THE UNIVERSITY OF MANITOBA

AN *IN VITRO* TERATOLOGICAL ANALYSIS OF NEURORETINAL EXPLANTS WITH METHOTREXATE

by

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STATEMENT ABOUT THESIS FORMAT

In the study of a specific *in vitro* teratological model, it is necessary to analyse the "possibilities, limitations and fallacies of this technique" (Saxen, 1966). The preliminary experiments performed were sufficiently distinctive to be presented as separate parts (I, II and III), each with a "statement" of its aims, "introductory review", "methods and materials" and "results and discussion". The "conclusions and recommendations" from each part contributed to the interpretation of *in vitro* teratological methods and specifically to the design and the analysis of the proposed *in vitro* teratological model considered in Part IV.

ABSTRACT

White Leghorn chick neuroretinal explants were adapted in an *in vitro* teratological study. Preliminary studies (PARTS I, II, III) established that:

1. The nutrient environment adapted did not adversely affect the inherent developmental potentialities of the chick embryonic tissues.

2. In the millipore filter culture technique adapted, the neuroretinal explants with associated mesenchyme maintained its pseudostratified organization. The adhesion mechanisms remained intact and facilitated the "elevator-like" movements of nuclei, characteristic of the ventricular cells of the early neural tube. The formation of distinct DNA synthetic, interkinetic and mitotic zones could conveniently be studied on the millipore filters. Proximity to the source of nutrients was the major limiting factor as could be determined by the observed gradient of DNA synthetic activity. The importance of the adhesive mechanisms was analysed and a variety of neuroepithelial responses (rosette-formations, neural folds, neuromery) could be followed after a transient depletion of the divalent ions. These studies also established the response potential of the neuroretinal explants.

The *in vitro* teratological model was analysed by studying its direct responses to methotrexate. Explants of four day old chick embryonic neuroretinas with associated mesenchyme were cultured on millipore filters on the Trowell platform. Medium 199 and CEE in humid air was used as the complete environment for culturing. *In vitro* cumulative labelling studied indicated that at least 95% of the

neuroretinal cells were capable of DNA synthesis after 12 hours of culture. Fifty percent of these cells had started DNA synthesis as soon as two hours after culturing. Experimental explants were exposed to an observed non-toxic concentration of methotrexate (5.5×10^{-7} M per ml medium) in medium 199 alone or with CEE. In view of the known action of methotrexate in the production of a thymidine-deficient state in cells, the DNA synthetic and mitotic responses were followed over long exposure periods (up to 16 hours). No direct stathmokinetic effects of methotrexate were observed throughout the exposure period since all mitotic stages were always seen. With prolonged exposure periods (8 hours, 16 hours), the mitotic index declined, but was directly related to the effects of methotrexate on DNA synthesis and cell death. After 8 hours exposure, the produced thymidine-deficiency in cells could be deduced by the readiness of cells to incorporate the exogenous labelled thymidine. The labelling index was significantly greater than the controls and the viability was well maintained. The associated mesenchyme and pigmented epithelium were less sensitive to the deleterious effects of methotrexate. With prolonged exposure periods to methotrexate (16 hours), the thymidine-rescued cells were unable to complete DNA synthesis. There was a progressive increase in detached labelled cells in phases of degeneration. A progressive decline in mitosis was observed in the intact epithelium that could only be related to a decline in DNA synthesis. No direct mitotic inhibition could be observed.

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STATEMENT OF THE PROBLEM

IN VITRO TERATOLOGY:

Knowledge of mechanisms involved in teratogenesis has been limited by the experimental methods used. Most teratological research is concerned with the effects of drugs on various species. The literature contain countless duplications of specific investigations with more controlled or slightly altered conditions. Furthermore, the production of malformations similar to that produced in humans has been reproduced only in some species.

The present in vitro teratological approach has been studied for several reasons:

1) Direct analysis of the mode of action of embryotoxic or other substances.

Interspecies differences in drug responses may result from variations in the fate of the drug (its absorbtion, distribution, excretion and biotransformation) ie. pharmacokinetic factors and from variations in the responding systems themselves ie. pharmacodynamic factors. Combinations of these factors compound the possibilities of various responses in the in vivo studies (Jacobs, 1968).

In vitro teratological methods have been adapted (Saxen, 1966; Daniels, 1967; Umansky, 1968) to eliminate many of the above-mentioned pharmacokinetic factors so that the embryonic tissue responses can be more directly related to the specific experimental exposure conditions. The in vitro studies supplement the in vivo studies as it distinguishes the direct from the indirect effects. Studies at the organ, tissue and cellular levels of organization reduce many of the interspecial

differences observed in the whole organism.

Since analysis of malformations observed at birth do not always reveal all the embryopathic responses, the direct observation of the development of a malformation is informative. Generalized necrotizing end responses mask many of the earlier direct effects on essential morphogenetic events such as proliferation, adhesions and motility. Menkes et al (1970) has observed this generalized response in malformations after treatments with a variety of agents (radiation, viruses and chemical injuries).

2) In the direct in vitro studies, the response potential of specific target tissues can be analyzed. These observations would aid in interpreting in vivo responses. During the determinative stages of morphogenesis, the undifferentiated territories are very sensitive to environmental influences. In this phase, however, a defect can be removed by the process of regulation (Rickenbacher, 1968). This process is distinguishable from regeneration which occurs in differentiated tissues. Unlike regulation, regeneration in embryonic tissues is considered to be more complicated and rare. It is possible that differences in regulatory capacity is also responsible for the different behaviour of various species against teratogenic factors.

The main objective of this study is to analyse and evaluate the use of neuroepithelial explants in an in vitro teratological study. The chick neuroepithelium provides an homogenous population of cells and is an ideal model tissue to study a number of morphogenetic events (proliferation, adhesive mechanisms, movements and regulatory capacity). The ventricular cells are the ultimate progenitors of all neurons and

macroglial cells in the CNS (Boulder Committee, 1970). Neuroretinal explants, still showing the ventricular stage, will be studied and is representative of early development of the CNS.

The analysis of this model in vitro system required an initial evaluation of the interaction between the explant and the culture conditions. Observations on the adapted in vitro culture method were made initially and the most favourable culture technique and environment was selected after considering the morphogenetic responses observed. The selected tissue (neuroepithelium) was then analyzed under the standardized culture method and also under experimental culture conditions to estimate its response potential in the "abnormal" environment. Finally, the model was tested by analyzing the mode of action of the antimetabolite, methotrexate.

The following series of experiments were performed:

Part I: Observations of critical morphogenetic events possible in an adapted in vitro environment.

Part II: Characteristics of this standardized culture method.

Part III: Sequential response potential of the neuroepithelium under specific environmental modifications.

Part IV: Analysis of a teratogen action (methotrexate) with this standardized in vitro model.

PART I

STATEMENT:

Many morphogenetic responses can only be studied *in vitro* and even then only under special culture conditions. In these studies special emphasis was placed on:

1) Description of the culture apparatus and method.

2) Observations of the range of developmental responses of the explants under the selected culture environment; namely medium 199 (9 parts) 10% chick embryo extract (1 part), humid air phase and a "dynamic" medium renewal schedule.

3) Consideration of the most favourable culture procedure and environment for the *in vitro* teratological analysis.

INTRODUCTORY REVIEW

Some of the underlying cellular interactions in morphogenetic responses (motility, aggregations, regulation and reorganization) can only be studied *in vitro*. The detailed review of the importance of the cell surface in morphogenesis by Curtis (1967) stresses than any environmental modification of the cell surface could abnormally affect morphogenesis. The "abnormal" culture environment is therefore a critical consideration in the *in vitro* teratological studies. The adapted culture technique and environment should be evaluated and standardized before such studies are undertaken (Saxen and Rapala, 1969). The inherent developmental responses of the explants should be supported but not challenged in the culture environment. Furthermore, these responses should be reproducible. Organotypic cultures favour normal differentiation of explants.

The procedure of culturing explants at the interface between medium and atmosphere is conducive for organotypic cultures but only when peripheral migration of explant cells is prevented. In these experiments, explants on millipore filters will be supported on stainless steel platforms as described by Trowell (1954).

In this essentially two-dimensional substration (millipore filter), organized growth responses can be followed under the specified culture conditions. However, the limitations on these responses, due to these culture conditions cannot be easily estimated in the study. Under experimental conditions, the explant responses may deviate from normal responses. It is necessary to eliminate the extraneous effects due to culture environment (medium, renewal, atmosphere). The culture conditions selected could possibly provide a restricted or an altered scope

for developmental expression. A means for estimating the environmental influences would be to challenge the explant responses in the unmodified environment.

By only altering the physical substratum of the explants, (to a three-dimensional substratum), the cells would be allowed to respond in a number of ways possible under the unmodified environment provided. Explants will be cultured on the collagen sponge supported on the Trowell platform. This combined Trowell-Leighton culture technique will at the same time, be analyzed as a method for the analysis of teratological events. The sponge culture technique is essentially a cell culture technique in which cellular interactions can be observed in a dimension comparable to the in vivo situation.

The technique facilitates a number of morphogenetic responses so that the limitations of the environment (medium, atmosphere, previous manipulations) and technique can be assessed. Any modulatory effects due to these environmental limitations can be followed.

METHOD AND MATERIALS

CULTURE METHODS

Stainless steel fine mesh platforms are the only special equipment required and can be obtained from the (Falcon Plastics. Becton, Dickenson & Co., Canada Ltd., Clarkson, Ontario). Triangular organ culture grids are made of non-toxic stainless steel cloth, 60 mesh, with a wire diameter of 0.0075 inches. The limbs of the triangular grid can easily be bent to adjust with various volumes of media. Routine cleaning for tissue culture glassware and Petri dishes is followed for the Petri dishes and grids,then the assembled grids and Petri dishes are sterilized in dry heat at 150^{0} C for 1 hour. Clean lens paper is cut into strips (3 x 1 cm), soaked in ether, ethanol and finally in several changes of distilled water. After drying in clean Petri dishes they are sterilized in dry heat at 120^{0} C for 1 hour, then covered with aluminum foil and stored in an ultraviolet cabinet. The final assembly of the sterilized equipment is carried out in the sterile cabinet. The complete medium is measured into the Petri dishes via a Swinney millipore filter (pore size = 0.2 micra). The platform had been previously prepared to be slightly higher than the surface of the measured medium. Medium is spread at the surface of the grid by the strong capillary forces of the fine mesh stainless steel grid and is maintained throughout the culture period at this level by the sterile lens paper which has been draped over this platform. The culture apparatus is then covered and set aside in the sterile cabinet (see Fig. 1).

Sterile porous Gelfoam sponge (size 12 x 7 mm) do not require preliminary treatment and are cut with the aid of supporting cover slips (Leighton, 1968) in the sterile cabinet. The porous, pliable, nonantigenic

matrix is obtainable from the Upjohn Company, Kalamazoo, Michigan, U.S.A. The sliced pieces (5mm in height) can be lifted by its strong electrostatic attraction for the strip of millipore filter. The millipore filter with the adherent sponge is then gently placed on the prepared culture platforms. The culture setup is then left to stabilize at room temperature in the cabinet as the microdissection procedure is carried out. Within half an hour the sponge would have shrunken a little as it has imbibed medium (see Fig. 1b).

The above procedure of combining the Trowell and Leighton techniques provide a modified diffusion system with many manipulatory advantages compared to the original three-dimensional culture technique described by Leighton (1968).

CULTURE MEDIUM:

The standard control culture medium (referred to henceforth as a "complete medium") consisted of chemically-defined medium 199, with glutamine (Gibco, New York). It was supplemented with a 10% chick embryo extract prepared from live embryos of the same chronological age as the explants. No serums or antibiotics were used. The complete medium was buffered (pH 7.4 - 7.6) and finally measured into the culture dishes as it was sterilized through (Gelman metrical) millipore filters (pore size = 0.2 micra). Five millilitres of medium was filtered into each culture dish. The air phase (of the covered culture dishes) appeared to be maximally humid after a few minutes in the incubator. The petri dish cover soon collected condensates on its inner aspect that persisted throughout the culture period.

FIGURE 1

CULTURE APPARATUS

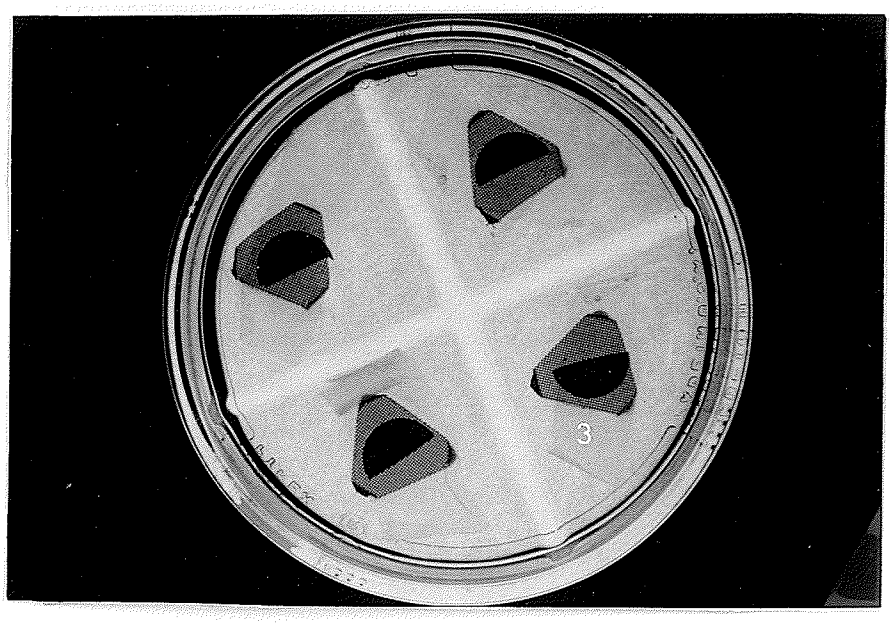
(a) Surface view of Trowell platforms in quadrant culture dish.

(b) Combined Trowell-Leighton culture technique adapted.

With prolonged culture periods and with increased metabolic activity, the pH changes are first observed in the gelfoam sponge. The millipore filter (pore size 0.45 micra) demarcates the distinct metabolic gradients in the sponge and main source of medium.

ABBREVIATIONS:

1. liquid culture medium
2. stainless steel grid platform
3. lens paper
4. millipore filter
5. gelfoam sponge
6. position of explant



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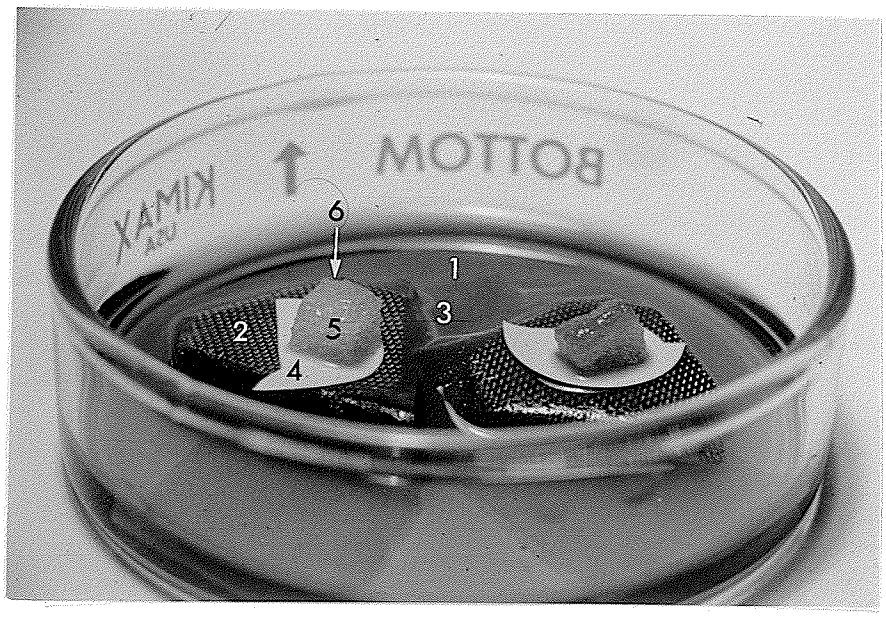


