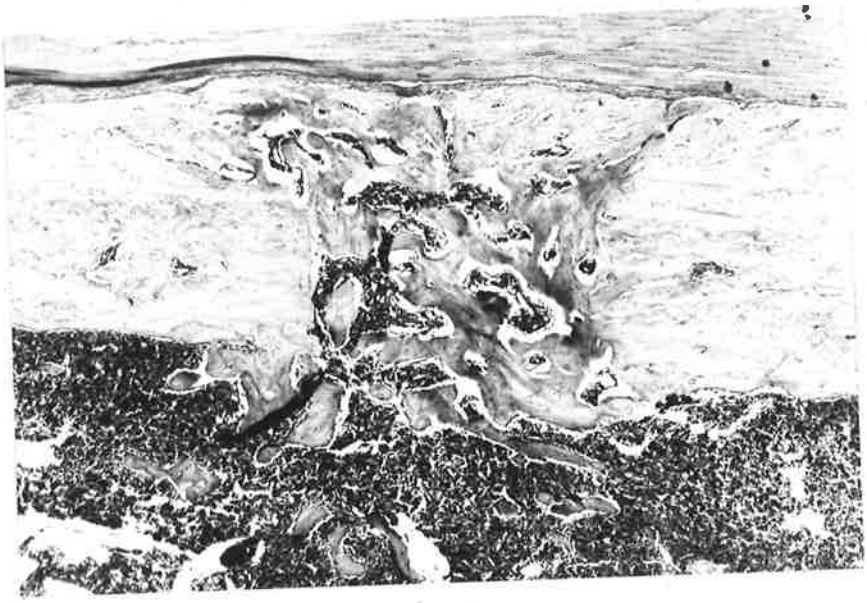


Fig. 7.53 Insert I

OM 3 weeks H & E x 100. Photomicrograph of the defect showing the internal and external callus. Internal callus is almost gone leaving only the bridging callus.

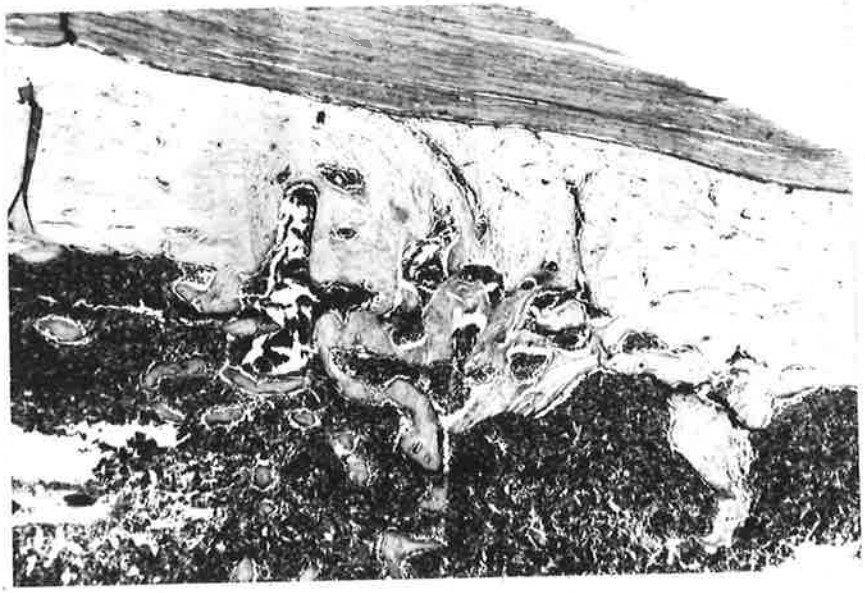


Fig. 7.54 Insert I

OM 4 weeks H & E x 25. Photomicrograph of the defect showing remodelling of the defect to be at an advanced stage.

Fig. 7.55 Insert I

OM 8 weeks H & E x 25. Photomicrograph of the defect showing that remodelling is almost complete. Note trabeculations of the callus still at right angles to the long axis of the femoral shaft.

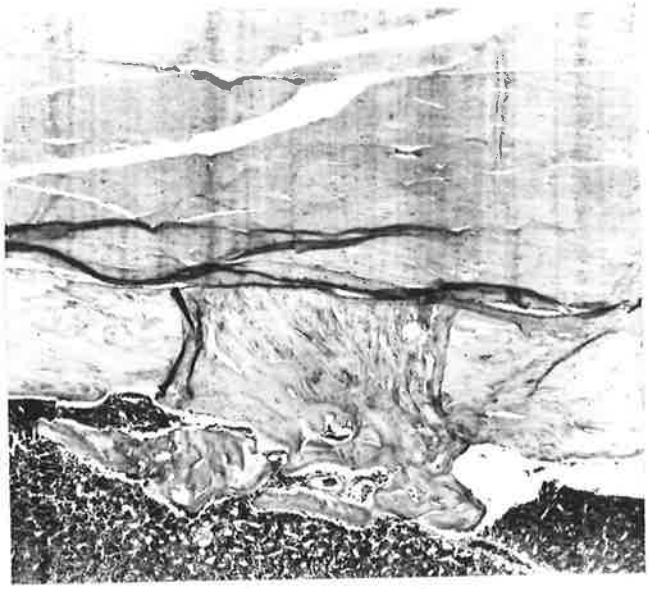


Fig. 7.56 Insert I

OM 12 weeks H & E x 25. Photomicrograph of the defect almost completely remodelled.

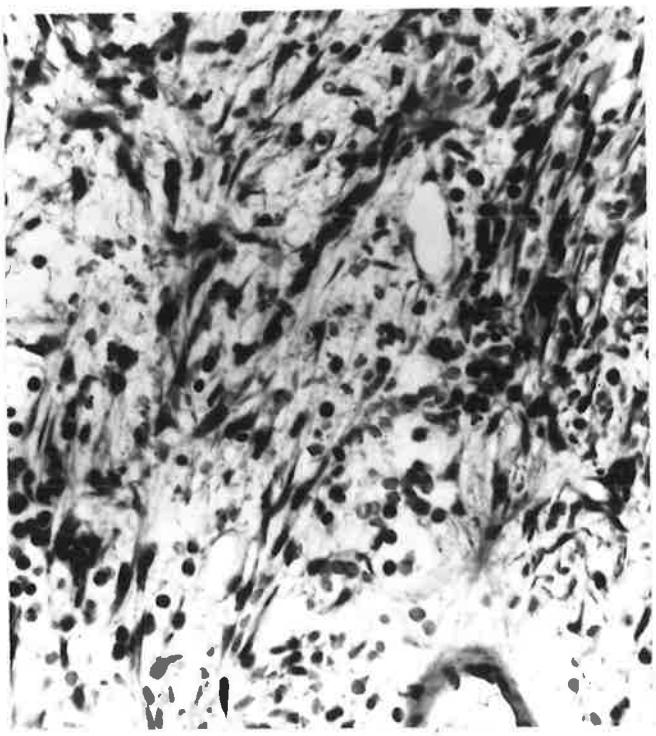


Fig. 7.57

OM 72 hours H & E x 250. Photomicrograph of the defect showing granulation tissue at the edge of the defect, with the fibroblasts appearing spindly and pyknotic.

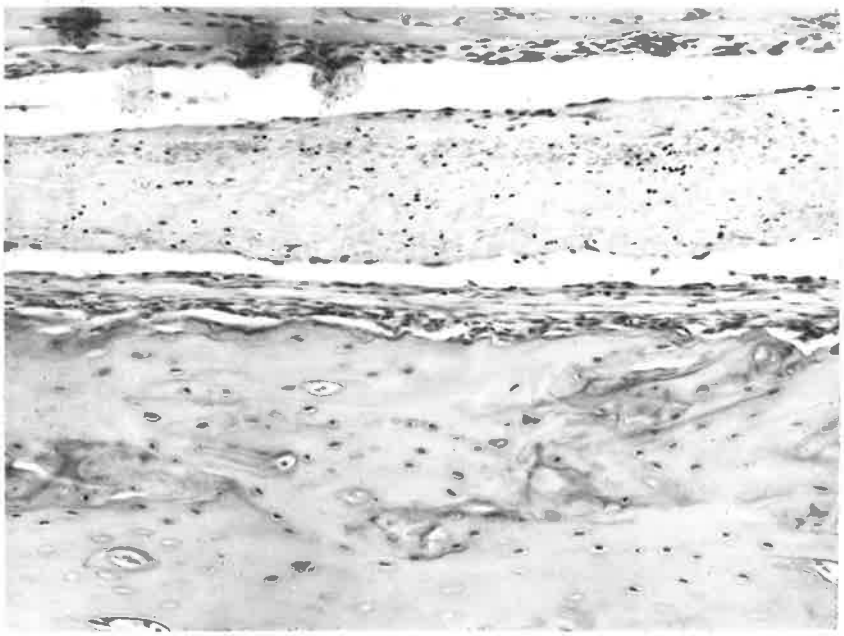


Fig. 7.58

OM 7 days H & E x 100. Photomicrograph of the defect showing the periosteal reaction.

(5) Remodelling

Remodelling of the defect could be seen to start by the 7th postoperative day and is the most important and prominent feature seen from 14 days - 3 months. No alteration in the remodelling process could be detected between the OM, MO and Control series (Figs. 7.53, 7.54, 7.55, 7.56).

Summary

The difference between the control and experimental series can be summarised as follows:

(a) Generally there is a haphazard arrangement of the granulation tissue, with spindly shaped cells, a decrease in number, intracellular oedema, poor staining and the persistence of an inflammatory response in the OM series up to 7 days (control Figs. 7.16, 7.20 and experimental Figs. 7.39, 7.40, 7.46, 7.57).

(b) Ground substance production was decreased in the OM series as demonstrated by the extent of staining with the PAS stain (Table T2 and hist ogram H1).

(c) Both the amount and extent of reticulin and collagen were reduced in the OM series, with collagen first appearing on the 5th day in the OM series and by 48 hours in the control series, (Tables T.3, T.4 and hist ograms H.2, H.3).

(d) The endosteal response seen on the control and OM series were essentially the same (Tables T.5 and hist ogram H.4).

(e) The subperiosteal reaction in the OM series was found to be initially less than that found in the control series but by 14 days the periosteal response was the same as that of the control series. (Figs. control 7.10, 7.21, 7.26, 7.28 and experimental 7.47, 7.48, 7.58, 7.49, and Tables T.6 and hist ogram H.5).

(f) The internal callus was found to be greatly retarded in amount in the OM series but the quality of bone formed did not vary. Internal callus formation could be seen in the control series by 72 hours and in the OM series by 5 days, with maximal internal callus being produced by 7 days in the controls and 14 days in the OM series (Table T.7 and histogram H.6).

The most important thing to note with this investigation relates to the fact that all of these changes were only present during the time of drug administration, namely between 12 hours and 14 days with no difference occurring between 14 days and three months.

D. EXPERIMENTAL SERIES - MO

All stages of healing in the MO series are comparable with the controls. No significant departure from the control series, either quantitatively or qualitatively was observed under the light microscope (see tables and hist ograms).

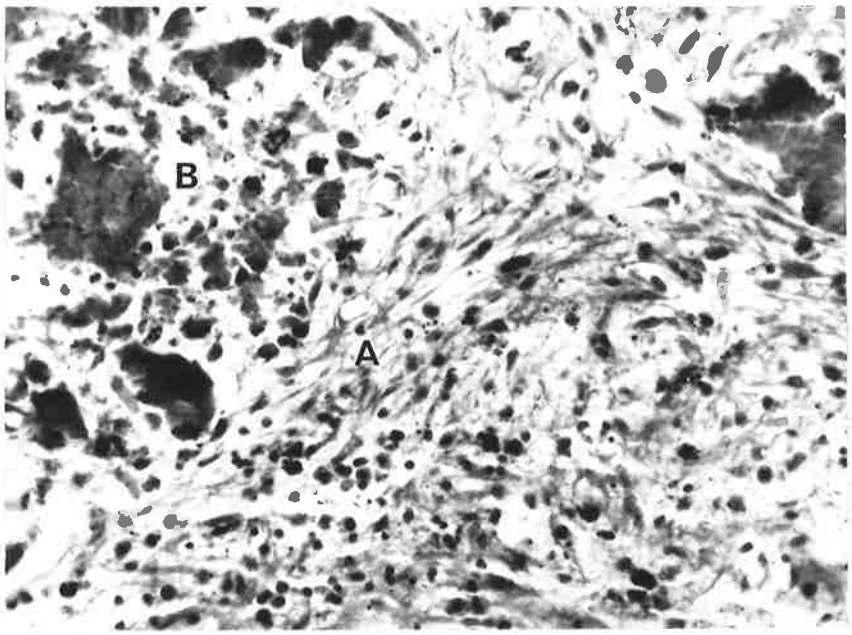


Fig. 7.59

MO 48 hours PAS x 250. Photomicrograph of the defect showing young granulation tissue (A), at the periphery of the defect, and bone filings (B) - Ref. Fig. 7.12 for comparison with the control.

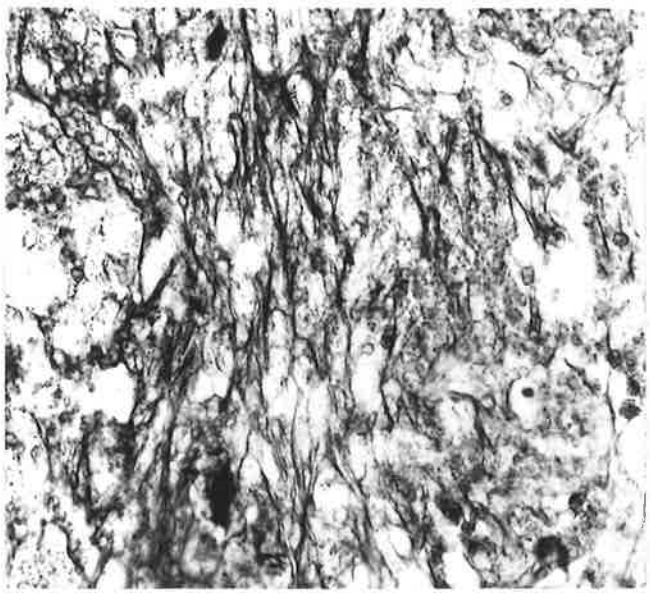


Fig. 7.60

MO 48 hours silver x 250. Photomicrograph of the defect showing reticulin fibre formation in relation to the granulation tissue. Ref. Fig. 7.15 for comparison with controls.

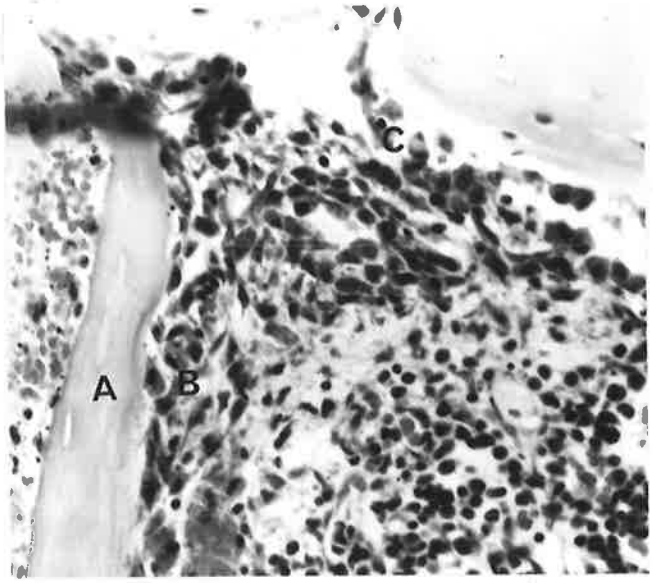


Fig. 7.61

MO 48 hours H & E x 250. Photomicrograph of the defect showing the endosteal reaction adjacent to the defect, bone spicule (A) endosteal cells (B) and (C). Ref. Fig. 7.13 for comparison with the control.

