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A DETERMINATIVE STUDY OF THE DIRECT EFFECTS
OF THALIDOMIDE ON CHICK LONG BONE EXPLANTS
DURING ACTIVE HISTODIFFERENTIATION

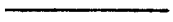


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ABSTRACT

Six and a half-day old paired chick femora were directly exposed to 10, 20, 30 and 40 mgm per ml suspensions of thalidomide in natural media for 2½ days. The experimental design was such that a rudiment in a pair served as a control for its contra-lateral homologue. Colchicine was added to control and treated culture dishes and all the explants were fixed after 8 hours of colchicine action.

The explants were systematically examined for matrix characteristics (metachromasia), cellular details (cell densities, mitotic indices) in each of the zones of transformation in an epiphyseal-diaphyseal direction in the growing end of the femur (the condylar end). In the fibrous and osteogenic perichondrium, the presence or absence of mitosis was recorded with all the suspensions of thalidomide tested, normal beta-metachromasia observed in the toluidine blue-stained sections, indicated no effects on matrix constitution. Furthermore, mitosis detected with colchicine was observed in the transformation zones, osteogenic and fibrous perichondrium in all the explants.

The difference in sample means (cellular densities, mitotic indices) in the transformation zones were not significant (95% level of significance) when tested with a 10, 20 and 30 mgm per ml suspension of thalidomide. The only significant difference in response was obtained in 40 mgm per ml suspension of thalidomide. The treated sample mean in the round cell zone (the first zone of transformation) showed a significant increase in cellular density ($+8.706 \pm 3.5$), whereas the densities in the flat cell zone and hypertropic cell zone were decreased. The decrease in the hypertropic cell zone (-8.69 ± 1.7) was significant.

Though during transformation mitosis was not affected, a high percentage of mitosis (9.9%) was observed in a disorganized culture of cartilage exposed to 10 mgm per ml suspension of thalidomide. With the same concentration, an extending resorption in the epiphysis at the head end of a femur was noted.

All four concentrations of thalidomide tested had no inhibiting effects on the mitotic activity of the various differentiated cells (Fibroblasts, osteoblasts and cartilage cells). The differentiative properties of these mesenchymally-derived cells appear to be affected since cellular density (involving matrix-cell relationships, cell hypertrophy during transformation) and active resorption during histodifferentiation were observed.

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I. INTRODUCTION

A Teratogenic Effect and Previous Investigations:

Many substances (insulin, nitrogen mustard and others) have shown a selective action on the limb bud when the embryo is treated with them at a particular sensitive stage of development. These chemoteratogens (Salzgeber and Wolff, 1964) can act by retarding (micromelia), altering (phocomelia) or arresting (ectromelia) the development of the limb bud. Ansell in 1950 (cited by Salzgeber and Wolff, 1964) pointed out that: "there is only a relatively small number of substances endowed with a selective teratogenic capacity, that is to say, for which an organic bud shows a selective sensitivity."

In humans, phocomelia could be regarded as a characteristic though not a typical feature of the observed defects recorded in the 20% teratogenosensitive woman (Cahen, 1966) exposed to thalidomide. The action of thalidomide has been shown to be less selective in producing malformations of the limbs in experimental animals than in man. Limb malformations in the rat chiefly, mice and hamsters are debatable and in particular they are not classically phocomelic but common reports include malposition (King and Kendrick, 1962) and malformations of the club-foot type (Dwornik and Moore, 1965).

With chick species, Karnofsky (1965) reported that they were unable to obtain significant teratogenic activity after thalidomide treatment in contrast to studies (Kemper, 1962; Boylen et al, 1963; Yang et al, 1963; Debock and Peters, 1963) that related thalidomide with the production of certain malformations. From general experience and their own trial experiments, Salzgeber and Salaun (1965) concluded that the

deposition of any kind of powder in the embryo in early stages (30-48 hours) produces malformations of the caudal region and limbs. Consequently, only embryos treated after the 50th hour of incubation were retained for examination. Furthermore, a quantitative study (Grabowski, 1966) has shown that even injection of sodium chloride solutions causes an increase in serum potassium and occasional maldevelopment, indicating that simply opening the egg and injecting saline is not an innocuous procedure. Salzgeber and Salaun observed between 20% and 50% malformations whereas Aleksandrov et al, (1966) observed 20% defective embryos after administration of thalidomide and some of its intermediate products. The latter concluded relative teratogenic specificity since the compounds acted comparatively uniformly on primordia of ectodermal origin but differently on primordia of mesodermal origin. This difference did not apply to the nature of the developmental defects but to its incidence. However, the studies of Salzgeber and Salaun have decisively demonstrated (Cahen, 1966) phocomelia similar to that of humans.

Of the limb anomalies in the chick, phocomelia - which rarely appears spontaneously (Salzgeber and Wolff, 1964) - has been reproduced with tochlorine, organic arsenicals, saponin and most consistently (90%) with the radio-mimetic substance nitrogen mustard. The critical stage for producing phocomelia appear to be stages 18-20, that is 3-3.5 days. Morphological lesions observed after 24-30 hours of pretreatment with the phocomelia-producing substance nitrogen mustard, revealed that the growth of the bud is inhibited and haematomata of varying sizes are seen at the centre of the bud or near its base (Salzgeber, 1966). Histological

studies over the ensuing days gave a detailed picture of the subsequent changes. The dense central mesenchyme with small cell nuclei was replaced by a vacuolated tissue with large cell nuclei. Forty hours after treatment the organization of the limb bud is characterized by the absence of the central mesenchymal condensation and the presence of lacunae of vacuoles and pycnotic cells. The chondrogenesis appeared to be inhibited or delayed since four or five days after treatment there was a reorganization of the limb rudiment. The distal structures appear, as do nodules of precartilagenous cells and muscle rudiments. Nerves and blood vessels enter the limb which is of reduced size. In cases where the mesoderm was extensively reduced, the buds degenerated resulting in ectromelia. These results suggested a specific action of the teratogen nitrogen mustard on the mesodermal structures which also appear to be selected by thalidomide.

Various Factors Related to the Production of a Similar Effect:

The relation of cause and effect is complexed not only by species differences (also discussed in chapter on the Metabolism and Pharmacology of Thalidomide) but also by the fact that these limb anomalies (ectromelia, micromelia and phocomelia), produced by specified chemoteratogens acting on developing limb buds, are comparable to those obtained through other factors, some of them indirectly, namely:

(i) Body Fluid Imbalances

Increases in body fluid may result in internal and external hemorrhages and in the formation of clear blisters (Grabowski, 1966). Hypoxia, various teratogens, lactic acid, egg albumen, calcium salts and dinitrophenol have produced blistering and hemorrhaging. In some cases, it is

the presence of a blister or hematoma adjacent to a rapidly developing organ that produces malformations rather than direct cytotoxic effects on target organs. Localized intraembryonic edema and hemorrhages in the caudal region, as is produced by strychnine, quinine salts, trypan blue, vitamin deficiencies, and by certain pituitary preparations (cited by Salzgeber and Wolff, 1964), may selectively destroy the limb to a greater or lesser extent resulting in ectromelia or hemelia.

(ii) Nutritional deficiencies

Many cases of acute, chronic or transitory nutritional deficiencies were related with a wide variety of soft tissue and hard tissue defects (reviewed by Johnson, 1965). Of the long bone anomalies, micromelia has in cases been related to nutritional deficiencies (Salzgeber and Wolff, 1964).

(iii) X-rays and Grafting Experiments

Limb malformations in the chick and in higher vertebrates have been reproduced by exposure of the developing embryos to ionizing radiations (reviewed by Kirrmann, 1964). Numerous experiments involving insertion of flakes of mica into the mesenchyme have revealed competition between the development of the tibia and fibula, which also occur in cases of limbs treated with chemical substances and in excision and re-grafting experiments of developing tissue to abnormal sites.

(iv) Genetic Factors

The majority of limb malformations induced by chemical factors (brachymelia, micromelia, - Landauer, 1954), are phenocopies of hereditary malformations which are mutations (reviewed by Gruneberg, 1963; Clarke Fraser, 1965). The experimental brachymelias and micromelias present

characteristics of spontaneous and hereditary achondroplasia and chondrodystrophy found in man, other mammals and birds (heterozygotic creepers). Homozygotic creepers show more marked defects in the fusion or absence of long bones. These characteristics also appear in phocomelic chick embryos obtained with nitrogen mustard and also with thalidomide.

Phocomelia in a mouse (Gluecksohn-Waelsch et al, 1965, cited by Gruneberg, 1963) is due to a simple recessive gene and in this case is considered (Gruneberg, 1963) not to be primarily due to a disturbance of cartilage histogenesis but is traceable to a membranous skeleton, that is a delay in precartilage formation. Also dominant hemimelia in the house mouse is reported to "produce effects more similar to those of thalidomide in man" (Clarke Fraser, 1965).

Phocomelia occur in varying severity depending on the extent to which the parts forming the limb are stunted. In less extreme cases, the terms micromelia (Landauer, 1936) or brachymelia (Ansell, 1944) have been applied to these malformations in the chick (examples cited by Salzgeber and Wolff, 1964). In the above, descriptions of the anomalies are taken directly from the reports and are referred to here only to indicate a relationship of limb anomalies to gene mutants. Phocomelia for the present study will mean "a deficiency in the intercalary regions", and hemimelia, "alterations in distal parts". Salzgeber and Wolff adapted this classification -- originally described by Geoffroy Saint Hilaire in 1836 -- for their teratological studies with the chick. This is also essential for the classification of human long-bone malformations described by O'Rahilly (1951).

Since the processes of development are the same in cases of brachymelia and chondrodystrophy, Wolff, in 1948, has raised the question as to whether a teratogenic factor may not also exert an influence on hereditary monsters and whether such malformations may not be due to the secretion of a teratogenic substance. The existence of lethal hereditary factors was mentioned as an argument in favour of this hypothesis. Such toxic factors would also be able to influence the appearance of a phocomelian species or breed with stunted limbs (Salzgeber and Wolff, 1964).

More Recent Methods of Investigation of the Problem:

In ovo studies (Jurand, 1966) on Brown Leghorn limb buds (stages 17-18) Hamburger and Hamilton, 1951) suggested that the primary cause of the thalidomide abnormalities is an injury of the endothelial lining of the axial limb artery. In cases of more prominent injury to the then-dilated axial artery numerous necrotic cells are found in the areas of the future skeletal blastemas, indicating that the dilated axial artery was the primary effect observed after the thalidomide administration. This vascular injury, probably, subsequently affected the developmental potentialities of the non-vascular part of the mesoblast tissue. The epiblast (apical ectodermal ridge area) a feature also observed in rats and humans (Blechsmidt, 1961), was insensitive to the drug and its closely related mesoblast cells showed a greater resistance than the rest of the mesoblast which was almost completely destroyed in extreme cases. Jurand cites these results as a possible explanation for Salzgeber and Salauns' phocomelia produced by thalidomide in chicks. In this case, the distal parts of the limb had a greater chance to remain undamaged than

the proximal parts. Numerous experiments on the limb bud of chicks (reviewed by Salzgeber and Wolff, 1964) attest to a preprogrammed interaction of ectoderm and mesoderm in the development of the limb. Though it is generally regarded (Salzgeber and Wolff, 1964) (Hamilton, Boyd and Mossman, 1964) that the apical ectodermal ridge and its related marginal vein are probably concerned in the successive stages in the proximo-distal differentiation of the limb bud, experiments (Bell et al, 1959; Amprino and Camossa, 1960, cited by Salzgeber and Wolff, 1964) contest the importance of the apical ectoderm in the distal differentiation of the limb bud. The reported inconsistencies following ectodermal removal may be explained (Hamilton, Boyd and Mossman, 1964) by the presence or absence, after such removal, of a refractile possibly non-cellular layer of material on the surface of the underlying mesoderm.

With regard to the effects produced by thalidomide (Jurand, 1966) it appears that with the intact ectoderm and mesoblast, the mesoblast showed selective sensitivity. At the ultra-structural level, the mitochondria in the endothelial cells of the dilated artery appear to be the most sensitive organelles. They became larger and more spherical and show only abnormally short peripheral parts of disrupted cristae followed by vacuolation. Furthermore, there was an increased number of lysosomes and the cytoplasm undergoes vacuolation. In the light of these findings two possibilities were suggested. First, the mitochondrial lesions are possibly the primary effect, anoxia inside the endothelial cells may be the first result, followed by a chain of changes leading to extensive necrosis of the surrounding mesoblast tissue, or secondly, the primary

effect may be the increase of the lysosomal population and that all other changes particularly those of mitochondria follow later. Mitochondria are known to lose their oxidative phosphorylative capacity and show extensive membrane damage when exposed to lysosomal enzymes in vitro. It suggested a potential increase in destructive processes.

The structural observations on the mitochondria are acknowledged to provide only limited information since mitochondria always show the same syndrome of changes regardless of the damaging factor, whether it is dietary deficiency, starvation, addition of toxic substances to tissue cultures (Frederic, 1958), radiation with x-rays or treatment with drugs. Necrotic changes in the nucleus (pycnosis and karyorrhexis) follow later as the last stage of cell death (Bessis, 1964) which is the end result of a complex of processes.

Present Objective:

There is no pharmacological or chemical correlation among the known teratogenic drugs. For the same teratogenic substance there is very little correlation of results among different species or different strains of the same species, even when they are tested with the same experimental procedure. This often accounts for the difficulties confronted with in the experimental production of foetal malformations and studies on the modes of action of the teratogenic drugs, as is exemplified in the case of thalidomide.

Following the production of phocomelia in the chick species by Salzgeber and Salaun (1965), Jurand's (1966) in ovo study suggested that the primary effect of thalidomide was via the defective vascular

system. Necrosis extended primarily from the endothelial lining into the proximal mesoblast in more severe cases, so that a direct action or indirect action is not clearly evident. Localized anomalies by definition must be ascribed to local causes. Whereas, in the case with systemic conditions the physiological mechanisms disturbed by the gene in question must either reside in the skeletal cells themselves (such as mesenchyme cells and its special forms, chondroblasts, osteoblasts, osteoclasts) or else that it must be of a humoral kind, which influences one or more of these cell types wherever they occur in the skeleton (Gruneberg, 1963). In other words, a systemic anomaly must potentially affect a phase of skeletal development as a whole, but need not necessarily do so everywhere to the same extent or at the same time. For instance, some cells or cell groups may be more sensitive than others. Similarly, where cells are susceptible during a certain phase of their life cycle, only this sensitive phase may not occur in all of them simultaneously. The decision as to whether the anomaly is conditioned intrinsically by the cells of the skeleton or whether it is initiated extrinsically could be reached by reciprocal transplantation or comparable organ cultures (Gruneberg, 1963; Salzgeber and Wolff, 1964; Clarke Fraser, 1965; Cahen, 1966).

The problem may be considerably reduced if the defective embryonic metabolism is known to be inherent or if it is not. Therefore, relying on structural expressions of metabolism (proliferation, cell details, matrix characteristics), the primary objective of this study is:

TO DETERMINE WHETHER THALIDOMIDE HAS ANY DIRECT EFFECTS ON CHICK LONG BONE EXPLANTS DURING ACTIVE HISTO-DIFFERENTIATION (THAT IS, STAGES - 6-6½ DAYS, HAMBURGER AND HAMILTON, 1951).

This will be ascertained from:

(1) The effects on the distinct zones of cells which are transformation stages in a cartilage cell life cycle (Gruneberg, 1963). Also on fibrous and osteogenic tissue surrounding the cartilage.

(2) The effect on the cartilage matrix constitution.

At this stage when transformation of cartilage cells are observed, the fibrous and osteogenic cells and osteoid formation are distinguished. Furthermore, at the lateral surface of the epiphysis there is a region where the cells are histologically more or less undifferentiated (Willmer, 1960). In this region (Figure 14), the small cells are strategically located to give rise to the round cells of the epiphysis and also the fibrous and osteogenic cells. There are, therefore, primitive mesenchymal cells in the explant (Willmer, 1960).

In vitro cultivation at earlier stages, 3-day-old limb bud, results in no further development than a cartilagenous skeleton (Willmer, 1960). Whereas, if the original tissue explanted is from a 6-day-old embryo, then it continues to develop more completely in that cellular hypertrophy, and osteoid occur in an organized manner (Fell and Robinson, 1929). Therefore, it seems that certain conditions are established during the 3rd and 6th days, which eventually allow the differentiation of cartilage cells and of the osteoblasts.

Studies on the mode of action in vitro, with insulin (Zwilling, 1954) parathyroid hormone (Gaillard, 1965), vitamin A (Fell and others) have testified to these substances being able to act directly on the limb tissues.

Concerning thalidomide, a study describing the influence of the drug and dioxane on the growth of chick embryo tibia in organotypic cultures was described by Franceschini and Mussa (1966). The organs were cultivated for eight days after addition of 3500 micrograms of thalidomide. They reported arrest in linear growth, near complete disappearance of the cartilage, matrix, disappearance of the morphological features peculiar to the diaphysis and practically complete arrest of mitotic activity.

For the present study, in view of the practical problems (described in Review of Literature) concerning culture media, duration of cultivation, criteria for indicating mitosis and concentrations of the drug, the following preliminary studies were performed so that thalidomide could be causically and unequivocally related to the observed responses in organ culture:

- 1). Normal in ovo histodifferentiation.
- 2). A selection of a favourable cultivation period under the present cultivation conditions used.
- 3). A wide range of concentrations of thalidomide exposure to the organ cultures.
- 4). Selection of a favourable concentration of colchicine to detect mitotic activity in organotypic cultures in vitro.

With information from these preliminary studies, the major study was performed using a small range of four concentrations of thalidomide over a short duration cultivation.

II. REVIEW OF THE LITERATURE

Criterion: Cellular Details

In the long bone explants cartilage cells are observed in three different states of cytodifferentiation (Fell, 1925). This is morphologically evident from their cytoplasmic-nuclear ratio and shape, namely round, flattened and hypertrophied. These cells have a functional distribution in the bone rudiment. Cells of the articular region are small and round and are characterized by a high nuclear-cytoplasmic ratio. Chondrocytes in the epiphyseal region are flattened and oriented more or less perpendicular to the long axis of the rudiment. In vitro, however, this orientation is not so distinct. The earlier the cultivation, the more apparent is the disorientation. In the third zone, the diaphysis, the cells are hypertrophied and highly vacuolated. Histochemically, it shows also an increasing amount of ribonucleic acid and glycogen over the proliferative cells and alkaline phosphatase and phosphorylase appear and accumulate (Cabrini, 1961).

Numerous in vivo and in vitro studies further indicate that these cells are distinctive and show selective sensitivity. In normal in vivo development cells in each of these zones have different fates. Articular chondrocytes persist into the adult stage. The flattened chondrocytes of the epiphysis proliferate and differentiate into hypertrophied cells which in turn eventually degenerate and are resorbed (Fell, 1925; Henrichsen, 1958). In vitro, the pioneering dissociation-reaggregation technique for embryonic organ cells of Moscona (1952) has proved invaluable in the evaluation of histogenetic capabilities of cartilage cells. This principle has been utilized (Okada, 1959) cited by Morris (1962) together with various histochemical and autoradiographic techniques, to investigate the regeneration of

cartilage matrix by the dissociated cells. Chondrocytes in each state of cytodifferentiation have also been cultured separately on a plasma clot (Stockdale et al, 1963). A metachromatically stained capsule around each of the flattened and hypertrophied cells was identified but not around the isolated articular chondrocytes. Also, the dissociated cells in culture indicated that mitotic figures are rare in hypertrophied cells and that cell death occurs throughout the entire population during the seventh and eighth day of culture. In comparison, articular and the epiphyseal flattened chondrocytes continued to proliferate for several weeks in cell cultures. The effects of the in vitro conditions on these events were uncertain and no results were available from these experiments concerning the synchrony of cell death in these two populations, nor was it known which population would show degenerative changes first.

Cooper and Lash (1964) observed that the reaggregated masses of cartilage cells in each of these states of differentiation are able to reconstitute cartilagenous tissue. The ability of hypertrophied cells to do likewise in vitro, was unexpected in view of autoradiographic data which indicated that hypertrophied chondrocytes exhibit low levels of radio-sulphate incorporation in ovo. These two investigators also studied inductive tissue interaction in vitro. Their results showed that chondrocytes obtained from each differentiative zone had different inductive properties. The flattened cartilage cells which undergo hypertrophy in vivo were found to induce chondrogenesis in embryonic somite mesenchyme in vitro.

Hypertrophy of cartilage cells and its functional interrelationships in normal and abnormal bone development was observed by Fell and

Landauer (1935). They have described the failure of cells to hypertrophy in the juxta-periosteal region in phocomelic limbs of the Creeper chick in ovo and reproduced in vitro. Usually the hypertrophied cells extend right to the periosteum in the diaphyseal region. In these cases the periosteal ossification was absent. These studies were performed in the mutant chick embryo, Creeper, which manifests an achondroplastic condition in which the long bones fail to elongate fully and undergo precocious ossification. The condition is inherited as a dominant and is lethal in the homozygous condition. In vitro studies with this species in growth restricting media indicated that the histological features were similar to those of the long bones of phocomelic embryos. From their studies, they concluded that an unspecific growth retardation due to a gross nutritional deficiency in an early stage of development was the chief cause of the phocomelic condition in the Creeper. More recently, Wolff and Kieny, 1957 (cited by Biggers, 1965), detailed studies using embryo extracts obtained from normal and defective chicks and conveyed that the genotype-environment interactions are more complex.

The behaviour of dissociated cartilage cells was studied by Moscona (1961). The cells were liberated from their matrix capsules by tryptic digestion and placed on a gyrorotatory shaker for twenty-four hours. The chondrocytes from the three different histodifferential zones behaved differently in their size, number and character of reaggregated patterns.

Besides the experimental evidence indicating a metabolic distinction of these round, flat and hypertrophied cells, one could also stress the academic interest, namely, inherent obsolescence concerning these cells and correlation of results with conditions in vivo.

The specific aging process of cells has been referred to as the "death clock" (Saunders et al, 1962), "normal degeneration" (Willier et al, 1955) and "inherent osolescence" by Cooper and Lash (1964).

Glucksmann (1951) and Saunders et al (1962) have reviewed cases in which cell death has played a part in normal vertebrate development and has shown not only that such processes are important where regression or transient structures occur, but that it is associated with events such as invaginations, evaginations, separation and fusion of embryonic parts, migration of rudiments, closure of tubes, formation of lumina and changes of shape. In the chick embryo the importance of cell death in the development of its appendages is well documented (Saunders et al, 1962; Biggers, 1964). It was observed that certain mesenchymal cells of the early chick appendage exhibit an irreversible progression towards the necrotic state-- "a death clock"--preprogrammed to facilitate the normal developmental structure of the limb. Willier et al, (1955) further implicates this "normal degeneration" as a possible cause of malformations, namely "Terata may arise as an extension or exaggeration of a normal degeneration or as a result of degeneration where none is normally involved". After considering studies on degeneration particularly on the chick, Zwilling (1964) stated that "degeneration is a constant feature of normal limb development. The existence of this degenerative material probably constitutes a threat to the rest of the limb tissue and in part explains why the denuded limb mesoblast is highly susceptible to degeneration when it is subjected to experimental procedures. This is exemplified by a normally occurring patch of degenerative tissue regularly found in a five-day limb rudiment

at the site of the future joint between the femur and tibiotarsus in vivo. It is, however, not certain (Fell and Canti, 1934, cited by Zwilling, 1964) that this necrosis is actually involved in joint formation. Zwilling attributes the extreme micromelia due to insulin injection into a four to five day chick embryo to massive degeneration at this joint region and adjacent parts of the long bones, i.e., "a spread of a normally limited and transitory focus of necrosis to healthy tissue", consequently producing micromelia. In the present in vitro studies, considerable care was observed to use only the explants that were carefully dissected and which apparently did not contain any joint tissue. This would be required for unequivocal descriptions, in view of the above-mentioned possibilities and chiefly because it was observed that explants that contained joint tissue showed histological degeneration that extended into the nearby epiphysis. This was not a central necrosis nor an environmental in vitro deficiency since mitosis was detected with colchicine in distal areas (Figure 19) that was adjacent to, but apparently not yet affected by, the extending necrosis.