(1)b

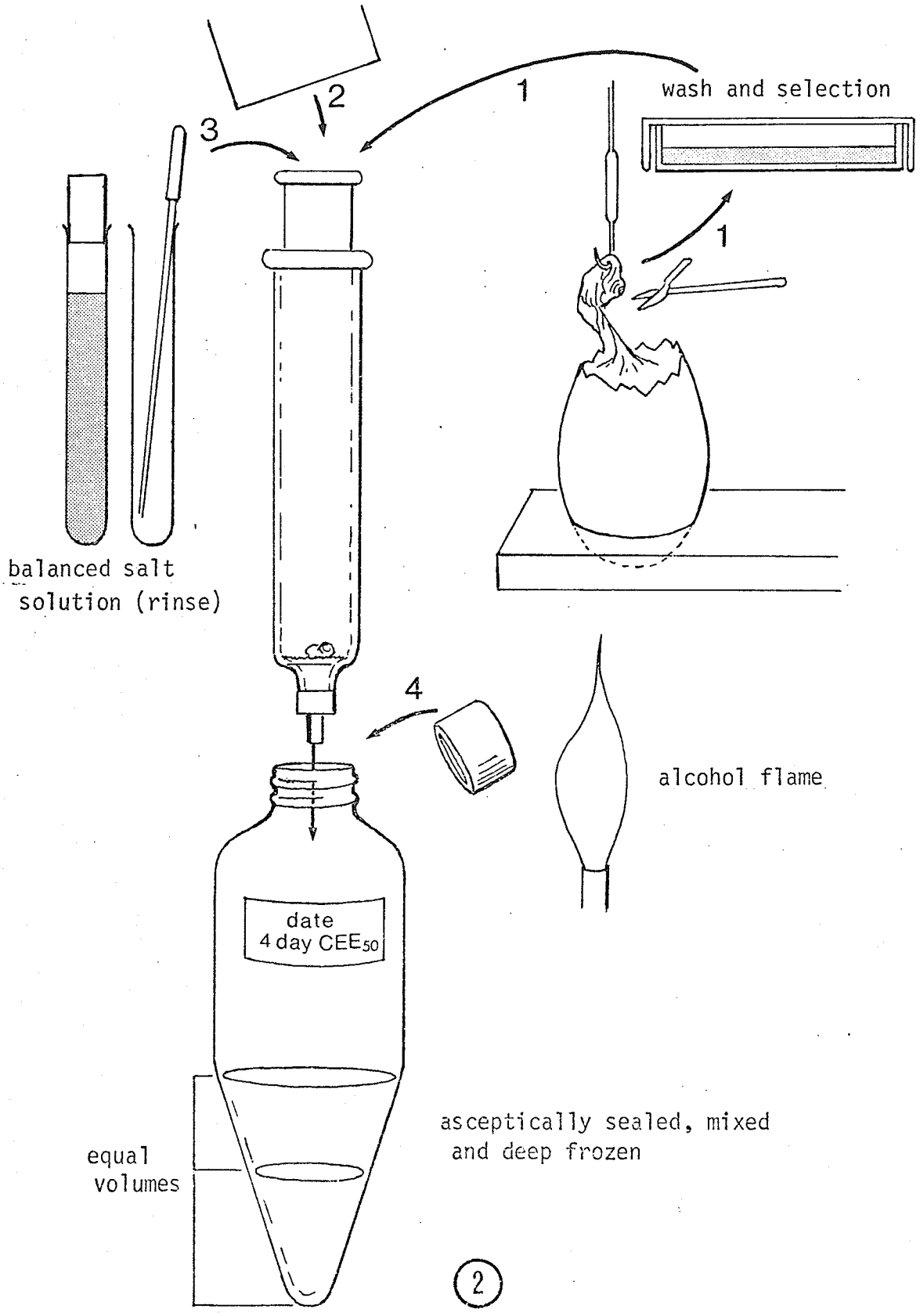
Since it is difficult to attain a universal standarization of chick embryo extracts (Parker, 1964), a strict routine in preparing the extracts was followed. The exact concentration of the embryo extract has been shown to be an important factor in determining mitotic activity (Jacoby et al, 1937). The embryo extract was prepared from 4 day old chick embryos as a 50% solution in Hanks balanced salt solution (HBSS) (Paul, 1960). The live embryos were washed to remove excess yolk and blood and aged according to Hamburger and Hamilton (1951). The embryo were then forced through the barrel of a sterile 20 ml syringe into a graduated centrifuge tube (Fig. 2). The syringe barrel base was previously fitted with a fine stainless steel grid before steam-sterilization of the whole apparatus. The non-toxic stainless steel grid cloth (60 mesh with a wire diameter of 0.0075 inches) ensured a fine maceration of the expressed embryos. The pulp was diluted to a 50% solution and centrifuged at 2000 rpm for 10 minutes. The supernatant was millipore filtered (pore size = 0.2 micra) to remove all cellular material, and finally, further diluted to make a 10% embryo extract. This solution was mixed with chemically-defined medium 199 in the ratio 1:9.

Medium replenishment was performed every forty-eight hours, at which time a fresh solution of embryo extract was prepared and used immediately. Embryos used in the extract preparation were of the same chronological age as the cultured explants. This procedure of maintaining a "dynamic medium" has been established by Gaillard (1935, cited by Grobstein, 1959) who observed better differentiation of tissues under these conditions.

FIGURE 2

PROCEDURE FOR THE PREPARATION

OF CHICK EMBRYO EXTRACT


1
2
3
4
wash and selection
balanced salt
solution (rinse)
alcohol flame
date
4 day CEE50
equal
volumes
asceptically sealed, mixed
and deep frozen

EXPLANTS:

Chick embryonic tissues (limb buds, mesonephric tissues, neuroretinal tissues, surface ectoderm) were dissected from 3 - 4 day old White Leghorn chick embryos. The explants (1 - 2 mm^2) were pipetted on to millipore filters or placed on the sterile gelfoam sponges in the culture dishes.

HISTOLOGICAL PREPARATIONS AND ANALYSIS:

All tissues were fixed in Carnog II at 4^o C for two hours. All gelfoam cultures were embedded on edge so that events in the explant and sponge could be simultaneously studied. Serial sections were cut at 5 micra and routinely stained with toluidene blue O (0.1% in 30% ethanol) and with hematoxylin and eosin. Qualitative descriptions of responses were recorded.

OBSERVATIONS

Clues as to the desirability of replenishment of the medium were estimated from histological observations of poor viability (cytoplasmic granularity and nuclear pycnosis over prolonged culture periods in the same medium. The presence and extent of pH changes were visually evident in media after 3 days so that the "dynamic" medium (5 ml) was replaced every 48 hours.

Explants cultured on millipore filters in this "dynamic media" showed excellent growth but the qualitative responses (cellular viability, proliferation, metachromasia) did not facilitate definite analysis of the actual or potential developmental expression of the explants. The adaptation of the three-dimensional gelfoam culture substratum of Leighton (1968), in the liquid organ culture environment of Trowell (1954), facilitated the following significant morphogenic responses of the explants in the same nutrient environment.

(1) Morphogenetic events that occur in the limb bud <u>in vivo</u> could also be observed under the environmental conditions used in this culture technique. The migration, self-aggregation and differentiation of the limb bud mesenchyme could be observed in the histological sections of the three-dimensional sponge cultures. Degrees of self-disaggregation and migration was always observed within and around the sponge pores. The first indications of differentiation was the formation of substantial aggregates in the loose three-dimensional network of mesenchymal cells (Fig. 3). Cartilage formed in aggregates as conspicuous three-dimensional whorls of cells (Fig. 4) within the loose mesenchymal cells or as nodules attached to the collagenous gelfoam trabeculae (Fig. 5).

In the same cultures, early stages of myogenesis were also observed.

At explantation there was no structural or staining differences in the mesenchymal cells of the limb bud. Migration of mesenchymal cells into the interstices of the gelfoam sponge was observed after 24 hours. In many interstices bipolar shaped cells with strong eosinophilic-staining cytoplasm were seen in clusters or aggregates. Konigsberg (1967) has characterized these cells as prospective myoblasts (Fig. 4). Various degrees of the aggregation and fusion can be observed as the aggregates have formed in many of the gelfoam pores (Figs. 4,6,7). Attachment of the fused myoblasts (myotubes) were observed to be at its free ends (Fig. 6) or throughout its surface to the gelfoam substratum (Fig. 7). The limited population of aggregated cells very likely limited the development of the formed myotubes. Incorporated nuclei do not replicate (Kornigsberg, 1967) so that further development depends on fusions of associated presumptive myoblastic-cells.

The culture environment proved suitable for the self-dissaggregation migration and self-reaggregation of the specific cell types. The surface interactions between cells in this environment apparently did not affect the normal differentiative states of the cells. The successful recognition and fusion of homotypic cell types suggest that the specific structure of the cell surfaces were maintained and not adversely changed. The fusion of presumptive myoblasts is thought (Okazaki and Holtzer, 1966) to be a recognition of the specific molecular architecture of the cell surfaces of these differentiative cells.

(2) Regenerative and other reorganizational responses that occur <u>in vivo</u>

under experimental conditions, were also observed. After two days of culture, mesonephric tubules were observed deep within the gelfoam pores (Fig. 8). A reconstruction of the morphology from serial histological sections indicated that the tubules were continuous with those in the explant. In the gelfoam pores the tubular epithelium was only observed in regions previously infiltrated with loose mesenchyme. It is possible that these observations indicate the continued growth of the tubules or the regeneration of the excised edges of the explant. The environmental conditions supported the continued development of these tissues.

Neuroretinal explants showed normal organized proliferative activity when cultured on a two-dimensional substratum but disaggregated in regions exposed to the three-dimensional surfaces (Figs.9,10).The typical reorganizational response of these cells in vivo (rosette-formations) could also be observed under these environmental conditions (Fig. 10).

The associated mesenchymal tissue (scleral mesenchyme) differentiated into cartilage as it normally does in vivo (Fig. 10).

CONCLUSIONS AND RECOMMENDATIONS

The culture conditions adapted did not adversely affect the inherent developmental potentialities of the chick embryonic tissues cultured. The following environmental conditions will be the standard culture conditions in subsequent experiments: medium 199 with glutamine (9 parts), 10% chick embryo extract (1 part), humid air phase, pH 7.4 - 7.6, renewal with "dynamic" medium every 48 hours, and the observance of similar explantation and manipulatory procedures. Under these conditions the following responses were obtained.

1. Limb bud mesenchymal cells were able to migrate, aggregate and differentiate to form cartilage, connective tissue and skeletal muscle. Scleral mesenchyme also developed specifically into cartilage. These *in vitro* responses parallel the normal phenotypic expressions of these tissues *in vivo*.

2. Reparative processes that occur *in vivo* under abnormal conditions were also observed in this environment. Excised mesonephric tubules continued growth and regeneration whereas the disorganized neuroretinal explants attempted reorganization in a similar manner as it does *in vivo*.

3. Though the combined Trowell-Leighton techniques were adapted to observe the possible explant responses in this particular nutrient environment, it was also considered as a technique for studying abnormal morphogenesis.

Self-disaggregation and self-reaggregation of embryonic tissues were observed in this technique. The provisions for migration

in the three-dimensional pores allows for intercellular contact between cells in a dimension comparable to the in vivo situation. The technique differs from other disaggregation: reaggregation culture techniques in that it depends on inherent motility and cell associations during histogenesis. The usual reaggregation technique, described by Moscona (1961 a), do not involve the initial active deformations of the cell surface, but depend on gyratory shakers to accumulate the spherical suspended cells. The importance of adhesiveness and motility in morphogenesis can only be indirectly inferred as it was in a recent in vitro teratological study (Ade and Akebak, 1968). The combined Trowell-Leighton technique could therefore provide a useful teratological technique which includes the early morphogenetic event of motility. Techniques that depend on motility, however, are highly susceptible to extrinsic influences (Moscona, 1961 b). Furthermore, this characteristic is not easily quantifiable and is therefore restricted to qualitative observations. Pilot studies were performed in order to obtain better environmental conditions in which this characteristic motility could be more consistent. The number of variables studied included: different media (Trowell T8, Scherer's maintenance medium, inactivated calf serum and with chick embryo extract combinations), 95% O_2 and 5% CO_2 gas phase, different culture durations and tissues of different ontogenetic ages.

In the cultures with good viability, the migratory activity was not improved over that observed in medium 199 plus chick embryo extract. Many extraneous factors were observed. In limb bud cultures, the interrupted surface epithelium frequently migrated and reconstructed multicompartmentalized vesicles. These extensive vesicles were distributed throughout the culture and frequently obstructed the migratory

pathways into the three-dimensional gelfoam pores (Fig.11). Degrees of migration of explant cells into the sponge were noted under many of these conditions and differentiation of these cells were noted in the migratory positions (Fig. 12). The number of factors that effect this morphogenetic response in vitro is considerable. Detailed studies (Lefford, 1964; Soukupova et al, 1970), have shown that the time before there is an initial migration from explants ("latent time") varies with the tissue type, ontogenic age and culture environment.

4. Considering the culture technique, environment and tissues that produced the most consistent results, it would be feasible to use the adopted culture conditions mentioned earlier with the two-dimensional millipore filter substrate. The neuroepithelial explants consistently maintained its inherent structure in this model and will be used in the in vitro teratological study.

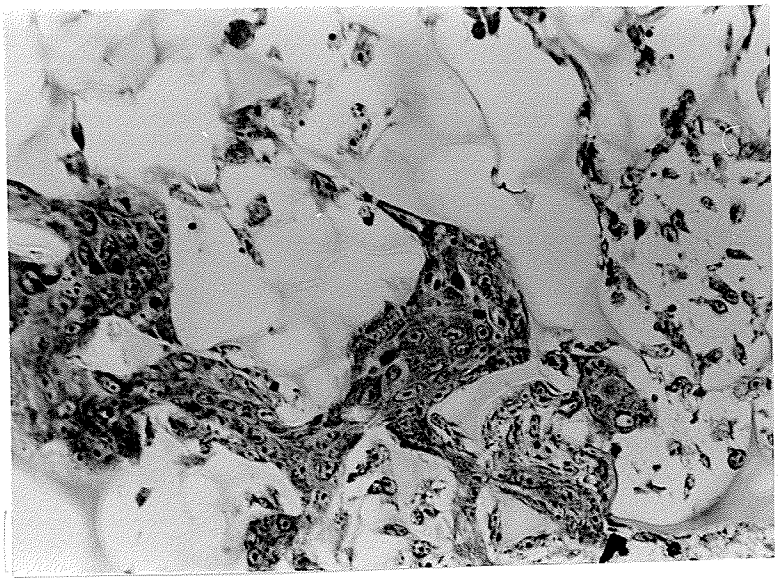
Detail standard responses of the neuroepithelium in this environment and technique will be studied in Part II.

FIGURE 3

Histological section through the Gelfoam sponge. Substantial aggregations of cells are observed in densely populated regions of the sponge.

(a) Migration of loose mesenchymal and epithelial cells in the pores of the sponge. x160

(b) Cell aggregations form in densely populated regions. x160



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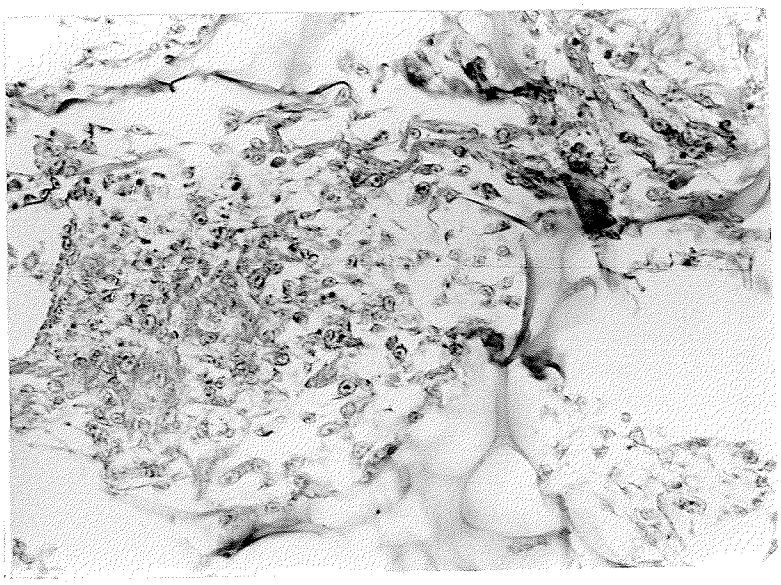

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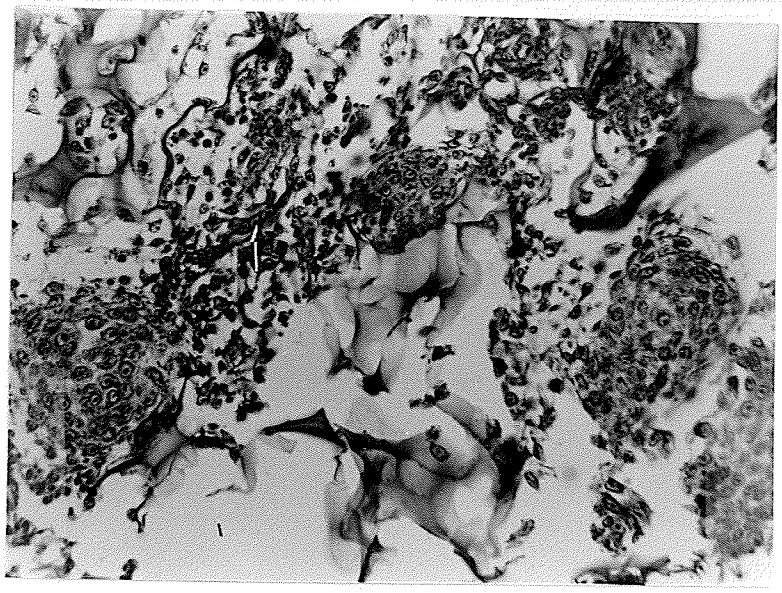
FIGURE 4 Histological sections through the gelfoam sponge. Stages in which the reaggregations of cartilage and muscle are seen. Three-dimensional whorls of cartilage within a region of loose mesenchyme. Stages of fusion of myoblasts can be seen between the cartilage nodules (arrow). x160

FIGURE 5 An irregularly-shaped cartilage nodule form on and around the collagenous trabeculae of the sponge. x81.2

FIGURE 6 Fusion of myoblasts (bipolar-shaped cells) in the formation of myotubes (arrow). x160



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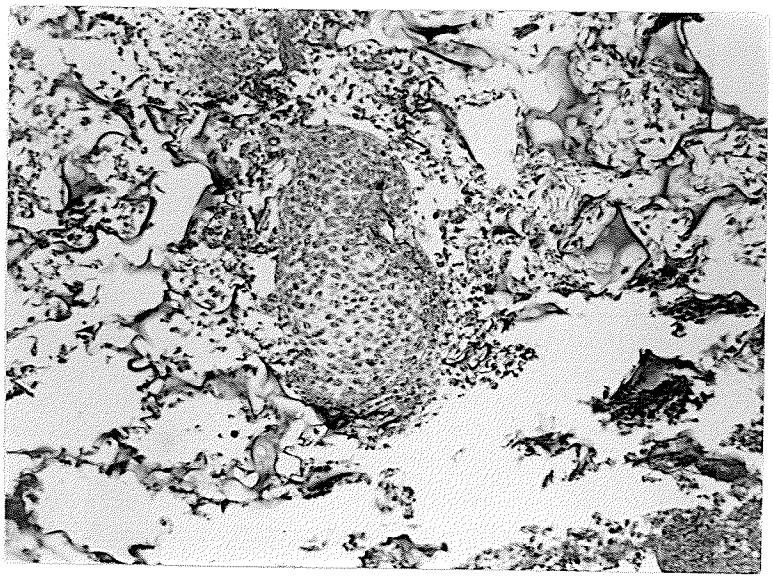