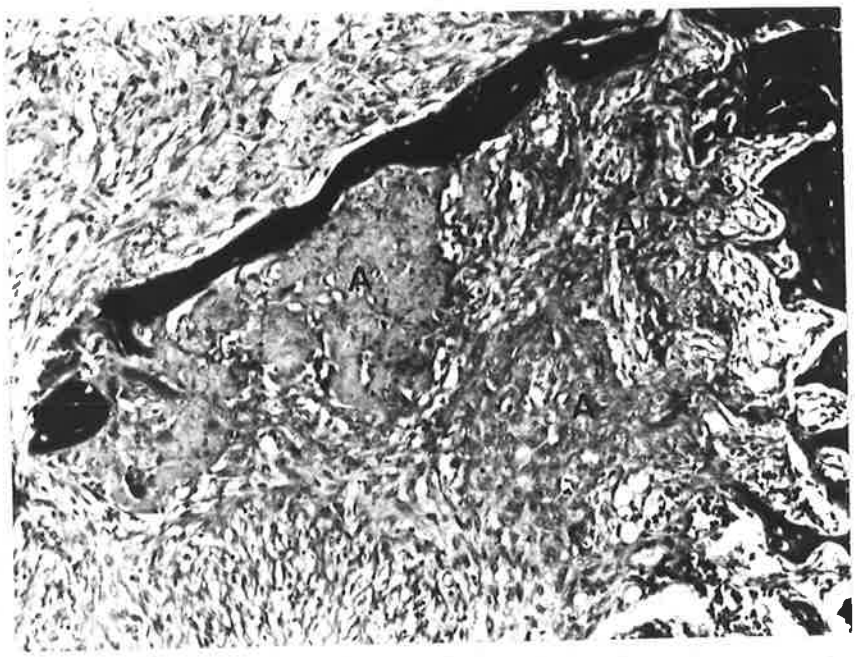


Fig. 7.62

MO 72 hours Van Gieson x 100. Photomicrograph of the defect in transverse section showing collagen formation (A) in relation to the cortical bone, bone spicule and bone mush. Ref. Fig. 7.18 for comparison with controls.

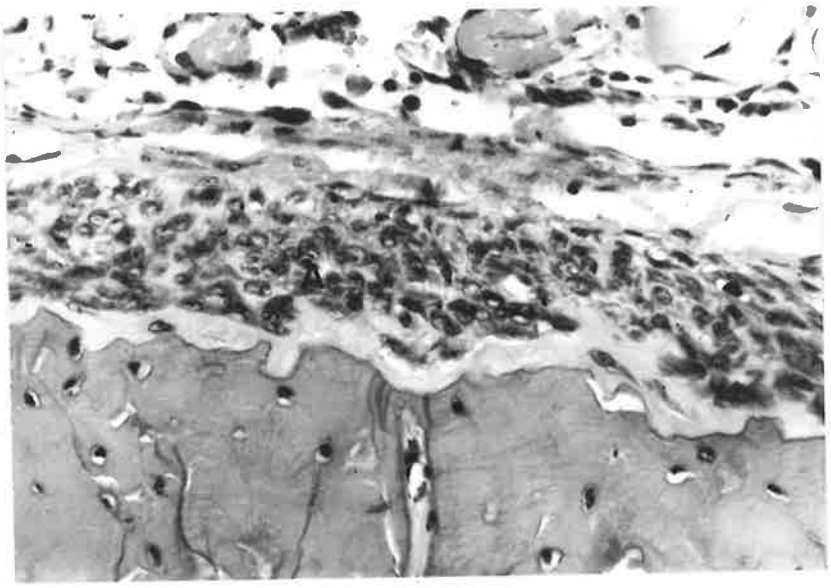


Fig. 7.63

MO 72 hours H & E x 250. Photomicrograph of the defect showing the periosteum (A) and early external callus formation. Ref. Fig. 7.21 for comparison with controls.

Fig. 7.64

MO 5 days PAS x 40. Photomicrograph of the defect showing granulation tissue and internal callus filling the defect. Ref. Fig. 7.22 for comparison with controls.

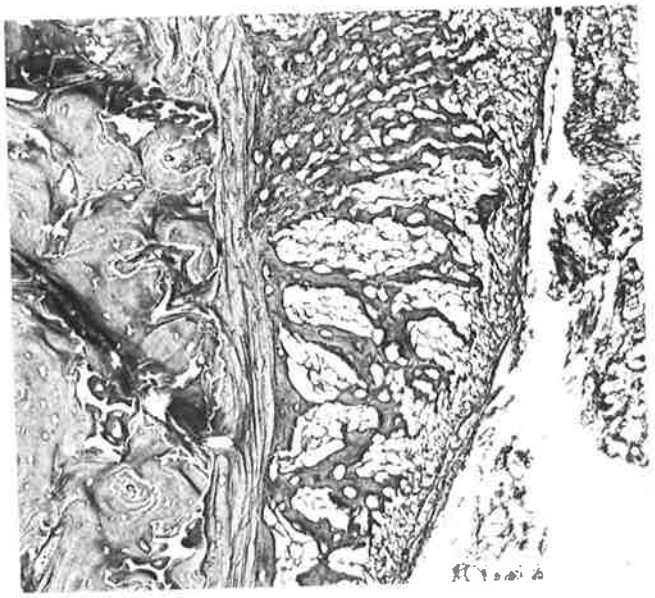


Fig. 7.65

MO 5 days silver x 100. Photomicrograph showing external callus formation. Ref. Fig. 7.26 for comparison with controls.

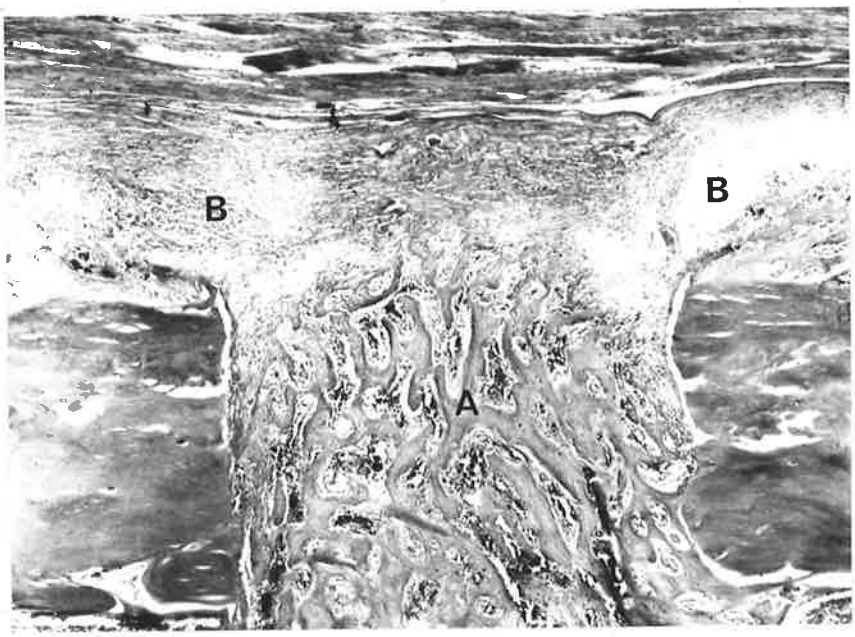


Fig. 7.66 Insert I

MO 7 days H & E x 40. Photomicrograph of the defect area showing the internal (A) and external callus (B). Ref. Fig. 7.27 for comparison with controls.

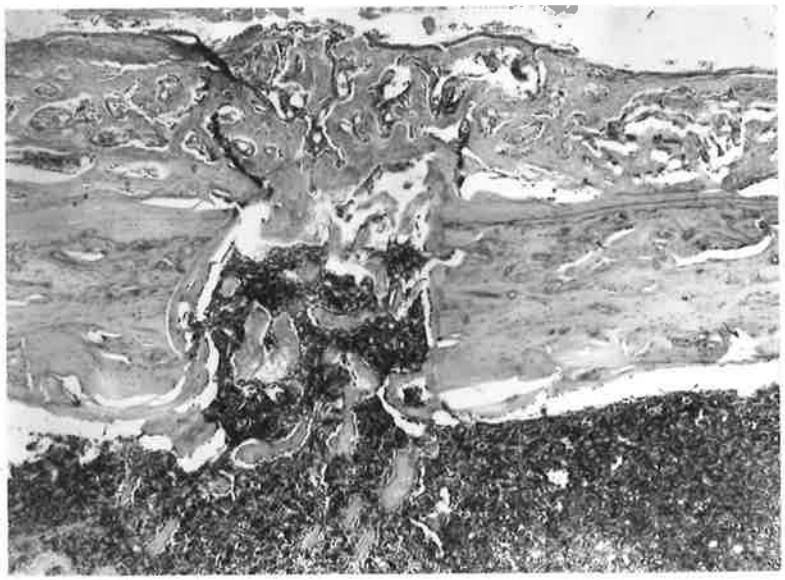


Fig. 7.67 Insert I

MO 14 days H & E x 25. Photomicrograph of the defect showing internal callus undergoing resorption.

Ref. Fig. 7.30 for comparison with controls.

Fig. 7.68 Insert I

MO 3 weeks PAS x 25. Photomicrograph of the defect showing the external (A) and internal (B) callus, note remodelling of the internal callus.

Ref. Fig. 7.32 for comparison with controls.

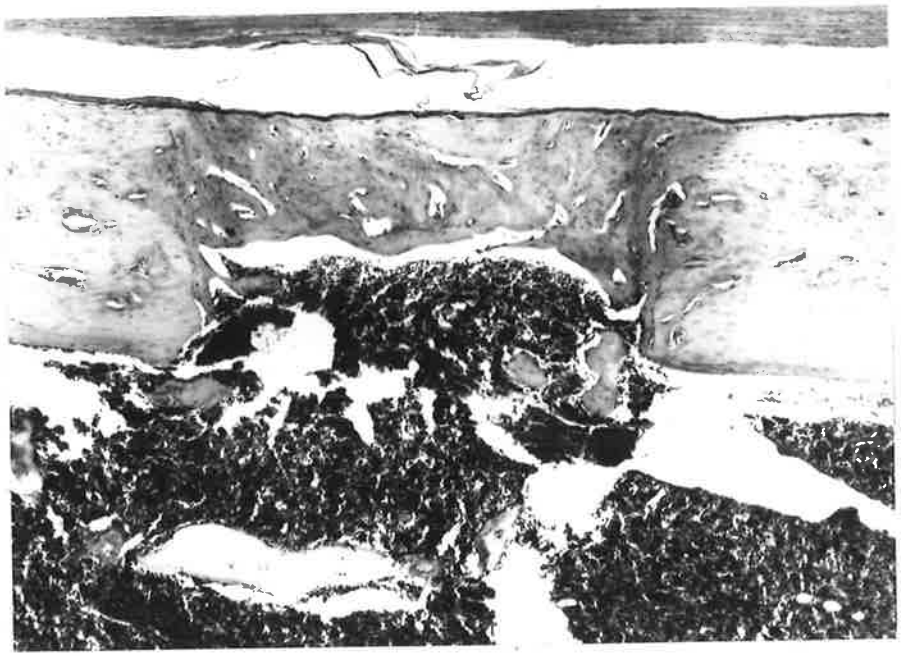


Fig. 7.69 Insert I

MO 4 weeks H & E x 40. Photomicrograph of the defect showing the defect to be almost healed and remodelled. Ref. Figs. 7.34 and 7.35 for comparison with controls.

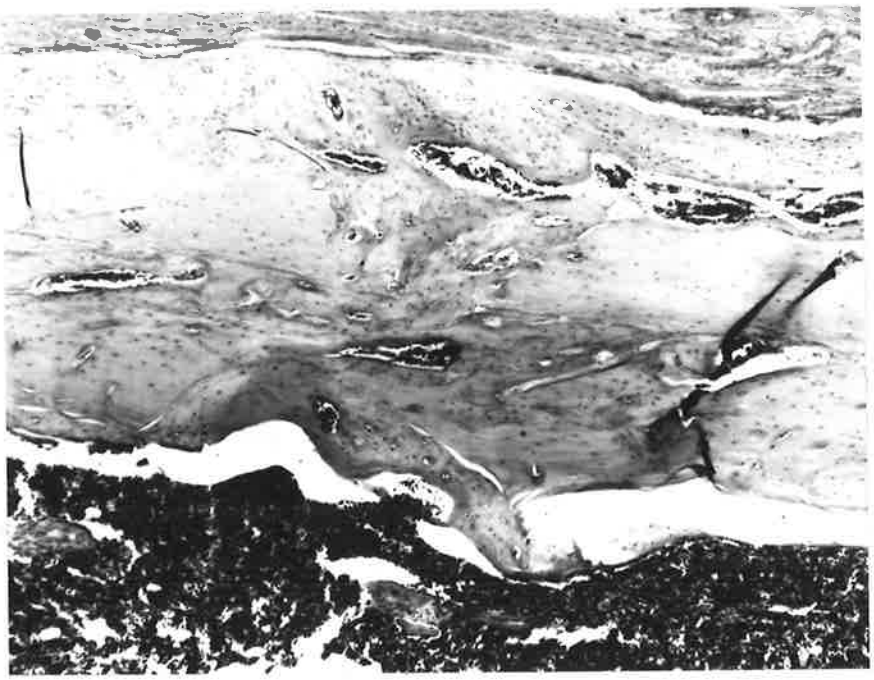


Fig. 7.70 Insert I

MO 2 months H & E x 40. Photomicrograph of the defect showing the defect almost remodelled. Ref. Fig. 7.36 for comparison with controls.

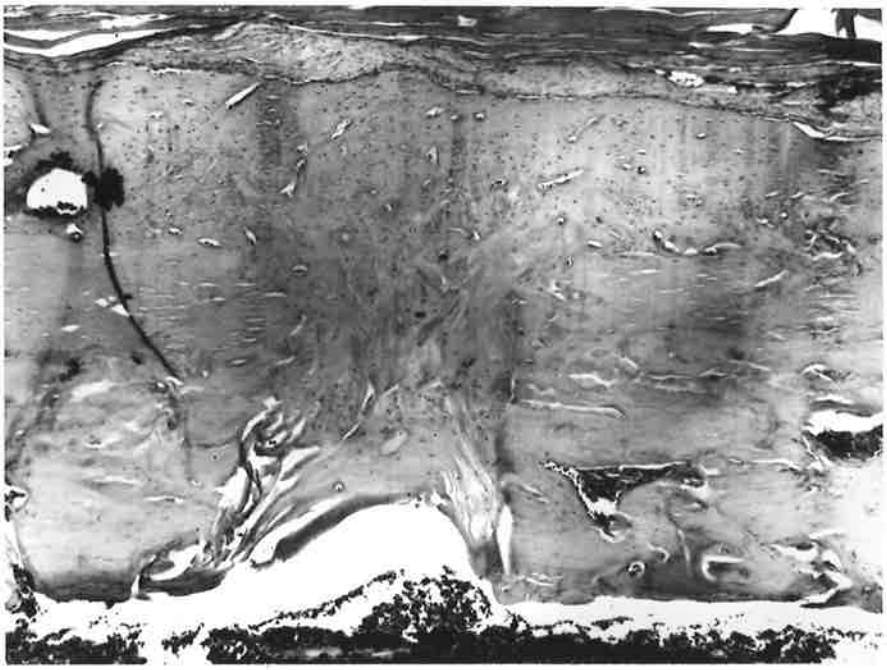


Fig. 7.71 Insert I

MO 3 months H & E x 40. Photomicrograph of the defect showing the defect to be almost remodelled. Ref. Fig. 7.37 for comparison with controls.