Inherent obsolescence is also exemplified at a later developmental stage in the mesenchymally-derived cells, the chondrocytes. For example, chondrocytes situated in the diaphysis of the long bone rudiment do not survive in ovo as long as those in Meckels cartilage, whereas the latter cells do not approach the longevity of articular chondrocytes. This indicates that the rate of obsolescence may differ within a particular organ and between different organs and has functional significances. In the above-mentioned examples, although cell death has been discussed as a normal developmental process - in the sense that it has teleonomic

value - it is not necessarily considered to be applicable to all cells (Cooper and Lash, 1964), but deserves attention in connection with problems of differentiation especially since this is an invitro study.

Correlations from culture conditions to in vivo conditions should take into account the new environment. Pertinent findings in this regard was indicated by the dissociation-reaggregation experiments of Glick and Stockdale (1964). Assays of the sulphated mucopolysaccharide content of reaggregated ten day chick vertebrate chondrocytes were performed. These were compared with values obtained from freshly isolated cells from fifteen day chick embryos. The results showed that not only the amount of chondroitin sulphate was reduced in the cultured cells, but that a new species of glucosamine-containing polysaccharide was synthesized in vitro. The characteristics of the three populations of cartilage cells observed in vivo, in vitro, mitotic ability, inherent obsolescence, behaviour and its organized relationship in the rudiment all suggest their distinct metabolic differences. A comparative investigation of an effect of a substance on this homotypically and heterotypically interacting system of cartilage and osteogenic cells may possibly provide information on the differentiative state that may be susceptible.

Criterion: Matrix Characteristics

The chemically complex matrix of the bone rudiment is an integral part of the culture and has to be produced and maintained by the constituent cells (Biggers, 1963). Its normal constitution can be evaluated by stain reactions. Histologically, metachromasia involves the staining of a tissue component by cationic dyes, for example, the thiazine dye,

toluidine blue O, so that the resulting colour of the chromotrope-dye complex is markedly different from the colour of the original dye or from its ordinary tissue complexes (Kramer and Windrum, 1933). According to Bungenberg de Jongs' theory (cited by Kramer and Windrum, 1955), a chromotrope of a high electronegative surface charge density and water molecular bonds between the then-aggregated dye complexes are essential factors for the resulting spectral absorption shift.

High molecular weight substances with free anionic groups for example acid mucopolysaccharides, nucleic acids, all fulfil the above conditions for metachromasia. Intercellular substance predominate in normal cartilage and in the hyaline type, the copious ground substance contain sulphated mucopolysaccharides in the A and C chondroitin sulphate forms. Another constituent of ground substance, hyaluronic acid, contain one dye bonding site in its carboxyl group, whereas the sulphated mucopolysaccharides contain two sites per dissaccharide unit, namely, carboxyl plus sulphate groups. As a consequence of these facts, the non-calcified embryonic cartilage is strongly metachromatic. Chromotrope metachromasia reaches a maximum when all sites are bound. The abundance of collagenous elements in bone matrix over available interfibrillar space accounts for negative metachromatic reactions in the osteoid stages (Tonna, 1963).

Significant metachromasia can be assured by observing certain technical precautions (Schubert and Hamerman, 1955). (See Chapter on Methods and Materials). For descriptive purposes, the notation of Michaelis, namely; alpha, beta and gamma, are often used for the resulting spectrum absorption shifts since it provides qualitative information in view of the above-mentioned requirements for metachromasia. The

designation, alpha, is described as orthochromatic and shows a blue hue while the metachromatic designations are beta (violet) and gamma (red).

The varying susceptibility of metachromasia in stained sections to alcohol dehydration is still the subject of an unresolved controversy (Schubert and Hamerman, 1955), dealing with the alleged ability of alcohol dehydration to distinguish between chromotropes containing ester sulphate from those which do not. Alcohol dehydration is known to extract thiazine dyes; therefore, acetone is often recommended for this purpose (Lillie, 1965). Though acetone is often preferred, metachromasia, due to sulphated esters of polysaccharides, are observed not to be destroyed by any reasonable exposure to alcohol and is thereby recommended as a controlled method to elicit significant irreversible metachromasia in such cases (Curran, 1964).

The information obtainable from such criteria can be visualized from various studies using the metachromatic reaction. Studies with certain biological agents at circulating levels have used this characteristic of the cartilage matrix effectively. Vitamins A, D, growth hormone, lathyrtic agents, papain have been shown to have characteristic and fairly specific effects on normal growth and metabolism. Other agents less specifically effective are vitamin C, cortisone and related compounds, thyroxine, parathyroid hormone, glucagon, insulin, and general nutritional level (Morris, 1962). Metachromasia featured in a series of experiments (Fell and Dingle, 1963; Fell, Mellanby and Pelc, 1956; Fell and Thomas, 1960) which led to an hypothesis on the mode of action of vitamin A and also led to the description of an acid protease

in normal cartilage. Metachromatic reactions were described as positive or negative, superficial loss or substantial loss from the different zones.

Hypervitaminosis A causes a rarefaction of bone which often causes spontaneous fractures (Fell, 1963). Fell and Mellanby (1952) utilized the organ culture method to determine whether vitamin A had a direct effect on the bone rudiment. After six days cultivation of tibia from the foetal mouse, a dissolution of the cartilage matrix in the treated explants was preceded by a loss of metachromasia. Degradation of cartilage matrix, being a less complex process than bone destruction, was investigated in an attempt to determine the cytological mechanism through which vitamin A acted. From radioautographic studies (Fell, Mellanby and Pelc, 1956), it was concluded that the effect of the vitamin was in two stages: first, the uptake of new sulphate was inhibited and then sulphate already incorporated was released. Studies on the action of vitamin A were resumed at the Strangeways Laboratory in 1960, when Fell collaborated with Thomas in comparing the effects of vitamin A and papain on cartilage in vitro. Thomas had earlier succeeded in producing a reversible collapse of rabbit ears with papain protease. Associated with this collapse was a narrowing of the intercellular partitions of the ear cartilage and loss of basophilia. This effect was strikingly similar to the vitamin A effect observed by Fell and Mellanby (1952), and this instigated the comparative study by the two authors (Fell and Thomas, 1960). Their working hypothesis was "that vitamin A may enhance the activity of a number of cellular enzymes, one of which resembles papain in its effects: such an enhanced hydrolytic activity might be due to the activation of enzymes to their greater production or their increased liberation through an increased permeability of the

cells or their organelles". Ensuing biochemical studies (Dingle et al, 1961; Fell and Dingle, 1963; Fell and Thomas, 1961) led to a tentative conclusion concerning the action of vitamin A.

Analysis of explants of cartilagenous limb bone rudiments from seven-day chick embryos showed that after six days, cultivation in the presence of vitamin A, the wet and dry weights, and hexosamine content, were about half those of the controls. Also, the amount of RNA was about forty percent, and the DNA about eighty percent of that in the controls (Dingle et al, 1961). Furthermore, earlier experiments (Fell and Thomas, 1960) had shown that the glycogen content of the periodic acid Schiff-stained sections was reduced. These experiments indicated that both cells and matrix had been altered by the vitamin A treatment. Biochemical studies showed that the lysed clot, on which Fell cultivated the rudiments, was due to the production of twice as much acid soluble nitrogen in the medium containing the vitamin A as in the control medium. Further studies (Dingle and Fell, 1961) described the proteolytic activity of normal limb cartilage from nine-day old chick embryos. The cartilage was pre-treated with distilled water to disrupt the cells and their organelles and then incubated at thirty-seven degrees centigrade in buffers ranging from pH one to eight. They observed that the metachromasia of the matrix was greatly reduced at between pH three and five; half the hexosamine content was lost (mostly as a polysaccharide of high molecular weight), and that the protein components were liberated into the buffer. These authors later isolated a proteolytic extract from cytoplasmic particles which had a pH optimum of three. From these results, it was concluded that normal

cartilage contains an acid protease. Since De Duve (1959) has shown that lysosomes contain cathepsins and other acid hydrolases, it was concluded that vitamin A affected the permeability of the cell membranes, artificially releasing the acid protease from the cytoplasmic particles into the matrix. The protein moiety of the protein-polysaccharide complex was digested, consequently a loss of metachromasia was evident. Extensive degradation of the matrix and almost complete disappearance of metachromasia and corresponding loss of hexosamine was obtained at physiological pH levels. The effect was more drastic though at pH six (Fell and Dingle, 1963). It is acknowledged (Dingle and Lucy, 1965) that the molecular structure of the lipoprotein membranes are affected. Hydrocortisone is also thought to act at the same level (Fell and Thomas, 1961), since it is observed that simultaneous addition of the substance greatly retards the vitamin A effects on the bone rudiments.

This example indicates how a negative metachromatic reaction of the matrix is obtained by a breakdown of the protein part of the protein-polysaccharide portion of the matrix. Metachromasia has specific requirements, therefore interpretations of a negative reaction, as instanced above, would have to be critical since this could be due to more than one reason (Kramer and Windrum, 1955; Schubert and Hamerman, 1955). In cartilage, the high molecular weight free anionic groups (sulphated mucopolysaccharides) are linked with a collagenous framework and the sum total normally gives a positive metachromatic reaction. The protein source in cartilage matrix could be either from collagen or more likely the sulphated mucopolysaccharide. Proteins may inhibit metachromasia not only by its effects on pH but also by acting as salt cations, by

sterically masking the anionic groups by binding with polysaccharides or by the formation of covalent bonds with anionic groups.

The involvement of collagen in this reaction is shown by its disruption by lathyrinic agents (Biggers, 1963, 1964). Biggers has compared the effects of three commonly known lathrogenic agents on chick embryo tibiotarsi in vitro. Lathyrism refers to the neurological disease in man caused by the consumption of the legume seeds *LATHYRUS ODORATUS*. These seeds produce two substances, namely; a diamino-butyric acid and a beta-aminopropionitrile (BAPN.) which causes a skeletal defect and other connective tissue lesions only in experimental animals. The many substances which mimic these actions are known as lathrogenic agents. It is relevant to the present study that one of the known lathrogenic compounds, beta-mercaptoethylamine, is broadly distributed throughout the tissues as part of the molecule of coenzyme A.

Collagen forms up to ten percent of the fresh weight of bone and cartilage (Harkness, 1961). In experimental lathyrism, the solubility changes of collagen are outstanding features of this defect (Gross et al 1963). Malformations of the skeleton and the structural failure of the blood vessels and fascia in lathyrism are attributed to the diminished tensile strength of the collagen, this in turn resulting from an increased proportion of collagen fibrils without firm intermolecular crosslinks. Gross et al (1963) and Harkness (1961) stress that collagen is constantly remodelled and that the chemically induced skeletal malformations could involve this remodelling during development. Since collagen and ground substance are intimately interactive during development (described by Schwarz, 1957), defective collagenogenesis could also affect metachromasia.

A metachromatically-stained matrix is, therefore, also indicative of a viable matrix. Biggers (1963) utilized a decline in the ratio of dry-wet weights or hydration values as a criterion of incipient degeneration (discussed under Culture Medium), whereas Zaaijer (1963) utilized the metachromatic staining reaction for this purpose.

Criterion: Proliferation

In a number of bone culture experiments (Gaillard, 1965; Lash and Whitehouse, 1961) it was reported that certain differentiative qualities were affected but that proliferation was unchanged. Gaillard, studying the mode of action of parathyroid hormone in organ culture of bone rudiments, obtained specific histological effects on the different populations of cells in the rudiment that was evidently correlated to the hormone administration. These experiments were repeated but in addition using protein synthesis inhibitors that are generally reported to be active at the messenger ribonucleic acid and ribosomal levels, namely, actinomycin D and puromycin respectively. On comparing the histological effects produced by these substances, he was able to correlate a greater blockage of the histological effects at the messenger ribonucleic acid level of protein synthesis than at the ribosomal level. The relevant observations from the report was that the proliferative activity was unchanged and this led to a speculation that the protein synthesis dealing with differentiative qualities was affected but that the synthesis associated with proliferation was not.

Lash and Whitehouse (1961) reported that cortisone and some related compounds depressed synthesis of cartilage matrix sulphated mucopolysaccharides and matrix growth, but that the cell proliferation was

not affected during induced cartilage formation in cultured chick embryo somites.

These indications of the possible use of organ cultures could be interpreted as providing support to Honor Fell's contention that proliferation and differentiation are not necessarily antagonistic events in organized cultures (Biggers, 1965). The literature of tissue culture contains in various forms statements that growth and differentiation are incompatible. The question of de-differentiation is often debatable under culture conditions where the production of specific proteins (differentiation) is often reversible. Strangeways (1924) states that it should be acknowledged that it is possible to upset potentialities of cells by the application of external influences. The points in his discussions implied a latency not the loss of those potentialities which enable cells to assume its specific structure and function. Weiss (1939) formally refers to this "loss of some actual functions without changes in potencies" as modulation. Under culture conditions, this term is preferable in contrast to true de-differentiation which is the loss of some actual functions with an increase in potential functions (Waymouth, 1960). In the various discussions on proliferation and differentiation (Gaillard, 1963; Franks, 1963; Waymouth, 1960), it is conveyed that though dividing cells do not show other functions at the same time, the suppression of proliferation of such cells might cause them to function depending on the environment. A minimal environment required for proliferation of a generalized cell type is required where quantitative data (mitotic rates) are sought. However, in organized growth proliferation and differentiation can potentially occur in this homotypically and

heterotypically interacting system (Grobstein, 1965) and therefore require more complex environmental conditions (culture medium, substrate) to facilitate this. The peripheral proliferation from organ explants is often observed and its suppression, which though is not the deliberate purpose in some cell behaviour studies, is facilitatory for organized cultures. It is a generality in organ culture studies (Parker, 1964; Morris, 1962; Willmer, 1965) that integrity of structure, histogenesis and functional activity is related to the degree to which peripheral migration from the explant fragment can be controlled. An expedient resorted to in earlier studies (reviewed by Parker, 1961) was the frequent transfer of the organ into fresh plasma clots at the expense of the outgrowth zone.

More recent advances to the organ culture technique originally described by Fell (Trowel, 1954; Chen, 1954) dispose of the plasma clot support and use tantalum grid supports or floating lens paper. Lens paper used in both techniques provide a favourable inert support at the liquid gas interface. Zwilling (1959) has demonstrated that different substances, through their effectiveness in limiting cell migration may control the degree to which cultured reaggregated embryonic cells are capable of reorganization, inductive interaction and ultimate cell differentiation. Furthermore, Weiss, L. (1960) studying essentially cell adhesion stressed the importance of the substrate and other environmental conditions affecting these properties in the control of cell migration. Taylor (1961) showed that silicone has poor spreading properties. Siliconization of the lens paper floats not only increased the manipulatory and floating practicality but also ensured the biological inertness of this explant support.

Preliminary studies (Chapter IV) have shown histodifferential features and relative proliferative activity that parallels that seen in vivo at a corresponding age. Connective tissue proliferation will be estimated (mitotic indices) from fibroblasts, chondrocytes and osteoblasts. In the central diaphyseal regions of the bone explants, the cartilage cells are highly differentiated (hypertrophied) so that estimations are chiefly obtained from the epiphyseal, growth and perichondrial zones. The relative densities of cells to matrix of the various populations of cartilage cells has provided information concerning transformation of the cartilage cells (Gaillard, 1965).

Culture Medium

(1) The physical and chemical constitution

The chemical and physical constitution of a medium should be conducive for normal growth and differentiation of the explant. A typical natural culture growth medium contains a balanced salt solution, serum and an embryo extract. This would be in equilibrium with a suitable gas phase which also aids in stabilizing the pH of the medium.

The balanced salt solution serves as a diluting and irrigating fluid, while maintaining tonicity of the cells (Parker, 1964). It also provides buffers to maintain a physiological pH range and finally to provide water and those inorganic ions needed for normal cell metabolism. Most balanced salt solutions include glucose as an energy source and the physiological pH indicator, phenol red, at non-toxic levels. The importance of the other two constituents of the medium have been shown to be specific for the material (type, age and species) and also type of experiment (cell or organ cultures). Parker and others in 1933 (reviewed by

Parker, 1964) succeeded in cultivating chick embryo tissues for extended periods both in heparinized plasma and also in serum, showing that it was possible to cultivate tissue in either of these constituents alone and that the plasma was not only useful as a supporting and stabilizing substrate. In a discussion on nutrient requirements for tissue maintenance, Morris (1962) concluded that "the only real function of the serum component in any tissue culture medium is to supply those necessary small molecular weight factors", namely, the amino acids. Serum, however, also contains various amounts of vitamins, carbohydrates, buffers, hormones, which makes it indefinite. Eagle (cited by Parker, 1964) has presented evidence that serum does contain one or more small molecular weight growth factors, either bound to serum proteins or formed from it on proteolysis. Furthermore, Biggers (1963) stated that the addition of ten percent chicken serum in a chemically defined medium will prevent excessive elongation of the bone rudiments and supposed that there may be substances present in serum which control and integrate the shapes of growing rudiments. The studies on the action of serum on cartilage and bone (discussed in the section on Osteogenesis in vitro) further stress the point that serum constituents could provide specific requirements for certain tissues so that generalizations are inept.

The importance of embryo tissue extract in promoting cell multiplication and growth was shown by Carrel in 1912 (Parker, 1964). More recently (Gaillard, 1955; Waymouth, 1954), it is believed that embryo extracts provide specific macromolecules or inducers required for differentiation besides these mitotic stimulants or "wound hormones".

Another important contribution of the classical embryo extract to the growth of cells lies in the fact that such extracts contain a high concentration of bound but easily available phosphate. Growth in an otherwise adequate medium can be stimulated in these cases (Waymouth, 1960). Waymouth (1954) has discussed the activity of the high molecular weight fractions in embryo extracts and deduced that the content of ribonucleic acid seemed more important than that of deoxyribonucleic acid. In Morris' (1962) discussion on embryo extracts, it is especially emphasized that different effects could be obtained with different types of cells with an embryo extract. According to Weiss' theory of feedback growth inhibition (cited by Poole, 1966), the hypothesized inhibitors and sites which bind inhibitors could be expected to be destroyed by the liberated enzymes of embryo extracts. Therefore, embryo extracts could be inhibitory or stimulatory to organ growth depending on whether the inhibitor or bonding site has been destroyed. However, the importance of the hypothesized tissue specific inhibitors for mitosis control has generally been studied only in fully grown animals. Since chick embryo extracts (CEE) will be used in these experiments, the discussion will focus on it and its effects on cartilage and bone. The principle intention in culture conditions is to provide a chemical and physical environment that would support but not challenge the conservative tendencies of the explant. It is suitable, therefore, that the media should be similar to physiological in vivo conditions at any time (Gaillard, 1955). For this reason, the media was changed every alternate day. A ten millilitre solution supported the cultivation of three explants for two days.

Since favourable histological details, such as mitotic activity, matrix characteristics and absence of necrosis were observed at the end of this period, it is apparent that the removal of metabolic wastes and the replenishment of minimal nutritional requirements are adequate with this routine. The balanced salt solution and serum provide these minimal nutritional requirements so that the embryo extract, the other constituent used in bone studies, seemed to be the constituent that could be varied and changed. Gaillard (1955) developed this technique now referred to as a "dynamic media"; that is, he utilized chick embryo extracts from progressively older embryos in successive media changes. He found that this method had very little effect on overall growth of the bone explant, but that it had a very profound influence on differentiation, particularly on ossification. The question as to whether adult extracts could be equally potent as that prepared from progressively older embryos is still an unsettled question (Biggers, 1965). Considerable advances have been made with the isolation of proposed effective molecules for cartilage induction from embryo extracts of different ages (Lash et al, 1964). Electrophoretic mobility and salt solubility experiments indicate that the chondroitin sulphates, isolated from chicks at stages 22-23 and others, have different stages of sulphation. The sulphated mucopolysaccharides seem to be essential for cartilage induction at least. A commercial preparation of crude chondroitin sulphuric acid (70% purity) was found to be an effective supplement to the chemically-defined medium 199, where it was observed to lengthen the survival of chick embryonic tissues. Endo (1960) and Ito et al (1963) have shown chick embryo extracts to be an

important constituent in media required for osteogenesis (discussed in section on Osteogenesis in vitro), not only in its concentration but also in the proportion with serum and balanced salt solution.

(ii) Biological and chemically-defined media

A chemically-defined media means "that the composition of the medium is rigorously known which includes exact information on the composition of the gas phase to which the medium is exposed. The medium is not chemically defined if biological fluids or partially purified biological products, such as dialysed serum, are included" (Biggers, 1963).

The complexity of natural or biological media makes it difficult to determine the nutritive requirements of cells and tissues and to isolate an effect due to a particular substance in the medium. Chemically defined media, being subject to rigorous analysis and being enzyme-free, would therefore be more suitable than biological media (Fell, 1963).

Since the original attempts to define a chemical medium (Lewis and Lewis, cited by Parker, 1964), success in demonstrating growth in such media was first acknowledged in 1953 by Wolff and co-workers (Biggers, 1965).

More recently, only a few successful media have been reported (Biggers, 1961; Gorham and Waymouth, 1965). Biggers designed a medium BLI which was reported to be more successful than that of Wolff, and this led to its further development, resulting in a medium BGJ (Biggers et al, 1961).

Comparisons were later made of biological and chemically-defined media utilizing seven day embryonic chick cartilage rudiments (Biggers, 1963).

The changes in length, wet and dry weights of tibiotarsi were compared after cultivation on medium BLI and in chicken serum supplemented with chicken embryo extract. The same amount of glucose was added to both

media so as to eliminate the possibility of differences being caused by uncontrolled variations in the concentrations of glucose of the two media. In the chemically defined media, the rudiments grew in length and showed an increase in wet weight and dry weight, in total deoxyribonucleic acid content, but the pattern of response was not the same as those in a biological medium. Although the rudiments cultivated on medium BLI elongated more than those cultivated on natural media, they were observed to grow significantly less in wet and dry weight. Macroscopically, the rudiment on natural media remained opaque, whereas the bone on the chemically defined media became translucent. These results have important implications concerning normal healthy growth of the bone. According to Biggers' dry and wet weight ratio or hydration values, a decline would be due to a slow hydration of the rudiment due to a rapid degeneration. Disruption of the intercellular matrix, an eventual phenomenon in vitro, would facilitate an uptake of water, resulting in the appropriate change in the ratio. The same bones become less hydrated as they grow in vivo (Biggers et al, 1961). This hydration phenomenon in vitro is detectable on the fifth day in both media. An advantage is noted in favour of natural media since this effect was somewhat greater on chemically defined media. In evaluating these studies, Fell (1963) after some reservation, was "sure that his conclusion that the rudiments became increasingly unhealthy after 5 days in chemically defined medium is correct, which means that this is the outside limit for experiments with such cultures".

Though chemically defined media has its advantages, it is sometimes possible to make "the best of both worlds" (Fell, 1963) by transferring the explants to chemically defined media for a short while after

previous cultivation in natural media. Dingle and Fell (discussed in section on Matrix Characteristics) had used this procedure very profitably to investigate the effect of vitamin A on bone rudiments.