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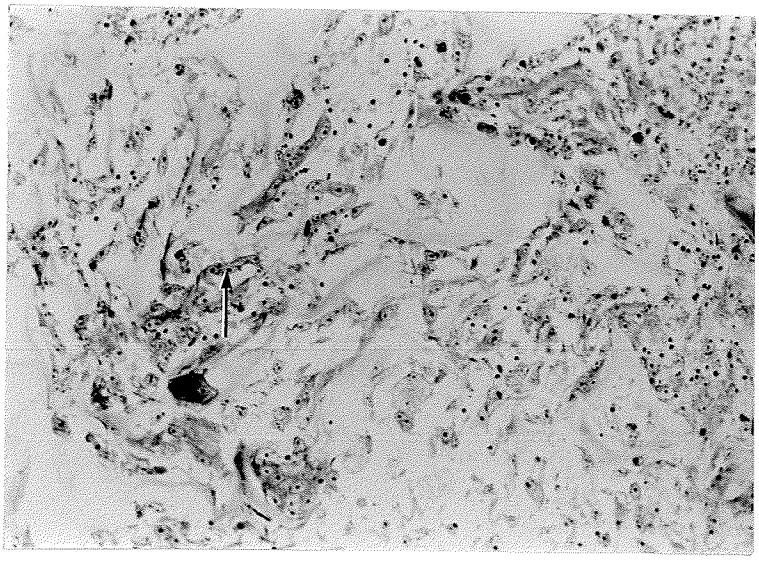

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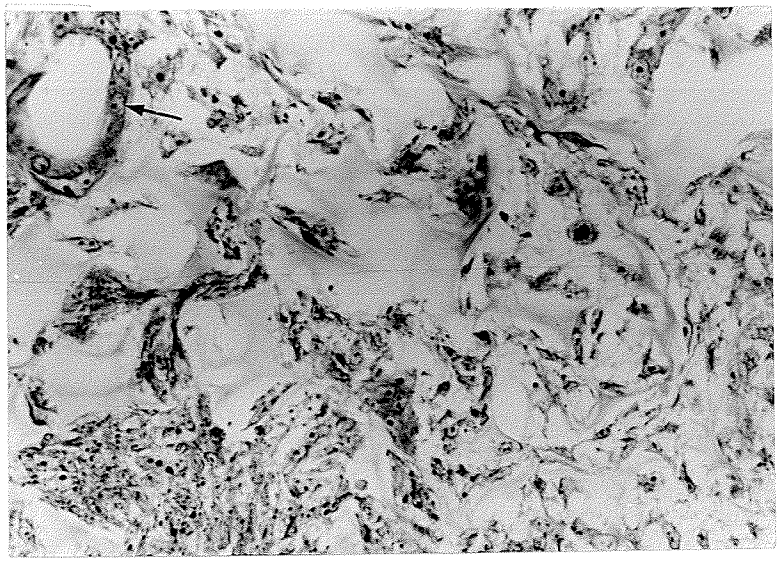
FIGURE 7 Histological section through a gelfoam sponge. Fusion of myoblasts and attachment throughout its surface to the collagenous trabeculae (arrow). x160

FIGURE 8 Histological section through a gelfoam sponge. Mesonephric tubules are seen deep within the gelfoam pores. The tubular epithelia are supported by associated mesenchymal cells that have infiltrated the pores. x160

FIGURE 9 Neuroretinal explant cultured on a two-dimensional substratum (millipore filter). The explant is well organized with all mitosis confined to the mitotic zone of the epithelium. x160



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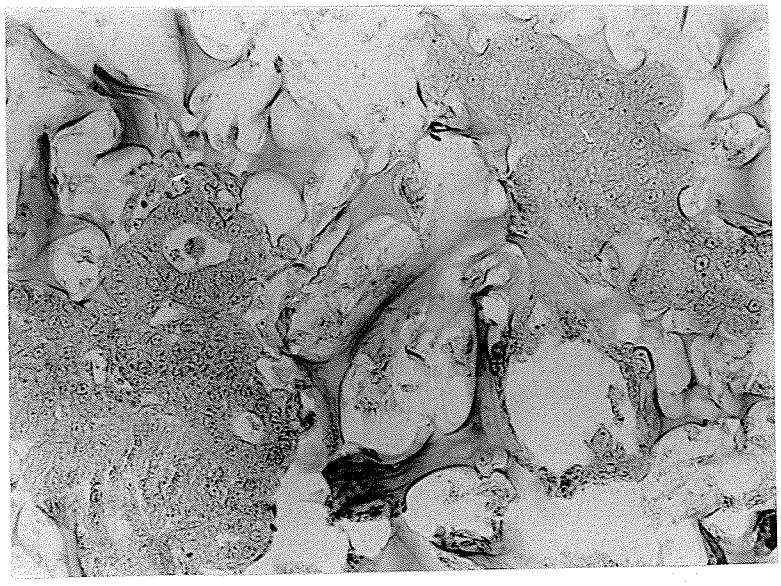

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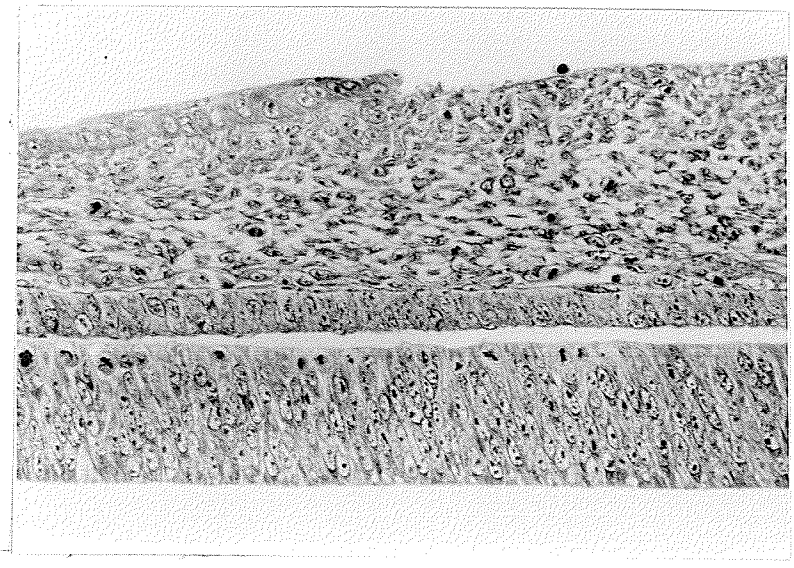

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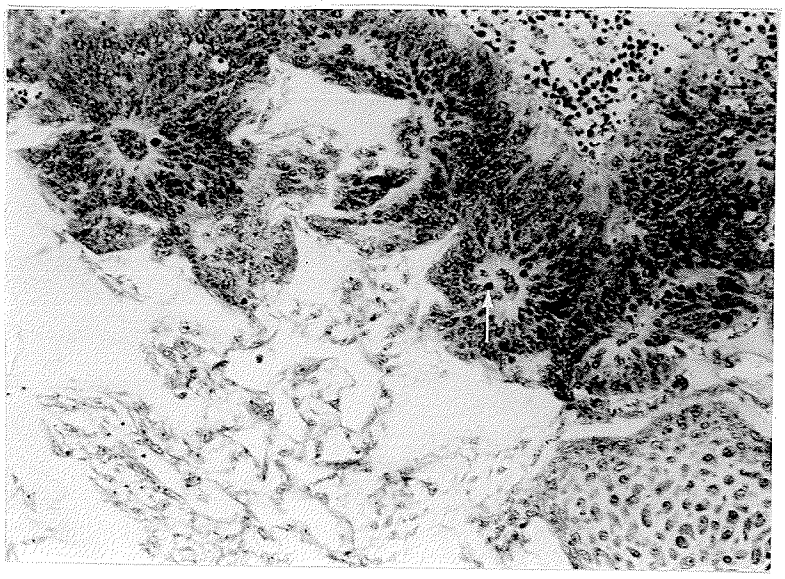
FIGURE 10 Histological section through the gelfoam sponge. Rosette-formations are seen in the reconstructed neuroretinal epithelium. Mitotic figures are localized toward the lumen of the rosette (arrow). Scleral mesenchyme differentiated into cartilage in the adjacent three-dimensional sponge pore. x100

FIGURE 11 Histological section through the gelfoam sponge. Epithelial vesicle formations are seen in migratory pathways. x100

FIGURE 12 Histological section through the gelfoam sponge. Cartilage differentiation is seen in migratory paths within the sponge. x160



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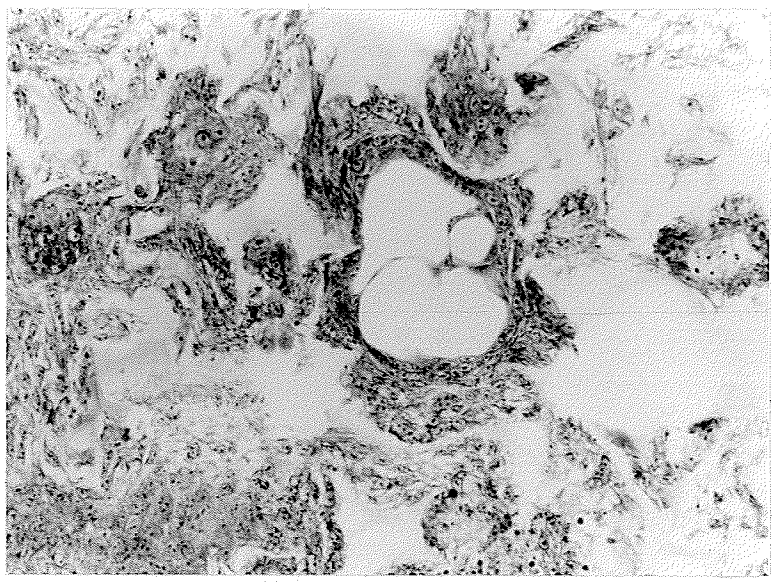
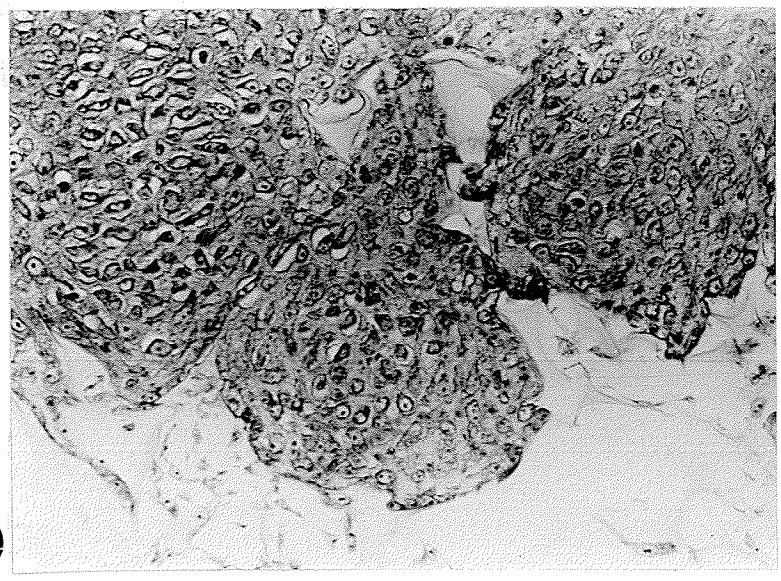


11



12

PART II

STATEMENT:

The culture system involves an interaction between the particular explanted tissues and the culture environment. Characteristics of the neuroepithelial responses in a standardized culture environment were studied. A critical evaluation of the responses and the analytic procedures are necessary so that unequivocal interpretations of ensuing experimental responses can be made.

The following responses were analysed throughout the culture period:

(1) Characteristics of incorporation of H3-thymidine into DNA synthesizing nuclei.

(2) Cell death and loss from the explant.

(3) Differential and morphological organizational responses.

INTRODUCTORY REVIEW

1. "LIFE HISTORY" OF EXPLANTS

Tissue and organ culture techniques are used experimentally to aid in the understanding of processes not easily studied in complex *in vivo* situations. These situations cannot be reproduced *in vitro* but can only be approached since the very act of explantation introduces abnormal surroundings. The explantation procedure and *in vitro* transfer represents a drastic change in the internal environment of the explant. Biggers (1963, 1965) described the duration of cultivation of bone rudiment explants in terms of its response "life history" *in vitro*. Viability changes in a tissue with abundant intercellular substance could be estimated by observing the quantitative changes in the dry weight: wet weight ratio throughout the culture period. Saxen et al (1968) reported more detailed metabolic changes in the predominantly cellular embryonic mesenchymal explants throughout the culture period.

The "life history" of the explant was considered in three major phases. The transfer of an explant to an *in vitro* environment involves movement of water, ions, intercellular substances (Biggers, 1965) and also the loss of some cells (Saxen and Rapola, 1969). This is known as the inital "shock phase" of explants and could possibly have serious "hangover effects" (Biggers, 1965). The stage is of vital importance in determining the subsequent phases of stabilization (restoration and adaption) and the rate of inevitable degeneration *in vitro*. Knowledge of these responses during the culture period are essential when studying normal developmental events and also specific responses to biological agents (Saxen and Rapola, 1969). The mitotic phases of cells

are susceptible during the initial culture period. The choice of valid analytic criteria is therefore important. The use of a labelling index would also be used in these studies. The mitotic time is considered to be about one tenth the duration of the DNA synthetic period and is more likely to be affected by a period of devascularization of culture experiments (Steel and Bensted, 1965). The use of a labelling index would also be a useful indication of metabolic synthetic activity during the culture period.

Saxen et al (1968) determined the initial metabolic responses in mesenchymal explants by following the one-hour incorporation of nucleic acids after various periods of culture. A considerable drop in an incorporation of these substances were observed after about an eight hour culture period. With longer periods of preculture, the incorporation rose more sharply, especially in induced mesenchymal explants. The viability of mesenchymal cells, under similar conditions in the same lab, were previously reported to be favourable during the initial 20 hour period of culture. This is also the period in which these authors observed the "metabolic lag period" and its initial recovery. Viability of the cells was considered to be unrelated to the early metabolic lag period. Even though the basic nature of these responses are not known, the assessment of these early responses was of interpretative value to these authors since the direction of subsequent differentiation seemed to be determined by this period of "low activity".

In order to further estimate the diffusional limitations of the environment a number of labelling studies were performed. Rajewsky (1965) has shown that labelled nuclei in cultured tumours are restricted

to a peripheral zone of the explants. These observations were reported to be independent of time of incubation. Rajewsky also studied the kinetics of incorporation throughout the culture period. Considering that labelled thymidine incorporation was a function of the number of synthesizing cells during the culture period, he observed that the time for 50% incorporation (t50) was about 70 minutes and the final incorporation being reached after 400 to 500 minutes. Similar incorporation characteristics were observed for other explanted tissues. Johnson and Bond (1961), Rajewsky (1965) and Steel and Bensted (1965) have all observed limitations in the depth of the labelling zone in their culture systems. This depended on the culture environment but also on the type of tissue cultured. The present studies will follow the characteristics of incorporation of the label during the culture period since these studies are performed on embryonic tissues in a stategically different arrangement of medium and atmospheric phase.

The normal cytokinetics and organizational responses of the neuroretina in vivo are importanct responses to study in vitro and will be considered.

2. CYTOKINETIC ACTIVITY OF THE NEUROEPITHELIUM DURING MORPHOGENESIS

The more recent analytic techniques of electromicroscopy cytophotometry, stathmokinetic methods and radioautography have provided ample evidence that the cells in the early neural tube form a pseudostratified epithelium (reviewed by Fujita ,1962; Watterson, 1965; Langman, 1968). The pseudostratification and interkinetic behaviour of the neuroepithelial cells presupposes that there is a firm attachment of the cells to the internal limiting membrane (ILM) and a relatively loose attachment

to the external limiting membrane (ELM). Ultrastructural studies have confirmed this assumption and a terminal bar system has been described at the ILM (Duncun, 1958). Fujita and Fujita (1963) confirmed these studies and also reported that the terminal bars remained visible in the rounded, dividing cells that have withdrawn toward the ILM. The orderly sequential release of cells from the ILM during differentiation results in the development of the normal cytoarchitecture of the wall of the neural tube. There is much speculation on the mechanism of release of the ventricular cells from the ILM. Langman (1968) considers that the mechanism would either involve the breakdown of existing terminal bars or the failure to form terminal bars at the last division before the peripheral migration. Considering the time of the first observation of detached cells ("neuroblasts") and the durations of the cell cycle phases from labelling studies, the author estimated that "neuroepithelial" cells must go through a mitotic division before giving rise to a "neuroblast". In this regard, the observation of mitosis preceeding specialization is a widespread observation in development and is exemplified also in myogenesis and intestinal renewal (discussed by Herrmann et al (1967)). Langman also considered another mechanism of release of cells from the ILM. In contrast to reports by Fujita (1962) and others, he reported a number of perpendicular and obliquely oriented mitotic spindles adjacent to the ILM. A parallel arrangement of the mitotic spindle is required to facilitate the continued attachment of the daughter cells to the ILM. Parallel orientation was the frequent arrangement of spindles, but the observation of some disoriented spindles suggested that detachment could be initiated in these instances. The rarety of these observations was attributed to the very short duration (10 minutes)

of the phase.