COMPARISON OF CONTROL AND EXPERIMENTAL SERIES - TABLES AND HISTIOGRAMS

Number of fibroblasts per high power field

	48 hours		72 hours		5 days	7 days
Specimen	Periphery of granulation	Deeper	Periphery of granulation	Deeper	Granulation uniform in structure	
C1	20	30	35	NA	116	All bone
C2	22	NA	60	NA	156	" "
C3	30	NA	70	40	157	" "
C4	30	60+	50	70	120	" "
C5	42	104	65	55	141	" "
C6	NA	NA	96	92	158	" "
Average	28.8	64.66	62.6	64.25	139.6	
OM1	22	17	16	NA	40	25
OM2	22	45	38	40	60	40
OM3	20	25	43	51	85	38
OM4	45	50	27	42	75	60
OM5	30	60	42	72	NA	36
OM6	25	40	30	NA	NA	NA
Average	28	39.5	32.6	51.25	65	39.8
MO1	30	100	50	60	110	All bone
MO2	25	40	45	50	150	" "
MO3	35	NA	60	55	160	" "
MO4	20	30	70	80	136	" "
MO5	24	20	50	70	140	" "
MO6	20	NA	90	NA	124	" "
Average	25	47.5	60.8	63.0	136.6	

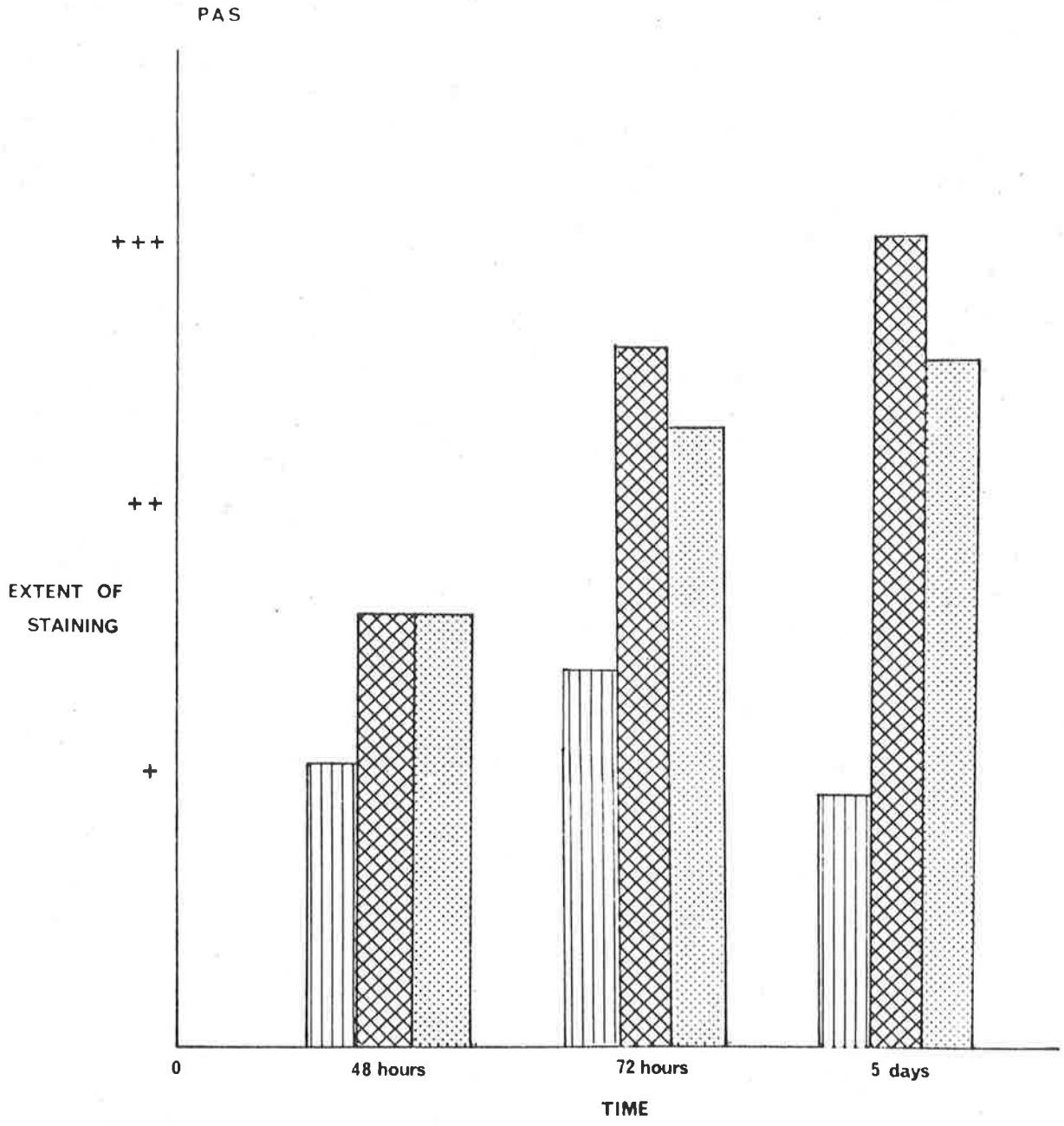
TABLE T.1.

P.A.S. Staining

Specimen	48 hours			72 hours			5 days			7 days		
	C	OM	MO	C	OM	MO	C	OM	MO	C	OM	MO
1	+	+	+	++	±	++	+++	+	+++	AB	AB	AB
2	++	+	++	++	++	++	+++	±	+++	"	"	"
3	++	+	++	++	+	++	+++	+	+++	"	"	"
4	++	+	+	++	++	++	+++	-	++	"	"	"
5	+	±	++	+++	-	++	+++	-	++	"	"	"
6	-	++	++	+++	-	+++	-	-	++	"	"	"
Average	1.6+	1.08+	1.6+	2.6+	1.4+	2.3+	3+	0.86+	2.5+	0	0	0

TABLE T.2

A.B: All bone.

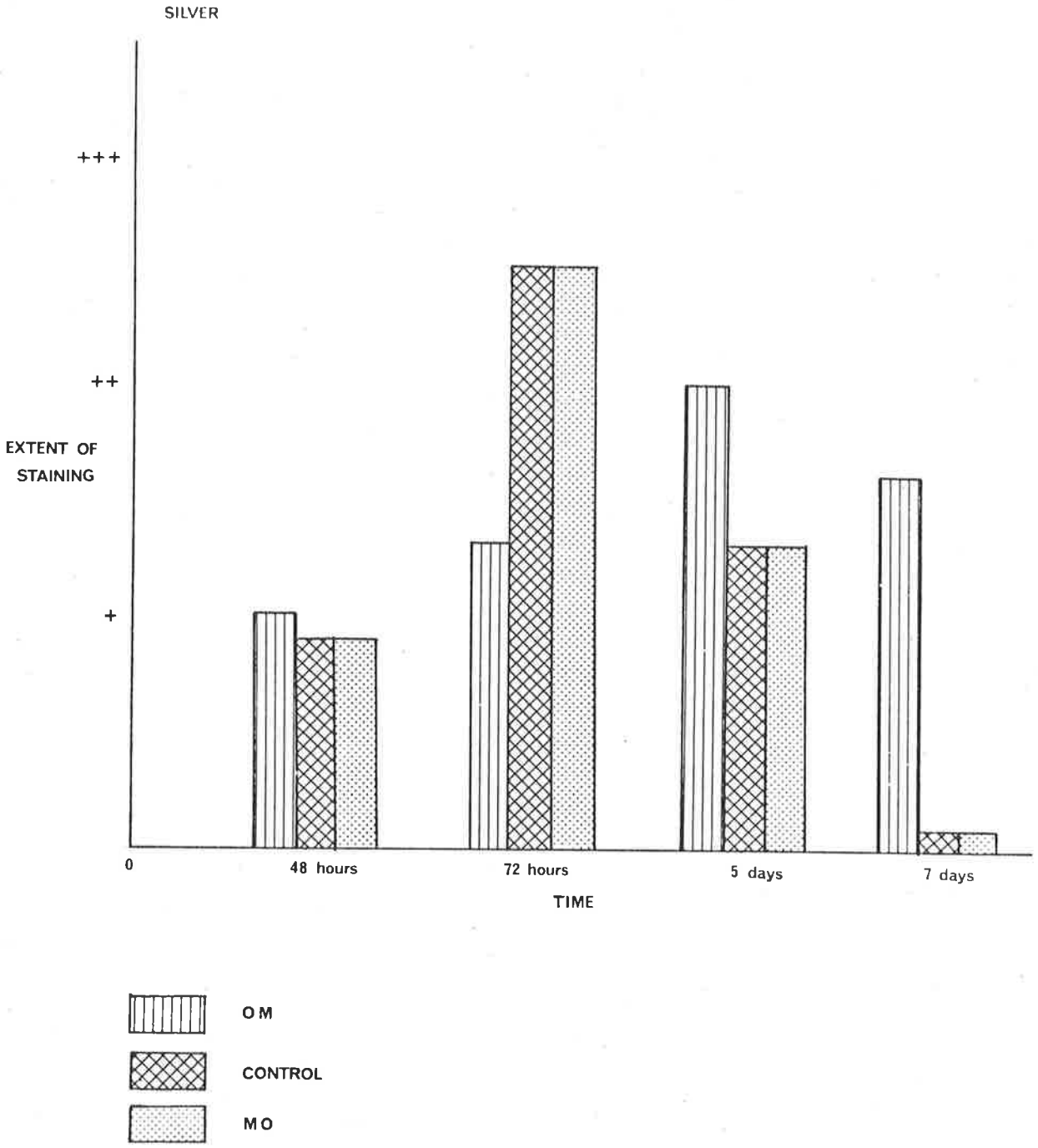


HIST OGRAM H 1

Silver Staining

Specimen	48 hours			72 hours			5 days			7 days		
	C	OM	MO	C	OM	MO	C	OM	MO	C	OM	MO
1	+	-	+	++	±	++	+	++	+	-	+	-
2	+	-	±	++	+	++	+	++	±	-		-
3	±	++	±	+++	++	+++	±	++	±	-	++	-
4	+	+	+	+++		+++	+		±	-		-
5	+	+	+	++	+	+++	+	++	++	-	++	-
6		++	+	+++	++	++	++		++	-	++	-
Average	.9+	1+	.9+	2.5+	1.3+	2.5+	1.3+	2+	1.3+	0	1.75+	0

TABLE T.3



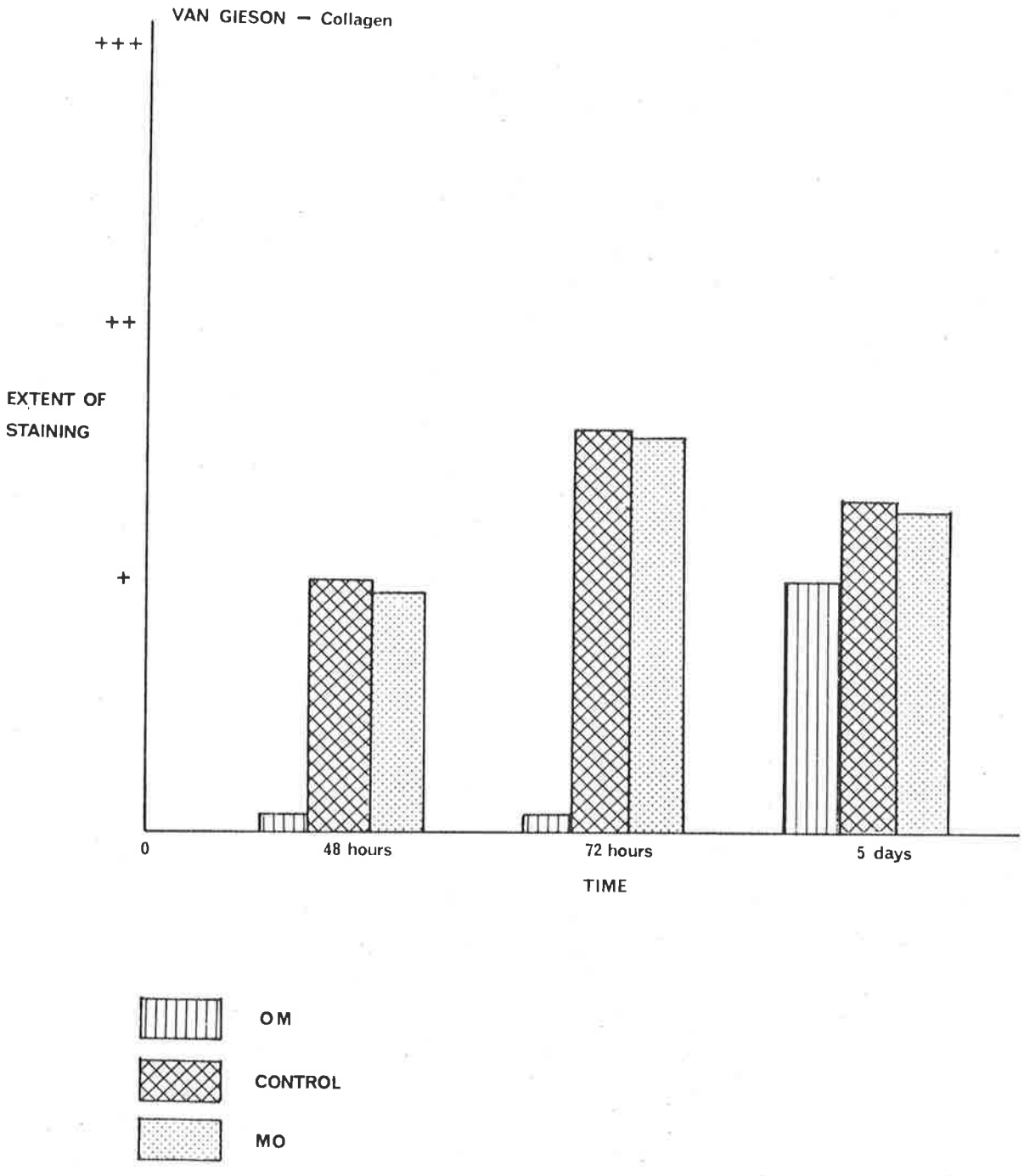
HIST OGRAM H 2

Van Gieson Staining

Specimen	48 hours			72 hours			5 days			7 days		
	C	OM	MO	C	OM	MO	C	OM	MO	C	OM	MO
1	±	-	+	++	-	++	+	+	+	AB	AB	AB
2	±	-	±	++	-	++	+	++	+	"	"	"
3	+	-	+	+	-	+++	+	-	+	"	"	"
4	++	-	+	+	-	+	+	+	++	"	"	"
5	+	-	±	++	-	+	++		++	"	"	"
6	+	-	+	++	-	+	++		+	"	"	"
Average	1+	0	.8+	1.6+	0	1.6+	1.3+	1+	1.3+	0	0	0