(iii) Duration of cultivation

As observed from Biggers' criterion of incipient degeneration, using dry and wet weight ratios, a translucency of the rudiments could be used as an indication of degeneration during cultivation. However, this macroscopic observation with the stereoscopic microscope is observed a couple of days after the wet:dry weight ratios indicate this onset of degeneration. Biggers describes the duration of cultivation of an explant in vitro as its "life history" in vitro. According to this description, there is usually a movement of water on transferring an explant to a medium. This also involves ions and proteins. Though it is generally assumed that stabilization is quickly maintained, this initial shock phase in its life history in vitro may have "hangover" effects (Biggers, 1965). After the initial shock phase, the cultured rudiments very gradually become hydrated as disorganization of the intercellular matrix progresses. The matrix becomes foamy and loses its ability to stain metachromatically with thiazine dyes. These degenerative changes occur in irregular patches which spread and coalesce until the entire rudiment is affected (Biggers, 1965). Throughout this process many chondrocytes remain viable though some lacunae may lose these cells.

In the present study, the viability and health of a rudiment was decided upon after examination of cellular details and matrix characteristics. A reasonable estimate of a duration of cultivation

for the major investigation was made from the criteria (discussed in chapter on Preliminary Studies).

The phenomenon of hydration as a criterion of incipient degeneration is of considerable practical importance since it is hoped that an accurate diagnosis of their ailments might lead to the prevention of their decline and the consequent prolongation of the cultivation period. Biggers has made important advances in this respect. The nitrite, benadryl, stabilizes the plasma membranes of cells and organelles under adverse conditions. This substance was added to the medium and the time of onset of the decline in the dry:wet weight ratio was recorded. Its inhibition by hydration supports the view that the primary damage involves the cells which are then unable to maintain the matrix between them. Biggers (1964) also extended this work and observed that the hydration produced by lathyrinic agents (defined in section on Matrix Characteristics) occur only in cultures that contain living cells, and is entirely suppressed if the explants are exposed to lethal doses of the compounds. With a sublethal dose the explants become wetter because dry matter is lost. He suggests that the lethal dose arrests the synthesis of the enzymes whereas the sublethal dose does not. In this case the hydrolytic enzymes are released into the matrix. These observations also suggested that other agents (hormones, drugs) may be more effective than benadryl in controlling the effects of mild cellular damage.

Following the bone culture studies with hydrocortisone (Dingle, Fell and Lucy, discussed in section on Matrix Characteristics), and benadryl (Biggers, 1963), Schryver (1965) and Reynolds (1966) continued investigations on the possible control of the progressive hydrating of

the bone explants after five days cultivation, on a chemically defined medium. Interpretations from Biggers' and Schryvers' work held that the hydropic explants in vitro had diaphyseal cells which showed excessive hypertrophy and was associated with a release of lysosomal enzymes into the matrix consequently resulting in a loss of its metachromatic characteristics. Schryver (1965), furthermore reported that high concentrations of hydrocortisone inhibited growth (length and dry weight) of the embryonic chick cartilage in vitro but that lower concentrations were much less inhibitory, this being practically nil after the first three or more days. The decreased water uptake calculated for rudiments treated with hydrocortisone was related to the effect of this drug on cellular hypertrophy which was then not as excessive as after the fourth day in vitro.

Time of treatment was observed to be critical since if treated at the same time of explantation, and before hypertrophy has proceeded, growth in length and increase in dry weight is inhibited. It is noteworthy that hypertrophy is an important event in relation to osteogenesis and elongation (Fell, 1925). Furthermore, if added after the fourth day when excessive hypertrophy and water uptake has begun, the hydrocortisone is less effective in controlling the process.

It is finally suggested by Schryver that the degeneration phenomenon is primarily due to leakage of acid hydrolases (acid protease has been characterized for avian embryonic cartilage discussed in section on Matrix Characteristics) inducing water uptake by the damaged matrix and cells resulting in the latter's distortion and excessive hypertrophy.

Reynolds (1966) of the Strangeways Laboratory communicated a somewhat different view concerning this hydration mechanism observed

for chick long bone rudiments in vitro. In agreement with reports from Schryver, Reynolds noted a fall in growth (decreased DNA synthesis was taken as decreased cell proliferation, decreased length and dry weight) with an increasing concentration of hydrocortisone. There was also a reduction of the excessive hypertrophy in explants, in the medium containing the hydrocortisone, but, in addition, there was an increase in metachromasia. Differences in staining techniques were suggested (Reynolds, 1966) to account for this metachromatic property not being detected in Schryver's results. In contrast to the previous reports, growth inhibition was observed to occur at lower concentration levels of hydrocortisone than that required to prevent terminal waterlogging in vitro. A view was expressed that part or all of the water of hydration may be associated with the synthesis of an abnormal matrix rather than with the cells since biochemical and histological data indicate this. The data was collected on the collagen percentage of dry weight and also the hexosamine content of the same dry weight. This was expressed as a collagen:hexosamine ratio. When the waterlogging occurs in the cultivation in synthetic media, an under-production of collagen and an over-production of hexosamine is calculated. Owing to the lack of ossification in such defined media (Reynolds, 1966; see also chapter on Osteogenesis in vitro), an abnormally large proportion of dry weight would be due to the hexosamine content. The effect of the hydrocortisone was to increase the relative collagen content of the explants. Although this raised the ratio of collagen to hexosamine, the ratio does not approach that observed for bones in ovo or cultivated on a plasma clot with embryo extract at any concentration of hydrocortisone tested. Here again is noted an advantage of the natural medium for cultivation. The under-production of

collagen in bones cultivated on synthetic media was also reported for chick tibia cultivated on media in which ascorbic acid was omitted (Jeffrey and Martin, cited by Reynolds, 1966). This effect of the vitamin C deficiency was confirmed by Reynolds, who observed that increased collagen synthesis occurred in seven day embryonic chick bones during culture in medium containing vitamin C, unlike those in the non-ascorbic medium. Only the rudiment in this vitamin C deficient medium became hydrated, whereas the other showed more ossified tissue and normal metaphase.

This new interpretation of the hydration phenomenon is regarded as significant since the culture medium BGJ used by Biggers and also by Schryver does not contain ascorbic acid. In the light of this recent information, the events in hydration is interpreted as follows (Reynolds, 1966):

A leakage of hydrolases is known to occur in chick bone rudiments cultivated in chemically defined media. Since vitamin C is essential for collagen synthesis, the osteoid formation which is generally observed to be low in chemically defined media, is inhibited in media BGH. There is noted an under-production of collagen at the onset of hydration. Under such deficient culture conditions, the leakage of hydrolases may cause a breakdown of collagen precursors rather than collagen incorporated in the matrix previously in ovo or in media with vitamin C. Hydrocortisone is known to stabilize cell membranes (and organelle membranes) and, hence, to inhibit the liberation of hydrolases. Since hydrocortisone under similar conditions increases the proportion of the total hydroxyproline-containing material in the rudiments and also the fact that this steroid

also inhibits excessive uptake of water suggested that the hydration of rudiments cultivated without the steroid may be correlated with either an inability to form collagen from precursors, or a lack of precursors. Any factor that influences the collagen: hexosamine ratio is likely to be important in the hydration phenomenon.

These investigations concerning the viability and therefore the significance of the experimental use of bone cultures in vitro have furthermore shown the underlying basis in the reliance of the matrix on its constituent cells.

It appears that the views expressed are at variance on the distribution of the excess water during matrix breakdown. One view holds that the diaphyseal cells and matrix become waterlogged (excessive hypertrophy and loss of metachromasia) whereas, the other, that it is predominantly initiated by the abnormally formed matrix.

(iv) Size of explant in relation to volume of medium

The size of the explant should be small enough to allow efficient diffusion of nutrients, gases and metabolic wastes. Franks (1963) suggested that the greatest diameter of the explant would fall in the range of 0.8 mm and 2.0 mm. Gaillard (1963) states that "to the actual optimal size of the fragment, it is considered to differ with the type of tissue". In the bone rudiment experiments, a six to seven day chick femur is approximately 2 mm in size and is observed to have a maximum growth of 4-5 mm after thirteen days in culture (Fell and Mellanby, 1955; cited by Biggers, 1965). The relevant point here is that considerable success has been reported with this material in organ culture. It is important to observe

the oft-mentioned fact that such discussions often depend on the efficiency of the diffusion system. In the plasma clot methods, this is often hampered. In the floating lens paper technique, the principle of keeping the explant at the gas:medium interface is one in which favorable diffusion could be maintained.

For the present study, femors were cultivated on liquid natural media for a short duration (discussed in Preliminary Studies and Methods and Materials).

Osteogenesis in vitro:

The earliest study of ossification in vitro (Fell, 1925) showed that calcification of osteoid tissue occurs only very slowly. Later studies chiefly with the chick long bone rudiments (review by Bigger, 1965) indicated that chondrogenesis and osteogenesis are closely interrelated in vivo and in vitro. The in vitro environment is an unnatural one and studies (Basset, 1964; Shaw and Basset, 1964, 1967) on the environmental and cellular factors affecting osteogenesis and chondrogenesis have shown how cellular activity and slight changes in oxygen supply and tensions could be stimulatory to different tissues of the bone rudiment. Their emphasis on slight environmental changes is evident by the unpredictable frequency in which osteogenesis and chondrogenesis occur in culture conditions.

In Basset's study (1964) an undefined population of cells was isolated from an outgrowth of a bone explant. These cells were subjected to various culture conditions so that the effects of tensions, oxygen concentration and media could be deduced from the histological structure

of the reformed tissue. His results stressed the interrelationship between the environment and the group of cells. A compaction of cells and a low oxygen tension would invariably produce cartilage whereas bone required a high oxygen concentration. Furthermore, if the compaction of cells were reduced under the same high oxygen concentration, a fibrous-like tissue resulted.

Besides the gaseous requirements for osteogenesis in vitro, the culture medial requirements has been the object of numerous studies (see also section on Culture Medium). The classical biological media of Fell utilized plasma and embryo extract in the ratio three is to one. More recently, Endo (1960) and Ito et al (1963), performed detailed studies on the histological and chemical development of chick embryonic femora cultivated on natural media. The intention of these studies was to "define" a natural media that would allow normal ossification to proceed physiologically.

This involved the preparation of various media containing Geys balanced salt solution (GS), chick embryo extract (CEE) and horse serum (HS). A control medium CEE:HS:GS in the ratio 1:2:7 was compared with the various constituents separately, in different ratios and concentrations.

From the experiments with either HS or CEE alone, the following conclusions were drawn: Growth in length of long bones was significantly accelerated in cell concentrations of HS over ten percent. The periosteum though often degenerated, so that no development of perichondral ossification resulted. As the same time, the cartilage cells showed healthy proliferation and hypertrophy. Chemically, the maximum values

of dry weight and hydroxyproline were low despite significantly promoted elongation. Furthermore, the amount of phosphorous was rather higher than that expected in view of the fact that there was an almost entire loss of periosteum where normal calcification would occur. The necessity for histological examination to complement the chemical determinations is shown in this study, since the von Kossa silver staining indicated that calcification was detected in the cartilage matrix which is abnormal for the embryonic chick long bones (Endo, 1960). Hanks (1949) has previously shown that calcium phosphate could precipitate in cell-free mixtures of serum and embryo extract. This calcification was closely associated with cells when present.

When cultivated in different dilutions of CEE, the elongation of the femur was inhibited in a thirty percent and in a twenty percent solution. Successful elongation was obtained in a ten percent CEE solution, although it was less vigorous than that of the controls. The dry weight, amount of phosphorous and the amount of hydroxyproline of the bones, however, were all far less than those of the control bones. This indicated that CEE alone was insufficient for chemical development of the bone even though it allowed at least tissue maintenance.

Finally, when cultivated in media containing ten percent CEE and varying concentrations of HS, the bones presented systematic fluctuations in their development. A medium containing CEE:HS:GS in the ratio 1:5:4 was observed to be optimal for chemical development that closely corresponded to their histological findings. The ossification in this case proceeded almost physiologically though much more slowly than in ovo.

Besides these environmental problems concerning osteogenesis in vitro, there are theoretical views concerning the calcification process in vitro (Henrichsen, 1956, 1958). Alkaline phosphatase has been linked with the normal calcification process in vivo (reviewed by Weidmann, 1963), but Henrichsen has presented evidences to support an hypothesis that alkaline phosphatase in vitro is a sign of degeneration and that calcification in tissue culture is thus a calcification of necrotic tissue. Various evidences were obtained from tuberculous lymph nodes, cartilage, stroma of rectal adenocarcinoma and kidney tubules in which alkaline phosphatase activity and calcification occur in vivo. In vitro, the enzyme occurs in fibroblasts on degeneration followed by calcification (Henrichsen, 1956). It is also known that both embryonic mammalian cartilage and embryonic avian cartilage contain alkaline phosphatase in the hypertrophied cartilage cells, but that only the mammalian cartilage calcifies in the embryonic stages. Avian cartilage normally calcifies later on in life after the first signs of marrow cavity formation (Fell, 1925). The concept, based chiefly on cartilage studies, points out the possibility that alkaline phosphatase might be an expression of degeneration in hypertrophied chick cartilage cells, whereas the presence of the enzyme in

osteoblasts is conceived of as an expression of a differentiation. Henrichsen does, however, concede that this question of nomenclature is purely semantic and that one can argue against the use of the word degeneration for a state which is a normal stage in the life of each cell from its formation from mitosis to the death of the cell or inherent obsolescence (discussed in section on Cell Details). The previous investigations of the calcification process in vitro, its possible theoretical significance and the fact that unphysiological calcification can occur, all stress that the calcification of cartilage is unsuited for this purpose. However, in the present study, calcification of cartilage does not occur during the embryonic stages in vitro neither does it occur in ovo. The earliest detectable normal calcification of the periosteum of chick femora is on the ninth day (Ito et al, 1963). The present in vitro cultivation of six to six and a half day old embryo femora was terminated at the chronological age (passage of time disregarding biological events) equivalent to a nine day embryo. The chief events studied involve chondrogenesis and perichondrial osteogenesis up to the formation of a calcifiable matrix (osteoid).

It is relevant at this point to affirm that though no calcification is detected in the osteoid, it does not mean that calcification has not commenced. Electron micrographs (Robinson and Watson, 1955) have shown that the earliest demonstrable calcification in osteoid time consists of crystals in the order of twenty to fifty angstrom units. As the light microscope will only show particles larger than 0.2 micra, that is, about a hundred times larger than the particles demonstrated in the commencing calcification of osteoid tissue, the negative von Kossa silver

staining does not preclude the formation of the initial crystal apatites. This is the first step in the calcification process (Cabrini, 1961; Henrichsen, 1958) in which calcium phosphate crystal apatite is formed in relation to the matrix when the phosphate ions become concentrated locally as a result of alkaline phosphatase activity. The second step of secondary crystal growth does not necessarily require the local hydrolytic splitting of ester-bound phosphate. Many experimental studies in vivo on skeletal tissue and other ossifiable tissue report findings based generally on negative stain reactions for calcification. Calcification is but one of a series of integrated steps in the highly organized process of bone formation or ossification (Weidman, 1963). In the embryonic long bone rudiment, the essential events leading to a calcifiable matrix or osteoid can be examined in vivo and also in vitro.

The metabolism and pharmacology of thalidomide:

The metabolic structure responsible for the teratogenic effect of thalidomide is not yet known (hypotheses reviewed by Cahen, 1966). The action of a drug observed in an animal is the resultant of a complex of drug transference processes (distribution, metabolism or biotransformation) which decides its effective concentration and form in a particular biophase (Van Os et al, 1964). Classifications of similar thalidomide studies under species, strains, doses and days, routes of administration have shown inconsistent results obtained with these variances so that data obtained from particular whole animals are considered (Ariens, 1964) to be too complex to be of use to interpret modes of action. The differences in toxicity of thalidomide has been ascribed to differences in metabolism (Cahen, 1966).

In order to emphasize the numerous metabolites of thalidomide it can be pointed out that it is a raceme (Schumacher et al, 1965a) and as such is a product made up of two enantiomorphous isomers and, therefore, is optically inactive. Consequently, some of its breakdown products will contain amino acid residues of the unnatural D-series. Any of its biological effects (embryotoxic, neurotoxic and sedative) could be due to the drug or its metabolites interfering with the normal metabolism of L-glutamine and L-glutamic acid. These amino acids are prominently involved in metabolism (Fabro et al, 1965).

Studies (Schumacher et al, 1965a) have reported that thalidomide was unstable in aqueous solutions at physiological pH values. The half life of the spontaneously-hydrolysed primary products was reduced as the pH was increased. All the substituted amide bonds of the molecule are sensitive to hydrolysis and at pH 7.4, twelve possible hydrolysis products are formed by splitting of these groups. Estimations of metabolic breakdown products in vivo from blood, urine and other samples are further complicated by the fact that the amide bonds present in the phthalimide and glutarimide rings of thalidomide exhibit slightly different pH sensitivities as regards to hydrolysis. This suggested (Schumacher et al, 1965b) that species differences in respect to the amounts of the hydrolysis products excreted may at least be partly related to differences in pH environment encountered by the drug in vivo. Other facts as would include time after dosing, tissue binding and the occurrence of hydrolases (in the gut or tissues) which may assist in the spontaneous hydrolysis (Ariens, 1964). This is exemplified in the following study. In the plasma and brain in rats, thalidomide, two primary and two secondary

hydrolysis products were identified. In the brain, the intact thalidomide appeared to be the main product. This is consistent with theoretical expectations, since the hydrolysis products of this raceme are carboxylic acids (which could be expected to be ionized at physiological pH values) and are consequently polar. These polar molecules are unlikely to cross the blood-brain barrier as readily as the lipophilic thalidomide. The hydrolysis products found in the brain are thought to be formed in situ from thalidomide by spontaneous hydrolysis.

Of the twelve possible hydrolysis products of this raceme, eleven can occur in (+) and (-) forms. The number of possible optically active products, including the (+) and (-) thalidomide is thus twenty-four. Boylen et al (1964) reported that 3- and 4- hydroxythalidomides produce abnormalities in chicks. This introduced another possibility that hydroxythalidomides could be teratogenic anabolites of thalidomide. If thalidomide is hydroxylated in vivo, this occurs in the 3- or 4- carbon positions, of the aromatic ring and possibly in three different positions (namely 3, 4 and 5) in the glutarimide ring. Disregarding hydroxylation of the glutarimide ring and considering only the hydroxylation of the aromatic ring, there are twelve 3-hydroxy and twelve 4-hydroxy metabolites possible, and twenty-two of these can occur in (+) and (-) forms, the two compounds not containing an optically active carbon atom being 3- and 4-hydroxyphthalic acids.

The number of possible metabolites of thalidomide is, therefore, very large for if any racemic metabolite underwent resolution the possible number of metabolites could be well over one hundred.

Besides the metabolites of thalidomide the intact form has also been extracted from the blood (15 minutes), brain (1 hour) in the urine and faeces (24 hours) in many species. Furthermore, since it has been isolated from foetuses and blastocysts, it is generally labelled as a latent carrier or even the active agent itself, because in this way all the possible metabolites are "smuggled" into the embryo, whereas they would not be able to penetrate this far if they were administered as such (Keberle et al, 1965).

Finally, because of the complex situation in vivo, efforts to interpret modes of action of a chemical substance are supplementarily studied with isolated organs or cells, microsomes, mitochondria or enzymes (Ariens, 1964). This information may give indications of the action of the drug in the living animal, but because its complicated drug transference is excluded, the information may not suffice to permit definite predictions of its action. The drug metabolism which usually leads to decreased toxicity by restricting the polarized metabolites to the extracellular fluid may also lead to intermediate bioactivation or increased toxicity. In this case, new pharmacological actions are introduced into its spectrum of activity (Ariens and Simonis, 1964). However, in view of the fact that intact thalidomide has been detected in the foetus and blastocysts and that it is capable of spontaneous hydrolysis, the above precaution would not stultify such a study. The intact substance could be directly applied to isolated organs under physiological conditions and the structural responses described.

III. MATERIALS AND METHODS

The Explant:

White Leghorn eggs (inbred at the University of Manitoba) were allowed to develop to 6-6½ days (stages 28-30 Hamburger and Hamilton, 1951) in a commercial incubator at between 37-38°C. Adequate aeration and humidification was roughly maintained. The limit of accuracy by this morphological aging (webbing and digit formations, relative lengths of visceral arches, feather germ distribution, number of scleral papillae and egg tooth appearance) is within half a day.

The femur which is the stoutest element during the chondral stage is the last of the long bone rudiments in the appendage to commence histodifferentiation. At the stage of explantation, chondrification has already begun (at 5.5 days) and the first layer of periosteal bone is in a stage of formation (details from Romanof, 1960). Figure 1 p.50 shows this rudiment in vivo, showing these characteristics and the extent of the separation of the femur from the pelvis by a dense cellular interzonal region at the presumptive articulation which normally begins on the 5th day. At this stage, a mesenchymal mass on the anterior side of the region of the junction between the femur and tibia has only given rise to a ligament which is known to appear on the 5th day and in which collagenous fibres are discernible at 6.5 days (See Fig. 1).

The patella, its other derivative, is distinguishable as an early condensation of mesenchyme (with many mitotic figures) and no articular cavitation is visible. These characteristics appear on the 8th day. Histological characteristics of the femur as originally described by Fell (1925), are seen at this stage. The hypertrophic cellular zone occupies one third of the total length of the long bone rudiment. The

Fig. 1 Histological characteristics of the chick femur at the time of explantation, seen in ovo. Toluidine blue O.X 16.

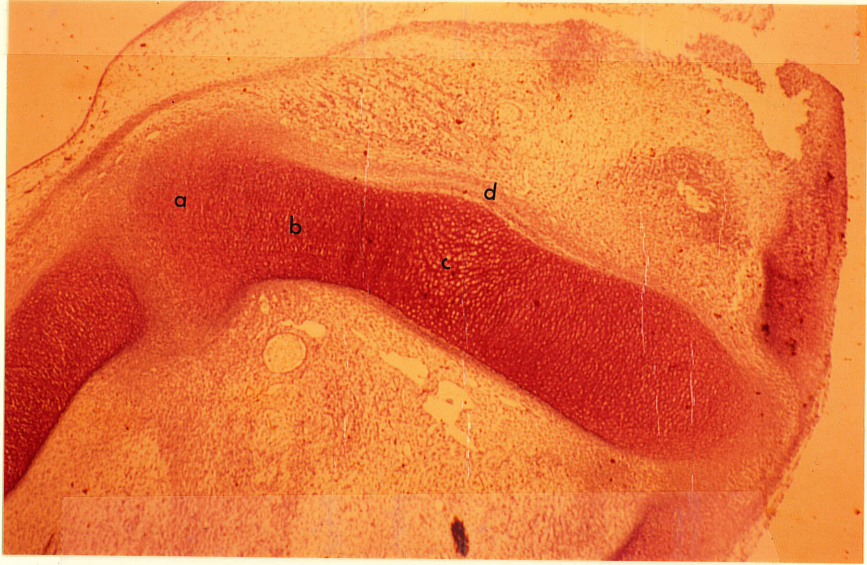
a = Round cell zone in the epiphysis.

b = Flat cell zone.

c = Hypertropic cell zone.

d = Fibrous and osteogenic perichondrium with a single layer of osteoid (negatively metachromatic) adjacent to the hypertropic cell zone.

Note the strong beta-metachromatic reaction of the cartilage matrix. Concentrations of mesenchymal cells are noted in the future joint regions.