3. SIGNIFICANCE OF THE STRUCTURAL ORGANIZATIONAL RESPONSES OF THE NEUROEPITHELIUM FOR TERATOLOGICAL STUDIES

The structural organization of the early neuroepithelium (4 day embryonic neuroretina) possible provides a basis for the EXPLANTATION of a number of its morphogenetic responses (neurulation, neuromere formation,differentiation). If the mechanisms involved in these major morphogenetic processes can be studied in the neuroepithelium, it would provide a model tissue for teratological studies. Several authors have considered various intrinsic and extrinsic factors possibly responsible for the major responses of the neuroepithelium. To be able to consider the neuroepithelial responses under the present experimental conditions, it is necessary to consider the major neuroepithelial responses in vivo.

(i) FOLDINGS AND NEUROMERE FORMATIONS:

The dramatic morphogenetic movements observed in the neuroepithelium during development are overt demonstrations of earlier cellular interactions and induction. Experimental studies have indicated that the associated mesenchymal tissues play an important role in determining these responses. Jacobson (Kallen, 1965) observed that neurulation occurred in the Ambystema neural plate even after it had been turned upside down. The folding had occurred in a way opposite to that inherent in the neuroepithelium. This interaction with the associated tissues in vivo is of prime consideration in interpreting in vitro studies since in many instances the neuroepithelium can be studied in complete isolation. Though the morphogenetic events can be studied directly in the

isolated epithelium, careful note will be made of responses when associated with mesenchymal tissues. The importance of tissue interaction in vitro was indicated by studies which suggest that the associated mesenchymal tissues is an important local factor in determining the proliferative activity of explanted embryonic epithelia (Wessels, 1963).

Various hypotheses have been considered to account for the morphogenetic movements of the neuroepithelium including regional distribution of cell death. Morphogenetic movements of the neuroepithelium are most evident in the neurulation process. Proliferation, changes in cellular shapes and adhesiveness are basic events that are considered during neurulation and throughout morphogenesis. In considering the forces responsible for abnormal movements in the neuroepithelium during specified developmental stages, it is essential to analyse the actual events during the particular morphogenetic period. For example, differential mitosis is not likely to be responsible for the normal folding of the neural plate in Amphibia and other species since mitosis occurs at a uniform rate in all its regions (Gillette, 1944, cited by Waddington, 1956). Any extraneous interference in the other morphogenetic forces during this period could affect this folding process. Differential growth normally assumes its importance in post-gastrula-neurula stages. Any production of abnormal proliferation or differential growth during the neurulation process would result in abnormal closures of the neural tube. The rationale in using the neurepithelium in the present dissertation, is to analyse all these morphogenetic mechanisms under experimental conditions.

Consideration of the importance in cell size and shapes in the

epithelium is an important factor that has been studied. The appearance of wedge-like cell shapes in the neural groove suggested (Waddington, 1956) that cytoplasmic fibrous structures might play a role, but these could not be verified because of the obscuring yolk in Amphibian cells. A significant observation made in these studies was the definite orientation of the nuclei of these cells and its effect on cell shape. Cell shape and adhesiveness are important mechanisms througout morphogenesis (Holtfreter, 1968). For example, the increasing surface area contact has been specifically observed in the morphogenesis of the notochord in the midline of invaginated mesoderm (Mookerjee et al, 1953). Time lapse cinematographic studies on these morphogenetic events has recently been reported for the neuroepithelium (Burnside and Jacobson, 1968). These authors reported that the newt neural plate cells retain their contact relationships with neighbouring cells during their movements in the neural plate. From their topographical observations and histological studies they were able to associate morphogenetic movements of the epithelium with regional differences in the amount of change of cell shape. The cellular heights increased as the dorsal surface area of the same cell decreased. The mechanisms of initiating and controlling these changes are unknown. Malfunction of the normal mechanism would affect the normal morphogenetic process of the neuroepithelium. It is important to affirm that this study was performed on the movements of the neural plate before the plate rolls into a tube. It does, however, represent changes that do occur in the orderly morphogenetic deformation of an epithelium. In this regard it could be applicable to morphogenetic movements other than the folding process exclusively. Since the whole process of neurulation is still not completely resolved all these aspects

(cell shapes, proliferation, adhesiveness) must be considered. Under abnormal conditions, as in the initiation of malformations, all these processes have to be considered since they are possible in a pseudostratified epithelium. The withdrawal of the loose cytoplasmic attachment of ventricular cells from the ELM during mitosis would produce a large change in the dorsal surface area of the cell. Serial section analysis (Stensaas and Stensaas, 1968, Shimada and Langman, 1970) indicate that the mitotic ventricular cells are spherical and have no processes. During the orderly "elevator movements" of cells between the ILM and ELM of the compact neurepithelium drastic changes in the two surface areas would not be expected. However, the effect of extraneous agents on these movements (eg. mitotic-arresting substances) would produce a large redistribution of the total surface areas between the ILM and ELM. This could result in a folding of the epithelium and is a consideration in the morphogenesis of the epithelium. Langman (1968) has considered this mechanism as being operative in the observed open neural tube in vincristine-treated chick embryos. This basic mechanism which deforms epithelia during morphogenesis has also been considered in the phenomenon of neuromery during normal and abnormal development of the neural tube (see Discussion, p. 59).

(ii) "OVERGROWTH":

Teratological studies on the Central Nervous System have chiefly focussed attention on the proliferation of the neuroepithelium (Fujita et al, 1964). It has, as yet, not been resolved whether the malformations could be initiated through a hyperplasia of the neuroepithelial cells (Patten, 1953 , Murakami, 1955). Patten (1953) described the myeloschisis in the lumbosacral region of an eight millimeter human

embryo by comparing rostro-caudal sections through the defect. The area comparisons, using an eyepiece grid, indicated that there was excessive neuroepithelial tissue in the wide open defect. Bergquist (1959) has suggested the "more exact term, 'hypermorphosis'" to describe the overgrowth of the neural tube. The more recent illustrations from human embryos by Patten (1968) clearly show excessive neuroepithelial tissue in malformations. These observations have stimulated much research since little is known of the malformative events at the tissue and cellular levels of organization. Whether the open neural tube was cause or result of the overgrowth has not been determined (Patten, 1968). The interpretation of the overgrowth has not been defined in terms of increased numbers or sizes of cells (Langman et al, 1966). Langman and coworkers have considered the significance of the disoriented mitotic spindles during morphogenesis of the neuroepithelium and stress that it is an important consideration in studies concerning "overgrowth". Mitotic spindles usually lie parallel to the ILM so that observations of any disorientation could be related to the detachment of cells from the ILM. Orientation of mitotic spindles is a significant growth criterion. In other studies it has shown to be a valuable diagnostic feature of early disorganizing tissues in neoplasia (Robbins, 1968). Orientation of mitotic spindles will be a criterion in these studies of the neuroepithelial responses in vitro.

The more frequent localization of these malformations in the lumbosacral region (Patten, 1953) and also reports of multiple cavitations in the human posterior neural tube (Criley, 1969) has stimulated research in the normal development of the posterior regions of the neural tube. Neurulation in this zone of the neural tube is much more complicated than

in the larger rostral region. While the open neural tube formation (by fusion of neural folds) occurs in the more anterior levels, Criley (1969) re-examined the older concept of normal closed neural tube formation in the more posterior levels. Closed neural tube formation occurs by canalization of a solid aggregation of neuroepithelial cells in the more posterior levels. The description from serial sections revealed a significant overlap zone in the lumbosacral level in which both types of neural tube formation occurred. In this zone, the open neural tube formation occurred dorsal to the closed neural tube formation. These observations are of significance in view of the frequency of malformations in this location and also the regenerative responses possible from both neural tube formative zones. The histological photographs of the normal development in the "overlap zone" published by Criley (1969) show structural similarities to the lumbosacral malformations illustrated by Patten (1953, Fig. 6, 7 and 12). It must be acknowledged, though, that this "overlap zone" has not been intensively studied in human development. There have been few reports of multiple cavitation in the posterior portion of the neural tube in an 8 mm human embryo (Criley, 1969). Lamire (1970) recently reported on the variations in the caudal neural tube in eight human embryos. He reported that "a major aspect of this development appears to be a process of 'cannalization' (accessory lumens coalescing from the differentiating caudal neural mass) which corresponds to such occurrences in the chick and rat".

METHODS AND MATERIALS

1. CULTURE PROCEDURES:

The culture method, nutrient environment and schedule of medium replenishment was described in Part I, (see Fig. 1). Four to five day old neuroretinal explants were cultured on millipore filters (pore size 0.45 micra) in medium 199 plus 10% chick embryo extract (9:1) in a humid air phase. Four explants were exposed to 5 millitres medium. The effects of variations in gas phase were not studied since good proliferative activity was obtained in the air phase with the neuroepithelial tissues (Part I).

Before culturing most of explants were evenly unfolded on the millipore filter by gentle pipette flushing with culture medium. Structural observations of the cytokinetic activity of the neuroepithelium was facilitated by spreading the epithelium on the surface of the millipore filter. In vivo studies on the cytokinetics of neuroepithelial cells are often hampered by the frequency of oblique sections usually obtained of the sacculated neural tube. Analysis of the cytokinetic responses of the neuroepithelium can be more confidently done in perpendicular sections of explants on millipore filters. In thin sections (3 micra) sufficient identification of entire cells can be made extending from the ILM to the ELM. Furthermore, observations on the importance of attachment devices can be made unlike in the in vivo situation where histological sections often give the misleading impression of disrupted attachment devices (example discussed under "neuromery" page 59).

2. RADIOAUTOGRAPHIC TECHNIQUE:

Cultures were exposed to labelled thymidine (Amersham Searle), Toronto, specific activity 5 curies per millimole) in a final dilution of 1 microcurie per millitre medium. Explants were instantly fixed in cold Carnoy II ($4^{o}C$)followed by two prolonged washes in absolute ethanol to remove the fixative and excess non-incorporated labelled thymidine from the explant. Serial sections were cut at 3 micra and mounted on alcohol-cleaned slides. Sections were deparaffinized and alternate slides were prepared for radioautography by dipping in Kodak NTB-2 emulsion.

EMULSION COATING (DIPPING PROCEDURE):

The improved liquid emulsion coating technique of Kopriwa and Leblond (1962) was used. These procedures were performed in a light-proof room without use of the Wratten safelight. A limitation in this manual dipping techniques is the difficulty in reproducing a consistently even emulsion coat. Uneven coating of emulsion could cause inaccurate counts since the emulsion thickness may vary around the range of penetration of the tritium particles which is considered to be about 3 micra (Rogers, 1967). This, however, is not a serious objection in labelled nuclei counts as it is in doing specific grain counts. If the dipping technique is standardized, a fairly uniform emulsion coat can be obtained in which the emulsion thickness is about 4 to 5 micra except around the edges of the slide (Leblond, 1959; Kopriwa and Leblond, 1962). A number of precautions suggested by these authors were followed. Sections were mounted on a region of the alcohol-cleaned slides that did not include the peripheral ¼ inch of the slide edges.

The deparaffined slides were kept in a warm ($40^{o}C$) humid

incubator for about 15 minutes prior to the dipping procedure. This facilitated the adhesion of an even coat of emulsion during dipping. The slides were dipped in a consistent manner in the undiluted liquid emulsion. The bubble-free liquid emulsion was maintained at 40°C in a waterbath. The excess emulsion was allowed to drain into the container and the back of the slide wiped clean with soft paper. The slides were then allowed to dry in a vertical position in a maximally-humid incubator at 30°C for 5 minutes. Ideally, the molten emulsion should gel before it begins to dry to prevent redistribution of silver halide crystals. Kopriwa and Leblond observed that a drying temperature of 28°C and 80% relative humidity for 30 minutes produced the best results as artifacts were negligible and emulsion thickness uniform.

EXPOSURE METHOD:

The dried, coated slides were transferred to black plastic boxes containing a drying agent, Drierite. The box was sealed with black adhesive tape and kept standing on edge in a larger plack plastic box. Preliminary studies indicated that a suitable exposure period in the refridgerator (4°C) was 5 days.

PROCESSING:

All the processing fluids were kept in a water bath maintained at 20°C with ice. This procedure eliminated many of the reticular cracks seen in the emulsion in earlier studies. The exposed slides were left at room temperature for a few minutes before being placed in Eastman Kodak Dektol developer (D-19) for six minutes and washed in distilled water (10 dips). Fixation was followed for 10 minutes in Eastman

Kodak acid fixer with hardner. The slides were then allowed to stand in distilled water before staining with Harris hematoxylin (5 minutes) and eosin. The dehydrated sections were mounted in Canada balsam.

3. EXPERIMENTAL DESIGN AND ANALYSIS

Characteristics of the "life history" of explants in the culture environment were estimated by following the characteristics of DNA synthesis, mitosis, pycnosis, cell detachments and organizational responses.

The percentage of cells capable of DNA synthesis (thymidine labelling index, LI) was microscopically determined in radioautographs. Counts, with the aid of an ocular grid and hand telly-counter, were done under oil magnification (1000 x). A cell containing 3 or more grains superimposed over its nucleus was designated as a labelled cell. If the total number of cells in a radioautograph was small, additional sections were counted to reach totals ranging from 500 to 1000 cells. Every 4th serial section was counted to avoid recounting sections of the same nucleus. All the other indices (MI, PI, disaggregated cells) were estimated as percentages of at least 1000 cells. Since the neuroepithelium is a relatively undifferentiated prolific tissue, the possibility of counting errors is not as considerable as would be the case inherent in tissues with a heterogenous distribution of cells and with low labelling indices.

The two basic experimental designs in which the above responses were studied were:

1. Addition of tritiated thymidine (H_3T) to culture dishes at various

intervals during the culture period. Explants were fixed after an hour H_3T exposure (Fig. 13).

2. Continuous exposure of cultured explants to H_3T with fixation of explants at various intervals during this exposure period. Colchicine was added to these cultures during the final hour prior of fixation so that the mitotic cells (viable cells) could be easily estimated (Fig. 14). Colchicine action would also prevent the increase in number of labelled nuclei which would otherwise have occurred because of cell division during that final culture period. In this regard it identifies recent DNA synthesis. 0.25 ml of a stock solution of colchicine (0.0125 g per 250 ml HBSS, pH 7.4 - 7.6) was added to 5 ml of medium. This concentration was found to be suitable to block all cells in metaphase in chick embryonic tissues in vitro (Daniels, 1967). Toxicity effects were only prominent after 10 hours of continuous exposure to colchicine. In the present study the exposure period was much shorter.

In continuous labelling studies it is possible to estimate the labelling rate of cells and also the percentage of cells in the population that is capable of DNA synthesis (Lamerton and Fry, 1963). In these studies it is also necessary to consider H_3T toxicity effects. Lajtha and Oliver (1959) considers than an important disadvantage of tritium is the high dose of radiation received by the cells containing and exposed to H_3T. Ninety percent of the energy of tritium disintegration is released within the nucleus so that deleterious effects are possible, especially in the highly prolific embryonic tissues.