Table T.4

A.B: All bone

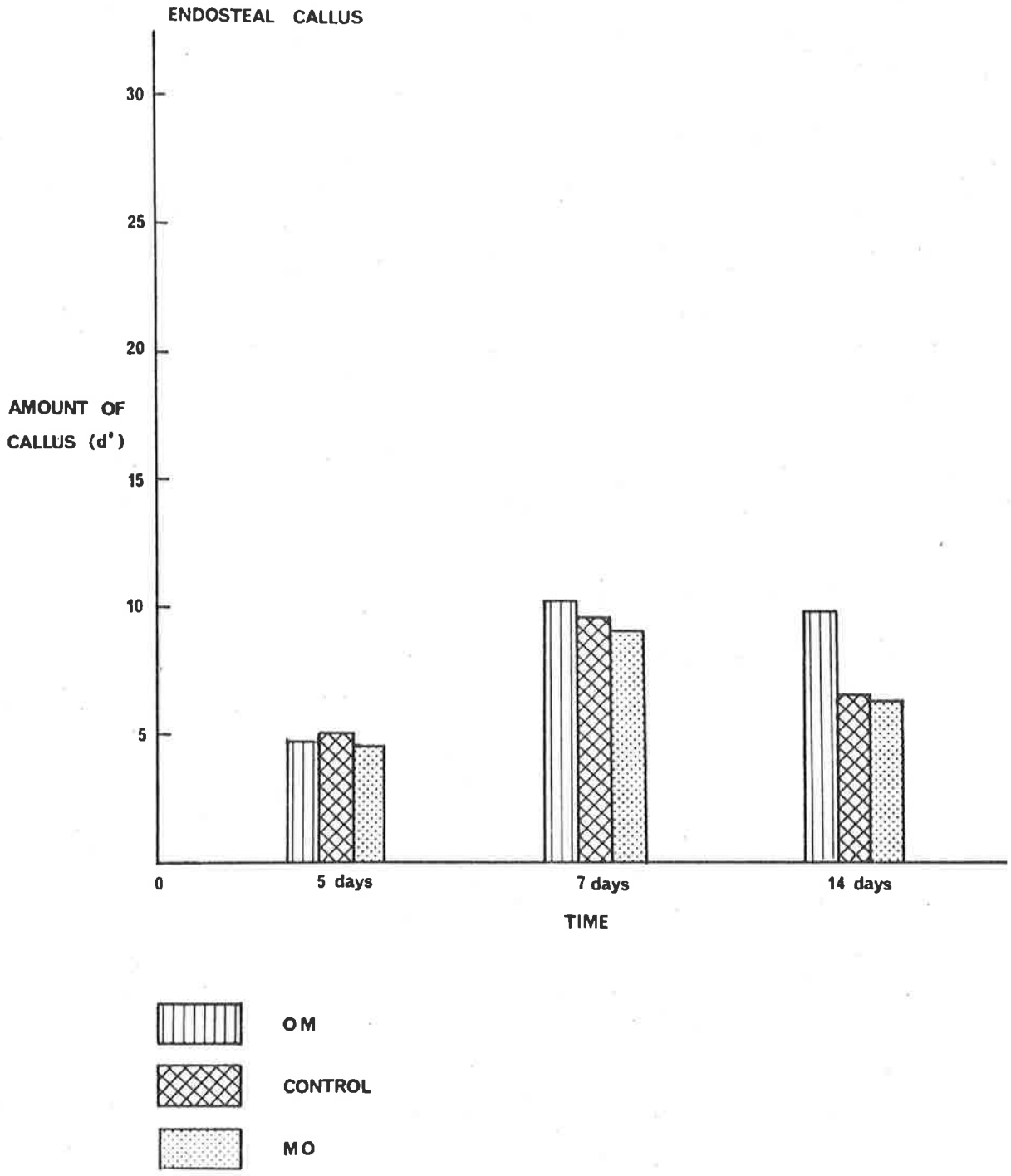


HIST OGRAM H 3

Endosteal Callus (d')

Specimen	5 days		7 days		14 days	
	d' left	d' right	d' left	d' right	d' left	d' right
C1	5	5	+	9	3	15
C2		4	5	15	8	7
C3	6	3	7	14	4	10
C4		6			8	4
Average	4.9		9		8.3	
OM1	-	6	4	4	7	9
OM2	4	4	20	25		15
OM3			8	7	10	14
OM4			8	5	4	6
Average	4.6		10.1		9.2	
MO1	5	5	6	7	7	8
MO2	5	4	5	12	6	6
MO3	6	2	10	11	10	9
MO4	4					8
Average	4.4		8.4		7.7	

TABLE T.5

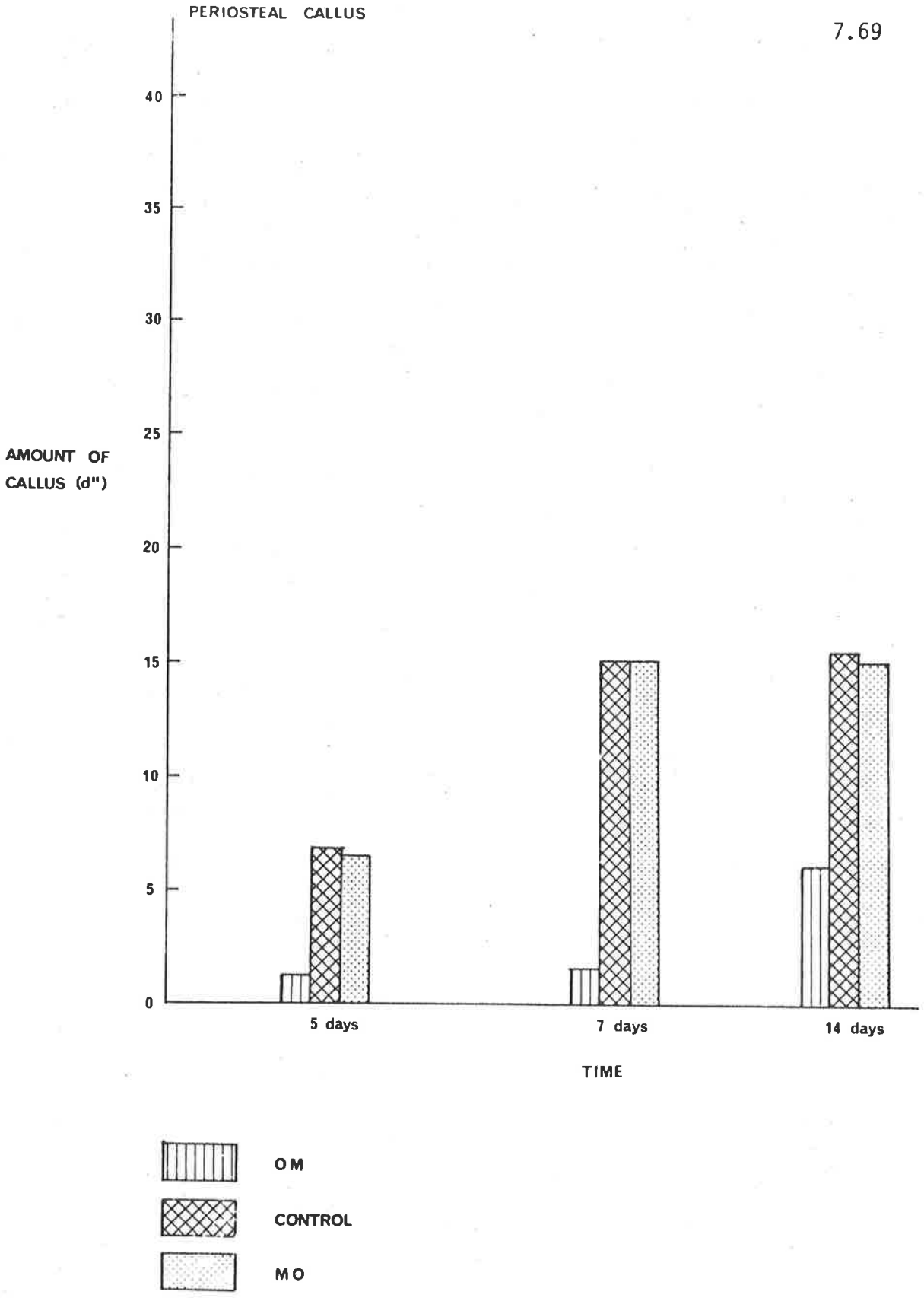


HIST OGRAM H 4

Periosteal Callus (d")

Specimen	5 days		7 days		14 days	
	d" left	d" right	d" left	d" right	d" left	d" right
C1	8	6	19	17	16	10
C2	14	7	10	17	25	15
C3	12		16	12	15	16
C4	9	4			15	15
Average	8.5		15.1		15.9	
OM1	5	-	4	-	5	8
OM2	-	5	8	3	4	10
OM3			-	2	8	
OM4						
Average	2.5		2.8		7	
MO1	7	8	17	20	16	25
MO2	15	6	14	12	15	16
MO3	13	-	12	16	15	10
MO4	8	5			15	15
Average	7.75		15.1		15.1	

TABLE T.6

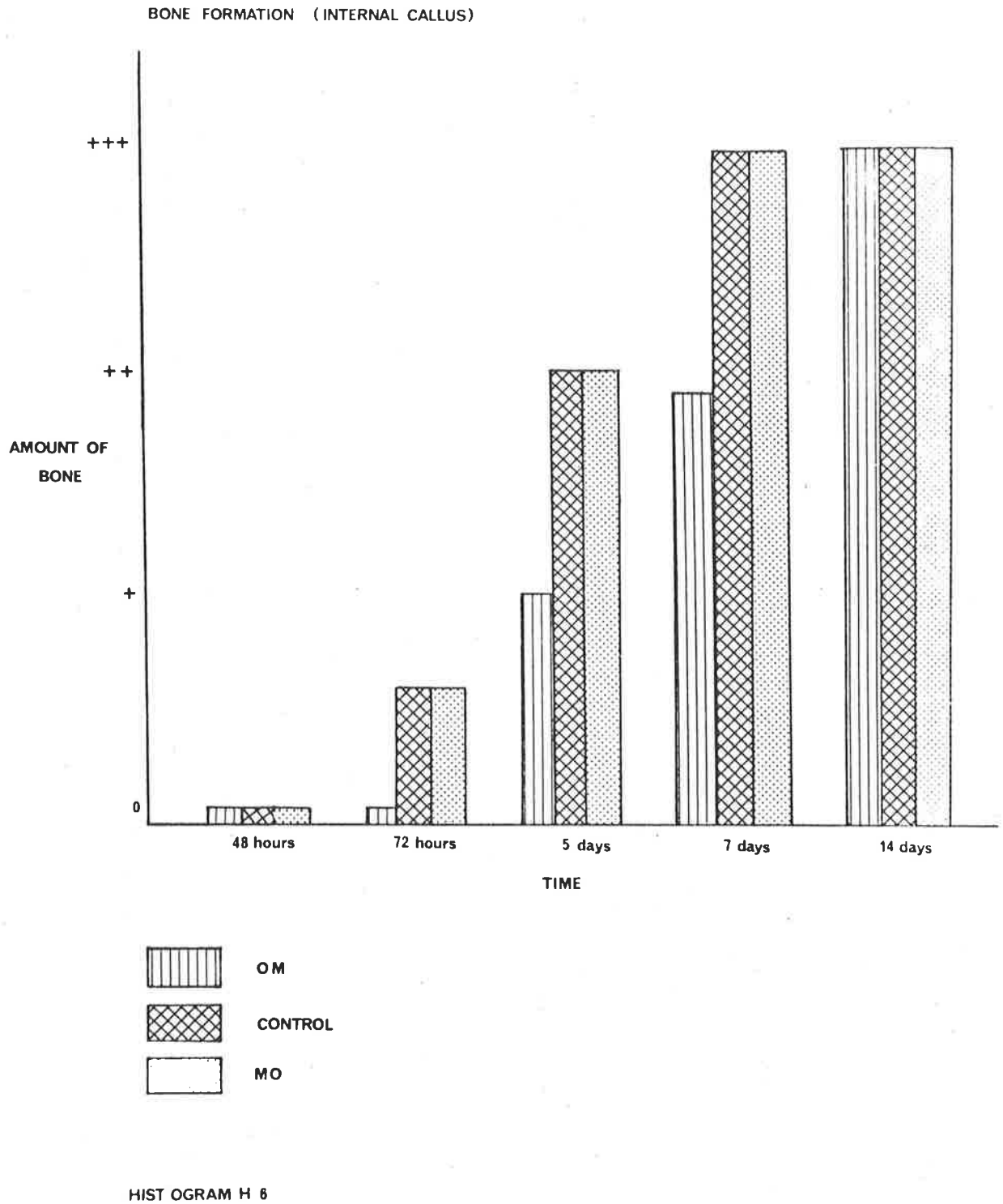


HIST OGRAM H 5

Internal Callus Formation

Specimen	48 hours			72 hours			5 days			7 days			14 days		
	C	OM	MO	C	OM	MO	C	OM	MO	C	OM	MO	C	OM	MO
1	-	-	-	±	-	±	++	-	++	+++	++	+++	+++	+++	+++
2	-	-	-	±	-	+	++	+	++	+++	++	+++	+++	+++	+++
3	-	-	-	±	-	+	++	+	++	+++	++	+++	+++	+++	+++
4	-	-	-	+	-	±	++	++	++	+++	+	+++	+++	+++	+++
5	-	-	-	±	-	±	++		++	+++	++	+++	+++	+++	+++
6	-	-	-	+	-	±			++	+++	++	+++	+++	+++	+++
Average				.6+		.6+	2+	1+	2+	3+	1.8+	3+	3+	3+	3+

TABLE T.7



HIST OGRAM H 6

DISCUSSION

A. HEALING OF THE CORTICAL DEFECT IN THE CONTROL SERIES

(1) Granulation Tissue Formation

(2) The fate of the Bone Filings and the Defect Margins

(3) Osteogenesis
- (i) Endosteal callus formation
- (ii) External callus formation
- (iii) Internal callus formation
- (iv) Remodelling

B. COMPARISON BETWEEN THE CONTROL AND EXPERIMENTAL SERIES

(1) The OM series
- (i) Granulation tissue formation
- (ii) Callus formation
  - (a) Endosteal callus
  - (b) External callus
  - (c) Internal callus
  - (d) Quality of the bone formed
- (iii) Remodelling

(2) The MO series

C. AN APPRAISAL OF THE PRESENT INVESTIGATION

(1) Effectiveness of Methotrexate Administration
- (i) Method of administration
- (ii) Duration of drug administration

(2) Histological and Histochemical Methods

(3) Analysis of Observations

CHAPTER 8

DISCUSSION

A. HEALING OF THE CORTICAL DEFECT IN THE CONTROL SERIES

The sequence of repair in this investigation follows the description of earlier investigations (ELY 1927, BOURNE 1944, PRITCHARD 1956, MELCHER & IRVING 1962, 1963, 1964, RADDEN & FULLMER 1969).

(1) Granulation Tissue Formation

Along with the proliferation of fibroblasts at 48 hours, an accumulation in and around the cells of a PAS positive material was seen. It should be pointed out that the PAS stain (after diastase) does not stain neutral mucopolysaccharides exclusively. It also reacts positively with mucoproteins and glycoproteins. For example, the PAS positive material seen perivascularly in the granulation tissue consists mainly of glycoproteins associated with the inflammatory exudate (GARDNER 1967). These perivascular accumulations have not been included in the present observations.

It is generally accepted that fibroblasts synthesise mucopolysaccharides (see Appendix I). In this investigation the PAS positive material in close association with the young fibroblasts, is taken to indicate the presence of neutral mucopolysaccharides.

It has already been pointed out that there was no positive reaction with the alcian blue stain for acid mucopolysaccharides. The negative staining with alcian blue was a constant feature throughout both control and experimental series and was thought to be due to the loss of acid mucopolysaccharides during processing. This aspect will be discussed more fully later.