①

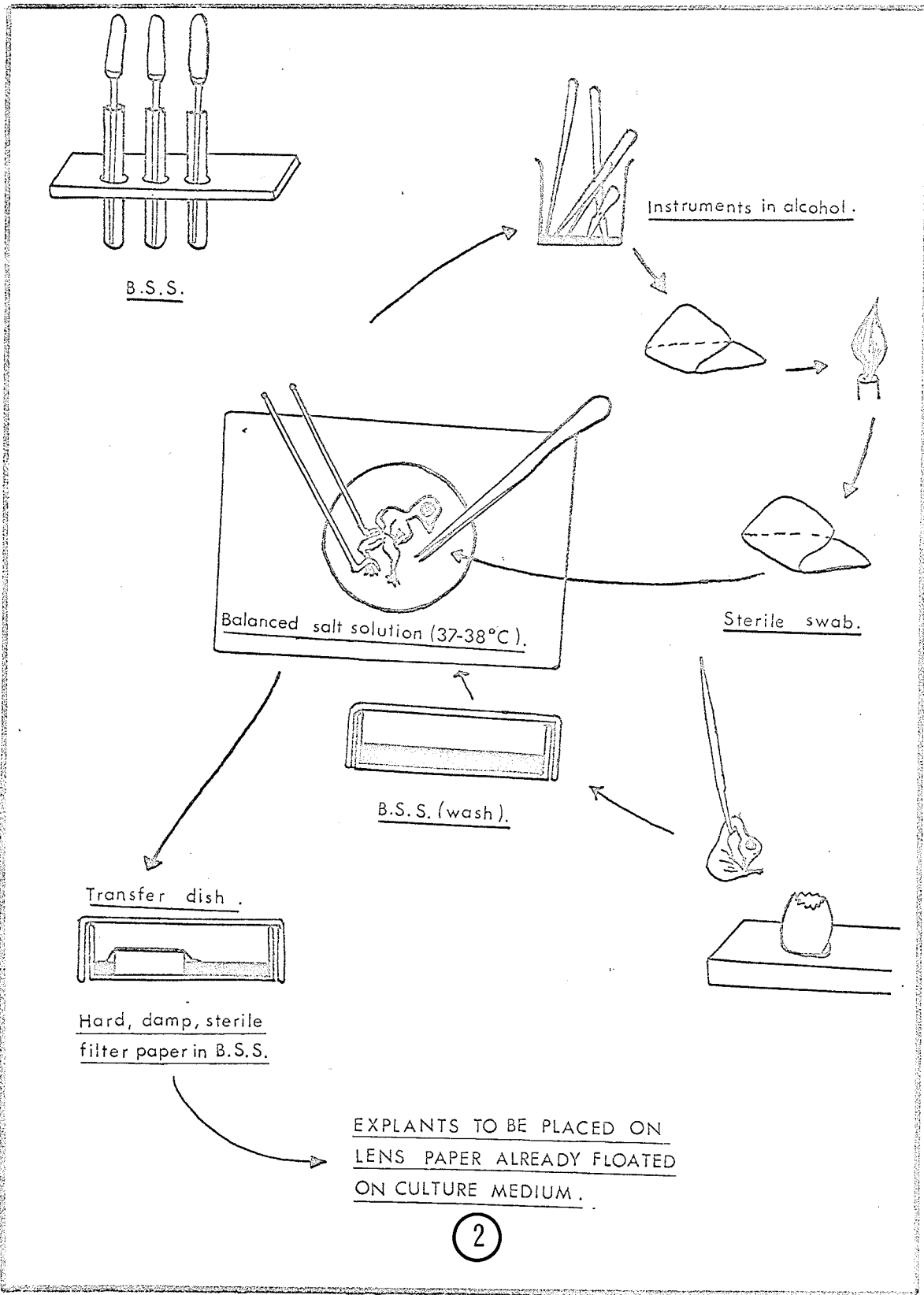
cellular hypertrophy is accompanied by changes elsewhere, namely; the first signs of ossification appear beneath the perichondrium surrounding the hypertrophic zone. This is seen as a very thin sleeve of osteoid between these regions which stains negatively metachromatic with toluidine blue and is also strongly eosinophilic. In the explantation procedure (Fig. 2 p.52), the femora at this stage were aseptically dissected out in balanced salt solution under a dissecting microscope. After removing as much muscle tissue as possible, the explant was gently rolled on a hard sterile filter paper moistened with balanced salt solution to remove any adherent soft tissue. This procedure, when followed, was not excessively prolonged, since previous histological studies showed poor perichondral development in some cases. The femora were kept in pairs as they were dissected from the embryos and systematically arranged on the floated lens papers so that they can be easily identified. In order to allow for variation between embryos, the femur from one side of the embryo was cultivated in one medium (experimental), and that from the opposite side of the same embryo in another medium (control).

In the preliminary studies, a total of 82 explantations were performed, whereas in the main study 48 explantations were performed (that is, 12 explants per group).

Culture Medium:

The culture medium consisted of a 6-6½ day chicken embryo extract (10%), heat inactivated calf serum (30%) and Hanks balanced salt solution in the ratio CEE:CS:HBSS, 1:5:4, (discussed in Chapter on Culture Medium).

Fig. 2 A diagrammatic representation of the explantation procedure.



The balanced salt solution contained phenyl red indicator at non-toxic levels and the pH was maintained at 7.2 to 7.6, using an isotonic, sterile sodium bicarbonate solution (1.4%). Gassing (5% carbon dioxide in air) also help maintain a physiological pH range.

The CEE was prepared, as described by Paul (1960), by homogenizing embryos of the same developmental stage under aseptic conditions (See Fig. 3). The 50% CEE was centrifuged at 3000 r.p.m. for 15 minutes and the supernatant fluid was used without delay. This 50% solution was diluted with HBSS to a 10% solution. All cellular material was finally removed from the supernatant by Swinney millipore filtration (pore size 0.45 micra). In the preliminary experiments, in which numerous sub-cultivations were performed, the CEE was prepared on the same day from embryos of corresponding age to the tissue under cultivation.

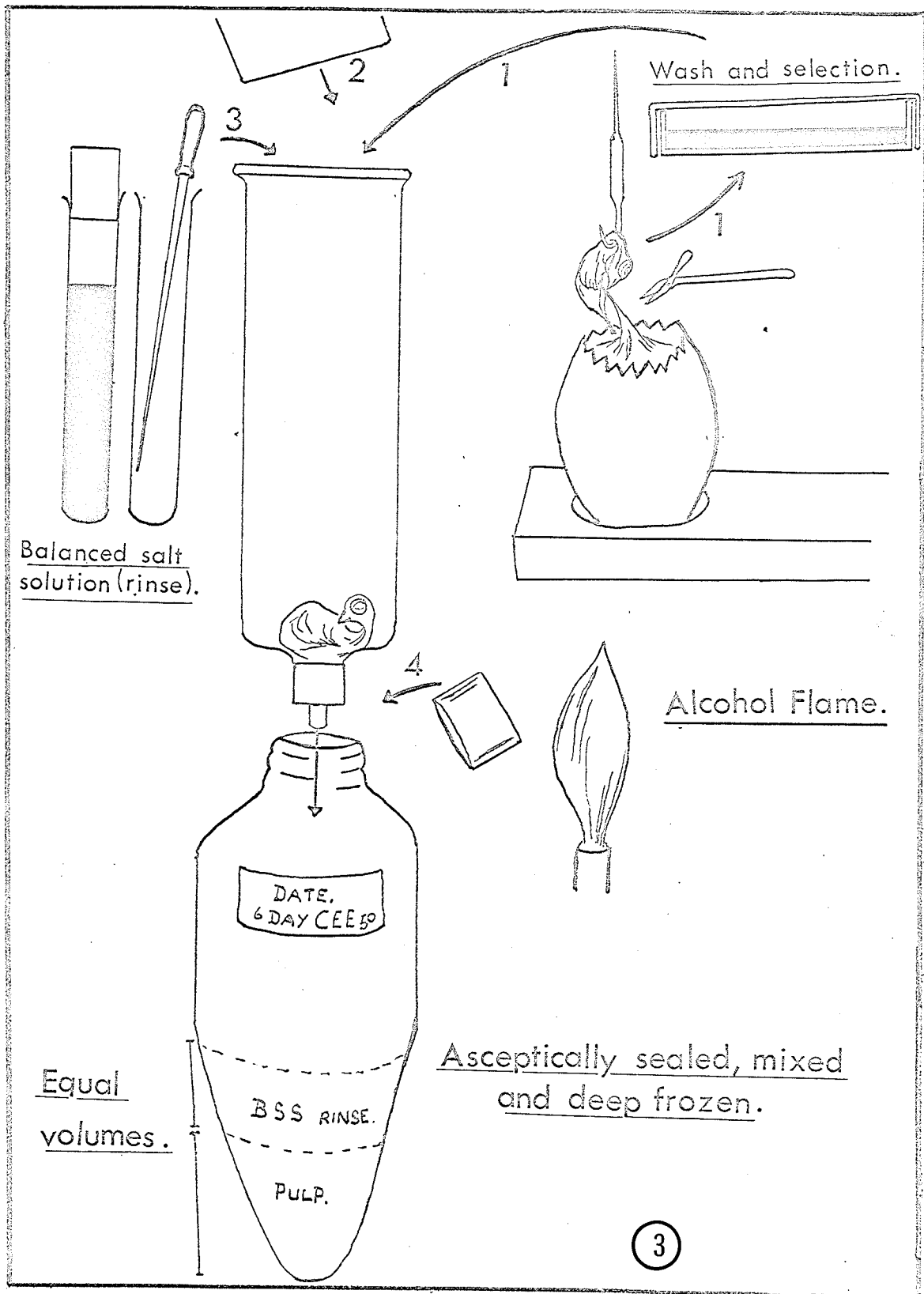
The calf serum was sterilized by means of a Seitz filter and deep frozen. Precaution against toxicity of mammalian sera, which is known to diminish at 4°C. (Parker, 1964), was ensured by heat inactivation in a waterbath at 55°C. for half an hour. Dilution of the serum to 30% was done with Hanks balanced salt solution.

As a precaution against bacterial contamination, penicillin (100 Iu per millilitre) was added to the filtered media.

Culture Technique:

The explants were cultivated at the interface of a liquid medium and gas (5% carbon dioxide in air) according to the floating lens paper technique for organotypic cultures (Chen, 1954). Small rectangles

Fig. 3 Procedure for the preparation of an embryo extract.



of sterile, siliconised lens paper were floated on the media. One rectangle per coded dish. The first of the three femora placed on the float was indicated by a cut edge of the rectangular lens paper. Ten millilitres had been measured into each dish as it was being sterilized through a Swinney millipore filter. Since sub-cultivation (Fig. 4 p.56) was performed every second day, each rudiment would have a provisional volume of medium of about one and a half millilitres per day. This volume would fall within the critical limits for this type of organ culture, as discussed by Fell (1963) and Gaillard (1963). Incubation temperatures was at 37.5°C.

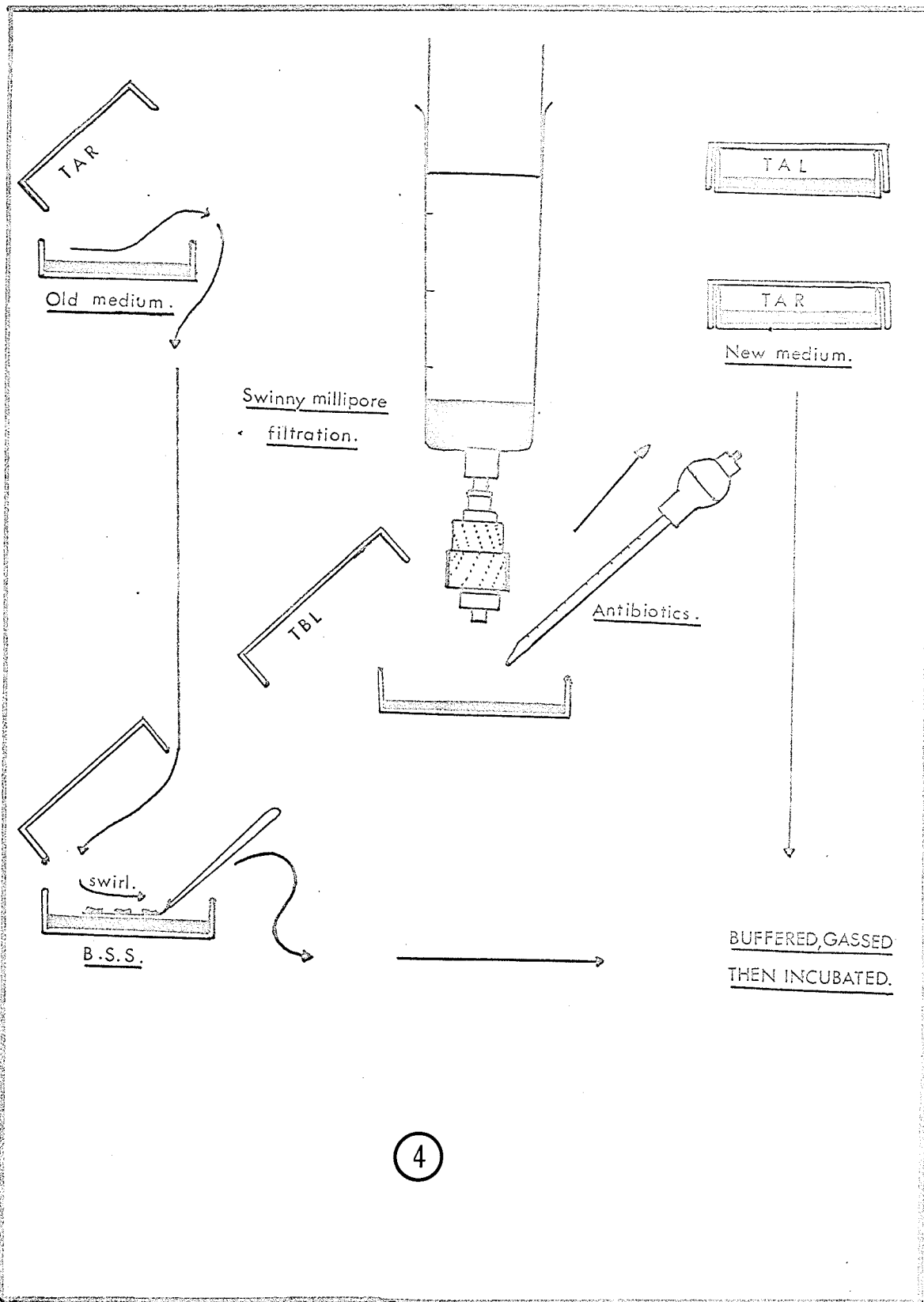
Histological Procedures:

Since in some of the studies, the degree of calcification was to be examined, the explants were fixed in absolute alcohol and later chiefly in Carnoy II (Curran, 1964). The use of these relatively non-aqueous fixatives reduces the small loss of calcium phosphate prevalent in aqueous fixatives. Carnoy fixation generally gave a much clearer picture of the cells. After 1½-2 hour fixation and two one-hour changes in absolute alcohol, the undecalcified explants were embedded in paraffin wax (M.P. 58°C.) and were all serially sectioned at five micra.

Histological details were always examined with Mayer's haematoxylin and eosin, whereas intercellular characteristics were observed by sections stained with toluidine blue O and Masson trichrome in cases. Calcium phosphate deposits, when examined for, were detected by the von Kossa silver reaction, together with a safranin O counterstain.

In the toluidine blue method, loss of metachromasia was minimised by observing a number of precautions. A compromise from a number

Fig. 4 Filtration of a culture medium and subculturing
 procedure.



of suggestions (Schubert and Hammerman, 1955; Lillie, 1965) was used and the critical dehydration process was done under microscopical observation. The final procedure decided upon for the main study was a prolonged staining (1 minute) with a diluter solution (0.1 percent toluidine blue O in 30 percent ethanol). After removal of excess dye in double distilled water, it was passed through 95% alcohol (2½ minutes) into two changes of acetone to complete dehydration without loss of significant metachromasia. The sections were finally mounted in permount.

Dosages of Thalidomide and Colchicine:

A selection of a concentration of a substance that corresponds to the top of the linear part of the sigmoid dose-response curve (when such data are available) implies that even a slight diminution of activity can be expected to lead to considerable influences on histological structures. Such a preference was made in a bone-organ culture study (Gaillard, 1965) with a hormone. The doses of thalidomide used in the in ovo studies with chick ranged from 0.2 to 500 milligrams per kilogram, and defects were inconsistently observed throughout this range (reviewed by Cahen, 1966). Kemper (1962) reported no relationship between the dose and 20% abnormalities observed. Furthermore, the only encountered report of an organ culture study of bone (Franceschini and Mussa, 1966) utilized a single large dose (3,500 micrograms) in an 8-day culture in a chemically defined media. The preliminary report included a disappearance of cartilage matrix among other degradative processes. In accordance with the results obtained in the preliminary studies (Chapter III) in which a wide range of doses were tested, it was decided to use four low doses, namely; 10, 20, 30 and 40 micrograms per millilitre. According to information received from the Stolberg-Chemie Gruenenthal (Salzgeber and Wolff, 1964)

the concentration of a saturated solution of thalidomide in distilled water at 37°C. is 106 micrograms per millilitre. The weighed out samples of thalidomide (1 mg, 2 mg, 3mg and 4mg) were well ground and dry sterilized at 100°C. before being incubated as a suspension in a phosphate buffered saline (5 ml.) an hour before 0.5 ml aliquots were added to the 10 ml culture media. The suspension of thalidomide was frequently shaken during its incubation. It has been shown (Schumacher et al, 1965a) that a buffered solution of thalidomide at pH 7 to 8 will contain within a period of 24 hours, thalidomide plus all its primary and secondary hydrolysis products. In a following paper (Schumacher et all, 1965b), it was confirmed that this situation also obtains in the tissues, blood and urine of animals dosed with thalidomide. The doses added to the culture media are comparable and fall in the range of doses used in the production of anomalies in ovo, example 2 mg per kilogram (Boylen et al, 1963), 0.3 mg per kilogram (Salzgeber and Salaun, 1963), and 0.2 ml of a 10-20 mg per ml suspension (Jurand, 1966).

The dose of colchicine (discussed in Chapter on Preliminary Studies) used to arrest mitosis was an aliquot (0.5 ml dissolved in phosphate buffered saline at 37°C.) per 10 ml culture medium.

Analysis and Statistical Treatment:

Estimations of Mitotic Index (MI), Pycnotic Index (PI) and Density.

Mitotic activity and density were calculated in cartilage cell zones of each explant and the presence or absence of mitosis was recorded for the fibrous perichondrium and osteogenic perichondrium (Figs. 26a & b).

Each experimental group for each concentration tested consisted of six pairs of explants (six left femora were treated whereas the right

femora were controls). For each zone of transformation of each explant, the average density of cells, mitotic index and pycnotic index were estimated by counting nuclei with a hand tally counter and a binocular light microscope fitted with an eyepiece graticule. In the mitotic counts observed under high power (450X), prophases were included with the interphase percentage. The constant random square areas were systematically counted for each explant in an epiphyseal-diaphyseal direction. In the hypertrophic cell zone, the most central area was not counted and, in this zone, counts were made as close to the transitional zone as possible.

To avoid recounting the same cell in sections, the nuclei observed in every fourth serial section was counted. After counting the total number of nuclei per square area, the same area was scanned for colchicine metaphases and pycnotic cells. For each explant, the average density of a transformation zone was based on five individual values and expressed as the number of cells per square area of the zone. The number of colchicine metaphases and pycnotic cells were expressed as a percentage of the total number of cells counted, namely; the mitotic index and pycnotic index for the zone in each explant. In each zone of each explant, the values obtained were compared with that of the corresponding zones of the control explant.

Therefore, for each transformation zone of each explant, the mitotic index (MI) represents the minimum number of cells that have entered mitosis during an eight hour period of colchicine exposure after two and a half days pretreatment in vitro with or without thalidomide. The pycnotic index (PI) represents the number of pycnotic cells observed

in the same field and, therefore, the same total number of cells in which the MI was calculated.

The density represents the average number of cells (interphase, colchicine metaphase and pycnotic cells) per square area. It reflects the previous proliferation in the zone up to the time the MI and PI were calculated. Information concerning the relative amount of intercellular substance could also be obtained from the density data.

Statistical Treatment:

The responses (MI, PI and Density) observed in the treated and control groups were compared as follows: The mean sample values of left femora (treated) were compared with the mean sample values of the right femora (controls). The significance for the difference of these values were tested by the Student t-test for paired samples where allowances have been made for small sample numbers.

The significance of the differences of the non-parametric data obtained from matrix characteristics (positive or negative metachromasia) and absence or presence of mitotic figures in the fibrous and osteogenic perichondrium were estimated by the sign test for paired data (Goldstein, 1964).

IV. PRELIMINARY STUDIES

PRELIMINARY STUDY 1. Normal in Ovo Histological Features of the Femur at the Same Chronological Ages of the Cultivated Explants in Vitro.

The normal in ovo histodifferentiation of chick long bones has been described by Fell (1925) and others (reviewed by Romanof, 1960). More recent interpretations of these histological features have been described by Henrichsen (1958).

The histogenesis corresponding to Hamilton and Hamburger aging of chick embryos (1951) at incubation temperatures of 37-38°C. was confirmed and the particular histological details corresponding to the time of explantation of the femora and at the time of fixation of the cultivated explants are described. The general histological details and also some particular characteristics have been described under Chapters on Materials and Methods, and also in sections on Cellular Details and Matrix Characteristics.

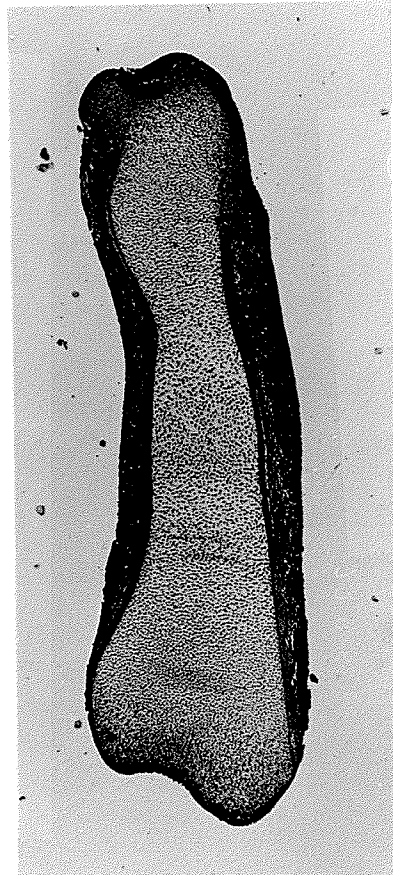
In the femur, as in other long bones, although the epiphyses are formed at both extremities, growth in length takes place mainly at one end called the "growing end", (Le Gros Clark, 1965). In the lower limb the growing end is nearer the knee joint region. The femur grows in length mainly from its condylar end. According to Lecroix (1951), this localization of the growing end is closely associated with the formation of joints and especially the attachment of ligaments. Figure 5 show the condylar and head ends of the femur indicating the distinguishing characteristics in the round cell and flat cell zones at both ends. In contrast to the head end, the condylar end, or growing end, show numerous cells which are uniform in shape in both the epiphysis and epiphyseal plate (metaphysis). Furthermore, the two cell zones in the growing end are more or less sharply distinguished, whereas the head

end show fewer epiphyseal round cells into which flat cells extend centrally and irregularly. Since cell density was one criterion to be used in the major study of cell densities, control calculations were made for both ends of the femur (Table I). The distal (condylar) end of the femora showed a greater density and a distinction in cellular transformation zones. It is evident, as is known, that the condylar regions showed a greater density of cells in all the transformation zones and also the percentage mitotic figures in the same areas were greater. The condylar region at an earlier developmental stage corresponding to the day of explantation (that is, 6-6½ days), showed a greater density and percentage mitotic figures than at the later stage (stage 35, 8½-9 days). This observation is consistent with the fact that there is a greater overall percentage increase in limb size at earlier stages of development (Romanof, 1960). It is also consistent with the mathematical models of Huxley's allometric or relative growth which is also based on the exponential decay of specific growth rate of organs with increasing age (Reeve and Huxley, 1945).