The toxicity effects of this culture environment will be estimated with the aid of the criteria adapted (specially the colchicine

FIG. 13 SCHEDULE OF EXPERIMENTAL EXPOSURE OF EXPLANTS

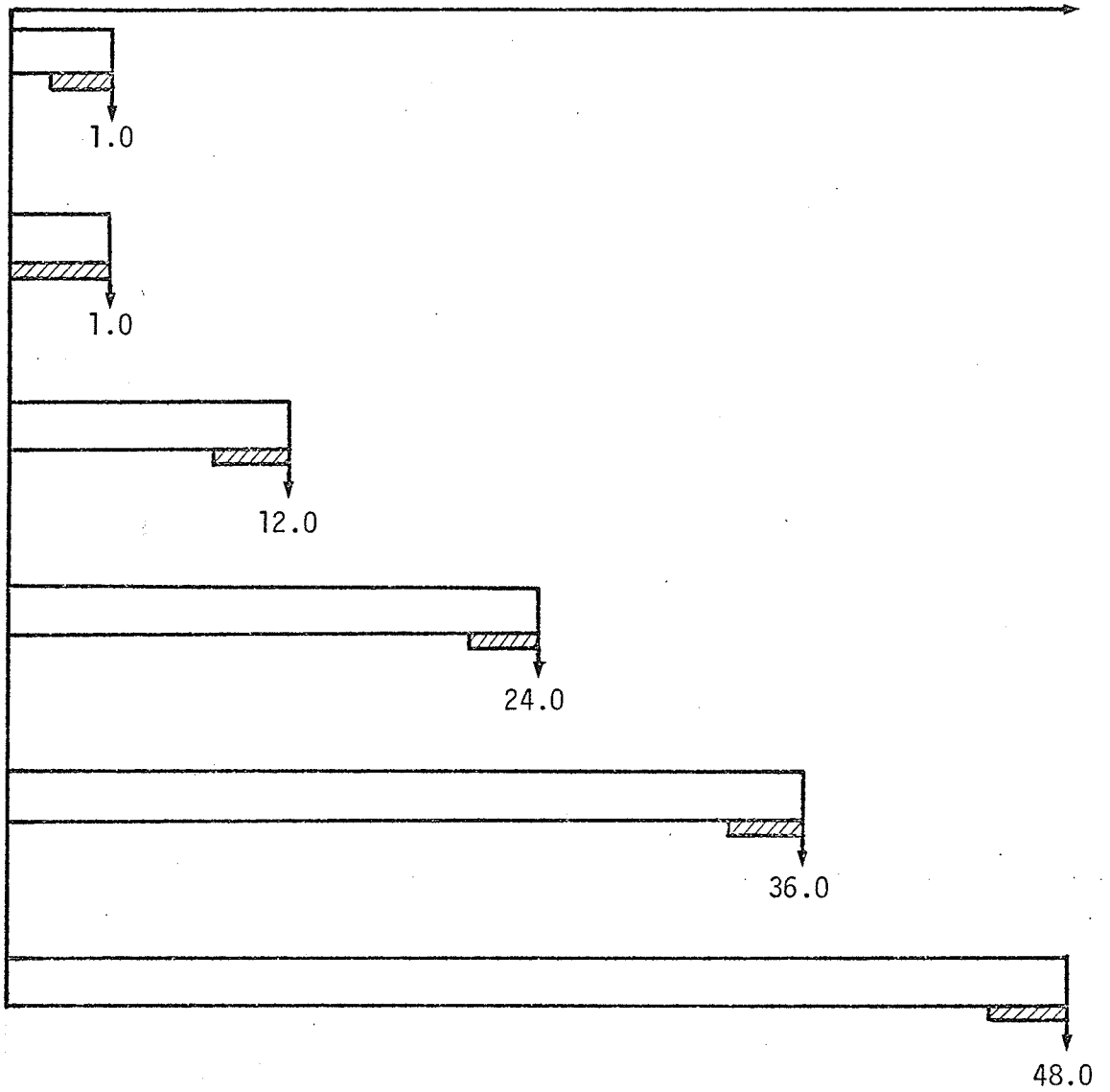
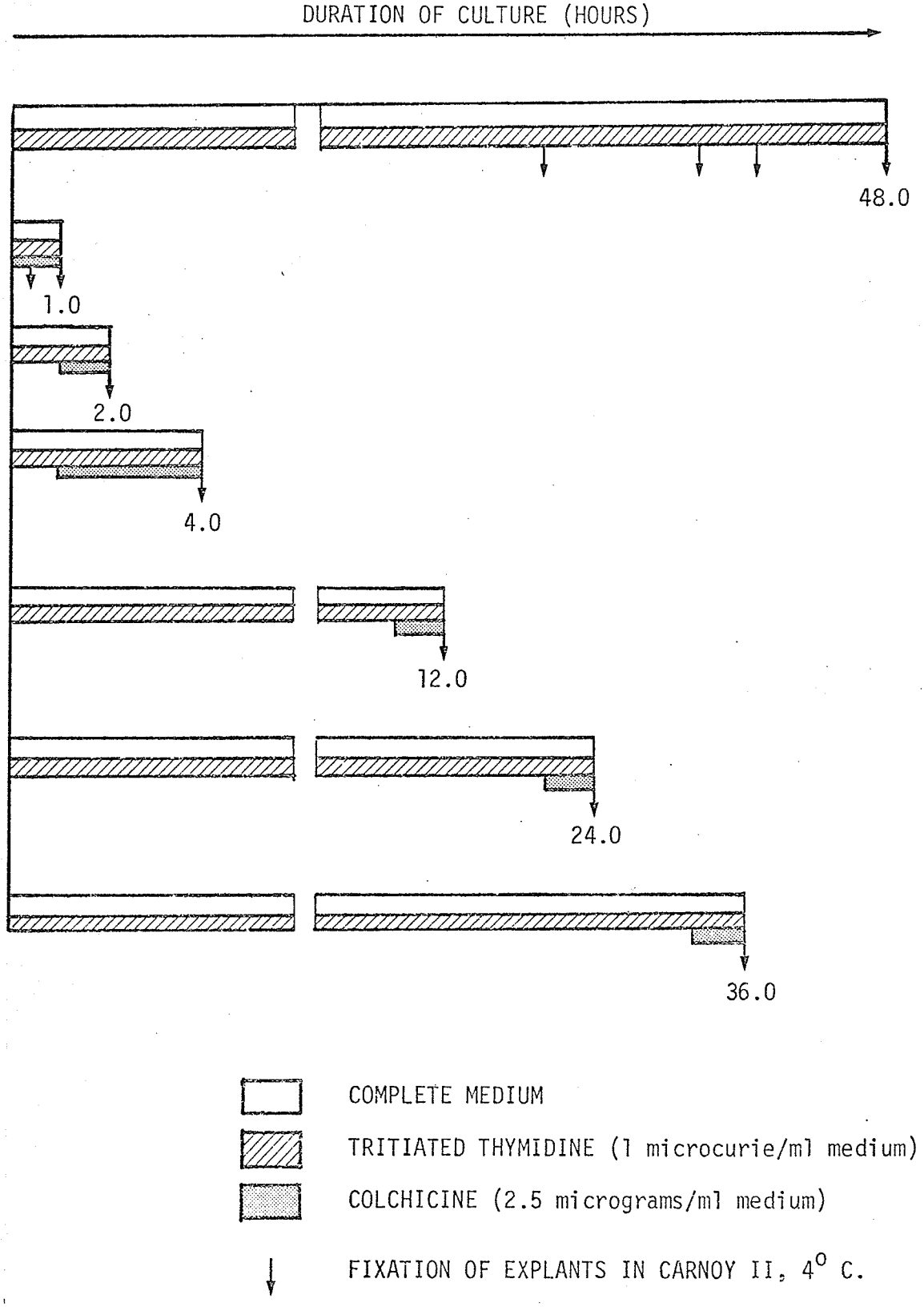


FIG. 14 SCHEDULE OF EXPERIMENTAL EXPOSURE OF EXPLANTS



arrested cells after continuous labelling). Comparisons can be made with cultures which received an hour pulse label at comparable culture durations.

RESULTS AND DISCUSSION

I. CHARACTERISTICS OF DNA SYNTHESIS AND VIABILITY THROUGHOUT THE CULTURE PERIOD:

(i) DISTRIBUTION OF SYNTHESIS THROUGHOUT THE EXPLANT:

Thymidine-labelled nuclei, indicative of DNA synthesis, were observed throughout all explants but the distribution and grain density varied. There was a characteristic pattern of labelled cells and also different degrees of labelling of the cells. A definite gradient of H3T incorporation was evident in the explants which was related to the proximity to the air and medium phases of the culture environment. A comparison of these features could be made in explants cultured as isolated neuroretinas only (Fig. 15), as folded neuroretinal epithelia (Fig. 16), with associated pigmented epithelia and mesenchyme (Fig. 17), and also in isolated scleral mesenchymal cultures (Fig. 18). In all these explants, the intensity of labelling (density of silver grains) was always greater in the tissue areas situated more proximal to the main source of nutrient medium. The regions of the tissues proximal to the air phase were structurally viable but relatively poorly labelled (fewer grains per nuclei). The density of grains decreased sharply with increasing tissue distance from the main source of medium as was evident in the triple folded neuroretinal epithelium (Fig. 16). Uniform intense labelling was obtained only in single layered epithelia, on the free edges of folded explants and with epithelia with very little associated mesenchyme. The millipore filter through which diffusion of nutrients occurred to the explant had a constant pore size of 0.45 micra. The above interpretations are based on diffusion through these specific filters.

Consideration of other limiting factors associated with the gradient of H3T incorporation in the explant would be the diffusional distance from the medium, gas phase and metabolic activity of associated tissues. Fluid imbalance at the explant: air interface could also be a factor. In vivo the explanted tissues are normally bathed in fluid whereas in the in vitro environment it is exposed to a humid air phase. Throughout the culture period the explants were covered by a film of fluid and the air phase was presumably maximally humid since fine condensates collected under the Petri dish glass cover. Estimates of the ionic imbalances at the explant: air interface cannot be made. In this regard, it is relevant to note that the surface cells were structurally viable and did incorporate H3T so that extreme ionic imbalances would be unlikely. Furthermore, single-layered epithelia and mesenchyme which were also directly exposed to the air phase were, in contrast, heavily labelled. The average thickness of the single-layered epithelia was ± 65 micra. Favourable optimum conditions for synthetic activity was also observed in explants of epithelium plus associated mesenchyme ranging from 65 - 130 micra thick.

The gradient of H3T incorporation also varied with the type of tissues cultured. The gradient was more extensive in mesenchymal explants (200 micra thick, Fig. 18). The structural and metabolic characteristics of the combined neuroretinal and associated mesenchyme explants could also determine the H3T incorporation characteristics. Diffusion through the relatively loose mesenchymal tissue would be expected to be greater than through the compact pseudostratified neuroepithelial layers. The importance of differential synthetic activity of these two tissues (plus pigmented epithelium) is visually evident in the labelling of the

explant in figure 19. The labelling intensity distribution in the double-layered explant cannot be explained on diffusional distance alone. The differentially-labelled zones of each epithelial layer was equidistant from the medium: air phase. Differences in synthetic activity of the tissues explains the differential labelling and is characteristically observed in cultures of the epithelium and associated mesenchyme (Fig. 18). Steel and Bensted (1965) and Rajewsky (1965) considered that H3T incorporation capacity in vitro would be determined by oxygen tension and QO_2 of the type of tissue studied. Johnson et al (1961) originally emphasized the importance of the gas phase in this regard. Considering Wasburgs' calculations, the possibility of oxygen concentration reaching zero at a certain explant level would vary as the square root of the external oxygen concentration. In an air phase the diffusion depth calculated to be approximately 100 micra (8 - 10 cells thick) and in pure oxygen the diffusion depth would be increased to only 230 micra. In experiments with solid tumour tissues, Johnson and coworkers had reported only a two-fold increase (ie. 200 micra) when 95% oxygen and 5% carbon dioxide was bubbled through the culture medium. Further observations (Rajewsky, 1965) confirm that the depth of the labelled cell zone increases with higher partial pressures but stress that it depends on the type of tissue cultured. The culture method used in the present study differs from that of Johnson et al and Rajewsky. The observations using the Trowell technique is based on an operationally modified confrontation of diffusion of medium and gas phases. The cultured explants were exposed to the air phase from above and medium phase from below. Diffusion gradients of air and medium were from opposite directions unlike in the solid tumour incubations in which both medium and gas diffused into the explant from one

direction. If the optimum gas and medium phase level can be estimated by the incorporation activity in the explant, than the more important limiting factor in the Trowell technique adapted with embryonic tissues would be the proximity to source of medium and pore size of the millipore filter used.

In the technique used the lens paper maintained a fine capillary layer of fluid at the level of the millipore filter throughout the culture period. The millipore filter (pore size 0.4 micra) prevented excessive wetting of the explants and favoured cultures of single-layered neuroretinal explants with little associated mesenchyme. With these provisions, the other local diffusional factors (gas phase) would not influence the inherent response potential of the explants. All cellular counts (PI, MI, and LI) were done in viable regions of explants proximal to the source of medium. These regions are easily identifiable in homogenously-labelled sections.

(ii) THYMIDINE INCORPORATION CHARACTERISTICS AND VIABILITY:

Addition of a sixty minute "pulse" of tritiated-thymidine to cultures incubated for various periods indicated that there was maximum DNA synthesis during the first hour of culture (Table I). The results from separate experiments (Tables I and II) indicated a similar range for DNA synthesis after the initial hour of culture. The mean labelling index ranged from 41.04 ± 9.6, 43.04 ± 6.5 and 46.52 ± 14.6. The labelling rate appeared to be much higher during the first 30 minutes of the hour culture period (Table II, 42.66% of 43.04 = 99.1%) than during the second 30 minutes (Table I, 27.34% of 41.04% = 66.6%). Pulse labelling at various intervals throughout the culture period indicated that there was a progres-

TABLE I

CHARACTERISTIC RESPONSES OF EXPLANTS THROUGHOUT THE CULTURE PERIOD: THE MEAN PERCENT DNA SYNTHESIZING CELLS (PULSE LABELLING INDEX, LI)* AT INTERVALS DURING THE CULTURE PERIOD.

HOURS OF CULTURE	NUMBER OF EXPLANTS	MEAN LI	MEAN MI	MEAN PI
1.0**	5	27.34 ± 1.3	4.60 ± 0.6	0.0
1.0	5	41.04 ± 9.6	2.83 ± 0.3	0.0
12.0	10	32.60 ± 7.2	5.66 ± 2.9	5.96 ± 7.2
24.0	5	31.08 ± 3.9	3.17 ± 0.4	6.18 ± 7.7
36.0	5	24.47 ± 5.6	4.36 ± 1.8	4.01 ± 2.1

* The mean mitotic (MI) and Pycnotic (PI) indices are also included.

± Standard deviation.

** Final 30 minute exposure to tritiated thymidine, unlike the other cultures which where exposed for the final 60 minutes.

TABLE II

CHARACTERISTIC RESPONSES OF EXPLANTS THROUGHOUT THE CULTURE PERIOD: THE MEAN PERCENT LABELLED CELLS (CUMULATIVE LABELLING INDEX, LI)* AT INTERVALS DURING THE CULTURE PERIOD.

HOURS OF CULTURE	NUMBER OF EXPLANTS	MEAN LI	MEAN MI	MEAN PI	PERCENT OF MAXIMUM INCORPORATION
SERIES 1					
18.5	7	77.76 ± 26.6	3.30 ± 0.4	2.70 ± 0.6	81.02
31.5	7	95.28 ± 1.9	5.09 ± 1.5	3.50 ± 1.5	99.27
36.5	5	95.16 ± 0.7	3.81 ± 1.4	3.79 ± 1.4	99.15
48.0	7	95.52 ± 1.1	3.31 ± 0.9	14.02 ± 8.2	99.53
SERIES 2					
0.5	6	42.66 ± 2.4	6.08 ± 0.6	0.0	44.40
1.0	11	43.04 ± 6.5	6.84 ± 1.6	0.0	44.84
4.0	10	47.26 ± 6.2	10.90 ± 4.0	5.58 ± 4.9	49.20
1.0	5	46.52 ± 14.6	8.68 ± 2.9	0.0	48.47
2.0	5	50.05 ± 3.9	4.73 ± 1.3	0.0	52.15
12.0	5	95.97 ± 1.4	3.24 ± 0.4	1.13 ± 0.9	100.00
24.0	5	93.65 ± 2.1	5.17 ± 0.8	3.91 ± 1.9	97.50
36.0	5	93.78 ± 2.4	2.95 ± 1.24	3.95 ± 1.4	97.70

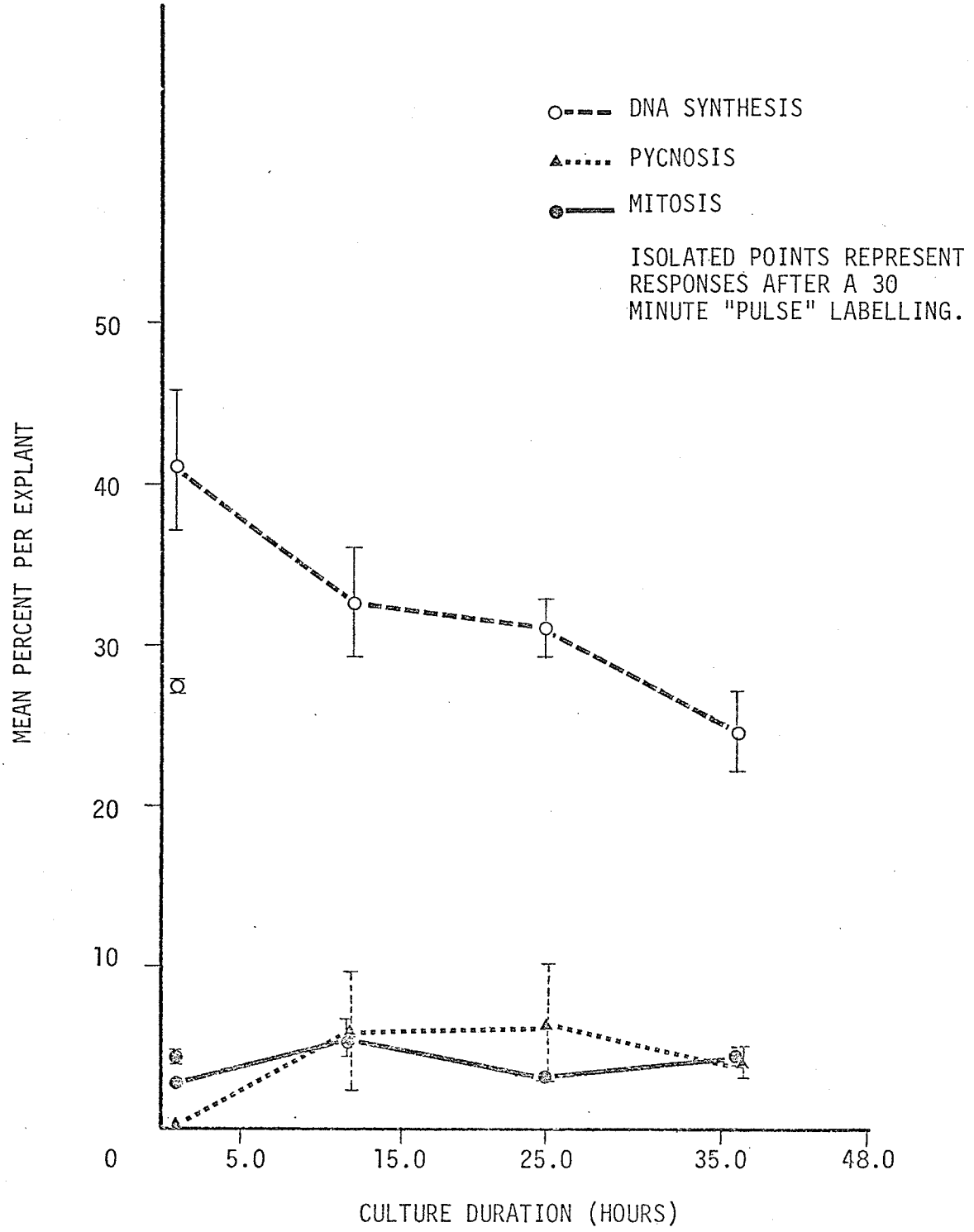
* The mean mitotic (MI) and pycnotic (PI) indices are also included.