By 72 hours the amount of intercellular substance between the young fibroblasts of the granulation tissue was almost at its maximum, and with time, the intensity and extent of staining decreased. At 5 days the

intensity of staining was maximal, however this measurement was clouded by the amount of new bone present in the wound. DUNPHY and UDUPTA (1955), while studying soft tissue healing in the rat, claimed that there was an accumulation of mucopolysaccharides in the ground substance prior to fibrilogenesis and that this build-up reached a peak by 5 days. The results of their work was based on histochemical staining procedures, assays of hexosamine and can best be summarised in the following graph:

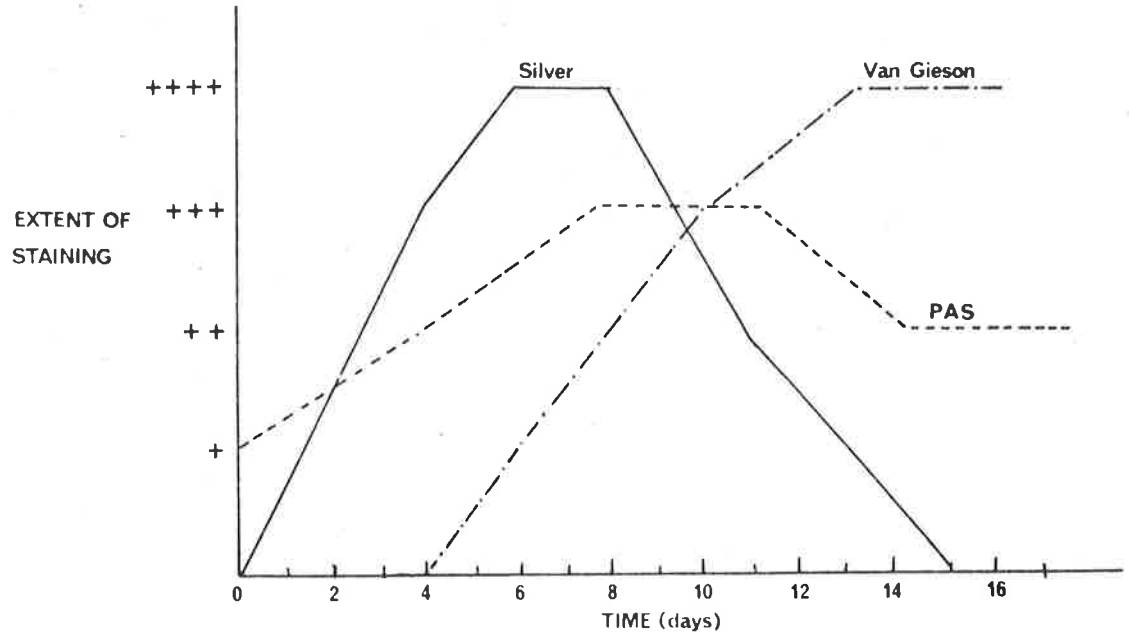


Fig. 8.1 Taken from DUNPHY & UDUPTA 1955.

In the present investigation, a similar pattern of mucopolysaccharides reticulin and collagen production could be seen and can best be summarised in the following graph:

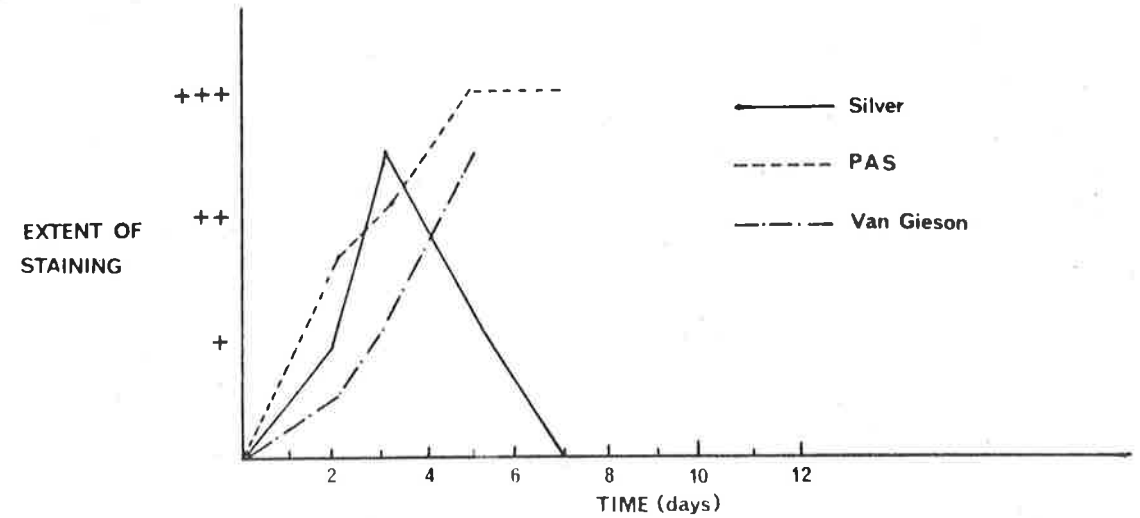


Fig. 8.2

As the gradings of the various staining reactions which constitute the above graph are arbitrary,no great emphasis can be placed on the accuracy of the findings. However, a general trend can be seen to emerge which follows that of DUNPHY & UDUPTA (1955) with the various staining reactions appearing a little earlier, the PAS reaction reaching a maximum by 5 days, reticulin by 3 days and collagen by 7 days.

A fine fibrilar pattern can be seen in association with the granulation tissue at 48 hours when the sections were stained with the silver stain (Fig. 7.15). As the granulation tissue matured, the reticulin fibres increased in number, with the individual fibres becoming thicker. The greatest concentration of these fibres could be seen at the periphery of the defect where later, collagen could be demonstrated with the Van Gieson stain.

At 72 hours the defect contained an abundant reticulin fibre network (Fig. 7.19) which by 5 days had been replaced by collagen (Fig. 7.24) thereby giving the impression of an inverse relationship between the reticulin and collagen formation. An observation also recorded by AMLER (1964) and CHAU (1968) while studying the alveolar extraction socket healing.

(2) The fate of the Bone Filings and the Defect Margins

In fracture healing and alveolar socket healing, resorption and removal of bony debris and dead bone are carried out. This resorption appears to be the result of inflammation within the wound. In the case of alveolar socket healing, infection, trauma, or the presence of a tooth root or food impaction were found to delay healing and to increase the amount of resorption seen (CHAU 1968). On the other hand, when the wound was uncomplicated by excessive inflammation, healing followed an orderly pattern with minimal resorption (SCHRAM 1929, SIMPSON 1960, CHAU 1968).

In the present investigation, no evidence of any resorption could be found. The bony spicules and filings produced at operation were tolerated and in many cases appeared to act as a nidus for new bone formation

within the defect, an observation also noted by RADDEN & FULLMER (1969). New bone was formed directly on existing bone and was also found to be laid down directly on the cut edges of the cortical plate without prior resorption.

The reasons for the absence of resorption most probably relate to the following:

(i) Minimal trauma produced at operation;

(ii) adequate coolant used at operation;

(iii) the wound was closed and therefore eliminated the possibility of food impaction or foreign body impaction and also minimised the chances of infection.

(3) Osteogenesis

Present knowledge indicates that the osteoblast is capable of elaborating a mucopolysaccharide-rich ground substance and a collagen precursor in early matrix formation (PRITCHARD 1972, also see Chapter 2). The PAS stain has demonstrated the presence of a PAS positive material in the interstitial area between the osteoblasts of the endosteal, subperiosteal and internal calluses. New bone formation has been demonstrated by routine H & E and Van Gieson staining.

(i) Endosteal callus formation

The first appearance of bone formation can be seen on the inner aspect of the cortical plate immediately adjacent to the edge of the defect. Early evidence of endosteal callus formation could be seen at 24 hours by the activity of the cells in the endosteum (Fig. 7.9). According to RADDEN & FULLMER (1969), new bone formation could also be seen endosteally on the inner aspect of the cortical plate directly opposite the defect. This new bone formation could not be seen on the control series but could be seen in some specimens on the OM series. It is unlikely that this new bone formation is due to the action of methotrexate but is more likely to be due to induction of bone formation or to the depth of the bur hole produced at operation.

(ii) External callus formation

Formation of the external callus is not as important in the healing of the cortical defect as in fracture healing. PRITCHARD (1964) stated that the size of the callus around an injured bone was related to the instability of the fragments. This could readily be seen when the femur fractured, as seen in isolated specimens in the present investigation. The first evidence of external callus formation could be seen at a distance from the edge of the defect at the same time as the endosteal callus. At 24 hours a thickening in the cambial layer of the periosteum could be seen (Fig. 7.10) with new bone formation occuring at 48 hours (Fig. 7.14). Gradually the external callus extended towards the margins of the defect and by 14 days had merged with the internal callus over the top of the defect (Fig. 7.30) a finding in agreement with RADDEN & FULLMER (1969), MELCHER & IRVING (1962, 1963, 1964), BOURNE (1944) and PALLASCH (1968).

MELCHER & IRVING (1962, 1964) and BOURNE (1944) have described the presence of cartilage formation in the external callus. In the present investigation, no cartilage could be found in the control series unless the femur was found to be fractured, in which case an abundant amount of cartilage formation was found. However, cartilage formation was found in the external callus in one specimen of the OM series when no fracture has occurred.

(iii) Internal callus formation

New bone formation could be seen at the periphery of the defect by three days and at this stage could not be distinguished from the endosteal callus. However, with time, this new bone the internal callus, was found to fill the defect and by fourteen days had merged with the externa callus over the top of the defect.

(iv) Remodelling

Remodelling of the wound is the last phase seen and is responsible for the functional reorientation of the tissues to withstand the stresses and strains demanded of them. Although active remodelling could be

seen as early as seven days (RADDEN & FULLMER 1969), definite evidence of remodelling in the present investigation could be seen between the seventh and fourteenth day (Figs. 7.27 and 7.30) with resorption of the medullary one-half to two-thirds of the internal callus. There appeared to be a decrease in the amount of trabeculation and an increase in the marrow space of the internal callus. This resorption continued to occur until it reached the inner aspect of the cortical plate. Here it stopped and the callus between the cortical ends of the femur remained intact and acted as a bridge across the defect. From this point onwards the external callus was remodelled with a thickening of the lamellae until they were parallel with the long axis of the femur. This had not occurred by the end of three months and according to MELCHER & IRVING (1969) continues up to twelve months or more.

B. COMPARISON BETWEEN THE CONTROL AND EXPERIMENTAL SERIES

The action of various cancer chemotherapeutic agents on wound healing has been reviewed in Chapter 7. In general, the conclusions reached by these workers are in agreement with the results found in the present investigation. However, some important aspects need to be emphasised. Most of the reviews in Chapter 7 were carried out on soft tissues using the alkylating agents. Some studies were carried out using the antimetabolites and in general, the conclusions were:

a. The drug interferes with wound healing.

b. This interference was due to a decrease in fibroblast production.

c. In high doses, abnormal fibroblasts were seen which resemble those seen in irradiated wounds.

d. The effects on wound healing were dose related and dependent upon the method of administration.

(1) The OM series

The early sequence of events follows the same pattern in both the experimental and control series. Initially a blood clot was formed,

followed by an inflammatory reaction, lysis of the blood clot and formation of granulation tissue by 24 hours around the periphery of the defect.

One difference noted in the early stages relates to the inflammatory reaction in the OM series which was more pronounced and persisted for a greater length of time. This increase in inflammation however, is not very marked. In some sections a large blood clot could also be seen. It is recognised that methotrexate has a toxic effect on the blood forming tissues of the marrow. However, it is unlikely that the drug would have any effect on these tissues at the time the clot was produced. This fact can best be explained in conjunction with the MO series as the animals in this series received a full dose of methotrexate before any operation was carried out. No difference in the size of the blood clot could be seen between the control and MO series and it was therefore concluded that the drug had no effect on the size of blood clot produced, while using the intraperitoneal method of administration.

(i) Granulation tissue formation

In the control series, early granulation tissue could be seen around the periphery of the defect, with the appearance of young fibroblasts by 24 hours (Fig. 7.8) and by 48 hours was well advanced in some sections.

The number of fibroblasts found in the OM series was less, with the general arrangement of the tissue being more haphazard than that of the control series, and in addition the individual cells appeared spindly and had pyknotic nuclei. Intercellular oedema was also present.

Although fibroblasts and ground substance appeared at the same time, the extent of the PAS staining was reduced in the OM series (see Table T.2, Histiogram H.1 and Figs. 8.3, 8.4).

In addition to the decrease in amount of ground substance production, reticulin and collagen formation was also reduced. Once again the production of reticulin occurred at the same time as that in the control

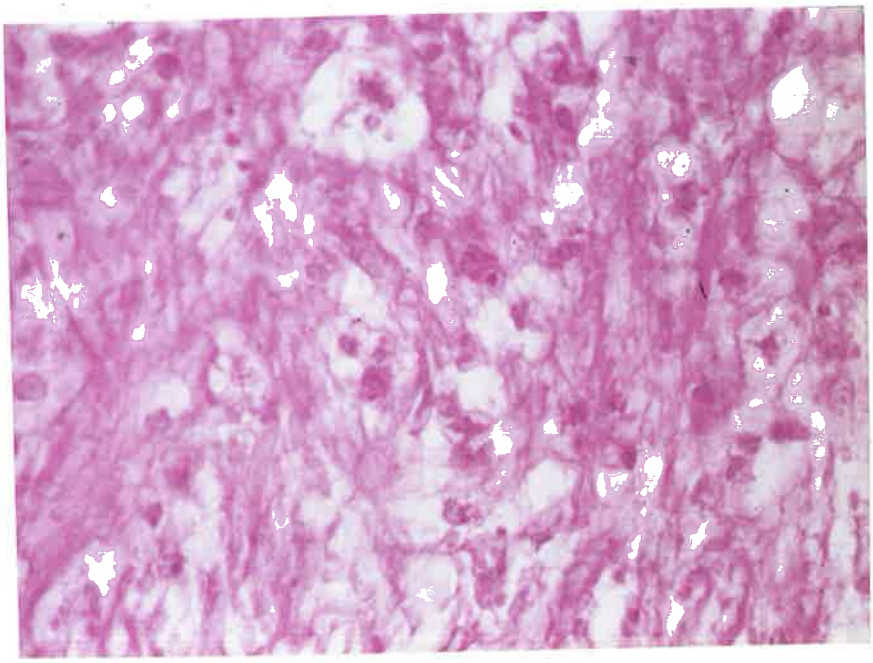


Fig. 8.3

Control 5 days PAS x 400. Colour photomicrograph showing the extent of PAS positive material in relation to the granulation tissue.

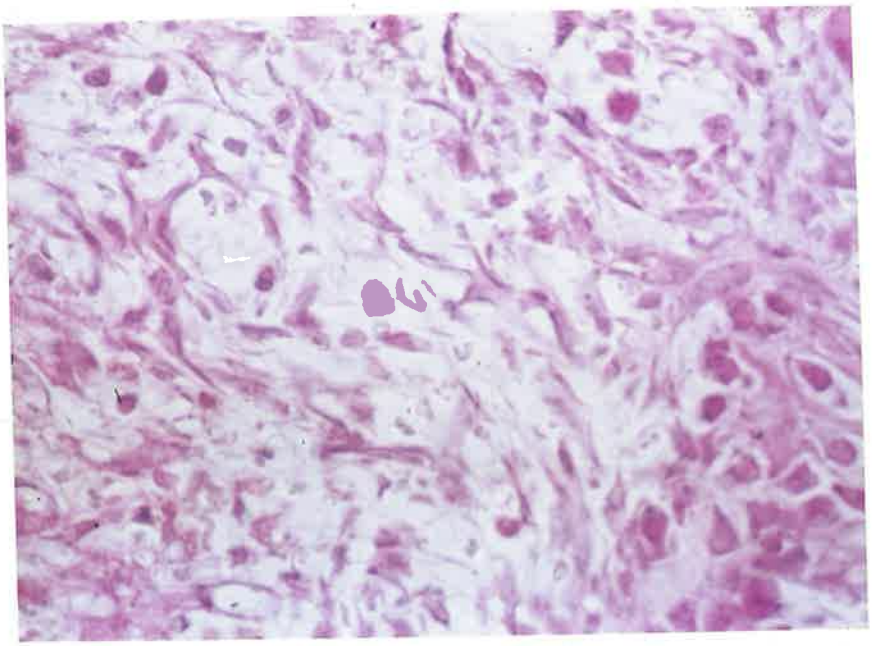


Fig. 8.4

OM 5 days PAS x 400. Colour photomicrograph showing the extent of PAS positive material in relation to the granulation tissue.

series but as time progressed, formation of this substance appeared to lag behind that of the control series (see Table T.3 and Histiogram H.2). Similarly, collagen was found to be reduced not only in amount but also in its initial appearance and distribution (see Table T.4, Hist ogram H.3 and Figs. 8.5, 8.6).