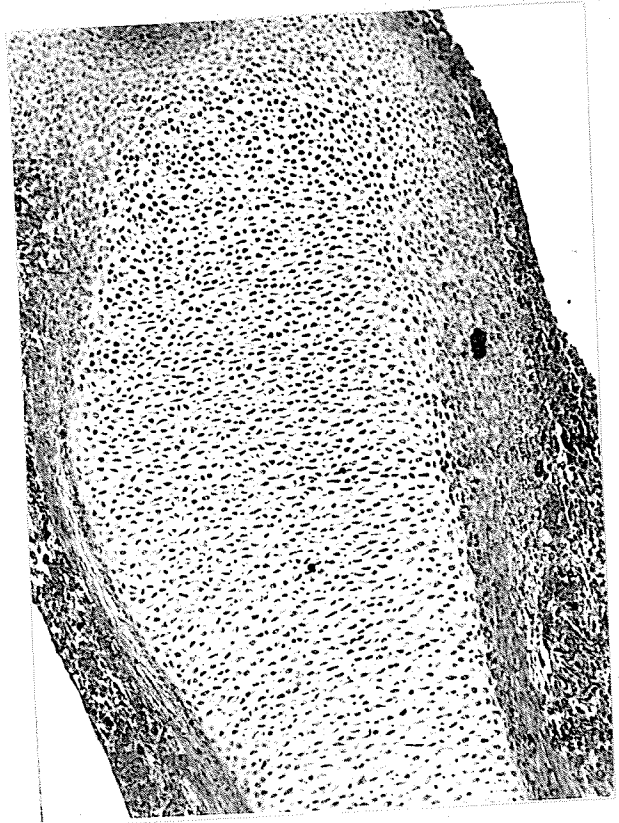
Recommendations

1. The stages described were the earliest stages in which all the zones of a typical bone rudiment are first observed and will be used in the ensuing *in vitro* studies.
2. The condylar ends of the femora showed the greater density and proliferative activity and was more distinctive. Data obtained from this growing end will be compared.

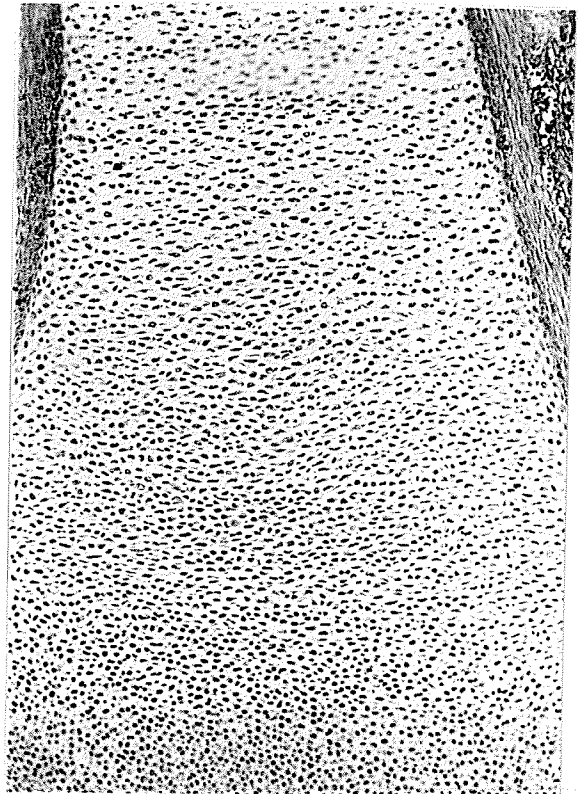
- Fig. 5 The proximal (head) end and distal (condylar) ends
of an explant.
- a. Head end. X 40.
 - b. Condylar end. Note the greater cellular density
and sharper distinction between the
two transformation zones. X 40.



5



a



b

TABLE 1

CELLULAR DENSITIES AND PERCENTAGE MITOTIC FIGURES AT
THE TWO ENDS OF A FEMUR IN OVO AT THE TIME EQUIVALENT
TO THE DAY OF FIXATION OF CULTURED EXPLANTS.

Number of Embryos	Developmental Stages	Region of Femur	Zone	Average Density	Mitotic Figures*
			Round Cell Zone	236.0 + -3.8	0.5085
		Head	Flat Cell Zone	141.0 + -3.7	0.7072
4	Stage 35 (8½-9 days)		Hyper- trophic Cell Zone	67.8 + -1.4	0.0
			Round Cell Zone	264.3 (278.0)** + -4.9	0.9079 (0.9353)
		Condylar	Flat Cell Zone	174.3 (207.4) + -5.8	1.262 (1.543)
			Hyper- trophic Cell Zone	70.3 (95.0) + -3.0	0 (0)

* Represents minimal values especially in the Flat Cell Zone.

** Values in brackets are corresponding values obtained from 6½ day embryos. That is, at the time of explantation.

PRELIMINARY STUDY 2. The Selection of a Favourable Duration of Cultivation of the Femora in Natural Media.

Whether an experiment is to last for hours, days or weeks, it is necessary to ascertain that during the operative period the tissue is in a normal viable condition (Fell, 1963). Quantitative analysis (Biggers, 1965) on seven day old embryonic chick rudiments on different culture media suggest that the system becomes progressively unstable after five days in chemically defined media and somewhat later in natural media (discussed in sections on Culture Media and Matrix Characteristics). The criterion used in the reported studies was that a decline in the dry weight:wet weight ratio would indicate a hydration that follows the loss of dry weight.

The method of analysis used in this study using a special natural medium (described in Materials and Methods) was qualitative and relied on cellular details and matrix characteristics.

From the experimental design (Figure 6) it is observed that all the explants of the same biological age were subjected to the same cultivation procedures. Explants were successively fixed after every two days, that is, on the day of sub-cultivation in a replenished dynamic media (discussed in Materials and Methods). Finally, these explants were sectioned and stained and then compared with the six control explants cultivated for ten days. Common unviable histological responses (pycnosis and loss of metachromasia) were estimated from the controls and regarded as 100% unfavourable characteristics. Pycnosis was recorded for each of the cartilagenous zones and classified as nil, completely pycnotic, occasional or grouped, so that a number of indices

were formed. A high coincidence or correspondence of experimental explants (of shorter cultivation periods) with the unfavourable characteristics shown by the controls (taken as 100) cultivated over ten days was taken to indicate the relative unsuitability of the particular duration of cultivation.

Pycnosis which predominated in the round cell zone and the flat cell zone of the explants (Figure 7) was reduced at each successive shorter duration of cultivation (the amount observed after $2\frac{1}{2}$ days is observed in figures for preliminary study 4 and the main study).

Concerning matrix characteristics, a constant feature in all the explants was a positive beta metachromatic reaction with toluidine blue O. However, this reaction in some cases was not present throughout the whole rudiment that normally shows this reaction. Negative metachromatic reactions observed in superficial epiphyseal cartilage areas (Figures 7 and 8) was not always obtained in deeper pycnotic cartilagenous areas (Figure 7). In some of these areas, a fainter positive reaction was observed. This seemed to indicate that, though a negative metachromatic reaction was indicative of matrix disorganization, it is not always present in all pycnotic areas immediately, but a gradual intensity of this negative reaction was observed extending from a less pycnotic area to a more pycnotic area. This might indicate a time lag between the two events (Matrix disorganization following pycnosis) which is indefinite from these trials as the onset of pycnosis is unknown. Figure 9 shows an extreme example in which the peripheral loss of metachromasia is gradual and associated with pycnotic cells (probably a karyorrhetic type of pycnosis as the small chromatin

clumps are separated from the intact nuclear membranes). This figure also provides evidence that the necrosis is not initially central as it would occur if the diffusion system provided by the culture technique was inadequate.

These are relevant observations for the main study in which an effect is to be related to a particular substance in view of the fact that it is known that a disorganization of the matrix (negative metachromatic reaction) could also be due to an active enzymic action brought about by its experimental release from viable avian cartilage cells. A true loss of metachromasia (discussed under Matrix Characteristics) was probably observed, since it is known that Avian cartilage does not normally calcify at these stages, but is actively resorbed by invading vascular tissue. However, since abnormal calcification can occur in vitro (Endo, 1960), this possibility was eliminated by observing a negative von Kossa reaction for calcium phosphates. In a control at a later stage of development, the black deposits of calcium phosphates were localized in the periosteal region, whereas the cartilage matrix stained an orange metachromasia with the counterstain safranin O, (Figure 10). Furthermore, serial sections stained with the Masson Trichrome stain for connective tissue showed that the cartilagenous areas (the positive Beta metachromatic areas) stained very faintly green, but in areas that were not metachromatic (also areas of osteoid, fibrous and degenerative areas) the reaction was enhanced (Figure 11). There was a complementation of negative metachromatic areas with strong Masson trichrome reactions, indicating a failure in the reaction with the collagen-sulphated mucopolysaccharide-complex (connective tissue plus

ground substance) but a strong connective tissue (collagen) reaction respectively.

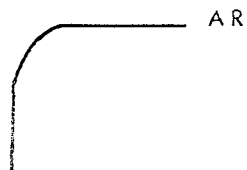
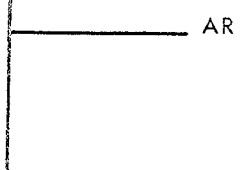
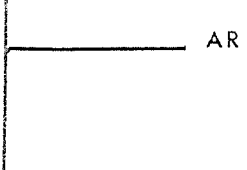
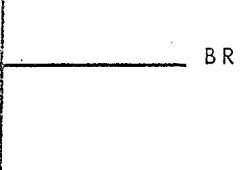

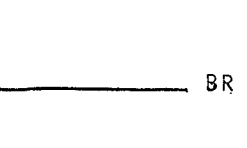
It was concluded that the matrix characteristics were maintained well over seven days in the natural media provided, though occasional pycnosis were observed in shorter periods. At about four to five days, there was a marked reduction in the number of pycnotic cells.

Recommendations

1. The shorter the duration of cultivation, the more normal the explant respond. In a shorter duration experiment, cell death associated with negative metachromatic reactions would still be equivocal but absence of pycnosis associated with negative metachromatic reactions would not be. Therefore, these two criteria could be informative if a duration could be selected in which cell death could not be attributed to duration of cultivation. Though the outside limit for the cultivation period appears to be 4-5 days, three days was decided upon since there is seldom much point in trying to maintain organ cultures for long periods because the explants usually react in a few days or weeks (in cases) to chemical agents, such as hormones, vitamins, carcinogens (Fell, 1963), and most synthetic processes are accomplished in a similar time.

2. An observation from histological studies of contaminated cultures revealed that groups of bacteria were associated with superficial pycnotic areas that gave a negative metachromatic reaction (Figure 8). This suggested that to ensure significant interpretations with these reactions, further precautions against contamination had to be taken.

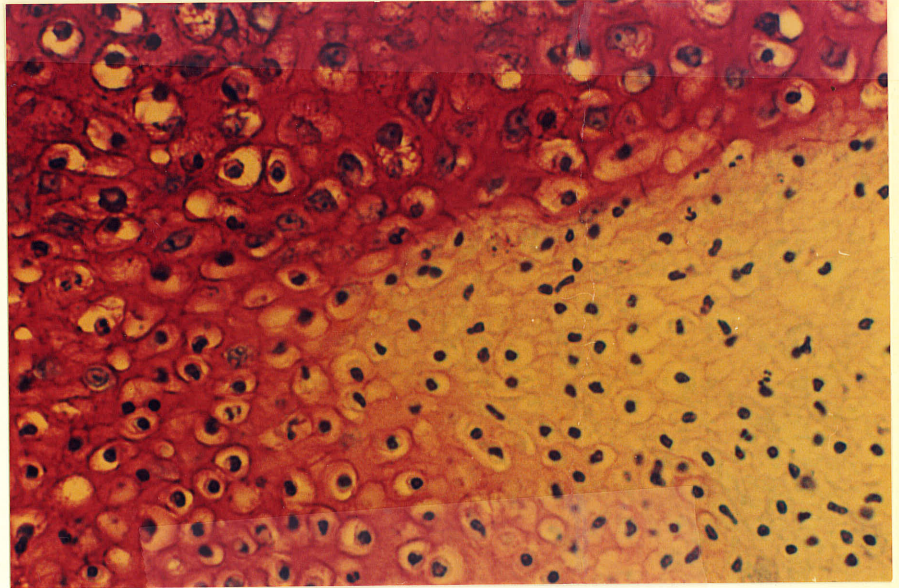
Fig. 6 Experimental design to study the histodifferential responses of parallel cultures maintained for various periods.

Embryo number	Left femora	Day of fixation	Right femora	Subcultivation in 'dynamic' media
1	AL →	0		7 day
2	AL →	2		9 day
3	AL →	4		11 day
4	BL →	6		13 day
5	BL →	8		15 day
6	BL →	10		—

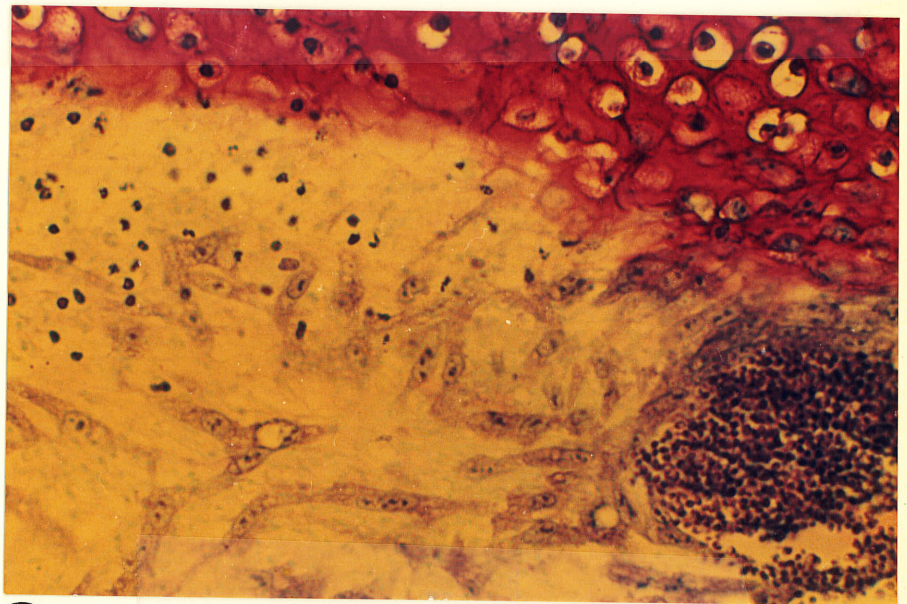
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Fig. 7 Pycnosis observed in the epiphysis after ten days cultivation in natural media. Note the loss of metachromasia of the matrix which extends initially from the predominantly pycnotic cartilage. A positive metachromatic reaction is still, however, evident in deeper cartilage which show many pycnotic cells. X 160.

Fig. 8 Superficial loss of metachromasia associated with pycnotic cells in an explant cultivated for 8 days. This particular example shows that such a loss of metachromasia was also observed in contaminated cultures. Note the fibroblastic growth around a group of bacteria in this area. X 160.



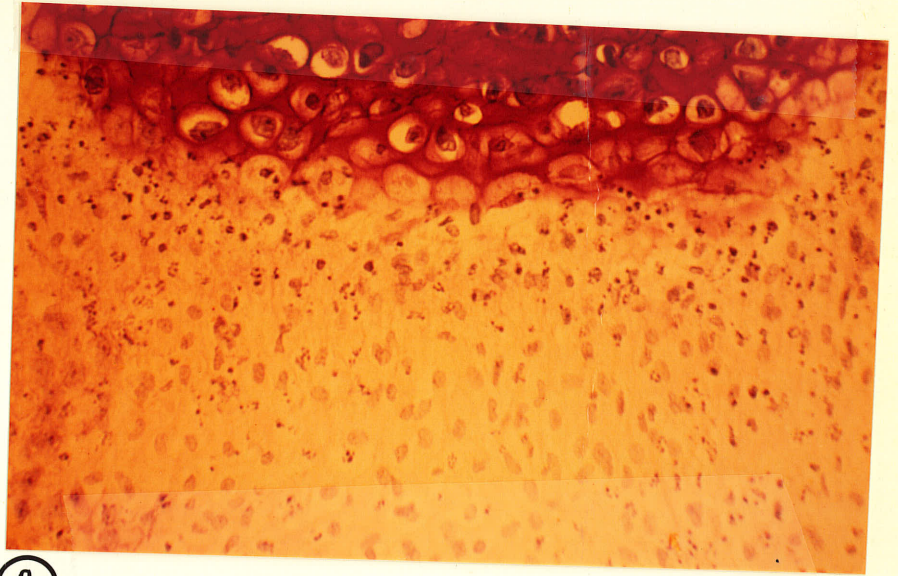
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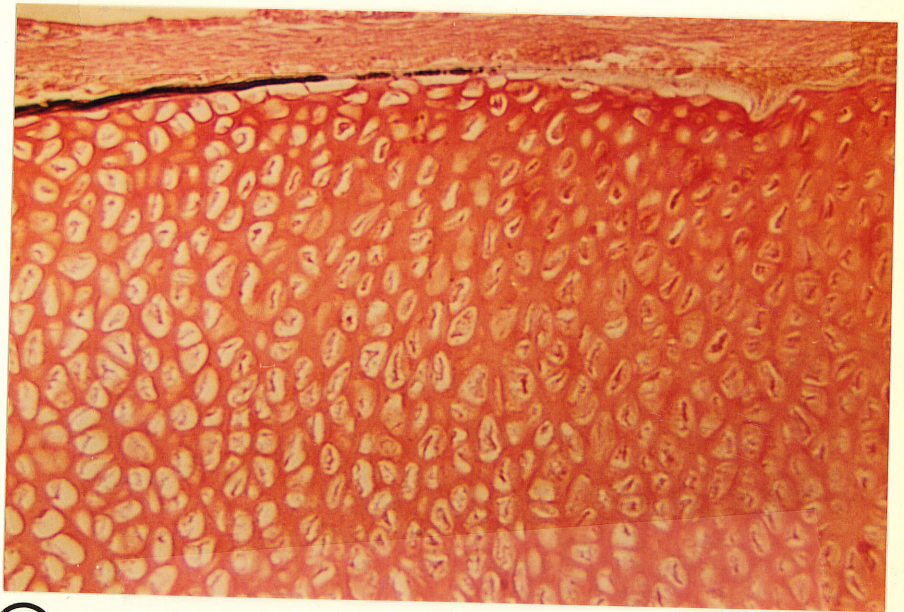
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Fig. 9 A long bone explant cultured for 7 days.
Note the peripheral loss of metachromasia which is
gradual and in the wake of extending pycnosis. X 128.

Fig. 10 Long bone cartilage from a ten-day old embryo showing
calcification only in the osteoid. Note that the
osteoid formation and its subsequent calcification
(normally observed at this developmental stage) extends
concurrently with the hypertrophy of the adjacent car-
tilage cells. X 64.



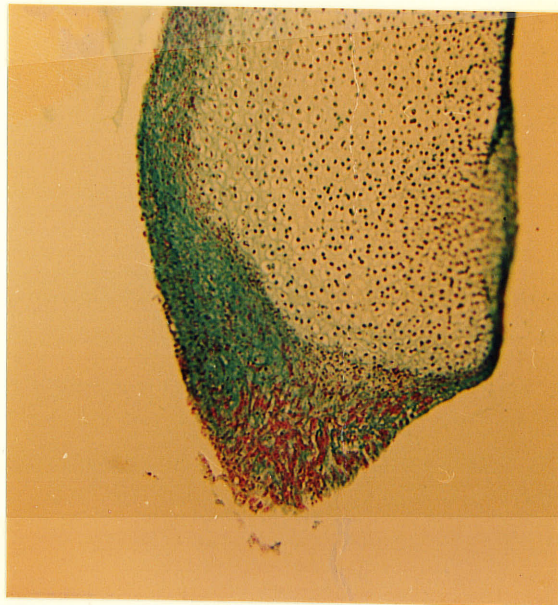
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10

Fig. 11 An enhanced connective tissue stain reaction in the periphery of a degenerating explant. These reactions corresponded with the superficial loss of meta-chromasia observed in toluidine blue stained sections.

Masson trichrome X 40.



②

PRELIMINARY STUDY 3. Observations of Histological Responses of the Explants Cultivated in the Presence of a Wide Range of Concentrations of Thalidomide.

Thirty six explants were cultivated for up to seven days in a natural media to which various doses were added, namely; 100, 200, 500, 1000, 2000, 4000 and 8000 micrograms of thalidomide suspended in balanced salt solution. In an organ culture study with tibial buds, Franceschini and Mussa (1966) observed almost complete disappearance of cartilage matrix and mitotic activity after eight days in the presence of up to 3,500 micrograms in the standard Wolff and Haffen medium.

The procedure and culture technique for this study was, as is described in the chapter on Materials and Methods. The explants were serially sectioned and stained with Halmatoxylin and eosin and also toluidine blue O.

With all the above-mentioned concentrations used, no explant had complete loss of matrix characteristics as observed in the metachromatically-stained sections. Pycnosis and some superficial loss of metachromasia similar to that observed in the Preliminary Study 1 (figures 7, 8, 9 and 11) were observed and since these effects were seen after prolonged cultivation (without thalidomide), these observations cannot be attributed to the thalidomide administration.

Recommendations

1. In view of the observed responses, lower concentrations of thalidomide (close to circulating levels) are recommended in a study of the effects on histodifferentiation. A criterion to compare mitotic activity (Preliminary Study 4) will also be used.

2. The shorter duration as established in Preliminary Study 2 will be used.

PRELIMINARY STUDY 4. The Selection of a Favourable Concentration and Exposure Time of Colchicine to be used to Describe the Mitotic Activity in Organotypic Cultures of Chick Long Bone Rudiments.

In the previous studies of organotypic cultures of long bones reviewed so far, proliferation was either described from visual comparisons or usually from DNA content. The quantitative analyses in which DNA content was calculated were made on a number of explants that were pooled to give a single value or made from single bones. Proliferation indicated by these values do not take into account the zone of the explant in which proliferation might predominate, neither can this possibility be verified. Figure 12 exemplifies a predominance of fibrous growth in the cultivated bone rudiment observed in histological sections.

In the present study, the conditions (concentration and exposure time) for the use of a mitotic inhibitor (colchicine) were estimated so that mitotic activity could be calculated for each of the interacting zones in the bone rudiment in vitro.

Calculations of mitotic activity with colchicine imply several known factors and they have a precise meaning only if certain conditions are fulfilled. For example, assuming that colchicine does not affect the normal mitotic activity, arrests all cells entering mitosis during its particular exposure time, and that the formed colchicine-arrested cells are identifiable at the time of fixation, the mitotic activity can be expressed as a percentage to be used as a mitotic index. In the case of a homogenous tissue (from the point of view of mitosis), these indices could be used to calculate mitotic rates. However, in the bone rudiment used, the interacting tissue zones are distinctive,

especially in mitotic behaviour (discussed in chapter on Cellular Details) so that mitotic indices are calculated for the different zones. Calculations of mitotic indices are considered (Bertalanffy, 1964) to be suitable for comparison between normal and experimentally modified tissues.

Concentration, time of exposure, mitotic stage, kind of cell, recovery, active treatment and the in vitro growth conditions all become critical to the formation of an arrested metaphase pattern, whether oriented or unoriented (Eigsti and Dustin, 1955). Furthermore, it has been reported that zones of certain tissues that show the highest mitotic activity are the most sensitive to colchicine, whereas in other zones the same concentration may yet enable mitosis to recover and to proceed to anaphase and telophase through the colchicine metaphase. The colchicine metaphases may remain intact for hours or even days in cold blooded animals, or it may undergo rapid fragmentation in mammals and avian species. In mammals, it is particularly rapid and it is therefore often difficult to know exactly how many metaphases with clumped chromosomes underwent degeneration. In these cases, they are usually destroyed in less than ten hours (4 to 6 hours has been used to estimate mitotic rates in mice and rat tissues). These uses refer to in vivo studies; however, in vitro, it is reported (Eigsti and Dustin, 1955) that colchicine may be left to act much longer than the times used for in vivo studies and up to 24 hours is often mentioned in studies with bone marrow.