± Standard deviation.

SERIES 1 Continuous exposure to tritiated thymidine.

SERIES 2 Continuous exposure to tritiated thymidine plus colchicine during the final hour of culture.

FIG.20 THE MEAN PERCENT CELLS IN DNA SYNTHESIS (60 MINUTES PULSE LABELLING), MITOSIS AND PYCNOSIS AT INTERVALS THROUGHOUT THE CULTURE PERIOD.



sive decline in the mean percentage of explant cells in DNA synthesis (Fig. 20). At no time throughout the culture period were there fewer than 20% neuroepithelial cells capable of DNA synthesis. The lowest value obtained was after 36 hours at which time the mean percent cells capable of synthesis was 24.47 ± 5.6. The progressive decline in the labelling of cells had its steepest gradient between the 2nd and 12th hours of culture. During this period there was a progressive accumulation of pycnotic cells (5.96 ± 7.2) but there was also an increase in mitosis (5.66 ± 2.9, Fig. 20). The mitotic values observed were expected responses in view of the high minimum number of cells in DNA synthesis observed previously and at intervals throughout the culture period. Synthetic activity during the interval (2 to 12 hours incubation) was not directly studied so that the rate of the decline and recovery characteristics cannot be deduced. The only observation made was that the decline in synthesis was less rapid and stablilized following the steep decline at 12 hours.

Studies by Saxen et al (1968) on mesenchymal explants have recorded a metabolic lag period after eight hours in vitro followed by a recovery with subsequent culture periods. The metabolic lag was indicated by a reduced incorporation of nucleic acids and amino acids. Cell viability was not a factor influencing the early decline in metabolic activity. The results obtained with neuroepithelial tissues indicate that viability must be considered as a possible factor in the progressive decline in synthesis. A gradual accumulation of pycnosis was observed with prolonged periods of incubation (Fig. 21). A loss of cells from the explant population is probably not a major factor since the mitotic activity was always higher than the value observed at one hour

(2.83 ± 0.3). These observations were probably related to the lower metabolic requirements of cells passing to and through mitosis compared to the metabolic requirements of the synthetic processes. Explants that were continuously exposed to H3T for four hours and to colchicine over the final three hours indicated a decline in DNA synthesis (Table II) but the progression of cells into mitosis was not affected. The mean percentage mitosis in ten explants was 10.9 ± 4.0.

A characteristic observed in the distribution of the pycnotic cells in epithelia was their accumulation under the external limiting membrane (Fig. 21). This was a consequence of the detachment of pycnotic cells from the ILM and their passive exfoliation toward the ELM. Caution is therefore necessary in interpretting the possible phase of the cell cycle in which cell death occurred since pycnotic cells move through the S-zone as it accumulates under the ELM. Many studies (Menkes et al, 1970) have reported cell death (pycnosis) in a specific zone of the neuroepithelium, specially the S-zone (DNA synthetic zone). In tissues in which the Intermediate zone is small, such designations are even more difficult.

In the cumulative labelling experiments, the rate of entry of cells into DNA synthesis was followed (Table II, Fig. 22). In vivo thymidine incorporation into DNA synthesizing cells occur for less than one hour because of the rapid catabolism and elimination from the body (Blenkinsopp, 1968). The time for 50% incorporation (t50) was considered to be of the order of a few minutes. In the culture system studied H3T incorporation occurred throughout the culture period (up to 48 hours). The cells were progressively more heavily labelled (Fig. 23). The viability of these heavily labelled cells could still be identified since

FIG.22a MEAN PERCENT LABELLED CELLS IN EXPLANTS AT INTERVALS THROUGHOUT THE CULTURE PERIOD (CUMULATIVE LABELLING)

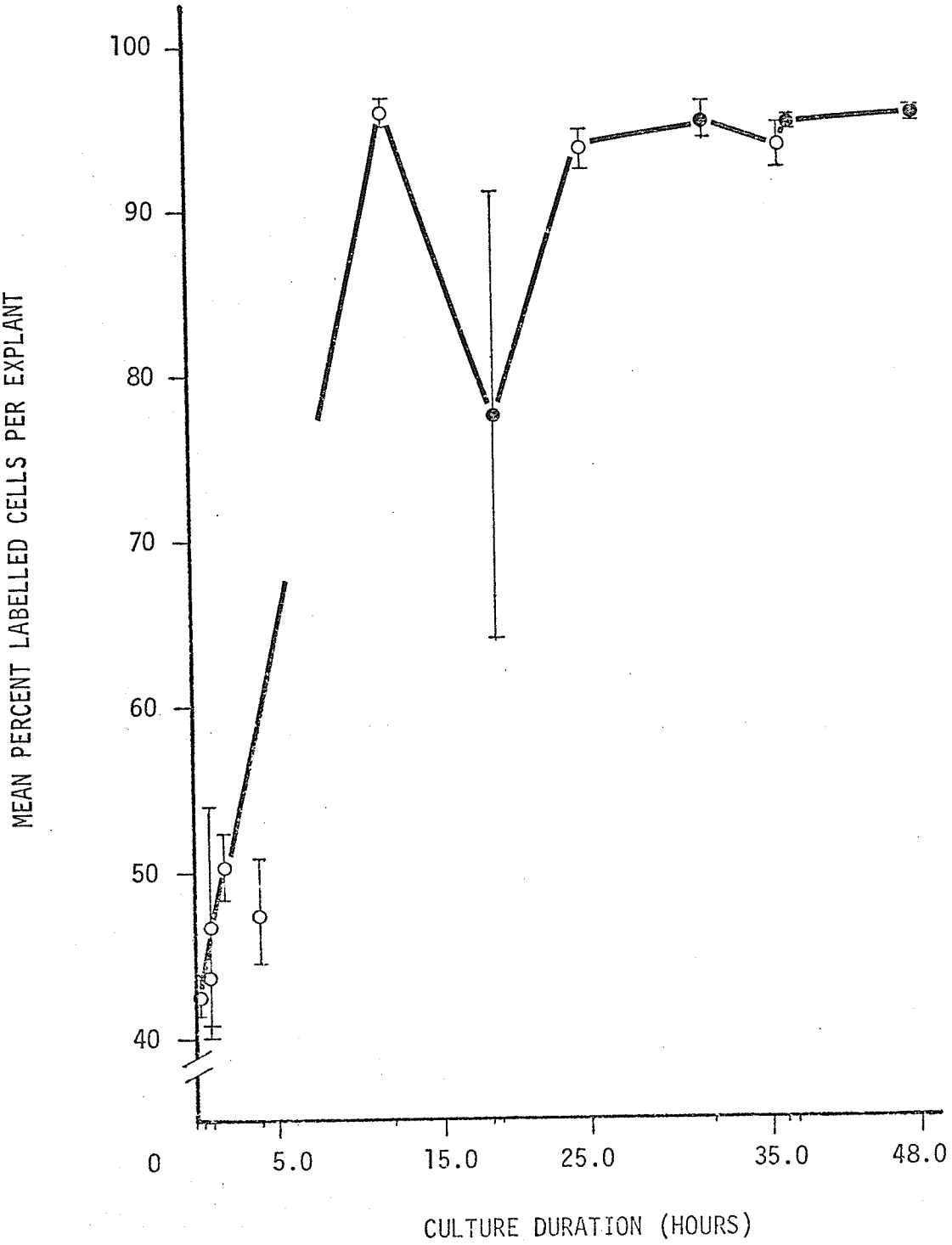


FIG.22b MEAN PERCENT PYCNOTIC CELLS IN EXPLANTS THROUGHOUT THE CULTURE PERIOD (CUMULATIVE LABELLING)

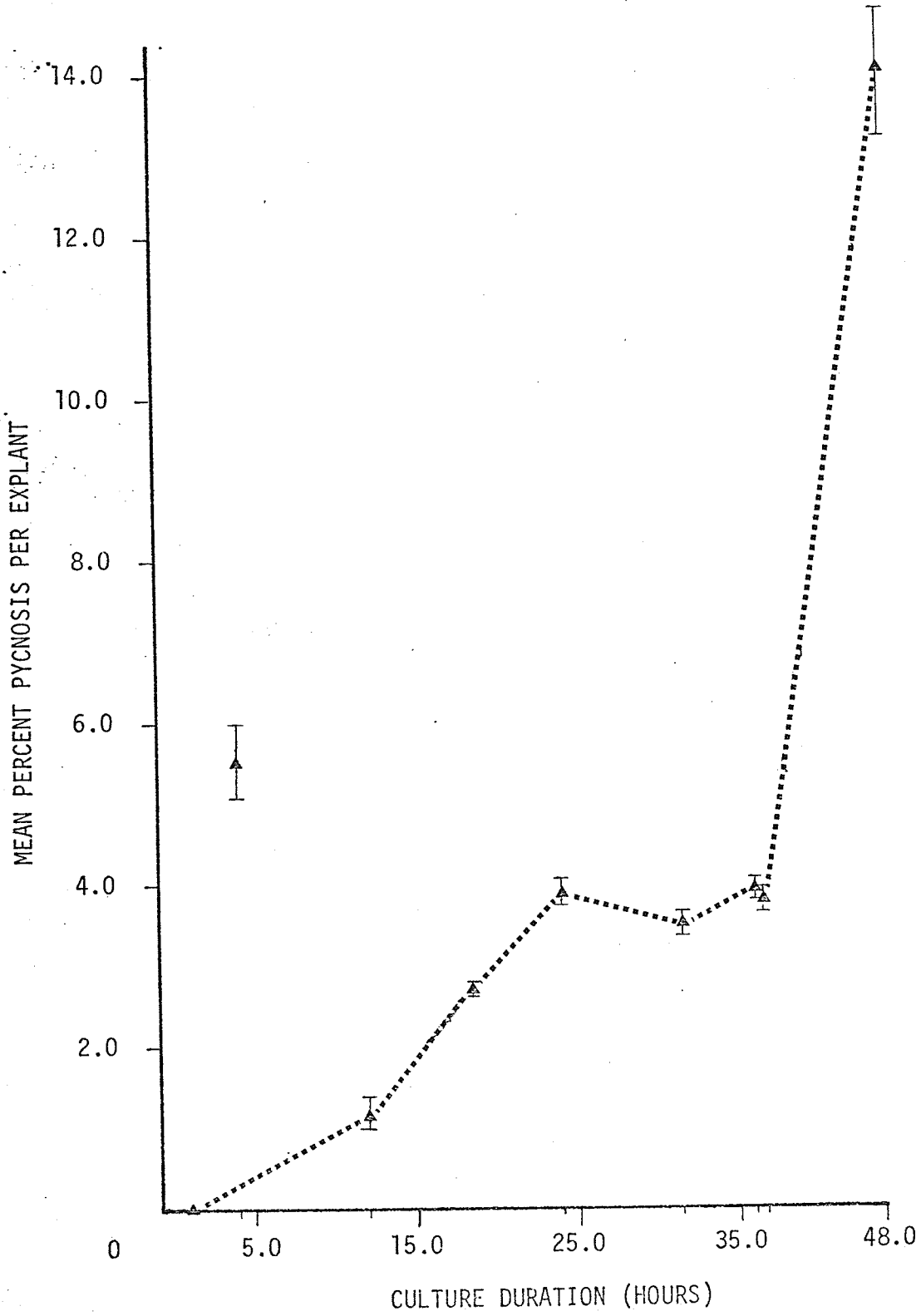


FIG.22c MEAN PERCENT MITOSIS IN EXPLANTS THROUGHOUT THE CULTURE PERIOD (CUMULATIVE LABELLING)

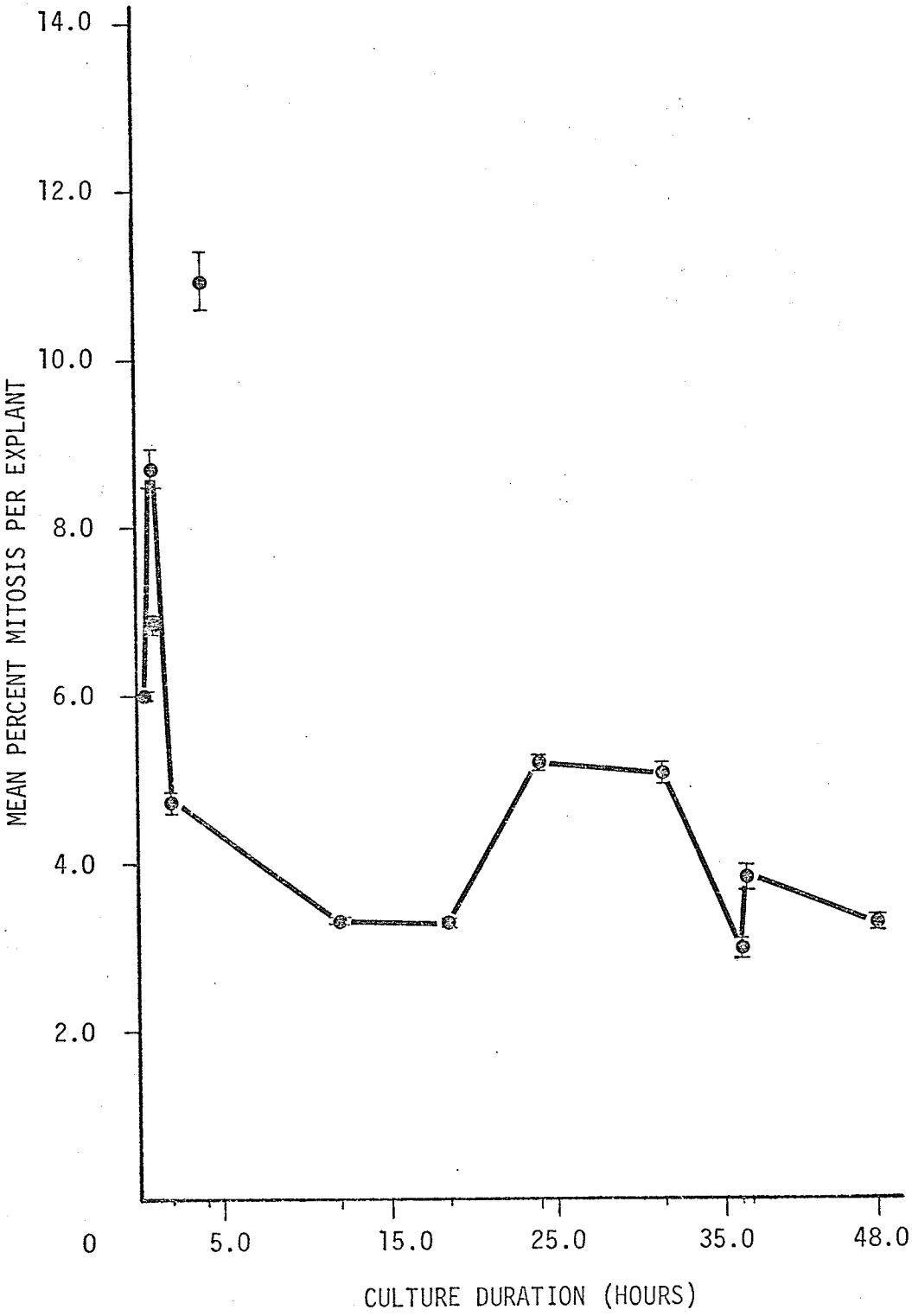
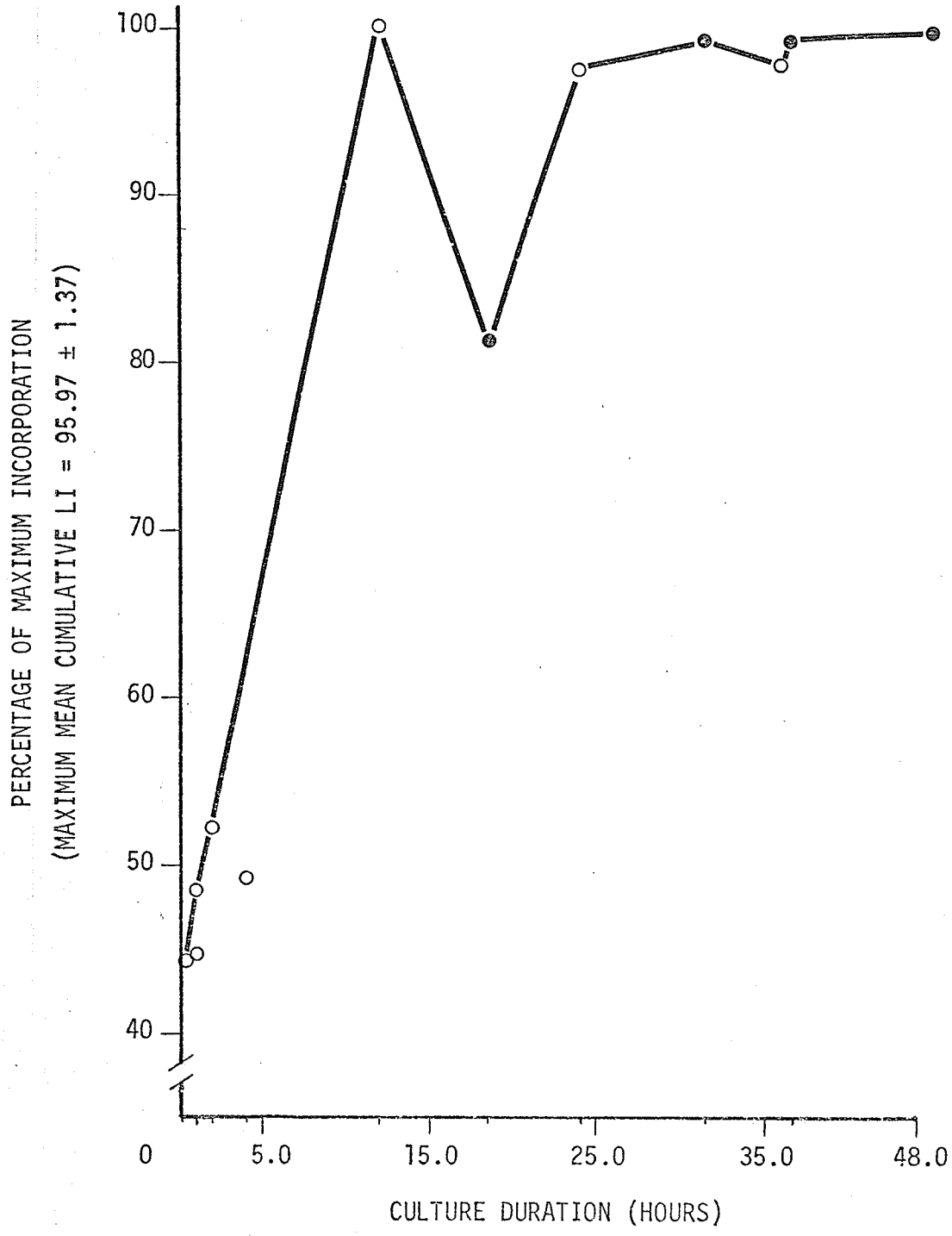