The overall picture suggests that there is a depression of granulation tissue, reticulin and collagen. This depression was not marked and was only found to occur during the time of drug administration, with recovery occurring rapidly once administration of drug had stopped.

(ii) Callus formation

(a) Endosteal callus

An endosteal reaction could be seen first at about 24 hours with the proliferation of the endosteal cells on the inner aspect of the cortical plate, immediately adjacent to the defect margin. Gradually the endosteal callus increased in size, with the endosteal and internal calluses being indistinguishable by the fifth postoperative day. Generally the endosteal callus of the OM series followed that of the control series. However, some differences were observed. In one specimen, a marked endosteal response was observed and appeared in conjunction with a large blood clot and an extensive inflammatory reaction which lasted until the seventh postoperative day. Measurements of the endosteal callus (Table T.5 and Hist ogram H.4) revealed that the endosteal response in the OM series was marginally greater than that of the control series. However, it was felt that this marginal difference could be explained by the single specimen which registered a marked increase.

(b) External callus

The production of external callus follows the same sequence as that of the control series, but the amount of callus produced in the OM series was found to be less than that found in the control series (Table T.6 and Hist ogram H.5).

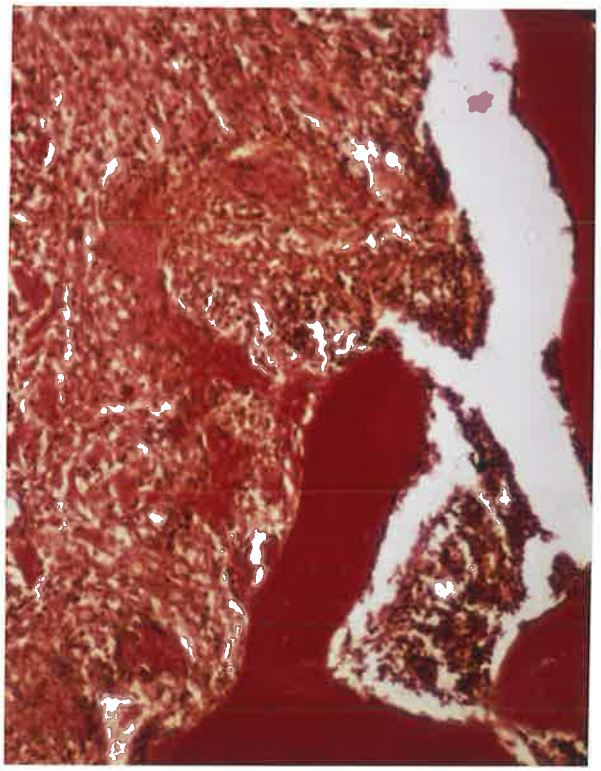


Fig. 8.5

Control 72 hours Van Gieson x 100. Colour photomicrograph showing the extent of collagen staining in relation to the granulation tissue.

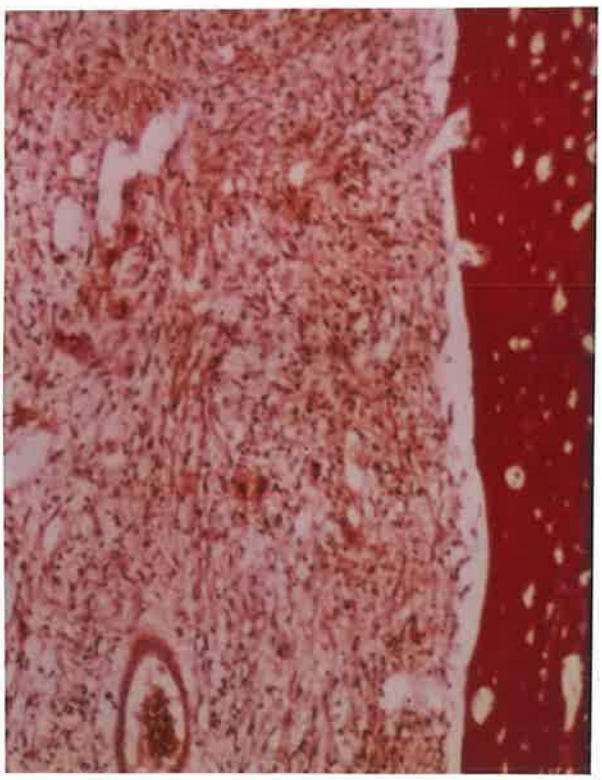


Fig. 8.6

OM 72 hours Van Gieson x 100. Colour photomicrograph showing the extent of collagen staining in relation to the granulation tissue.

(c) Internal callus

Internal callus could be seen around the periphery of the defect by the fifth postoperative day, but was not as extensive as that found in the control series. This was a constant feature and could be seen in all of the OM specimens up to the seventh day (see Table T.7 and Histogram H.6). The internal callus filled the defect from below upwards and was found to be completed by the fourteenth day when no difference could be seen between the control and OM series.

(d) Quality of the bone formed

While there is a definite difference in the quality of the granulation tissue produced between the control and OM series, there was no such difference noted in the new bone formed.

This was so, even in the early phase of bone formation up to the seventh post operative day, during which time methotrexate was being administered.

No explanation can be offered for this observation except to postulate that there is a difference in sensitivity to methotrexate between the fibroblasts and osteoblasts. Such an explanation does not appear very likely.

(iii) Remodelling

From fourteen days until three months, remodelling took place. No difference could be seen between the control and OM series.

(2) The MO series

No difference could be seen between the control and MO series, as demonstrated in Chapter 7.

This fact and the rapid return to normal after cessation of drug administration seen in the OM series led to the conclusion that methotrexate has no long term deleterious effects on bone healing. Recovery was so rapid and the return to normal so quick that the MO series exhibited no change even during the very early stages.

In summary, the results of the OM series show a consistently depressed reaction in all phases of wound healing during the period of drug administration. Initially a greater inflammatory reaction was encountered followed by a decrease in the number and arrangement in the fibroblasts of the granulation tissue, with the individual cells having a spindly shape and pyknotic nuclei. In addition to these features, intracellular oedema was also noted. Following this, the amount and distribution of the ground substance was delayed as demonstrated by the PAS stain.

Reticulin fibre formation was also depressed as too was collagen formation, which showed the greatest change. Callus formation was depressed in the external and internal areas, but the endosteal callus was not affected in the OM series. Perhaps this lack of depression in the endosteal callus formation can be explained by the profuse periosteal blood supply which may have a greater effect on subperiosteal bone formation by delivering a relatively greater amount of drug to the active cells of the periosteum, whereas the marrow is supplied by a single nutrient artery and therefore the relative amount of drug reaching the marrow space was correspondingly less.

C. AN APPRAISAL OF THE PRESENT INVESTIGATION

(1) Effectiveness of Methotrexate Administration

That methotrexate produces only a mild depression of wound healing is a little surprising, as the basis for its therapeutic use is the cytotoxic and growth inhibitory effects it has on rapidly proliferating cells. The results of the present investigation are not as clear-cut as would have been expected. The following sections attempt to explain this inconsistency.

(i) Dosage and method of administration

A number of conflicting statements regarding the effective dosage of methotrexate required for retardation of wound healing in experimental animals are found. KIEHN et al. (1962) stated that methotrexate did not affect a primary wound, or the take of a skin graft, or the formation of

granulation tissue unless the dose exceeded the LD_{50} in mice. CALNAN and DAVIES (1965), using rats, concluded that wound healing was depressed significantly when a dose of 0.3 mg/kg/day was used for 5 days and that considerable depression was found with a dose of 0.9 mg/kg/day for 5 days. Both these doses are below the LD_{50} as established by the present study. The dosage used in the present study is 0.25 mg/day in animals weighing between 200 gm., and 300 gm., therefore giving an equivalent of 0.8 mg. to 1 mg/kg/day for 6 days. Thus it can be seen that the dosage used in the present study is almost the same as the higher dosage used by CALNAN and DAVIES, who obtained a considerable depression. However, their results were assessed by using tensile strength estimations and not a histological one.

FREIREICH et al. (1966) stated that the toxicity of anti-cancer agents is cumulative and that the LD_{10} in small animals is equivalent to the maximum tolerated dose (M.T.D.) in man. The LD_{10} for the rat is 0.58mgs/kg. given daily over 5 days and via the peritoneal cavity, and the M.T.D. is the most often used dose schedule for the chemotherapy of cancer. The dose used in the present investigation is therefore almost double that of the LD_{10} and the M.T.D. in man (FREIREICH et al. 1966).

It is therefore difficult to explain the minimal retardation of wound healing found in the present investigation unless the plasma concentration of the drug did not reach the necessary levels.

Here again, there are conflicting viewpoints in the literature regarding which is the most important factor in the effectiveness of methotrexate.

GREENWALD (1972) found that the effective administration of methotrexate in rats depended upon the duration of contact of the drug with the cell, rather than the amount of the drug in the blood stream. On the other hand, GOLDMAN and FYFE (1974) believed that the cancerocidal activity of methotrexate depends upon its intra-cellular concentration.

GOLDMAN and FYFE stated that this concentration must be high enough for it to compete successfully with dihydrofolate reductase and thus to inhibit D.N.A. synthesis. At least 0.05ug/ml. appears necessary to bring about this inhibition. Since the intracellular concentration of methotrexate is about 0.1% (FREI et al. 1975) of the extracellular concentration, a plasma level of a thousand-fold is required to raise the intracellular methotrexate concentration to therapeutic levels. According to ROSENFELT (1975) and BENDER (1975) these concentrations should be maintained for at least twelve hours for any effect to be noticed.

In order to obtain and maintain these high concentrations, various methods of administration have been devised. These methods have been enumerated in Chapters 4 and 5. Clinically the best method appears to be by continual arterial infusion in high doses in conjunction with the administration of citrovorum factor systemically to counteract toxic side effects. Secondly, the drug could be administered by continuous intravenous drip, but the maximum dose using this second method, is limited by the general toxicity of methotrexate. The impracticality of these above methods in the present investigation has already been discussed in Chapter 5.

An intermittent daily intra-peritoneal administration was therefore used. This method of administration not only suffers from the same problems of the other methods of systemic administration, namely that the maximum dose is limited by toxicity of the drug, but also other problems as well. The blood level obtained by intraperitoneal absorption is not likely to be sustained at an adequate level unless the drug is administered at frequent intervals.

However, as explained in Chapter 5, the dosage is cumulative as far as the LD_{50} is concerned, so that the daily total dose for LD_{50} for a 6 day course lies somewhere between 1 mg/kg. and 2 mg/kg. Therefore, by using a more frequent injection, the dosage per injection would have to be reduced, with a corresponding decrease in the resultant blood level attained, thus defeating the object.

In the present investigation, the blood level of methotrexate over a twenty four hour period is not known. The blood level of methotrexate can be determined by the use of a fluorescent technique (FREEMAN 1957, SALASOO et al. 1976). The unavailability of equipment did not permit this aspect of the problem to be investigated.

It is likely that for a significant period of time, the blood level attained in this study was inadequate.

(ii) Duration of drug administration

The duration of drug administration in this study has been arbitrarily chosen to be six days. According to RADDEN and FULLMER (1969) and MELCHER and IRVING (1964), callus formation had almost reached a peak by the sixth-seventh day and was at a maximum by twelve days. For this reason, it was decided to administer the drug over a six day time period. In order to calculate the amount of drug to be given in each daily dose, the LD_{50} for this type of administration was calculated and has been presented in Chapter 5. However, in retrospect, the drug could have been given over a greater length of time, which might have produced a greater response. However, as pointed out in Chapter 5, intermittent daily doses of methotrexate is cumulative in relation to the LD_{50}. Thus lengthening the time of drug administration would mean a reduction in the individual daily dose, with a resultant reduction in the blood levels obtained.

(2) Histological and Histochemical Methods

Limitations in staining the various substances which appear during healing relate to the type of tissues being stained. Most histochemical procedures use fresh frozen sections. As the material used in this investigation was bone, decalcification was necessary. This was carried out using acid solutions (Formic acid, Nitric acid) or by using chelating agents. RADDEN and FULLMER (1969) have used a chelating agent. They decalcified their specimens at 4 degrees centigrade in an effort to preserve histochemically active substances and obtained favourable results with this method.

However, time did not allow us to use this technique, particularly with the number of specimens used.

The PAS stain is a well recognised histochemical stain (see Chapter 5) which has been used to demonstrate the neutral mucopolysaccharides, mucoproteins and glycoproteins of which there are small quantities present in connective tissues and bone (HERRING 1964, ANDREWS and HERRING 1965). However the most important mucopolysaccharides in the ground substance of bone are the acid mucopolysaccharides. Alcian blue has been used to stain the acid mucopolysaccharides in the present investigation (for details see Chapter 5 and Appendix I). The sections seen stained poorly with alcian blue while those with PAS stained adequately. The most likely explanation for this poor staining relates to the harshness with which the tissues were treated during processing; first fixation then decalcification in acid and finally paraffin embedding, each stage taking its toll on the substances being stained. No doubt considerable leakage of these materials occurred during processing.

(3) Analysis of Observations

An attempt has been made in the present investigation to quantitate the results obtained. As detailed in Chapter 7, fibroblast number, shape and arrangement of the granulation tissue have been specifically compared. Staining intensity and extent of ground substance (PAS stain), reticulin (Silver stain) and collagen (Van Gieson stain) have been graded arbitrarily as +, ++ and +++, tabulated and visually presented by the use of histiograms. Similarly, the endosteal, external and internal calluses have been measured or graded according to extent and amount and have also been presented visually by the use of histiograms.

These measurements and presentations have been used to illustrate descriptions of the control and experimental series and in conjunction with photomicrography, give a more definite picture of the observations made in the present investigation.

There has been no attempt at statistical analysis of these measurements and observations, as the number of samples in each group is too small for valid analysis.

CHAPTER 9

SUMMARY AND CONCLUSIONS

An experimental investigation designed to investigate the effects of an anti-cancer chemotherapeutic agent (methotrexate) on bone healing has been carried out in the Sprague-Dawley rat. A cortical defect in the mid-shaft of the femur of the rat has been used as the experimental model.

Histological and histochemical methods have been used and have provided information regarding the histogenesis of various tissue components in the healing wound. In addition, they have provided a means of comparing the experimental and control series as to the appearance, amount and distribution of the various components in the healing wound.

The present study confirms the findings of previous studies on cortical defect healing by the formation of endosteal, periosteal and internal calluses. No cartilage formation could be detected in the control series but one specimen of the OM series exhibited cartilage formation at the leading edge of the advancing subperiosteal callus. Cartilage formation was also found when the femur was inadvertently fractured.

No resorption of bone was found in the present investigation and the bone filings within the defect were accepted and found to provide a nidus for new bone formation.

The administration of methotrexate was found to retard healing during the time of administration. No long-term effects were observed. Also, recovery was found to occur immediately after cessation of drug administration, with all of the OM specimens showing the same advancement of healing by the end of two weeks.

Retardation seen in the early specimens of the OM series included a decrease in the number of cells in the granulation tissue; the presence of spindly shaped fibroblasts with pyknotic nuclei; intracellular oedema and

poor staining properties. The ground substance (PAS stain) was found to be depressed in amount and distribution, as were the intercellular fibres, reticulin and collagen.