With these requirements in mind and the fact that growth in vitro is often uncontrolled, or controlled by factors not ordinarily

important in vivo, example nutritional factors and non-lethal dissection trauma; the primary objective of this preliminary study was to select a concentration that would arrest in the metaphase all cells entering mitosis in all the zones of the explant.

The materials and methods are as described in Chapter III and the duration of cultivation was the same as that to be used for the major study, namely; two and a half days. A compromise concentration of colchicine was selected from in ovo studies (cited by Eigsti and Dustin, and by Diwan (1966) and various lengths of exposure were tested in view of the fact that this is an in vitro study and reports that the number of arrested mitosis increased over a longer exposure time in the chick species (cited by Eigsti and Dustin, 1953). Since in the major study the mitotic index is to indicate the number of cells dividing at a certain time, it would be advantageous for the comparative study to accumulate as many mitotic colchine figures as possible if the earlier arrested figures could be maintained in vitro.

A stock solution (0.0125 g per 250 ml) of colchicine was prepared on the day of experimentation by dissolving the measured quantity in phosphate buffered saline. Various working solutions of this stock solution was made up by dilution with phosphate buffered saline. By means of a Caulfield safety pipettor and separate pipettes, 0.5 millilitres of the working colchicine solutions (at 37°C.) was added to each experimental culture media. Figure 13 shows a design of the experiment in which the effects of three exposure durations (4 hours, 8 hours, 11 hours) was tested for each colchicine solution (0%, 10%, 25%, 50%, 75%, 100%).

The histological sections were systematically examined and the number of colchicine metaphases and other figures were examined for each explant. As can be seen in the experimental design, the number of arrested metaphases per 100 cells seen after 4, 8 and 11 hours' cultivation on each colchicine solution could be compared. These values were obtained for the round cell zone and flat cell zones, whereas the presence was also noted in the perichondral zones.

A common feature in all the explants (except those cultivated in 0%) was the presence of a ball type colchicine metaphase. At the lower concentrations, the colchicine metaphases were present in fibrous perichondrium, osteogenic perichondrium and round cell zones, but not detected in the flat cell zone (Figures 14, 15). Furthermore, the number of colchicine metaphases varied as pycnotic cells were frequent and there was no correlation in the number of colchicine metaphases and the increased duration of exposure.

The most favourable concentration appeared to be the 100% stock solution, since prophases and colchicine metaphases were abundant in all the zones of the rudiment and no other mitotic figures were observed. Furthermore, there was an increase in the number of colchicine metaphases as the exposure time to colchicine was prolonged. Figure 16 shows numerous ball type colchicine metaphases with dark clumped chromatin, no nuclear membrane and surrounded by a halo of light cytoplasm. With this concentration and after eleven hours of exposure to colchicine a high percentage (11%) of colchicine metaphases was observed in an isolated case in the flat cell zone (Figure 16). Though in this figure no pycnotic or fragmented metaphases are observed, at this long

exposure time various stages of fragmentation of arrested metaphases were observed in the round cell zone and also flat cell zone (Figures 17a,b,c). The flat cell zone usually appeared to have more pycnotic or very late colchicine metaphases (Figure 17C) because of the orientation of the cells.

Though there was an accumulation of colchicine metaphases in the round and flat cell zones in the longer exposure times, there was an increase in the number of pycnotic and fragmented colchicine metaphases which introduce a difficulty in classification.

A subsequent experiment using the 100% concentration with exposure times of 4, 8, 9, 10 hours indicated that at nine hours a few pycnotic cells still hindered classification of colchicine metaphases and pycnotic cells. At four and eight hours this was reduced, though the number of colchicine metaphases was not as high.

Conclusions and Recommendations

1. The concentration decided upon (100% stock) was not insufficient since it arrested mitosis in all the zones and no other mitotic figures (anaphase and telophases) were observed.
2. Though there was an accumulation of colchicine metaphases with increasing exposure time, there was also an increase in the number of fragmented cells which are often difficult to distinguish from pycnotic cells that are known to occur sporadically in organ cultures.
3. To reduce this interpretation problem, the duration of exposure would be considerably reduced for the main study (8 hours) and a pycnotic index would be calculated concurrently with the calculations of the mitotic indices in the same field of cells.

4. The demonstration of arrested mitosis, especially in the distal areas of the explants (Figure 18) is further proof that the diffusion system provided by the culture technique is adequate and special proof that over this decided cultivation period, there was organ culture not mere organ maintenance.

Fig. 12 A long bone explant cultured for ten days and
showing a predominance of fibrous growth.

Safranin O. X 16.



12

Fig. 13 Experimental design to estimate and select a concentration and maximum exposure time of a stock solution of colchicine for cultures of embryonic chick femora.

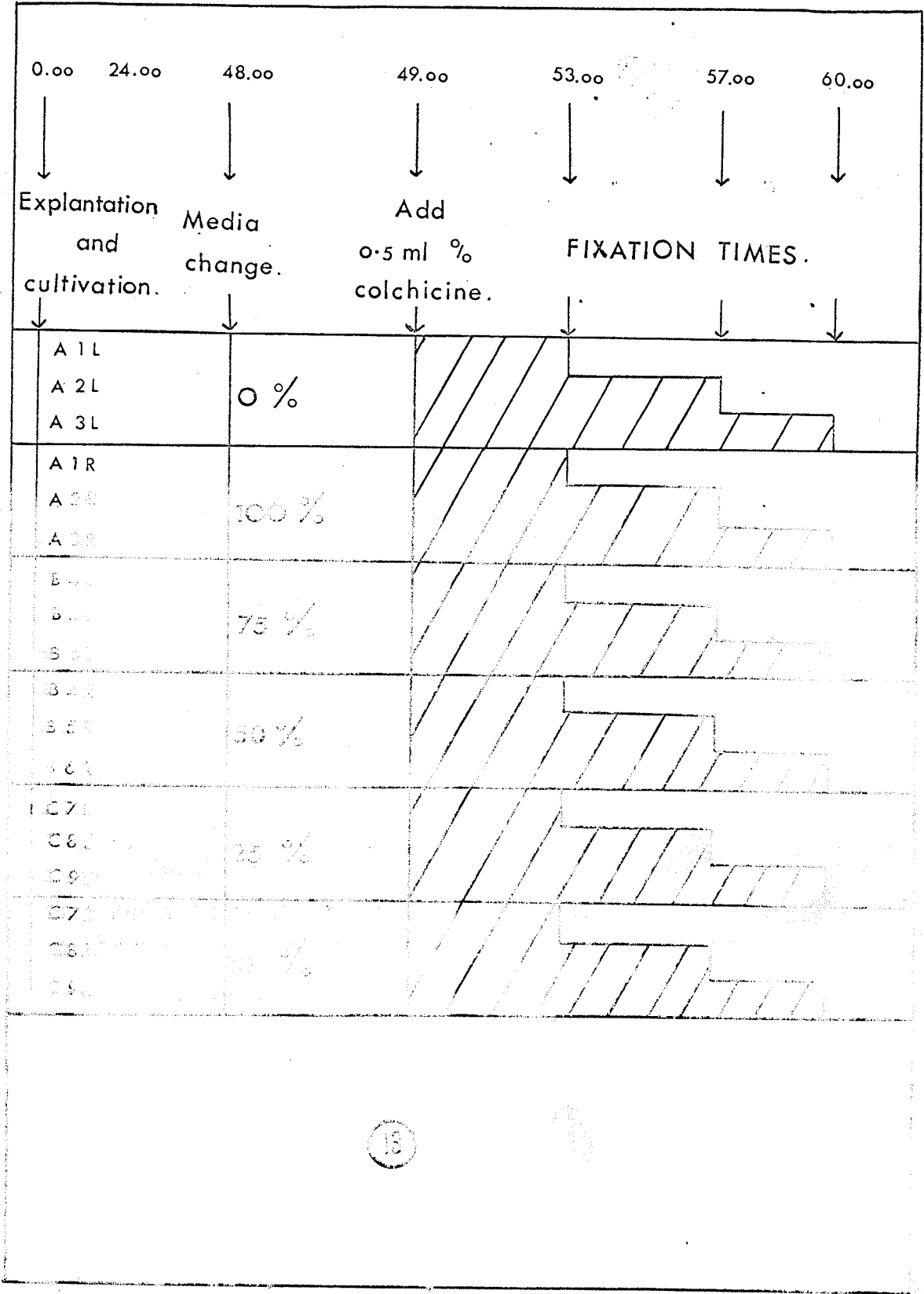
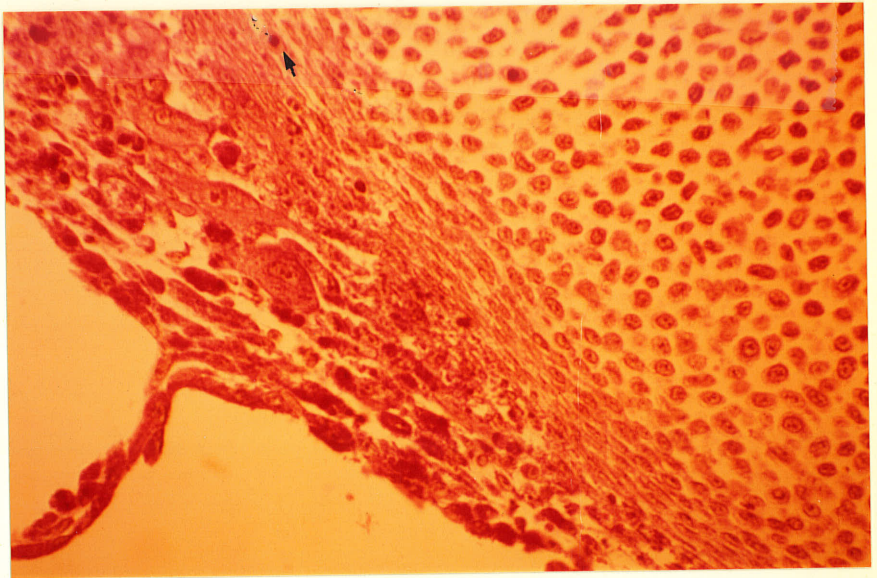
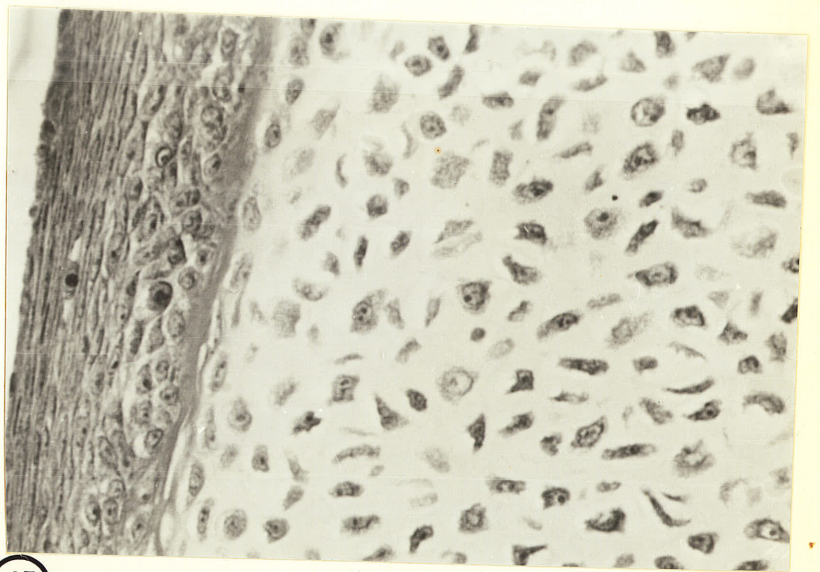


Fig. 14 Ball-type colchicine metaphases in the round cell zone and also in the perichondrium. Note (arrow) a colchicine metaphase in the region of the perichondrium that is strategically situated in relation to the fibrous and osteogenic perichondrium and the round cell zone. X 204.8.

Fig. 15 Colchicine metaphases in fibrous and osteogenic perichondrium. The tapering osteoid marks the transitional region of cartilage transformation in which no definite C-metaphases are observed. X 160.



14



15

Fig. 16 Numerous ball-type C-metaphases in the flat cell zone. Note the clumped chromatin and absence of nuclear membrane. X 128.



16

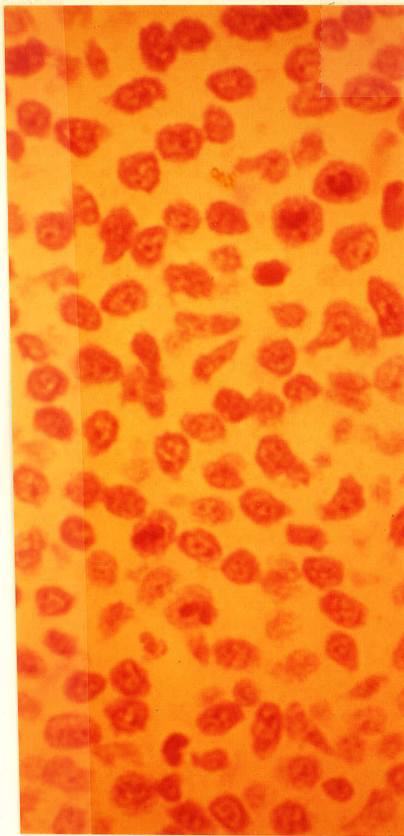
Fig. 17 Stages of fragmentation of colchicine-arrested
metaphases.

a and b - in the round cell zone.

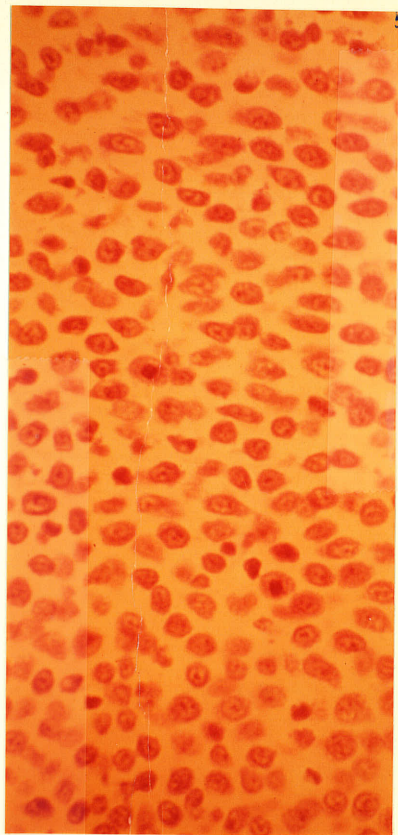
c - in the flat cell zone.

Note in this region they appear as pycnotic cells.

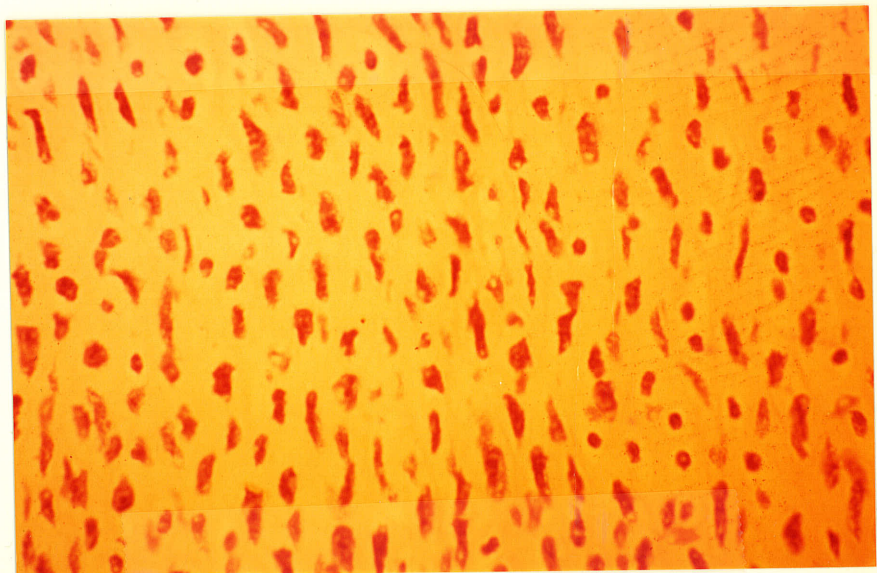
X 256.



a

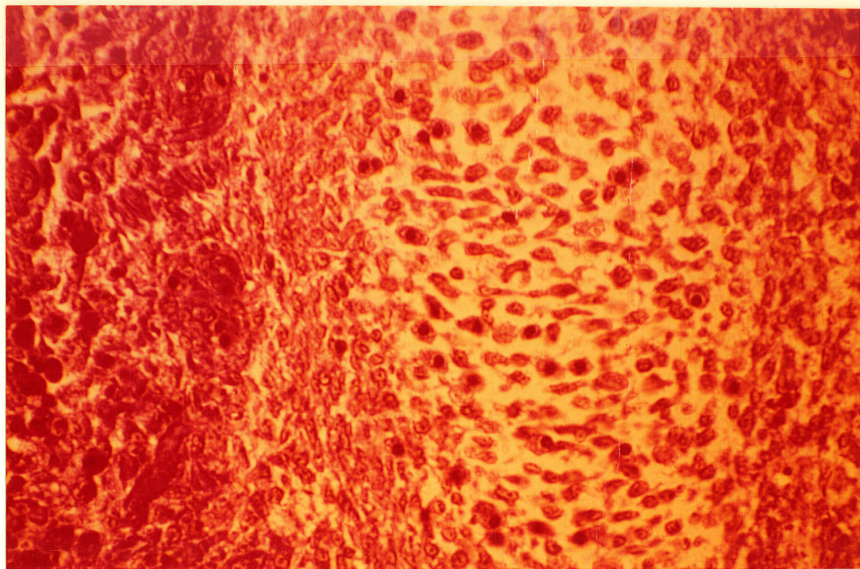


b



c

Fig. 18 Colchicine metaphases in the flat cell zone of cartilage cultivated with a considerable amount of attaching tissue. It is evident that the diffusion system was efficient. X 128.



18

RESULTS OF MAJOR STUDY

RESULTS

The differences in responses of paired femora cultured for $2\frac{1}{2}$ days with and without the doses of thalidomide revealed, first of all, positive basophilia (observed in the haematoxylin and eosin stained sections) and positive beta-metachromasia indicative of normal viable matrix characteristics in all the explants (the stain reactions are exemplified in Figure 5 and Figure 1, respectively). In a few isolated cases, however, explants unavoidably had some attaching joint tissue. In such cases (Figure 19a) necrosis extended from the joint region into the epiphysis. Many viable cells and some colchicine-metaphases were observed in regions not affected by the necrosis and in some cases normal counts (example density counts) could be made in areas. In Figure 19b, good cartilage development with numerous colchicine metaphases are observed alongside the necrotic joint regions.

The deletion of such necrotic zones in some samples resulted in some sample sizes not being equal (note degrees of freedom in Table VI). Consequently, in calculating the standard error of the mean differences (the difference between the control and treated sample means) in the Student t-test for sample means, modifications were made accordingly (Goldstein, 1964) for samples of unequal sizes with the pooled variance estimates.

The quantitative data (mean densities, mitotic and pycnotic indices) were compared for each transformation zone in control versus treated explants. The significance of the difference was calculated by comparing the magnitude of this observed difference (i.e., in sample means) with an

estimate of the standard error, and referring to the t-distribution in the Fisher and Yates tables, where modifications have been made for small samples.

In accordance with the recommendations from Preliminary Study 4, a pycnotic index had to be added to the criterion of mitotic index, since occasional pycnoses were observed in the same field in which the mitotic counts were made (Figures 20a & b).

The values obtained for each concentration of thalidomide tested (I = 10 mgm per ml, II = 20 mgm per ml, III = 30 mgm per ml, IV = 40 mgm per ml.) are shown in tables II, III, IV and V. The significances of the differences in responses are shown in table VI.

The mean difference in sample responses (Table VI) usually corresponded in the density and mitotic index values in a particular zone, indicating that when a decrease in cell density was observed, the mitotic activity observed at the end of the experiment showed a lower value. The results were not always as consistent. In the treatment with 20 mgm per ml thalidomide, there was an observed increase in density (20.7 ± 13.05) but a decrease in mitotic activity (0.484 ± 0.37); whereas, with the 30 mgm per ml thalidomide treatment a decrease in density but an increase in mitotic activity was calculated for the flat cell zone. Both these results showed high standard errors and were insignificant ($p > 0.05$) (discussed in section on Discussion).

Irrespective of the type of response, the decreases or increases in sample differences observed were not significant at the 5% level of significance in all the explants cultured in 10 mgm per ml, 20 mgm per ml and 30 mgm per ml thalidomide suspensions. The only significant results

were observed with a 40 mgm per ml suspension of thalidomide.

Figures 21, 22, 23, 24, 25 compare the cell densities, mitotic and pycnotic indices for each of the zones of transformation treated with the four concentrations of thalidomide. The values of the pycnotic indices are noted with the graphical comparisons of mitotic indices and are especially noteworthy with the first three concentrations (10, 20 and 30 mgm per ml thalidomide) in which insignificant differences were calculated.

Assuming that a larger fraction of the classified pycnotic cells (Figures 20a & b) were actually ball-type colchicine metaphases that were already destroyed, this fraction even if added to the observed treated values for mitotic indices could only show the following changes: In figures 22 & 24, there is very little difference between control and treated values and the difference in mitotic indices was such that if the observed difference in pycnotic indices (pycnotic indice (treated) - pycnotic indice (control)) were added to the mitotic indices of the treated groups, it would only reduce the observed difference in responses in control and treated means. The insignificance of the results obtained in the round cell zones with concentrations I, II, III and IV, and in the flat cell zone with concentrations I and II, could probably not be changed. However, in the flat cell zone in explants treated with concentration II (30 mgm per ml thalidomide), a very high pycnotic index was observed compared with the mitotic index in the treated sample (Figure 24). Therefore, in this zone it is possible that the insignificance of the observed increase in mitotic index could be erroneous.

The significant results obtained with a 40 mgm per ml concentration of thalidomide was in the increased cell density in the round cell zone ($p = 0.05$ but $p = 0.01$) and in the cell density in the hypertropic cell zone ($p = 0.001$). The mitotic indices in the round and flat cell zones were reduced but insignificant ($p = 0.05$).

In the fibrous and osteogenic perichondrium, the presence of colchicine metaphases were recorded in all the explants cultivated in treated and control samples. The osteogenic perichondrium was well developed even in the treated explants and showed many colchicine metaphases, so did the fibrous perichondrium (Figures 26a & b). There was, however, in an isolated case, a region of the perichondrium that was necrotic in a treated explant, but this could not be related to the thalidomide administration, since this area was associated with extraneous necrotic tissue (Figure 27). In this figure, many colchicine metaphases are seen in the viable perichondrium, whereas the necrotic perichondrium is on the opposite side which has the excessive adherent tissue.

Good mitotic activity was also observed in the perichondrium in Figure 28C. In this particular explant, cultivated in 10 mgm per ml thalidomide, an active resorption of the epiphysis was observed. In serial sections (Figure 28a & b), a hollowing of the cavity in the viable epiphyseal cartilage was noticed. The invading connective tissue contained many colchicine metaphases which were also observed in the round cell zone that was affected. This resorption was observed in the head end of the femur and the mitotic activity extended from the perichondrial end in this region.