FIG.22d CHARACTERISTICS OF ACCUMULATION OF DNA-SYNTHESIZING CELLS THROUGHOUT THE CULTURE PERIOD (CUMULATIVE LABELLING)



many colchicine-arrested metaphases were observed at intervals during the culture period (Fig. 24). The fluctuation of the mean percentage cumulative labelling index after the maximum labelling was reached was not considerable (Fig.22a) and could be explained by the progressive accumulation of pycnosis (Fig.22b). The concentration and stability of the tritiated thymidine in the medium was not studied and this could also contribute to the maximum labelling values obtained at 18 to 48 hours (Fig.22a). The maximum number of neurepithelial cells capable of DNA synthesis was 95.52 ± 1.1 and 95.97 ± 1.4 (Table II). Therefore, the explants used consists of at least 95% ventricular cells since these are the only cells capable of DNA synthesis in the neuroepithelium (Boulder Committee, 1970). The rest of the cells of the neuroepithelial explants that do not synthesize DNA comprises differentiated detached cells that are easily identifiable under the ELM (Fig. 23) and also pycnotic cells. The earliest culture incubation period at which the maximum number of labelled cells were detected was at 12 hours. Figure 22d is a graphical representation of the rate of accumulation of labelled cells throughout the culture period. The maximum amount of labelled cells was taken to be 100% and each mean percentage cumulative labelling index was plotted as a percentage of this value (see Table II for values). Extrapolation for the time it takes for 50% incorporation (t_{50} values) indicated that it occurred between the first and second hours of culture (48.47 to 52.15% respectively). This value is similar to the t_{50} value (70 minutes) reported for solid tumour H3T incorporation <u>in vitro</u> (Rajewsky, 1965). The final incorporation of H3T in the solid tumours was reported to occur after 400 to 500 minutes. In cultures of the neuroepithelial explants, the maximum mean percentage labelled cells was

observed at 12 hours but values were not monitered at earlier periods. The lag in the accumulation of DNA synthesizing cells after the first two hours is supported by the pulse labelling study (Table I) which indicated a sharp decline in the mean percentage of neuroepithelial cells capable of DNA synthesis at 12 hours (32.60 ± 7.2). This period at which the synthetic "lag" occurs is comparable to the period observed for tumours (Rajewsky, 1965) and for mesenchymal explants (Saxen et al, 1968). It appears to be a characteristic synthetic response of explants in vitro.

(iii) VIABILITY DUE TO THE EXPERIMENTAL EXPOSURE CONDITIONS:

Apart from the incipient degeneration commonly observed over prolonged culture periods, the possible toxicity effects of the colchicine and tritiated thymidine must also be considered. All explants (Table II) were exposed to colchicine only for the final 60 minutes of culture. The colchicine-arrested cells in the specific mitotic zone of the neuroepithelium facilitated counts of mitotic indices and also the identification of the viability of heavily labelled cells in explants continually exposed to H3T. The possibility that colchicine affected the rate of DNA synthesis during this brief period was estimated by prolonging the combined exposure periods to H3T and colchicine. Ten explants (Table II) were exposed to H3T for four hours and to colchicine for the final three hours. The mean percentage cumulative labelling index was 47.26 ± 6.2 which represented a small decline to the value observed at 2 hours (50.05 ± 3.9). Furthermore, the mitotic index was high (10.9 ± 4.0) and these observations were monitered during a period of probable synthetic "lag" (the greatest synthetic activity was observed

after the first two hours of culture).

If the DNA synthetic activity was not considerably reduced after 3 hours exposure to colchicine, the concentration used (2.5 micrograms per ml medium) over 60 minutes should not be a serious factor in the observed synthetic responses. Depression of DNA synthesis after treatment with colchicine in vitro at concentrations of 5 micrograms per ml and above has been reported in human lymphocytes (Fitzgerald and Brehaut, 1970). In the reported studies the reduced DNA synthesis was followed by the expected reduction in mitotic indes. The magnitude of the observed depression was related to increased concentration of colchicine (5 micrograms to 15 micrograms per ml medium) and also to longer periods of exposure.

Under the present culture conditions any deleterious effects of the colchicine exposure was not evident in the slight decline in labelling index and large percentage mitosis. The increase in mean percentage of pycnotic cells (5.58 ± 4.9) was the only indication of possible deleterious effects. Pycnosis throughout the culture period did not rise excessively. In separate experiments the mean percentage pycnosis after 36 hours was 48 hours of continuous exposure to H3T that the pycnotic index increased rapidly (14.02 ± 8.2). Many heavily labelled cells were detached and in stages.

2. DIFFERENTIAL RESPONSES AND MORPHOLOGICAL ORGANIZATIONAL RESPONSES:

(i) CYTOKINETIC ACTIVITY:

Confirmation of the normal cytokinetic activity was obtained in the neuroretinal explants that is similar to the activity reported to occur throughout the early neural tube and retina (Fujita, 1962). DNA synthesis is restricted to ventricular cells that form a distinct labelled zone of DNA synthesis (Fig. 25). At the end of this phase, cells increase in volume and descend toward the internal limiting membrane as it enters into the mitotic zone. After an oriented division at this zone, daughter nuclei would then ascend as it enters G_1 and S phases. The zone of nuclear migration between the S-zone and M-zone is known as the interkinetic zone (Fig. 25). The mitotic zone is emphasized in figure 26 where all the cells entering the mitotic phase has been experimentally arrested by means of a stathmokinetic agent, colchicine.

The descent of the nucleus towards the internal limiting membrane at mitosis is considered to be a consequence of the rounding up phenomenon of the cell at mitosis and its attachment to the membrane by means of terminal bars. The suggestion (Kauffman, 1969) that the nucleus migrates possibly by means of the microtubules is unlikely not only because of the direct observation of the rounding-up of the cell at this time, but also due to the observations that the colchicine-arrested cells do in fact retract all the way to the internal limiting membrane. The effects of colchicine on microtubules have been widely studied, and the specific binding of this substance to the microtubular proteins of nervous tissue has been used to study axonal flow mechanisms by its characteristic blocking of this pathway (Kreutzberg, 1969; Weiss, 1969). In the event

that migration of the nuclear material by means of the microtubules does occur, then the nuclear material should not withdraw all the way to the internal limiting membrane with the prolonged exposure to the colchicine in these studies.

(ii) NEUROMERY:

In the experimental continuous exposure of explants to colchicine (up to 3 hours), a number of significant morphogenetic responses of the neuroepithelium could be studied. The characteristic bulging of the neuroepithelium (neuromery) could be accentuated in developing 3 day old chick embryos by addition of colchicine onto the embryos in ovo (Fig. 27). Similar responses was produced in the neuroretinal explants cultured in the presence of colchicine. Neuromery is an organizational response of the neuroepithelium that occurs in vivo and in vitro and its observation has attributed much to the analysis of normal and abnormal responses of the neuroepithelium.

The phenomenon of neuromery in the early neural tube has been reported for a number of species including man (Kallen, 1962). These distinctive sacculations of the neural tube have an orderly spacial and temporal appearance in the neural tube and reflects mitotic patterns (Kallen, 1962). Kallen, in a number of reports, has carefully analysed neuromery. In his 1962 report he made a detailed study of the neuroepithelium in colchicine-treated chick embryos. The thickness of the non-arrested cell layers showed a significant arrangement in the neuromeric bulging. Kallen (1956) accounted for the neuromery by considering Sauers' 1935 explanation for the interkinetic movements of the cells in the neuroepithelium. The bulging observed would then appear to be a

direct cause of the redistribution of the cells during the interkinetic movements in the epithelium and also by the formation of more cells in the centre of the neuromere. There was a marked accumulation of mitotic cells around the centre of the neuromere (see also Fig. 24).

From his studies with Ambystoma, it appeared that the associated mesodermal structures were not the primary cause of the neuromery but likely acted as a general mitotic stimulant. The fact that neuromery do occur in the isolated neuroepithelium in culture (Fig. 24) is supporting evidence for the above view. Kallens' explanation of the neuromery on the basis of the interkinetic movements of cells in the epithelium would also explain the ephemeral nature of these bulges during normal development. Although colchicine emphasizes existing mitotic patterns and therefore accentuates existing neuromery, its possible effect on shrinkage of the epithelium and embryo as reported by Overton (Watterson, 1965) must also be considered as an independent contributing factor. In the present in vitro study using minute explants, the possible independent shrinkage effects of colchicine are reduced or eliminated. Another factor contributing to the neuromere response would be the increase in surface area by the mitotic cells, specially the accumulated colchicine-arrested cells. In figure 24, the highly turgid colchicine-arrested cells definitely contribute to the increase in the surface area of the neuromeric bulge. Considering the above mechanism observed in experimental studies, it is possible that these are grades of deformations of the neuroepithelium throughout morphogenesis and also throughout the culture period in the present studies.

The interpretation of the observations of neuromere-like

bulges in the cultured neuroepithelial explants is based on the same mechanism of redistribution of cells as described by Kallen. Since the neuroretinal explants do show these basic responses inherent to the neural tube, it should be a suitable model tissue to study early neuroepithelial responses under various conditions. The method of culturing the neuroretinal explants on a millipore filter facilitates observations of deformations of the epithelium due to changes in surface area or cytokinetic activity. Neuromere-formation is more conspicuous in species where the neural tube has a wide lumen. The spreading of the explants on a millipore filter would favour such observations if it could be produced. More important however, the fact that both the cytokinetic activity and adhesions can be studied, is a significant factor. This is exemplified in the following observations of explants cultured on millipore filters. Neuromere formations were only observed in explants with disproportionate mitotic activity and never in explants with uniform mitotic acitivity. Figure 26 shows an explant with a single row of colchicine-arrested mitotic cells indicative of uniform mitosis. No bulges can be seen in these explants compared to the explant shown in figure 24 Secondly, microscopical examination of explants on millipore filters facilitates observations of attached cells and estimations of their numbers. In vivo studies are often complicated by the plane of sectioning,especially in the event of neuromeric bulgin. Kallen (1962), has illustrated that tangentially-sectioned neuromeric folds often give the impression of overgrowths into the ventricles. Such illustrations have in the past (Paff, 1939) been described as "excessive amounts of tissue". Furthermore, the highly turgid accumulation of colchicine-metaphases often give the appearance of detached cells.

In the millipore filter studies the colchicine-arrested cells can be distinctly seen as they fill up the mitotic zone of the epithelium (Fig. 26). The slender cytoplasmic processes of neighbouring cells can be seen in between these turgid cells. As a second row of turgid mitotic cells accumulate, the attachment of the turgid cell can still be seen even though the cell cannot be drawn all the way to the internal limiting membrane. The bulging pressure of the accumulated metaphases seen in these figures attest to the stability of these adhesive mechanisms.

EMBRYONIC CNS TERMINOLOGY ADAPTED

The older terminology used to describe the developing CNS became invalid when it was confirmed by Watterson (1965) that the early vertebrate neural tube was a pseudostratified rather than a stratified epithelium. Terms such as "germinal cells", "neuroblasts", "spongioblasts", "mantle" and "marginal layers" were previously defined based on inaccurate developmental descriptions. The "Boulder Committee" (1970) proposed a revised terminology which is based on the corrected sequence of CNS development. The adaptation of this revised terminology is to facilitate a more consistent form of communication between researchers.

To avoid misinterpretation of the various authors referred to in this dissertation, their reported usage of the terminology will be presented in parenthesis. Descriptions of the explants studied in this dissertation will adhere to the suggestions of the "Boulder Committee" (see Fig. 25). The four fundamental developmental zones of the neural tube were defined as the "ventricular, subventricular, intermediat and marginal zones".

The Ventricular zone is defined "as the space to which this to-and-fro nuclear movement is confined. The ventricular cells are the ultimate progenitors of all neurons and macroglial cells of the CNS and the zone will become attenuated and eventually will disappear as its cells become transformed".

The Marginal zone is "recognizable shortly after formation of the ventricular zone as a cell-sparce layer composed of the outermost cytoplasmic parts of the ventricular cells".

It has no primary cell type of its own and the nuclei of the

ventricular cells do not enter it. The inner parts of this zone is eventually occupied by intermediate and subventricular components, so that it is considered reasonable to designate "only an outer layer of fairly constant form" as the marginal layer.

The Intermediate zone is the 3rd developmental zone and is established by "some progeny of dividing ventricular cells" which migrate outward to form this zone at the junction between the ventricular and marginal zones. "The early cellular occupants of the intermediate zone are immature neurons that are destined never to divide again, at least under the usual conditons of life".

The older terminology not acceptable to the "Boulder Committee" include:

(i) "Matrix cells" - This term does not distinguish between ventricular and subventricular layers. It is also more commonly used to describe connective tissue intercellular materials.

(ii) "Primitive ependyma" - is an inaccurate term because the zone is an analage of much more than the ependyma. "Ependyma" and "subependyma" are inaccurately applied at embryonic stages when no ependyma exists. Use of the terms "ventricular" and "subventricular zones" are advocated. It is also considered possible that the ependyma arises directly from the subventricular zone.

(iii) "Neuroepithelial layer" - Many authors (Langman, 1968) use this term in reference to the specified ventricular zone. The "Boulder Committee" restricts its usage to "a more general connotation" as for example, to characterize a CNS cell as opposed to a mesenchymally-derived

cell.

In the present dissertation, the term will be used only where the above general connotation is meant. The interchangeable usage of the terms "neuroepithelial" and "neuroretinal" is therefore purposive. Usage of the term "neuroretinal" will be for reference to the specific type of neuroepithelial tissue used in these experiments.

(iv) "Neuroblasts" - Since neuroblasts are post-mitotic cells, the use of the suffix "-blast" was not acceptable. Langman and co-workers (1968) designate the unlabelled cells that first appear at the end of the second day of development under the ELM, as "primitive neuroblasts". These have a distinctive morphology comprising a large round nucleus with pale nucleoplasm and dark-staining nucleolus. The cells are comparable to the "immature neurons" described by the "Boulder Committee" that come to lie in the intermediate zone (see designation in Fig. 25). The committee, however, defers proper designation for immature nerve cells until exact identification is made of a specific proliferating cell whose progeny exclusively differentiates into neurons.

The neuroepithelial explants used in these experiments correspond to the first three developmental stages (V, M, I zone formation) described for the Central Nervous System. The neuroretinal explants studied can be considered a fair representative of the neural tube epithelium since it simulates a stage common to the development of all regions of the early neural tube. In these explants (Fig. 25), the intermediate zone is already present. According to Langman (1968), this zone of cells under the ELM appear immediately after closure of the neural tube.

CONCLUSIONS AND RECOMMENDATIONS

1. The inherent cytokinetic activity of pseudostratified neuroepithelia has been observed in neuroretinal explants throughout the culture period (48 hours). The "elevator-like" movements of nuclei in the epithelium facilitates direct analysis of the phases of the cell cycle. The responses observed with the neuroretina would therefore be representative of developmental neuroepithelial responses.

2. The epithelium provides a homogenous asynchronous populations of cells for analysis. At least 95% of the neuroretinal cells were capable of DNA synthesis by 12 hours of culture. These represented the attached ventricular cells of the neuroepithelium. The differentiated detached cells located under the ELM did not incorporate H3T even after 48 hours of continuous exposure to the label. Pycnotic cells usually accumulate under the ELM and many of the labelled cells in this differentiative zones were actually detached moribund cells in degrees of disintegration.