Early bone formation in the subperiosteal and internal callus was also found to be retarded, but by fourteen days no difference could be seen between the control and the experimental OM series. Bone formation in the endosteal callus was not affected. This lack of depression may be related to the relatively poor blood supply of the marrow cavity.

From a clinical point of view, certain conclusions may be drawn from the results of this investigation.

Firstly, methotrexate does not appear to have any long-term effects on bone healing and is therefore different in this respect to radiotherapy. Thus patients who have received chemotherapy in the past, pose no special problem to oral surgery.

Secondly, the deleterious effects of methotrexate on bone healing appears to be active only when there is an adequate blood level. Thus bone surgery is best avoided during the active phase of treatment, particularly when a high dosage intra-vascular infusion is used.

In cases when oral surgery is required prior to commencement of chemotherapy, it seems desirable to allow for a period of healing for ten days before methotrexate is given.

Of course these clinical conclusions suffer the danger of inappropriate extrapolation of results from animal experimentation to the human situation. Therefore these conclusions, based on extrapolations, must necessarily be guarded.

Many questions still remain unanswered. This investigation deals with methotrexate given intraperitoneally. Whether a higher dose given by a different method of administration would give widely different results is not known. This investigation studied the effects of methotrexate alone and therefore the results presented here cannot be extrapolated to include

other cytotoxic agents. Further, the synergistic action of methotrexate and radiotherapy in the retardation of wound healing is not known. The results of the present investigation cannot therefore be extended to include the above clinical situation.

All of these unanswered questions deserve further experimental investigation.

APPENDIX I

THE GROUND SUBSTANCE IN WOUND HEALING

The formed elements of connective tissue are embedded in a matrix of amorphous ground substances having the consistency of a viscous fluid or gel containing carbohydrate-protein substances.

A. The function of Mucopolysaccharides

The carbohydrate-protein substances of ground substance can be divided into:

1. Glycoproteins (Sialo proteins)
2. Mucopolysaccharides (Protein polysaccharides)

The glycoproteins only occur in small quantities in connective tissue and have been isolated from bovine bone (HERRING 1964, ANDREWS and HERRING 1965).

The mucopolysaccharides or protein polysaccharides are important constituents of connective tissue and include the chondroitin sulphates A, B, and C, keratan sulphate, heparan sulphate, hyaluronic acid and chondroitin.

These mucopolysaccharides are characteristically long in molecular form and contain from 150 to 1000 sugar units. The sugars are of two main types and alternate along the chain. One type of sugar is a hexosamine and the second type is a hexuronic acid. The detailed biochemistry of these mucopolysaccharides may be found in standard texts of biochemistry and will not be repeated here.

These flexible chains are randomly distributed in three dimensions with negative charges on the side chains repelling each other, thereby forming an open network. There are numerous hydrophilic groups (including ionised carbosyl and sulphate groups) which allow the network to hold water. The complex can be considered as a molecular net allowing penetration of

ions and smaller molecules, but excluding the larger molecules and proteins, which collect outside the complex (KENT 1967). In behaving in this manner three effects are obtained:

1. In the extra-cellular environment where flow occurs, the movement of high molecular weight substances is favoured against that of lower molecular weight substances.
2. By letting smaller molecular weight substances through, an accumulation of the high molecular weight proteins occurs thereby encouraging fibre formation and deposition.
3. The macromolecules of collagen are forced between adjacent sheets or nets of ground substance, this having some effect on the fibre arrangement.

Therefore, the primary function of the ground substance is to provide an extra-cellular milieu which permits the subsequent formation and maturation of fibres as the tissues develop. In support of this is the fact that conditions which lead to abnormal mucopolysaccharide formation also lead to defective connective tissue formation as in the case of scurvy (KENT 1967).

Of the mucopolysaccharides, hyaluronic acid and the chondroitin sulphates are of particular importance. In embryonic pig skin, for example, hyaluronic acid and chondroitin sulphate B (dermatan sulphate) are present in a ratio of 5:1. This ratio is reversed with maturation. In human tissues, maturation is also marked by a decline in the amount of hyaluronic acid and an increase in the amount of chondroitin sulphate. KAPLAN and MEYER (1959) showed that in aging rib cartilage a third stage of maturation occurs in which keratan sulphate replaces chondroitin sulphate.

The relationship between these mucopolysaccharides and collagen formation is important, for in wound healing, the same process (namely, hyaluronic acid --→ chondroitin sulphate --→ keratan sulphate) is repeated as collagen is being formed.

B. Mucopolysaccharides in Wound Healing

Histological studies of wounds carried out by STEARNS (1940), DUNPHY and UDUPTA (1955) show a regular sequence of events leading to complete closure. After early proliferation of fibroblasts, an amorphous matrix appeared which had staining properties of the acid mucopolysaccharides. Metachromasia with toluidine blue was found to reach a peak between the fourth and sixth day and then decline in intensity.

Using hexosamine as an indication of mucopolysaccharide content in the healing wound, EDWARDS, PERNOKAS and DUNPHY (1957) found that the hexosamine content was extremely high as early as six hours after the implantation of polyvinyl sponges. At this stage no connective tissue formation had occurred. GRILLO, et al. (1958) found the hexosamine content of open skin wounds was at a maximum immediately after wounding. DUNPHY and UDUPTA (1955) found that in healing wounds, after the so-called 'lag phase', the collagen content of the wound rose rapidly while the hexosamine content declined. On the basis of these studies, it was considered that early production of the mucopolysaccharides was necessary for production of collagen (De VITO 1965).

Later, however, it became apparent that one of the major sources of hexosamine in the early healing wound was from the serum glycoproteins carried into the wound in the initial inflammatory exudate (GARDNER 1967). Thus hexosamine estimation is not a true indication of mucopolysaccharide content (BENTLEY 1969).

By selective removal of serum glycoproteins using Ecteola cellulose columns, BENTLEY (1965) was able to extract three fractions of mucopolysaccharides from eight day old granulation tissue. These consisted of:

1. Hyaluronic acid
2. Chondroitin 4-sulphate
3. Dermatan sulphate.

Changes in the mucopolysaccharide content during healing have been studied by DORNER (1967), BENTLEY (1967), ANSETH (1961), ANTONOPOULOS et al. (1965), WHITE (1966). The general pattern which seems to emerge from these studies, is that in a wound the less highly charged mucopolysaccharides such as hyaluronic acid appear in the early stages, while the more highly charged mucopolysaccharides appear later. The time at which these changes occur depends upon the tissue being tested.

It is more difficult to study the mucopolysaccharides in fracture callus and to make any comparison with this time sequence, for this tissue not only forms collagen but also calcifies it (BENTLEY 1969).

C. The effects of Mucopolysaccharides on Cell Growth and Differentiation

BALAZS and HOLMGREN (1950) suggested changes in the mucopolysaccharides effected cell growth and differentiation. Their results were based on histochemical staining methods, using toluidine blue, after making aqueous extracts of granulation tissue at different times during wound healing. They also showed that three day extracts had a stimulating effect on fibroblast cultures, whereas inhibition was found with the four to fifteen day extracts. It was postulated that the predominance of non-sulphated mucopolysaccharides in the early wound stimulated fibroplasia whereas the production of collagen and differentiation of cells was stimulated by chondroitin sulphate. The same conclusion was reached by CAMPANI et al. (1959) who studied radioactive sulphate uptake in granulation tissue.

These works suggested that the mucopolysaccharides play an important role in the control of collagen synthesis in granulation tissue.

APPENDIX II

THE SYNTHESIS OF COLLAGEN

Although the early histologists were impressed by the increase in fibroblasts and collagen as healing progressed, the cells received the main emphasis of attention and were thought to be responsible for wound strength (HARVEY 1929). It is now clear that although in the first few days wound strength depends upon fibrin and epithelial adhesion (VAN WINKLE 1969), after this 'lag period' collagen is by far the most important factor. The structure of collagen is well known (PIEZ, WEISS and LEWIS 1960, PIEZ, EIGNER and LEWIS 1963, RAMACHANDRAN 1967, GRANT and PROCKOP 1972) and need not be repeated here.

Collagen synthesis in healing tissues and in normal connective tissues follows a similar course (GREEN and GOLDBERG 1963).

It is clear that fibroblasts synthesise both collagen and the mucopolysaccharides (GREEN and HAMERMAN 1964, BENTLEY 1969), and that the two processes are related (ROKOSOVA-CMUCHALOVA and BENTLEY 1968), although control of the two are separate (GREEN and GOLDBERG, 1965).

A. The intra-cellular synthesis of Collagen Precursors

By using labelled proline, the sequence of collagen formation in the fibroblast has been studied (REVEL and HAY 1963,ROSS and BENDITT 1962, 1965).

Early workers in this field found that rats fed with labelled proline produced collagen containing both labelled proline and hydroxyproline. However, a polypeptide intermediate which contained more proline and lysine than collagen was found (GOULD 1968, KIVIRIKKO and PROCKOP 1967, LUKENS 1966, PROCKOP 1970). This intermediate was called 'Protocollagen' by GRANT and PROCKOP (1972). The proline and lysine of 'Protocollagen' was then hydroxylated to hydroxyproline and hydroxylysine. This process re-

quires oxygen, ascorbic acid, and α ketoglutarate as well as enzymes such as protocollagen proline hydroxylase and protocollagen lysine hydroxylase (HALME, KIVIRIKKO and SIMONS, 1970, RHOADS and UNDENFRIEND 1970, HURYCH and NORDWIG 1967, HAUSMANN 1967, MILLER 1971, KIVIRIKKO and PROCKOP 1967). A number of other enzymes have also been found to be associated with collagen synthesis (MILLER 1971, BOSMAN 1968, SPIRO and SPIRO 1971).

As with other proteins, the peptide bonds linking the amino acids together in collagen are synthesised on ribosomal complexes containing m-RNA. The α chains of collagen are assembled as continuous peptides on the ribosome complex and the α_1 and α_2 chains are synthesised at the same time (VUUST and PIEZ 1970).

After release from the ribosomes, they pass to the extracellular space either directly (ROSS and BENDITT 1965, SALPETER 1968, COOPER and PROCKOP 1968), or into the Golgi vacuoles to be excreted from the cell by reverse pinocytosis (REVEL and HAY 1963). However, there is no general agreement as to how this actually occurs.

B. The extra-cellular formation of Collagen Fibres

The basic molecular unit of collagen is known as "tropocollagen" and has the dimensions of a rod 15 Å in a diameter and 3000 Å long (GRANT and PROCKOP 1972), and consists of three polypeptide chains that are coiled into a unique rigid helical structure.

It would appear that the first collagen formed by the connective tissues extra-cellularly is of a larger form than tropocollagen and it has been referred to as "procollagen" (BELLAMY and BORNSTEIN 1971). This transport form of collagen does not appear to aggregate into fibres under conditions that induce fibre formation in tropocollagen. The "procollagen" is converted to tropocollagen by an enzyme "procollagen peptidase" (LAPIERE et al. 1971, BORNSTEIN et al. 1972, VEIS et al. 1972, NIMNI 1973).

The spontaneous aggregation of tropocollagen into fibrils is produced by the interaction of the side chains on the tropocollagen molecule.

The rate of collagen synthesis is important, as the availability of collagen monomers would have to exceed that being utilised in order to allow fibre formation to continue. If the monomer pool dropped below a certain value then fibre formation would cease.

A number of theories have been proposed to explain the way in which tropocollagen molecules are linked together to form collagen, giving rise to the 640 Å periodicity. The "quarter-stagger" theory was widely held by SCHMITT et al. (1955), HODGE and SCHMITT (1960), PETRUSKA and HODGE (1964), HODGE (1965). However, others have suggested that aggregation of fibrils into a fibre occurs by random selection (GRANT et al. 1965, COX et al. 1967).

Individual collagen molecules increase in diameter with age due to aggregation of further molecules to the surface of the existing structure. Stabilization of the fibre is due to cross linking of the macromolecules by oxidation of the lysine residues (BORNSTEIN et al. 1966), and as the molecule becomes packed together, intra- and inter-molecular bonds are formed resulting in a cross-linked insoluble fibril, the final diameter being characteristic of the particular tissue.

C. The Maturation of Collagen

The increase in tensile strength of a healing wound parallels that of collagen formation up to twelve to fifteen days (DUNPHY and UDUPTA 1955). However, as HOWES (1929, 1939) has shown, the tensile strength of the wound at this point, has only reached a small percentage of its ultimate strength. During this later increase in tensile strength, there is no increase in the amount of collagen formed (JACKSON 1969).

In fact, work by LEVENSON et al. (1965), PEACOCK (1966) and DOUGLAS et al. (1969) has shown that the tensile strength of a wound continues to increase after collagen content of the wound has returned to normal.

Three basic mechanisms have been put forward to explain this development of strength in a wound:

1. Increase in the amount of collagen.
2. Increase in the extent of cross-linking of collagen.
3. Some sort of interaction between collagen and ground substance.

The main histochemical events in scar maturation is the progressive inter- and intra-molecular cross-linking (PEACOCK and VAN WINKLE 1970). LEVENSON et al. (1965) and PEACOCK (1966) have shown that introduction of methyl links increased wound strength without increase in collagen content.

Little evidence has been produced to show that ground substance has an effect on wound strength except the work by FORREST and JACKSON (1971) which suggested that an interaction between collagen and proteoglycan or glycoprotein may be of some significance after one month.

D. The Remodelling of Collagen

Collagen synthesis was found to reach a maximum by twenty to sixty days and return to normal after about one hundred and fifty days (WOESSNER 1961). From these experiments it can be seen that collagen is being removed from the wound as it is being laid down. This was thought to be due to the action of collagenase (GROSS and LAPIERE 1962, GRILLO et al. 1969, RILEY and PEACOCK 1966, 1967). Collagenase has been found in wounds as early as fourteen days and as late as thirty years (RILEY and PEACOCK, 1966, 1967). This synthesis and removal would enable collagen fibre remodelling to take place.

APPENDIX III

EXPERIMENTAL METHODOLOGY

A. Surgical Anaesthesia and Post Operative Care

For surgical anaesthesia, pentobarbitone (Nembutal-Abbot) was used following the manufacturers' recommended dosage of 1 ml of the 6% solution (Vetinary 60 mgs/ml) per 5 lbs. body weight.

This agent was administered as an intra-peritoneal injection. The dosage given was calculated using the following formula:

$$\text{Anaesthetic dose} = \frac{1 \text{ ml/5lbs. body weight}}{5 \text{ lb} = 2250 \text{ gms.}}$$

The Nembutal was then diluted 1:9 with saline for injection and hence the dose administered was thus calculated as follows:

$$\text{Dose} = \frac{X \times 9}{2250}$$ where X = the weight of the rat in grams

The rats were first placed in an ether chamber until they became drowsy and manageable. Pentobarbitone was then administered by intra-peritoneal injection. The rats initially recovered slightly from the ether, but within 10 minutes all had reached sufficient depth of anaesthesia from the pentobarbitone, and were ready for operation.