Furthermore, with the same concentration of thalidomide (10 mgm per ml)

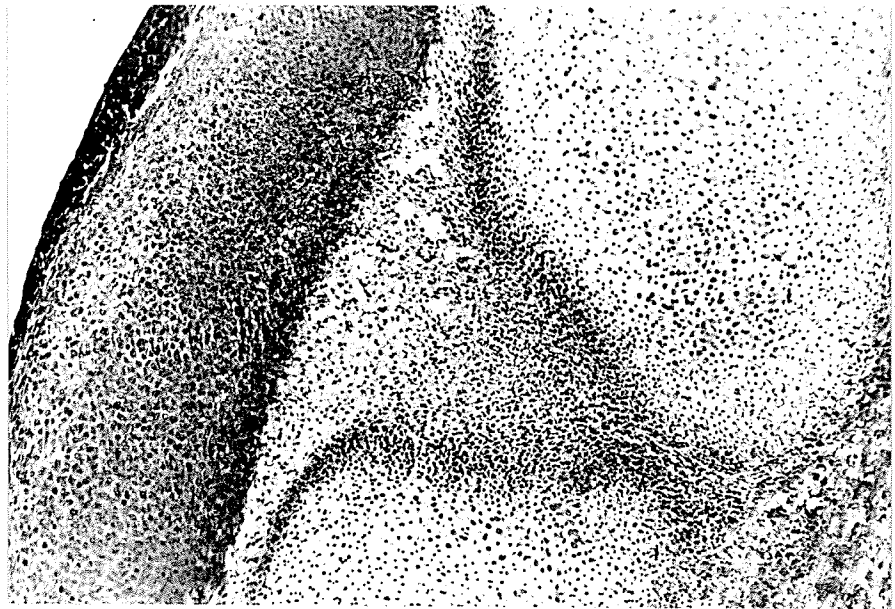
a very high percentage of colchicine metaphases (9.98%) was observed in a disorganized culture attached to an explant (Figure 29).

Fig. 19 Necrosis in the joint region of an explant cultivated with thalidomide.

- a. Low power of the explant.
- b. High power showing many C-metaphases in cartilage adjacent to the necrotic region. X 40.

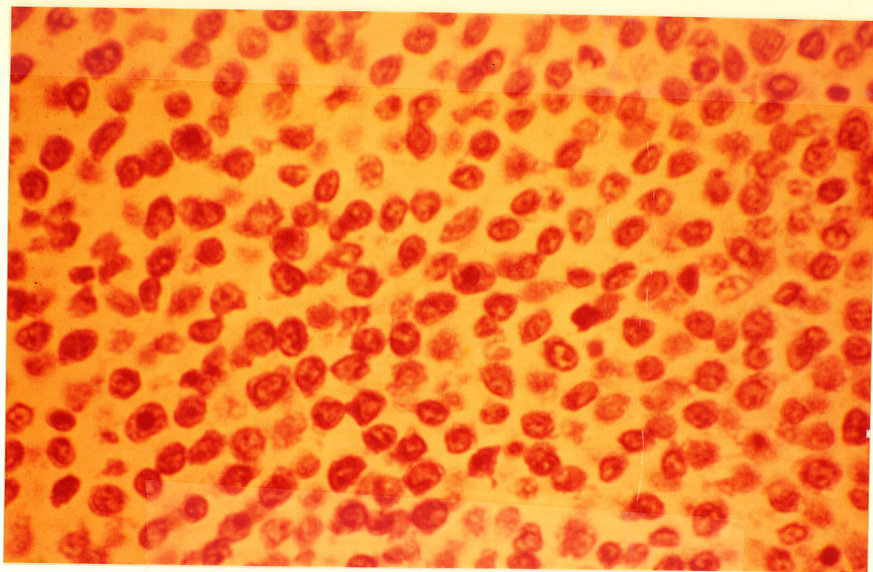


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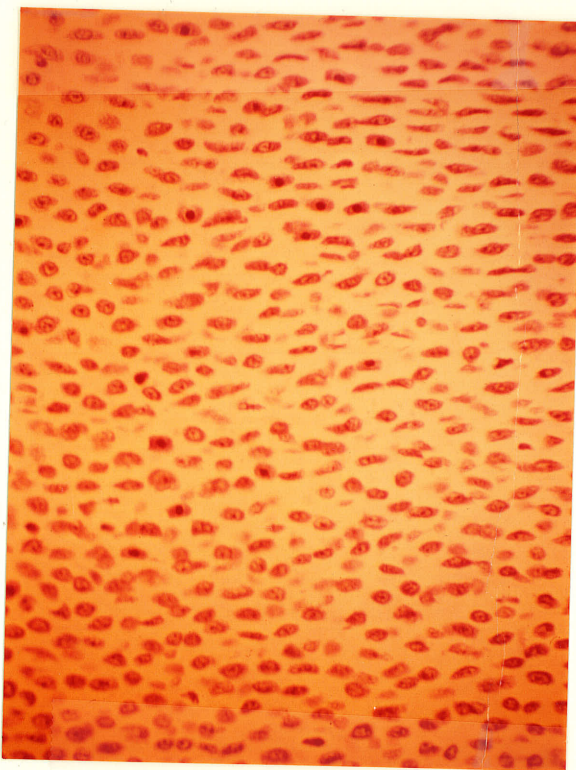


b

- Fig. 20 Colchicine metaphases and classified pycnotic cells
in a field in which cell density, mitosis and pyc-
nosis were calculated.
- a. In the round cell zone X 256.
 - b. In the flat cell zone X 128.



a



b

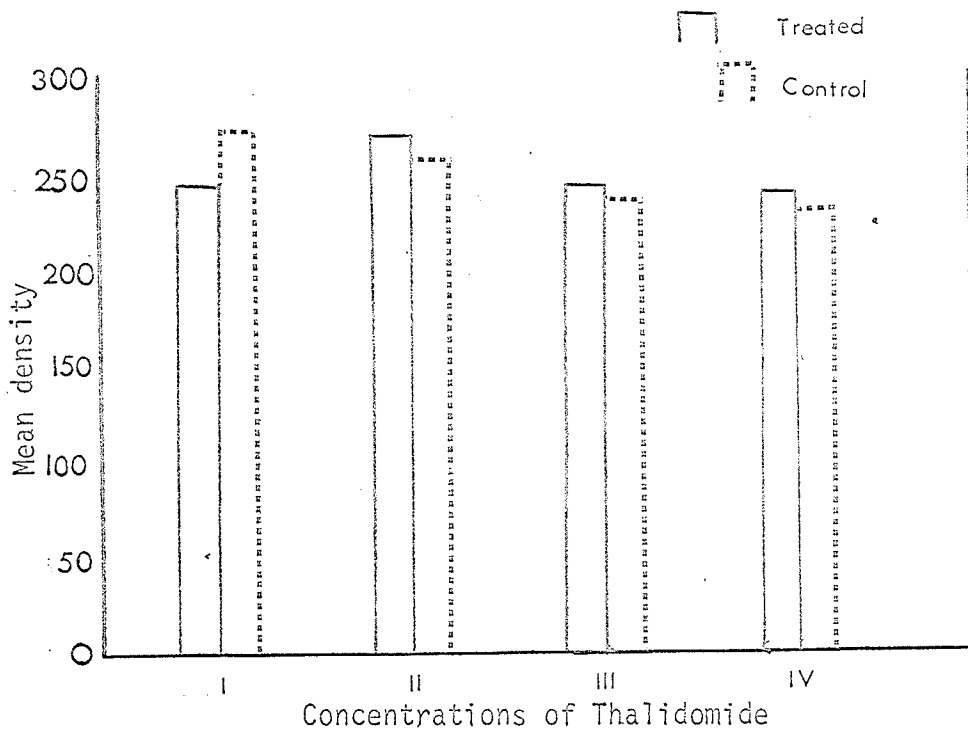


Fig. 21 Comparison of cell densities in the round cell zones.

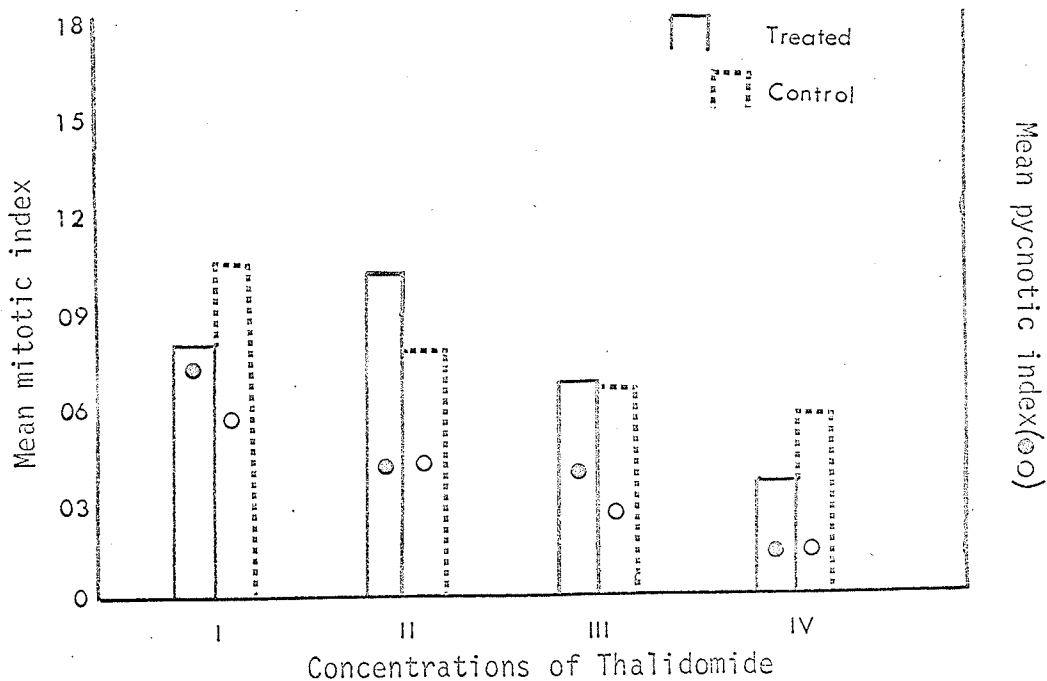


Fig. 22 Comparison of mitotic indices (together with pycnotic indices) in the round cell zones.

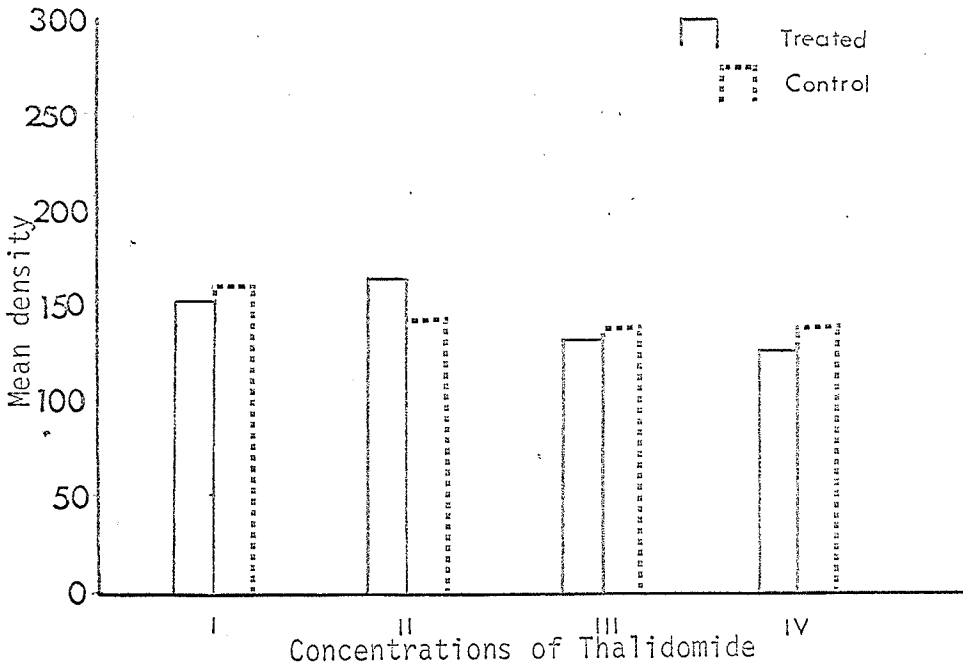


Fig. 23 Comparison of cell densities in the flat cell zones.

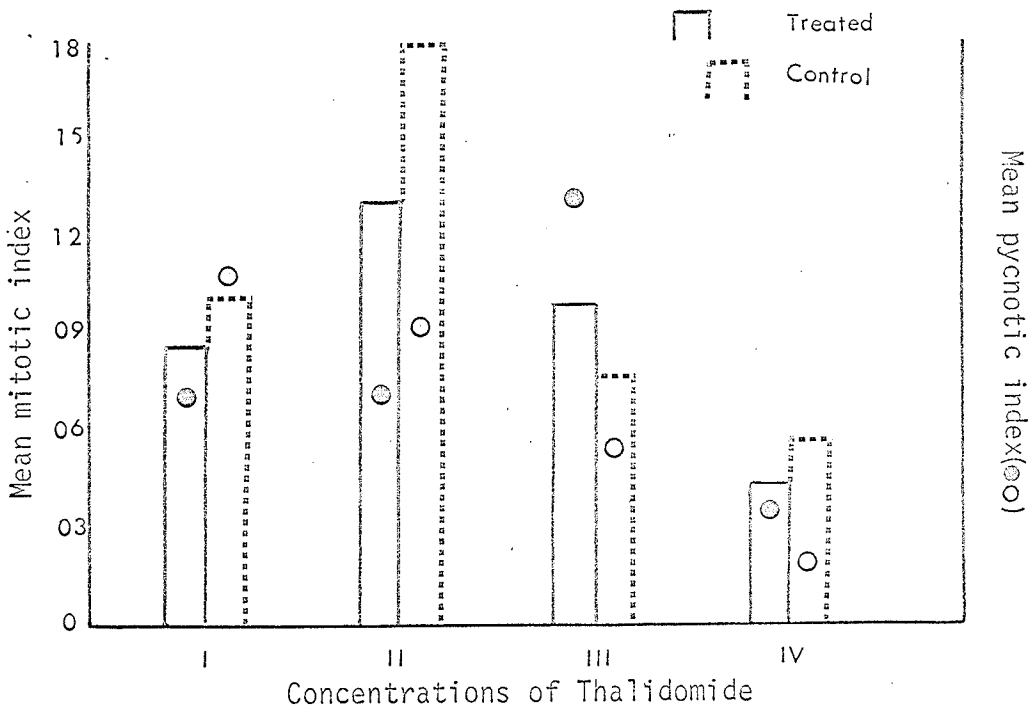


Fig. 24 Comparison of mitotic indices (together with pycnotic indices) in the flat cell zones.

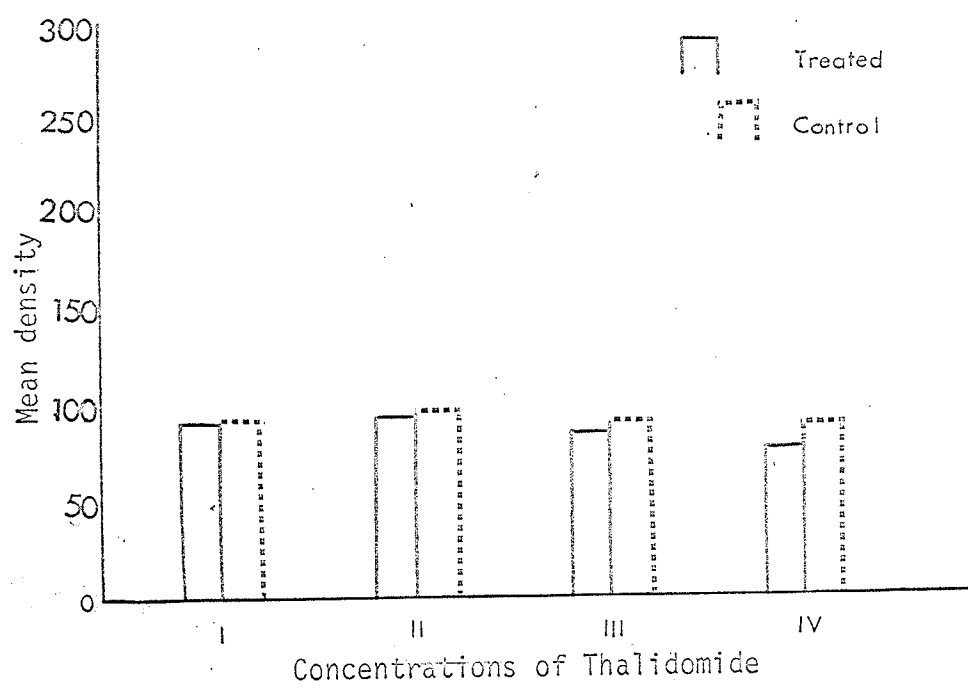
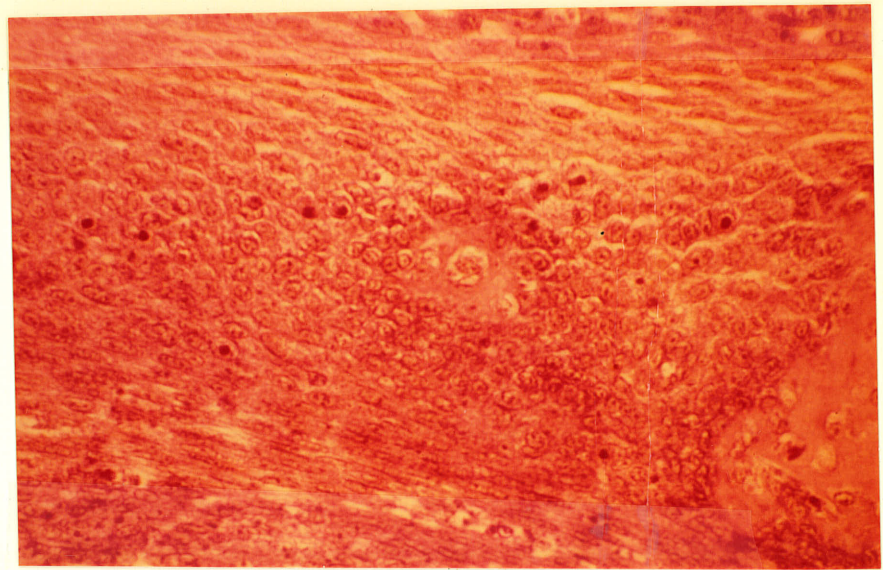
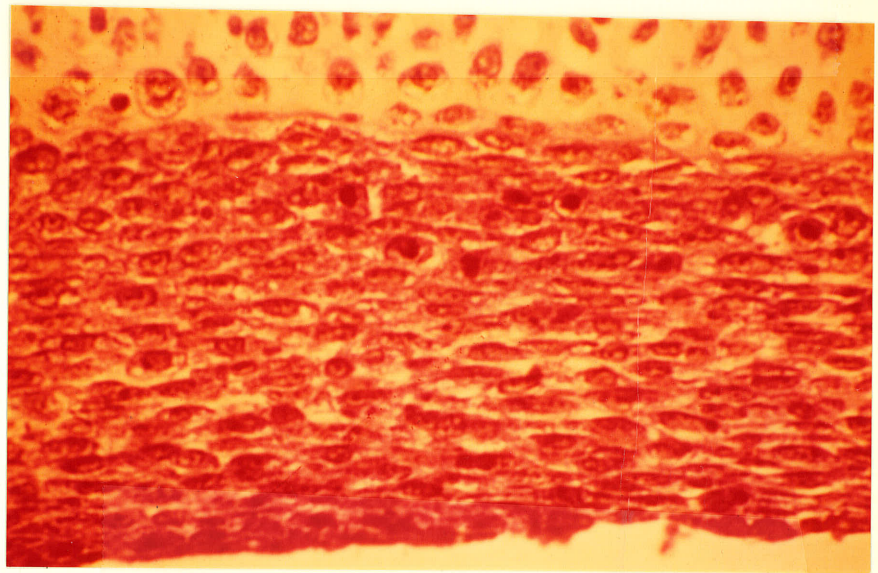


Fig. 25 Comparison of cell densities in the hypertrophic cell zones.

- Fig. 26 Colchicine metaphases in the fibrous and osteogenic perichondrium in explants cultured with thalidomide.
- a. Tangential section through the osteogenic perichondrium. X 128.
 - b. C-metaphases seen also in the fibrous perichondrium. X 204.8.

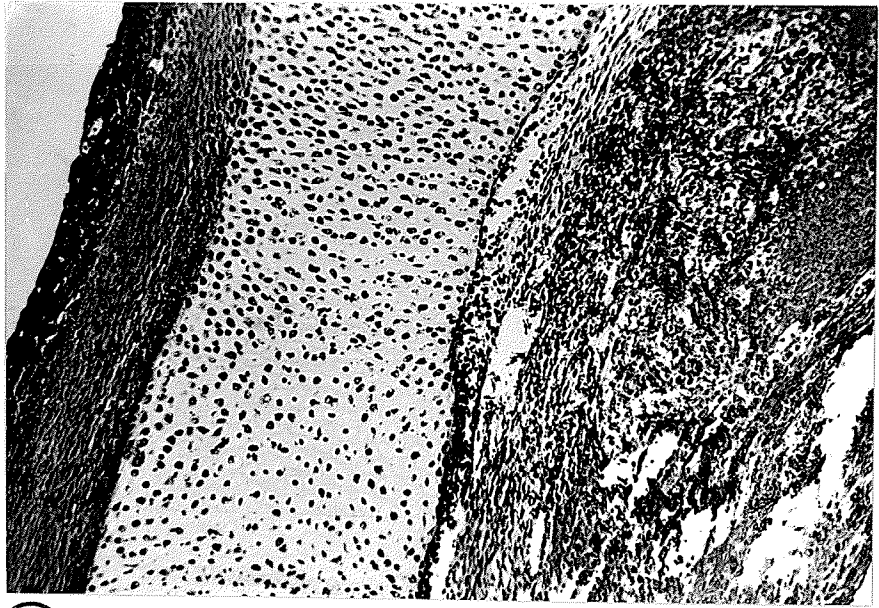


a

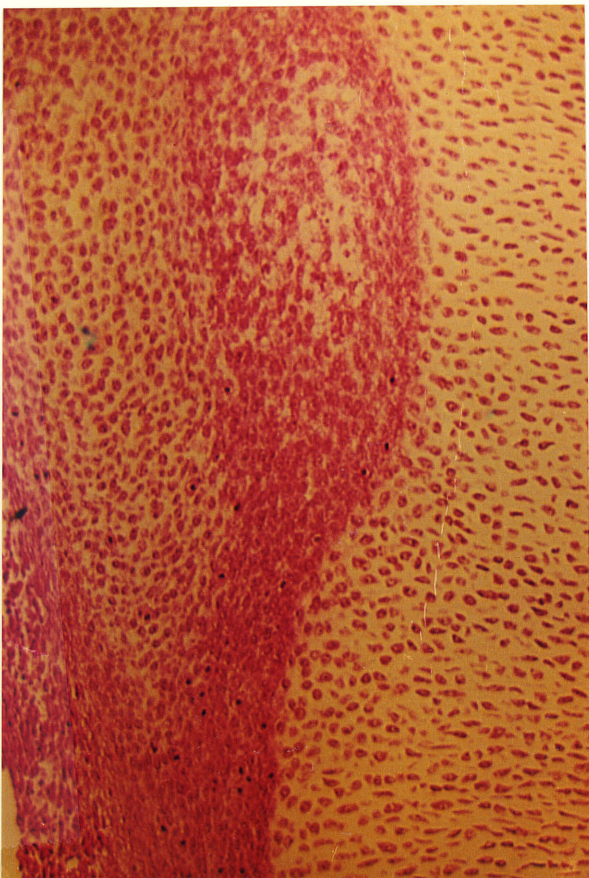


b

Fig. 27 Colchicine metaphases in the viable perichondrium and necrosis on the opposite side with excessive adherent tissue. X 40.