B. Method Wound Production

Instrumentation:

1. Fine tipped sucker
2. Scissors
3. Scalpel handle and No. 15 blade scalpel
4. Periosteal elevator
5. Engine, hand piece and No. 3 inverted cone bur
6. Suture holder and 3/0 black silk suture
7. Fine mosquito artery forceps

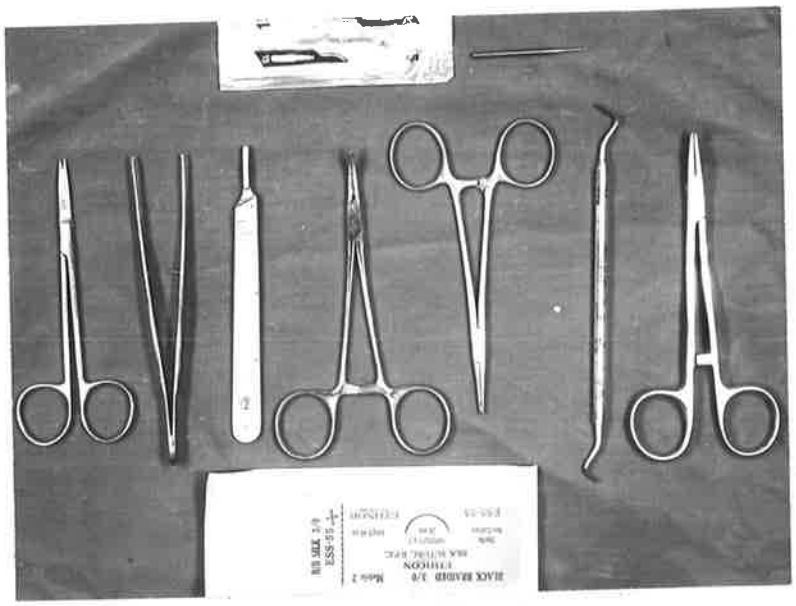


Fig.III.1 INSTRUMENTS USED IN THE OPERATION

(1) Positioning of the rat

The operating table consisted of a flat board, with hooks placed on either side. To these hooks the legs were attached by the use of elastic bands.

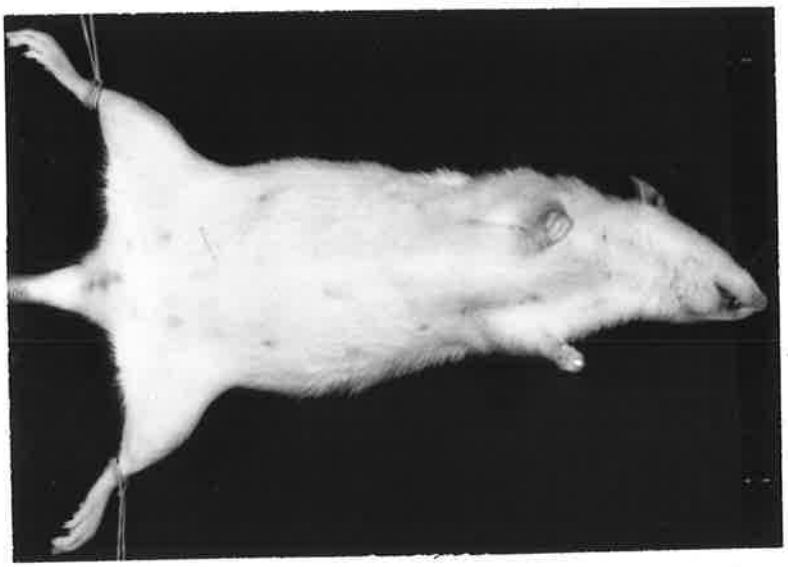


Fig.III.2 OPERATING TABLE WITH ANIMAL IN POSITION

(2) Method of operation

An incision was made through skin on the inner aspect of the rat's thigh. Blunt dissection was then carried out to expose the musculature in the area and more importantly to clearly demonstrate the femoral neurovascular bundle. Once this was isolated and retracted out of the way, an incision was made through muscle and

down to bone. Care was taken to make sure the incision through muscle ran parallel with the fibres of the muscle. Once this had been achieved, a definite cut through the periosteum was carried out and then the periosteum was elevated.

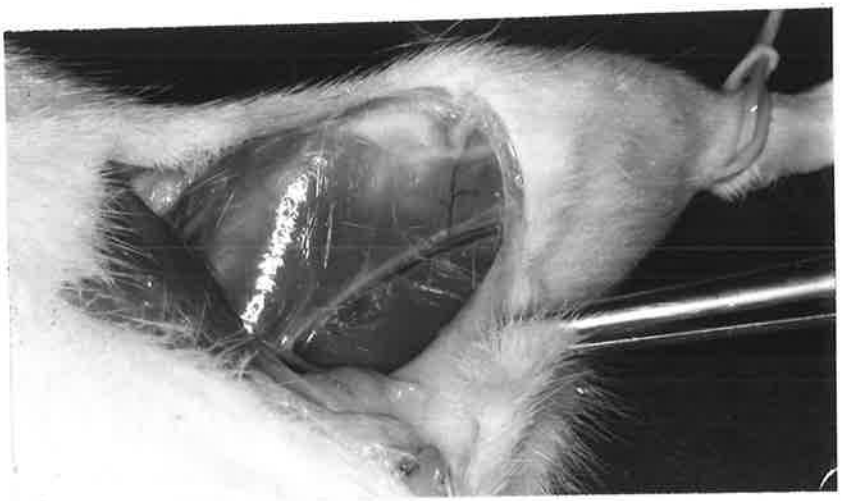


FIG.III.3 PHOTOGRAPH SHOWING THE FEMORAL NEUROVASCULAR BUNDLE

A drill hole was made in the midshaft of the femur with a No. 3 inverted cone bur, with adequate water coolant. Care was taken to irrigate the defect after the drill hole had been made in order to remove all debris from the surgical site.

A suture was placed through the muscle directly over the drill hole which also acted as an aid in orientation of the specimen at the time of blocking in wax.

Closure was effected in layers using 3/0 black silk suture.

Fig.III.4 PHOTOGRAPH OF THE DRILL HOLE BEING CREATED IN THE MID SHAFT OF THE RAT FEMUR WITH A No. 3 INVERTED CONE BUR IN A DENTAL HAND PIECE.

Fig.III.5 PHOTOGRAPH OF THE MUSCLE SUTURE IN POSITION.

Post operative care involved suction, protrusion of the tongue to ensure a good airway and the placement of the head in a suitable position for drainage of any secretions. Recovery from the anaesthetic was gradual and took about 2 hours to complete.

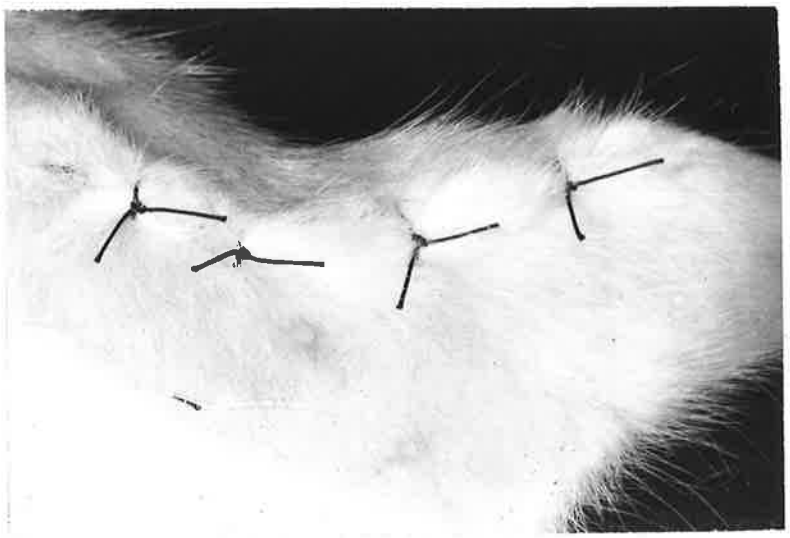


Fig.III.6 PHOTOGRAPH OF THE FINAL SUTURED WOUND.

C. Method of Removal of the Femur for Processing

Sacrifice of the rats was effected by intraperitoneal injection of the undiluted 6% saggital solution, or in an ether chamber.

Access was gained to the head of the femur by an extended incision in the area of the operation. The head of the femur was then dislocated from the acetabulum and the muscles severed, and the leg was removed en bloc. The femur was then separated from the tibia and fibula and asso-

ciated muscles were trimmed, making sure that the periosteum and some muscle over the wound remained, along with the suture for later orientation.

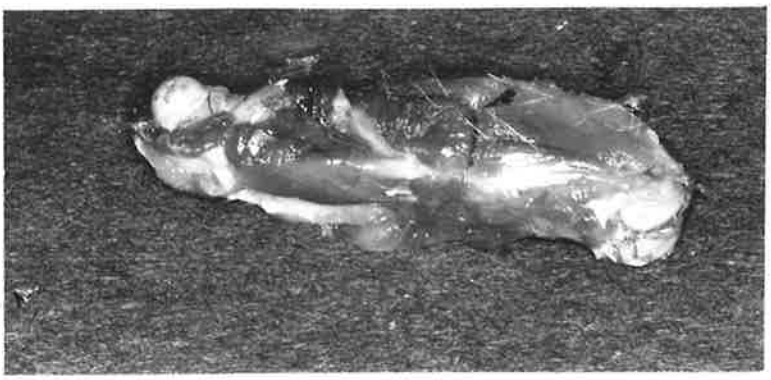


Fig.III.7 REMOVAL OF THE LOWER LIMB EN BLOC.

Those specimens being used for longitudinal sectioning were then processed as such. However, those specimens being sectioned transversely were cut back further in order to reduce the amount of cutting on the microtomes.

APPENDIX IV

DIET

All animals used in the present investigation were fed on the following stock diet:

Ground wheat	40.0%
Ground barley	18.0%
Bran & pollard	12.0%
Meat & bone meal	9.6%
Ext. soya meal	6.2%
Fish meal	6.2%
Milk powder	3.0%
Brewers yeast	1.0%
Salt	1.0%
Molasses	3.0%

Vitamin supplement per kilogram of feed

Vitamin A3	3928 I.U
Vitamin D2	928 I.U
Vitamin B6	1.5 mgs
Vitamin B12	0.2 mgs
Vitamin B	3.4 mc gms
Vitamin E	1.2 mgs
Vitamin K (menadione)	0.5 mgs
Pantothenic acid	0.5 mgs
Choline chloride	25 mgs

APPENDIX V

HISTOLOGICAL AND HISTOCHEMICAL TECHNIQUES

A. Tissue Fixation

Neutral buffered formol saline

Formaldehyde solution	100 mls.
Sodium dihydrogen phosphate	3.5 gms
Di-Sodium Hydrogen Phosphate	6.5 gms
Tap or distilled water	900 mls.

The legs of the experimental animals were fixed for three days at room temperature.

B. Decalcification of the Hard Tissue

Decalcification solution

Formic acid	340 mls.
Sodium formate	68 gms.
Distilled water	1650 mls.

This reagent produced slow decalcification with anything up to two weeks being required for the specimens. The advantage of this material lies in its safety. Prolonged immersion, within reason, appears to do little harm to the specimens. Roughly 100 mls. of reagent was used for every gram of specimen with the solution being changed daily. Decalcification was effected at room temperature.

Determination of the end point of decalcification was carried out using radiography.

C. Embedding Procedures

After decalcification, the specimens were neutralised in sodium sulphate solution for about 12 hours before embedding. The specimens then went through the following reagents:

37°C	1.	Alcohol 70%	1 hour
	2.	" 80%	" "
	3.	" 90%	" "
	4.	" 95%	" "
	5.	" absolute	" "
	6.	" "	" "
	7.	" "	" "
	8.	Absolute alcohol and Methyl Salicylate (1:1)	2 days
	9.	Methyl Salicylate and Celloidin 0.5%	2 days
	10.	Celloidin 1%	2 days
60°C	11.	Wax + Methyl Salicylate (1:2)	1 hour
	12.	" " " (1:1)	" "
	13.	" " " (2:1)	" "
56°C	14.	Wax	" "
	15.	"	" "
	16.	"	overnight

The specimens were then placed in a vacuum for one hour for evacuation of air bubbles and were subsequently blocked in wax at 56°C.

Sections were cut on a rotary microtome at 7μ. Each 5th section was retained through the series for the following staining procedures:

D. Staining Procedure used in the Present Investigation

1. PAS stain with prior diastase digestion
2. Alcian Blue stain at P.H. 2.3 and 0.9
3. Haematoxylin and Eosin stain
4. Van Gieson stain
5. Silver stain of Lillie
6. Mallory's Aniline Blue stain

1. PAS stain with prior diastase digestion

Diastase Digestion

(i) Sections to water

(ii) Place slides in preheated diastase solution for 1 hour at 37 degrees centigrade

(iii) Stain with PAS or other stain

Periodic Acid Schiff (Ref. LYNCH et al. (1969) Medical laboratory technology and clinical pathology. pp. 1088-1089.)

(i) Sections to water

(ii) Oxidise in 0.5% aqueous periodic acid

(iii) Rinse in tap water, then distilled water

(iv) Place in Schiff's Reagent for 15 mins.

(v) Wash in running tap water (5-10 mins.)

(vi) Counter stain with Mayer's haematoxylin.

(vii) Differentiate in 1% acid alcohol.

(viii) Blue in running tap water

(ix) Dehydrate clear and mount.

2. Alcian Blue Stain (for Acid Mucopolysaccharides
Ref: STEEDMAN, H.F., Quart J.
of Microscience (1950), 91-477).

(i) Sections to water

(ii) Alcian Blue (30 mins.)

(iii) Rinse in distilled water and then tap water

(iv) 1% neutral red (5 mins.)

(v) Dehydrate clear and mount

3% acetic acid gives a PH of approx. 2.3, 2 drops of CHCl; substituted for glacial acetic acid will give a PH of 0.9. Both PH's were used in the present investigation.

3. Haematoxylin and Eosin

Procedure

(i) Sections to water

(ii) Haematoxylin for 10 mins.

(iii) Wash in water (10 mins.)

(iv) Differentiate in acid alcohol

(v) Wash in running tap water 20 mins.

(vi) Eosin 5 mins.

(vii) Wash in tap water briefly

(viii) Dehydrate clear and mount.

Reference - Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology, p. 76.

4. Van Gieson Stain

(i) Sections to water

(ii) Stain with Weigert's haematoxylin (10 mins.)

(iii) Wash well with tap water

(iv) Rinse in distilled water

(v) Stain with Van Gieson solution

(vi) Rinse in distilled water and then in 95% alcohol.

(vii) Dehydrate clear and mount.

Results:

Nuclei - brown black

Collagen - deep red

Muscle, cytoplasm, blood cells, fibrin - yellow.

5. Reticulin fibres were stained with the silver stain of LILLIE, R.D. (Stain technology 21; 69-72 1946).

(i) Deparaffinised in Xylol, then through absolute alcohol, 90% alcohol, 70% alcohol.

(ii) Washed in tap water for 1 minute

(iii) 0.5% potassium permanganate - 2 minutes
190 ml. of 0.5% pot. perm.
10 ml. of 3% sulphuric acid, made just before use.

(iv) Washed in tap water

(v) Treated with 5% Oxalic acid - 5 minutes

(vi) Washed in tap water

(vii) Applied Ferric Chloride - 2 minutes

(viii) Washed in tap water 3 minutes

(ix) Washed in de-ionised water - 2 changes

(x) Immersed in diammonical silver nitrate solution - 3 minutes.

(xi) Drained and rinsed quickly in distilled water

(xii) Flooded with 10% formalin for 1-2 minutes - until section turning black.

(xiii) Washed in running water for 3 minutes

(xiv) Toned in 2% acid gold thiosulphate for 2 minutes

(xv) Fixed in 5% sodium thiosulphate for 2 minutes

(xvi) Washed in water

(xvii) Dehydrated in 2 changes of absolute alcohol

(xviii) Cleared in Xylol

(xix) Mounted in Zam.

Diammonical silver nitrate solution.

1 ml. of concentrated ammonium hydroxide in small flask titrated with 10% silver nitrate until faint permanent tupidity remained. Diluted with equal parts of distilled water.

Reference - Manual of Histologic Stain Methods of the Armed Forces Institute of Pathology, p. 75.

6. Mallory's Aniline Blue Stain

(i) Sections to water

(ii) 0.5% Acid Fuchsin (5 mins.)

(iii) Transfer directly to Aniline Blue (30-60 mins.)

(iv) Dehydrate clear and mount.

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