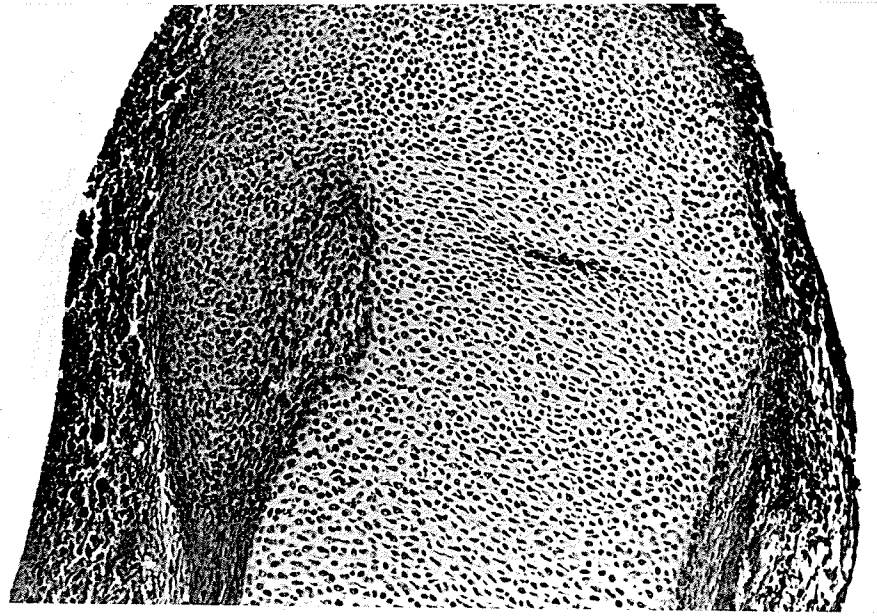


27

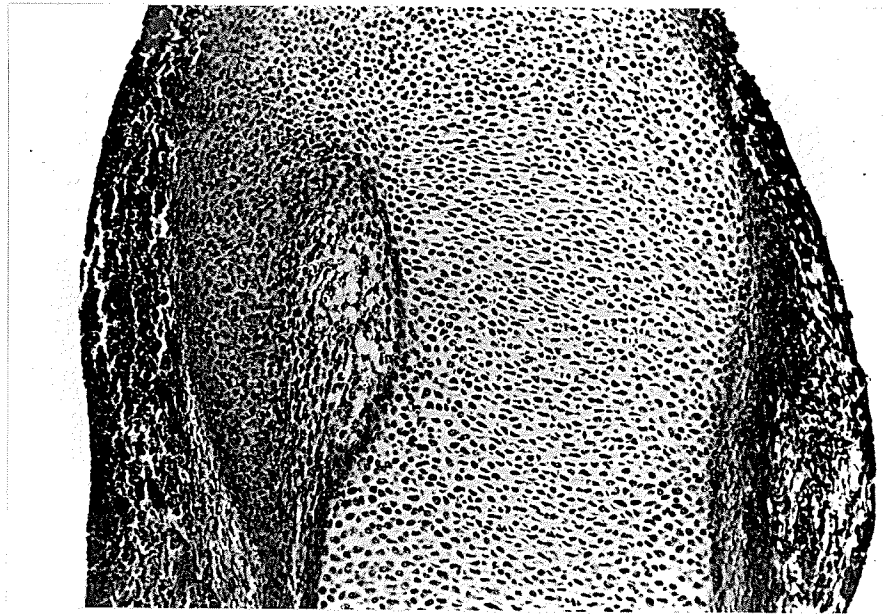


28 c

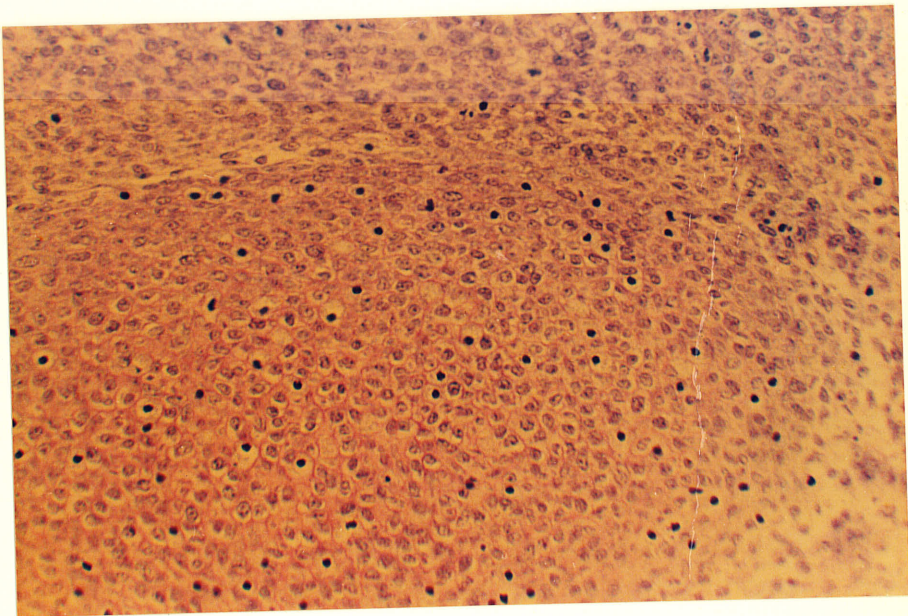
- Fig. 28 Resorption in the epiphyseal region of an explant cultured with 10 mgm per ml thalidomide.
- (a) and (b) show cavitation observed in serial sections. X 40.
- (c) Note numerous C-metaphases in the resorption site extending from the perichondrium. X 64.



a



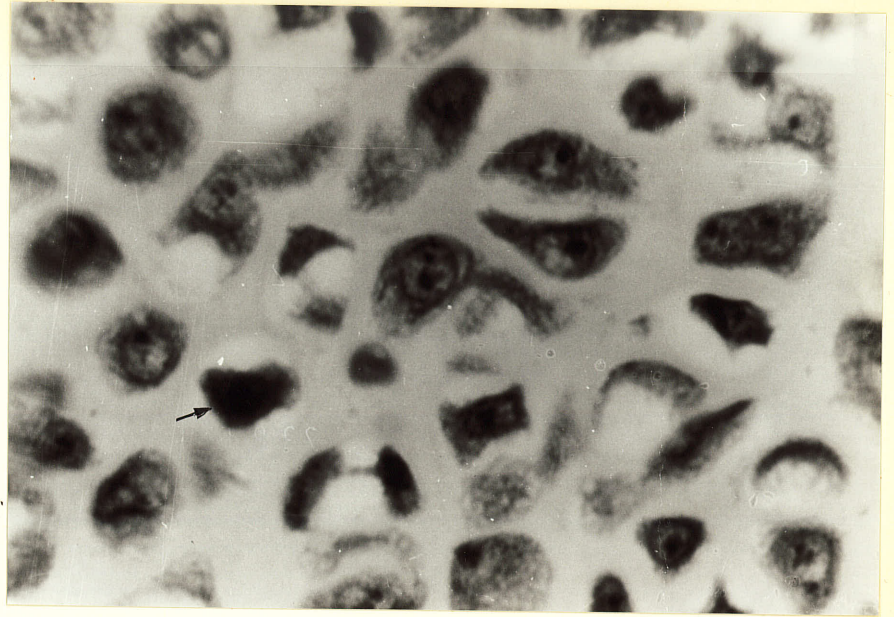
b



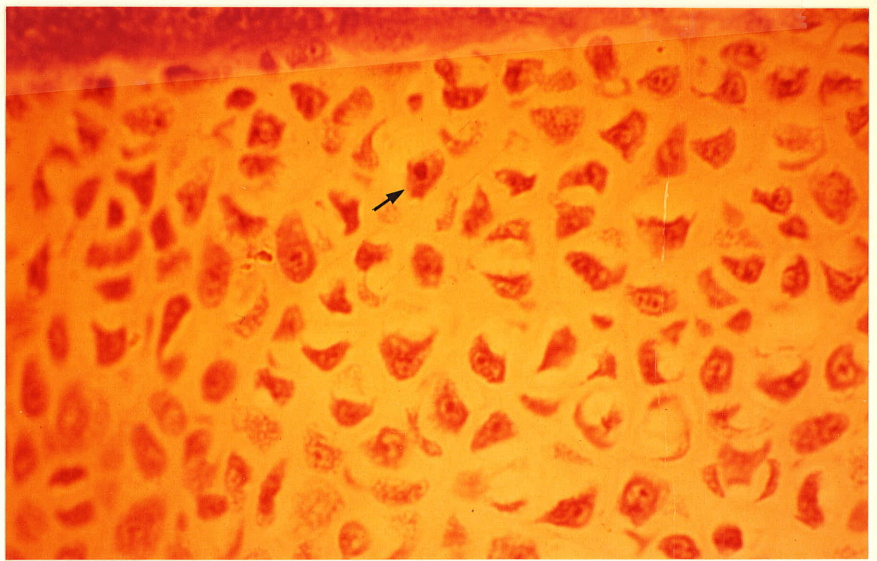
29

Fig. 29 Numerous C-metaphases in disorganized cartilage cultured with 10 mgm per ml thalidomide. Note the metaphasia in the intercellular regions. X 100.

- Fig. 30 Cell densities in the hypertropic zones of treated and control explants.
- a. Control explant showing a C-metaphase and many recently hypertrophied cells. X 256.
 - b. Treated explant also showing a C-metaphase with larger hypertrophied cells noted in lower power magnification. X 160.



a



b

DISCUSSION AND SUMMARY

DISCUSSION

Relying on criteria such as cell density, mitotic indices, there were observed no significant change in the transformation from the round cell zone to the hypertropic cell zone of the chick long bone cartilage when treated for 2½ days with 10, 20 and 30 mgm per ml thalidomide. The concentration of 40 mgm per ml was related to a significant increase in density in the round cell zone and a highly significant decrease in the density in the hypertropic cell zone in the same sample. The increased density in the round cell zone was associated with a decrease in mitotic activity in the same zone and also in the flat cell zone (the second zone of transformation). Though these changes in mitotic activity were insignificant when compared to the controls, the observation that mitotic activity was reduced in the treated group does not support the possibility that the increased density in the round cell zone was due to a predominance of mitotic activity in this zone. The contribution of the cartilage matrix to the increase in cell density was not determined in this approach. However, the normal matrix characteristics were recorded, in that good basophilia and beta-metachromasia were observed in control and treated explants, and also rudiments in ovo. The only possibility is that there was a quantitative delay in matrix production and this would require a modified experimental approach.

The significant decrease in cellular density in the hypertropic cell zone is more complicated since cellular hypertrophy contribute considerably to the density values in this zone. The matrix characteristics were normal in treated and control explants. In the hypertropic cell zone in both the treated and control explants, cells that appear to be

colchicine metaphases are seen close to the transition area (Figure 30a & b). Though the visual comparison (Figure 30a & b) is subjective, it appears evident that cellular hypertrophy has occurred in vitro as the transitional zone shows many such cells. The cellular hypertrophy-matrix relationship would require quantitative studies for matrix production and histochemical studies on the hypertrophy of cells to explain the reduced cellular density observed in this zone after a treatment of 40 mgm per ml thalidomide.

The suggestion that a differentiative quality appears to be affected during transformation is further supported by the following observations:

(i) The reduction or increase in mitotic index observed was not significant in the transformation zones and it is noteworthy that in all the explants mitosis was always detected with all the concentrations of thalidomide tested. Mitotic activity was also always present in the fibrous perichondrium and also in the osteogenic perichondrium.

(ii) A striking example of apparently undisturbed mitotic activity was observed in a disorganized culture of cartilage exposed to a 10 mgm per ml thalidomide suspension. In this culture (Figure 29), 9.9% of 951 cells counted showed colchicine metaphases. There was little intercellular substance, but sufficient to give a beta-metachromatic stain reaction with toluidine blue O.

Regarding the effects of thalidomide on mitosis, studies (Roath et al, 1963; Lindahl-Kiessling and Book, 1963) on leukocyte cultures were conflicting in that an inhibition and an absence of inhibition were

reported respectively. Furthermore, cultures of Ehrlich ascites tumour cells in the presence of thalidomide showed an increase in mitotic activity. These studies suggest, as Jurand (1966) points out, that the effect of thalidomide is not specific for any particular stage of cell division and is more related to retardation of development and not inhibition.

(iii) Another observation made in an explant cultivated in 10 mgm per ml thalidomide was an active resorption of epiphyseal cartilage, extending from the perichondrium toward the round cell zone (Figure 28a & b). In this figure, the cavitation reconstructed from serial sections showed a gradual resorption. At the initial point of the cavitation, numerous colchicine metaphases were aligned extending from the perichondrium toward the cavity (Figure 28c). Normally, the avian epiphysis is invaded by vascular tissue much later on in development in ovo. Willmer (1960) describes this junctional zone (the distal limits of the perichondrium between the round cell zone and flat cell zone), as the undifferentiated zone of a bone rudiment that could give rise to fibroblasts, osteogenic cells and chondrogenic cells. The observation that the resorption and localization of mitotic activity extend from this region suggest an initiation of destructive processes from this area containing undifferentiated cells. Resorptive activity usually involves the breakdown of cartilage matrix due to liberated cathepsins and hydrolases from viable chondrocytes (also discussed in review on Matrix Characteristics), or clastic activity by cells containing lysosome-like hydrolytic enzymes (Hancox and Boothroyd, 1963). Jurand (1966) described an increase in lysosomal population in the endothelial cells and in advanced cases in mesoblast cells of avian

limb buds treated with an 0.2 ml of a 10 to 20 mg per ml suspension of thalidomide. He suggested the possibility that the primary effect due to the thalidomide treatment was an increase in destructive processes during development.

In a study of the effect of hyperoxia on chick cartilage in organ culture, Sledge (1964) described the surprising observation of the presence of a few typical osteoclasts in an oxygen-treated group. These were abnormal at this stage of development (8-day old embryos), as osteoclasts do not appear to be present in areas other than the medullary cavity of older embryos (Tanzer and Hunt, 1963), although Fell (1925) has described osteoclasts in periosteal bone in younger embryos.

It should be noted that the observation of active resorption was only seen in two cases and the confirmation of lysosomal activity (osteoclasts) was not made. This would require histochemical staining. The short duration of this study proved to be advantageous, since a gradual effect could be noted, whereas, over a longer duration study by Francheschini and Mussa (1966), with thalidomide, practically complete mitotic arrest and disappearance of cartilage matrix was reported.

The observations made in the present study were made on transformation on bone rudiments taken from six and a half-day old embryos, after complete transformation of cartilage had just been initiated in ovo. The forms and functions of tissues of the body emerge by what is actually not formation but progressive transformation of the initial system (Weiss, 1965). It is possible, as suggested by the studies of Lash (1964) that thalidomide could affect the tissue interacting during the initiation of chondrogenesis at an earlier stage of development.

In conclusion, this determination of the direct effects of thalidomide on active histodifferentiation (cell densities and mitotic activity in the cartilage zones) indicated that mitotic activity was not inhibited and that certain differentiative qualities, possibly involving matrix production, cellular hypertrophy and cartilage resorption could be directly affected by thalidomide. These direct effects could be conclusively determined in further studies, involving additional investigation techniques with these organ cultures, cartilage and bone rudiments taken from younger embryos and also other areas of the body.

SUMMARY

1. Six and a half-day old chick femora were directly exposed to four concentrations (10 mgm, 20 mgm, 30 mgm and 40 mgm per ml) of thalidomide suspensions in natural media for two and a half days.
2. Matrix characteristics (metachromasia) were normal in all the treated explants and mitosis was always observed in fibrous and osteogenic perichondrium. The mitosis, as detected by the colchicine method, was always present in the round and flat cell zones of cartilage and was not significantly affected by any of the thalidomide suspensions.
3. Significant results were observed only with the 40 mgm per ml suspension of thalidomide. The cellular density of the round cell zone was increased, whereas the density in the last zone of transformation (the hypertrophic cell zone) was reduced. The mitotic activity in the round cell zone was insignificantly reduced and the matrix characteristics in both the zones were normal.
4. Though the thalidomide suspension (10 mgm per ml) had no significant effect on cartilage transformation, it was associated with an active resorption in epiphysis in the head region of the femur and also with observations of high mitotic activity in disorganized cartilage.
5. Mitotic activity was not affected during active histodifferentiation whereas the differentiative qualities appear to be affected and require additional techniques for verification.

TABLE 11

CELLULAR DENSITIES, MITOTIC AND PYCNOTIC INDICES AND MATRIX CHARACTERISTICS
 IN THE TRANSFORMATION ZONES OF THE FEMORA AFTER 2 1/2 DAYS CULTIVATION IN THE
 PRESENCE OF 10 MCM PER ML THALIDOMIDE IN NATURAL MEDIA

Explants	Zone	Total Cells	Mean Density*	Total Colchicine Metaphases	Mean MI (%)	Total Pycnotic Cells	Mean PI (%)	Presence of C-Metaphases in FP. OP. in Matrix	Positive Beta-Metachromasia
6 Treated	RCZ	5364	242.5 +	34	0.7850 +	43	0.7172	6/6	6/6
	FCZ	4027	-10.8 152.05 +	17	-0.41 0.8587 +	30	0.712		6/6
	HCZ	2004	-6.9 91.46 +	0	-0.2 0	49	4.28		5/5
6 Control	RCZ	4194	-7.7 271.3 +	50	1.1490 +	17	0.5506		5/5
	FCZ	2549	-12.1 157.32 +	20	-0.25 1.0152 +	17	1.0853		5/5
	HCZ	1559	-12.3 92.94 +	0	-0.5 0	0	0		6/6
			-6.8						

+ Standard error of the mean.
 * eyepiece graticule of constant sq. area

RCZ Round Cell Zone
 FCZ Flat Cell Zone
 HCZ Hypertropic Cell Zone

FP Fibrous Perichondrium
 OP Osteogenic Perichondrium

CELLULAR DENSITIES, MITOTIC AND PYCNOTIC INDICES AND MATRIX CHARACTERISTICS
 IN THE TRANSFORMATION ZONES OF THE FEMORA AFTER 2 1/2 DAYS CULTIVATION IN THE
 PRESENCE OF 20-MGM PER ML THALIDOMIDE IN NATURAL MEDIA

Explants	Zone	Cells Counted	Mean Density	Colchicine Metaphases	Mean MI (%)	Pycnotic Cells	Mean PI (%)	Presence of C-Metaphases in FP. OP.	Positive Beta-Metachromasia in Matrix
6 Treated	RCZ	6434	267.6 +	65	1.0098 +	26	0.4079	5/5	5/5
	FCZ	2997	-7.9		-0.29				
	HCZ	1724	164.025 +	34	1.3182 +	18	0.7305		4/4
6 Control	RCZ	6150	-12.3 94.22 +	0	-0.33 0.0 +				5/5
	FCZ	2363	-3.1 256.56 +	45	0.7770 +	26	0.4132	6/6	6/6
	HCZ	1505	-5.2 143.32 +	31	-0.13 1.8022 +	15	0.9104		6/6
			-6.4 96.86 +	0	-0.18 0.0				5/5
			-6.2						

+ Standard error of the mean.
 - eyepiece graticule of constant sq. area

RCZ Round Cell Zone
 FCZ Flat Cell Zone
 HCZ Hypertropic Cell Zone
 FP Fibrous Perichondrium
 OP Osteogenic Perfochondrium

TABLE IV
 CELLULAR DENSITIES, MITOTIC AND PYCNOTIC INDICES AND MATRIX CHARACTERISTICS
 IN THE TRANSFORMATION ZONES OF THE FEMORA AFTER 2 1/2 DAYS CULTIVATION IN
 THE PRESENCE OF 30 MG/ML OF THALIDOMIDE IN NATURAL MEDIA

Explants	Zone	Total Cells	Mean Density	Total Colchicine Metaphases	Mean MI (%)	Total Pycnotic Cells	Mean PI (%)	Presence of		Positive Beta-Metachromasia in Matrix
								C-Metaphases	FP OP	
6 Treated	RCZ	7230	240.93 +	49	0.66248 +	28	0.3760	6/6	6/6	6/6
	FCZ	3900	-8.9 133.5 +	36	-0.13 0.9968 +	37	1.3278			6/6
	HCZ	2542	-6.7 87.24 +	0	-0.59 0 +					6/6
6 Control	RCZ	4975	-7.0 235.3 +	305.5	0.6509 +	15	0.2532			5/5
	FCZ	3449	-3.9 135.5 +	21	-0.07 0.7734 +	14	0.5454			5/5
	HCZ	2221	-8.5 88.11	0	-0.25 0.0					5/5

+ Standard error of the mean.
 * eyefiece graticule of constant sq. area

RCZ Round Cell Zone
 FCZ Flat Cell Zone
 HCZ Hypertropic Cell Zone
 FP Fibrous Peticochondrium
 OP Osteogenic Periochondrium

TABLE V

CELLULAR DENSITIES, MITOTIC AND PYCNOTIC INDICES AND MATRIX CHARACTERISTICS

IN THE TRANSFORMATION ZONES OF CHICK FEMORA AFTER 2 1/2 DAYS CULTIVATION IN THE

PRESENCE OF 40 MG PER ML THALIDOMIDE IN NATURAL MEDIA

Explants	Zone	Total Cells Counted	Mean Density	Total Colchicine Metaphases	Mean MI (%)	Total Pycnotic Cells	Mean PI (%)	Presence of C-Metaphases in FP OP	Positive Beta-Metachromasia in Matrix
6 Treated	RCZ	6627	237.26 +	23	0.35305 +	8	0.1382	6/6	6/6
	FCZ	3812	-4.5 126.95 +	16	-0.05 0.4371 +	13	0.3581	6/6	6/6
	HCZ	2345	-7.0 78.033 +	0	-0.07 0.0 +				6/6
	RCZ	5247	-1.3 229.56 +	30	- 0.5506 +	7	0.13108		5/5
	FCZ	3326	-6.2 137.76 +	17	-0.10 0.5716 +	5	0.1943	6/6	6/6
	HCZ	1822	-9.2 86.72 +	0	-0.11 0.0 +				
			-1.14		-0.0				

+ Standard error of the mean.

* eyepiece graticule of constant sq. area

RCZ Round Cell Zone
 FCZ Flat Cell Zone
 HCZ Hypertrophic Cell Zone
 FP Fibrous Perichondrium
 OP Osteogenic Perichondrium

THE STATISTICAL SIGNIFICANCE OF THE DIFFERENCES IN
RESPONSES OF EXPLANTS CULTURED WITH AND WITHOUT THALIDOMIDE

Conc.	Zone Compared	CELLULAR DENSITIES			MITOTIC INDICES			Level of Significance	
		Degrees of Freedom	Mean Diff. in Response*	Standard Error of the Mean Diff.	Level of Significance	Degrees of Freedom	Mean Diff. in Response*		Standard Error of the Mean Diff.
10 mgm./ml.	RCZ	8	-28.8	16.325	> 0.05	8	-0.3640	0.488	> 0.05
	FCZ	9	-5.27	13.535	> 0.05	6	-0.1565	0.618	> 0.05
	HCZ	8	-1.48	10.369	> 0.05	8			
20mgm./ml.	RCZ	9	+11.04	9.195	> 0.05	9	+ 0.2328	0.294	> 0.05
	FCZ	7	+20.7	13.057	> 0.05	4	-0.4840	0.374	> 0.05
	HCZ	8	-2.64	6.979	> 0.05	8			
30mgm./ml.	RCZ	9	+5.63	10.464	> 0.05	9	+0.0116	0.159	> 0.05
	FCZ	10	-2.0	10.822	> 0.05	8	+0.2234	0.772	> 0.05
	HCZ	10	-0.87	7.945	> 0.05	10			
40 mgm./ml.	RCZ	9	+8.706	3.5834	< 0.05	9	-0.1975	0.109	> 0.05
	FCZ	9	-10.81	11.424	> 0.05	9	-1345	0.412	> 0.05
	HCZ	9	-8.69	1.786	< 0.001	9			

+ sign refers to an increase over the control mean.
 - sign refers to a reduction compared to the control mean.
 * eyepiece graticule of constant sq. area

mgm = micrograms

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