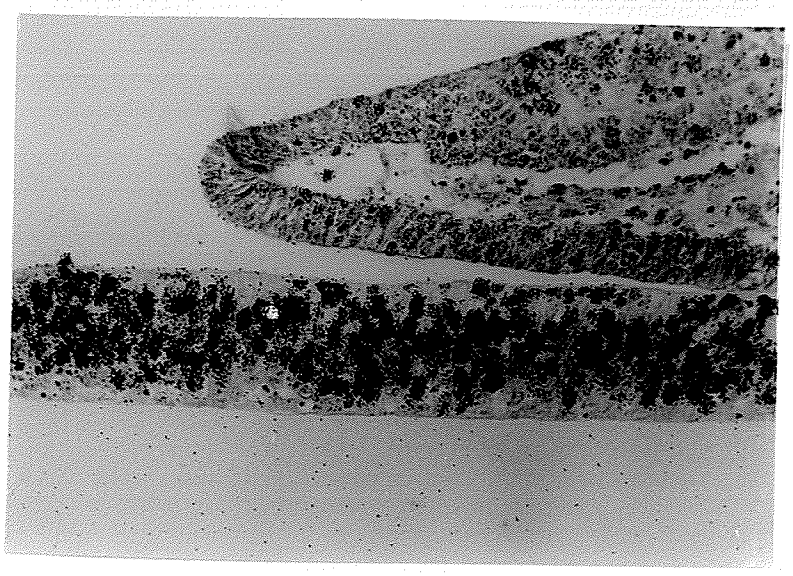
3. There was a progressive decline in DNA synthesis with prolonged culture periods but at no time during the 36 hours of culture duration were there less than 20% neuroepithelial cells in DNA synthesis. The maximum synthetic activity (50% incorporation) was reached after two hours and was followed by a steep decline in synthetic activity. After 12 hours the decline was less steep even though it was associated with a progressive increase in pycnosis. These characteristic synthetic responses of the explant "life history" provides the basis for the design of the teratological study with methotrexate (Part IV). Other recommendations obtained from this study includes:

(i) The use of radioautographs for the identification of viable synthetic zones that were in optimal culturing locations. The use of single layered neuroretinal epithelia with little associated mesenchyme were seen to be homogenously labelled and least affected by the diffusional limitations inherent in the environment.

(ii) The use of the analytic criteria adapted in this study namely; labelling index, mitotic index, pycnotic index, percentage disaggregated cells were informative in the analysis of the highly organized epithelium. Additional criteria such as the distribution of mitotic phases and orientation of mitotic spindles are relatively easily studied in this epithelium and were significantly associated with organizational responses eg. neuromery, detachments and foldings.

FIGURES 15 - 18

Radioautographs of neuroretinal explants cultured on millipore filters. A gradient of incorporation of labelled thymidine is seen related to the proximity to the main source of nutrients. This can be seen in the isolated neuroepithelium (Fig. 15), in the triple folded isolated neuroretina (Fig. 16), in the neuroretina with associated pigment epithelium and mesenchyme (Fig. 17) and also in regions of the isolated mesenchyme (Fig. 18). x100



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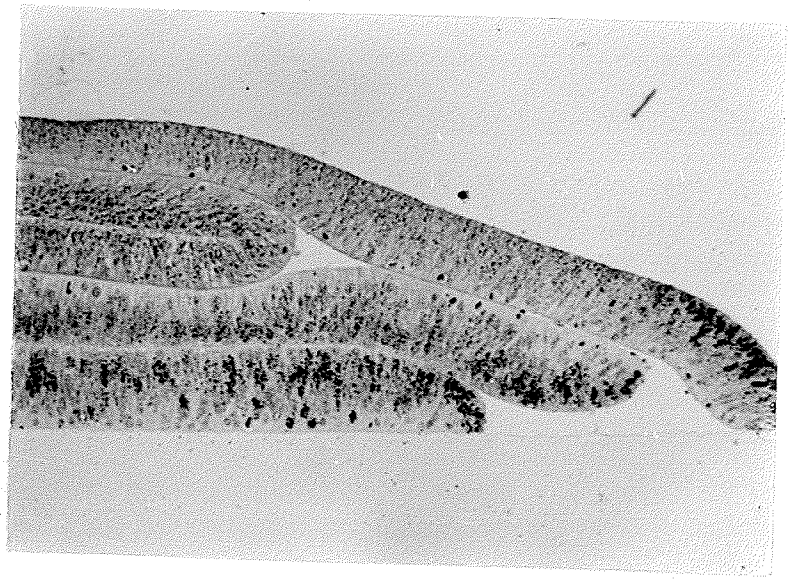

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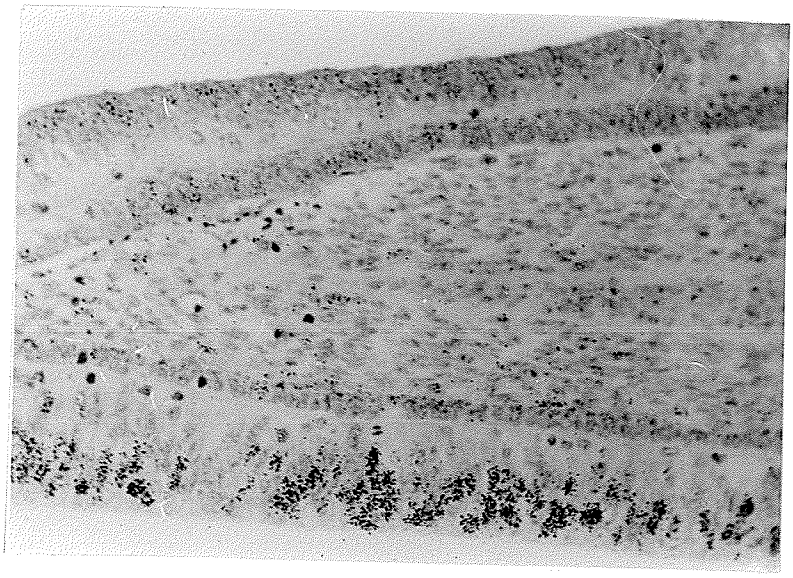

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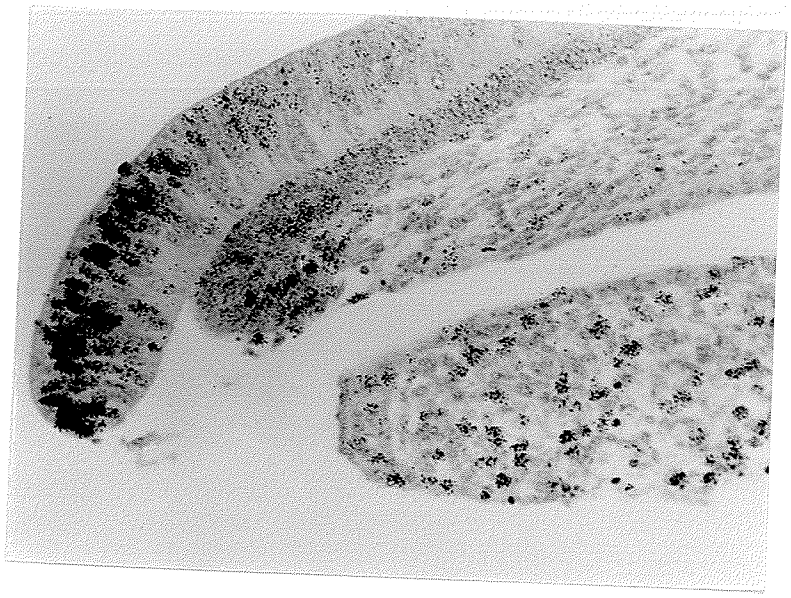
FIGURE 18 Radioautograph of a neuroretinal explant cultured with associated mesenchyme. The gradient of labelling in the mesenchymal region is more extensive than in the epithelial region. x100

FIGURE 19 Radioautograph of a folded neuroretinal explant with associated tissues. A differential degree of incorporation of labelled thymidine is related to the tissue type proximal to the source of nutrients. x100

FIGURE 21 Histological section through an explant cultured for 48 hours. Pycnotic cells accumulate in the region under the external limiting membrane (ELM). x100



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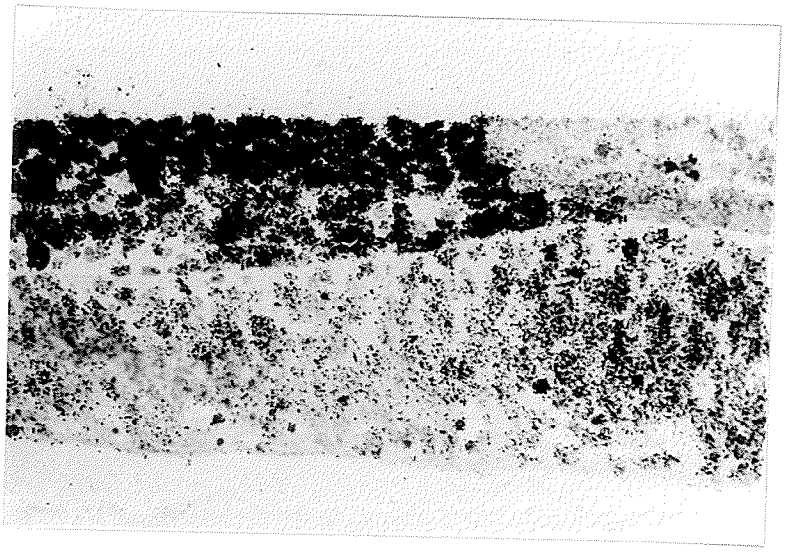

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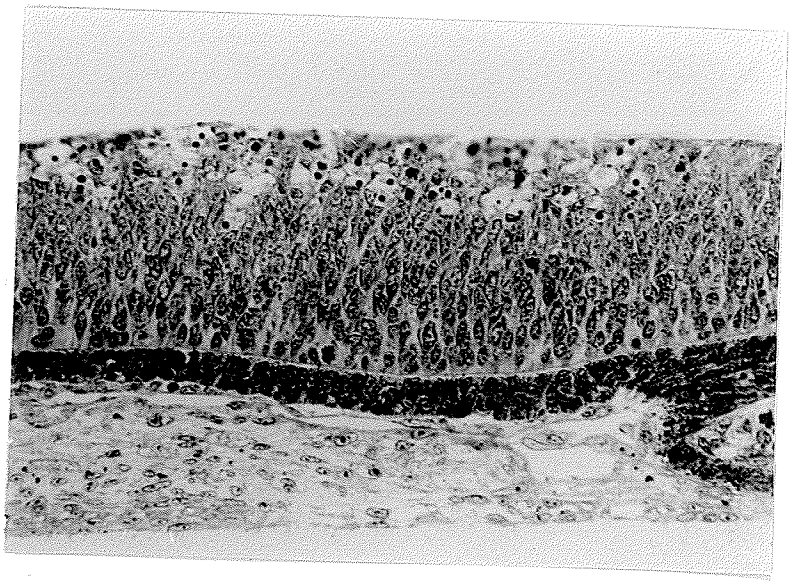

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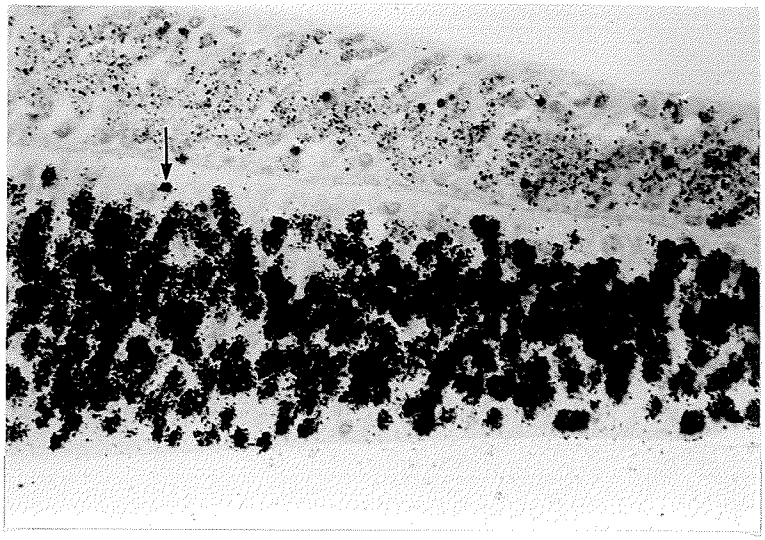
FIGURE 23

Radioautograph of a neuroretinal explant that has been cultured in the presence of labelled thymidine for up to 48 hours. The cells under the external limiting membrane do not become labelled, the occasional labelled cell (arrow) are detached labelled pycnotic cells. Many mitotic figures are heavily labelled. x128

FIGURE 24

Neuroretinal explant that has been continually exposed to labelled thymidine for 36 hours plus colchicine during the final hour of culture. The cells remain viable since colchicine metaphases (arrows) accumulate along the free internal limiting membrane resulting in neuromere formation. x128



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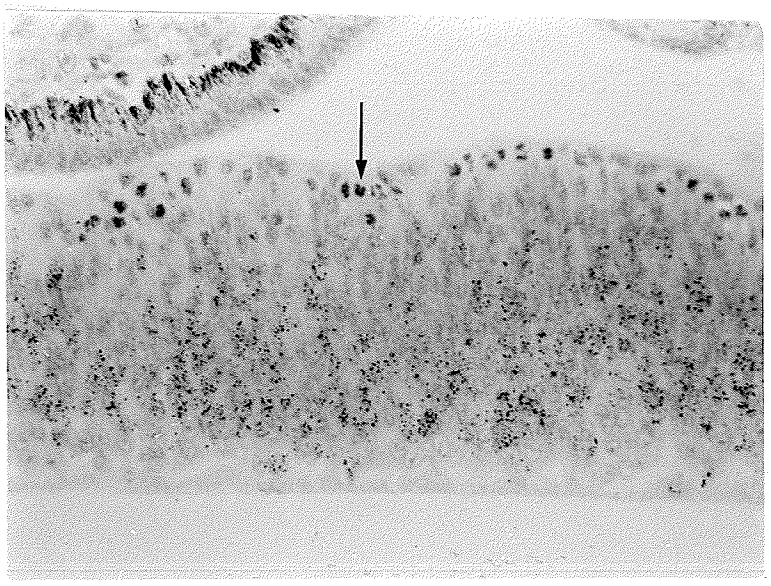

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FIGURE 25

Autoradiograph of a cultured neuroretinal explant with the adapted descriptive terminology recommended by the "Boulder Committee" (1970).

ELM - External limiting membrane

ILM - Internal limiting membrane

V - Ventricular zone: comprising the pseudostratified columnar cells

M - Marginal zone: outermost, cell-free layer of fairly constant form.

I - Intermediate zone: with unlabelled cells

Sv - Ventricular zone of DNA synthesis

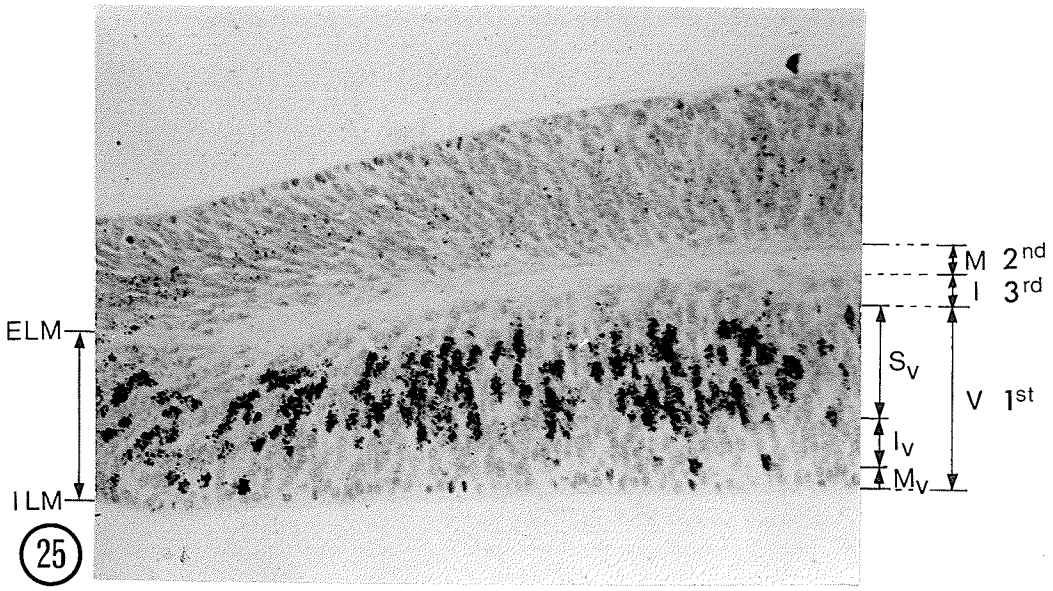
Iv - Ventricular zone of interkinetic movement

Mv - Ventricular zone of mitosis

1st, 2nd and 3rd - sequential developmental stages of demarcation of fundamental zones

FIGURE 26

Histological section of a neuroretinal explant exposed to colchicine for 3 hours. The turgid metaphase-arrested cells accumulate in a distinct mitotic zone. No neuromere-formation is seen. x128



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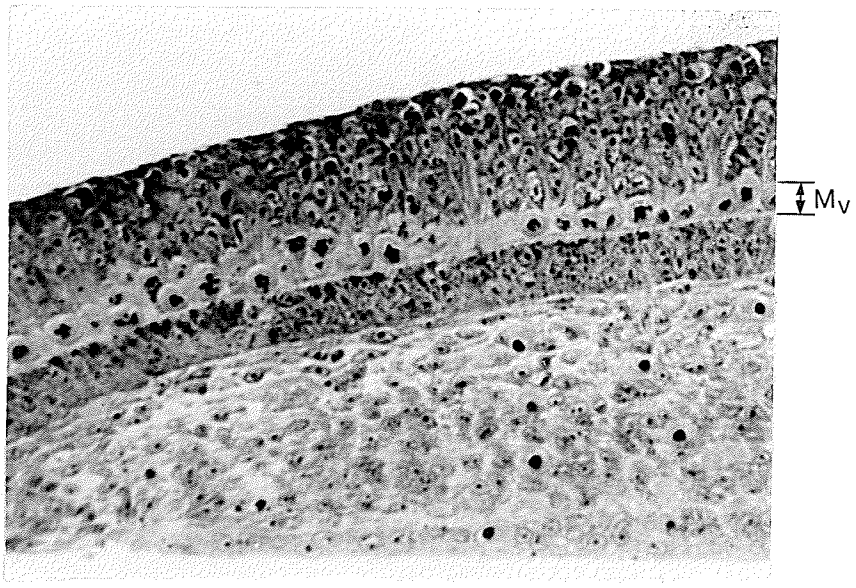
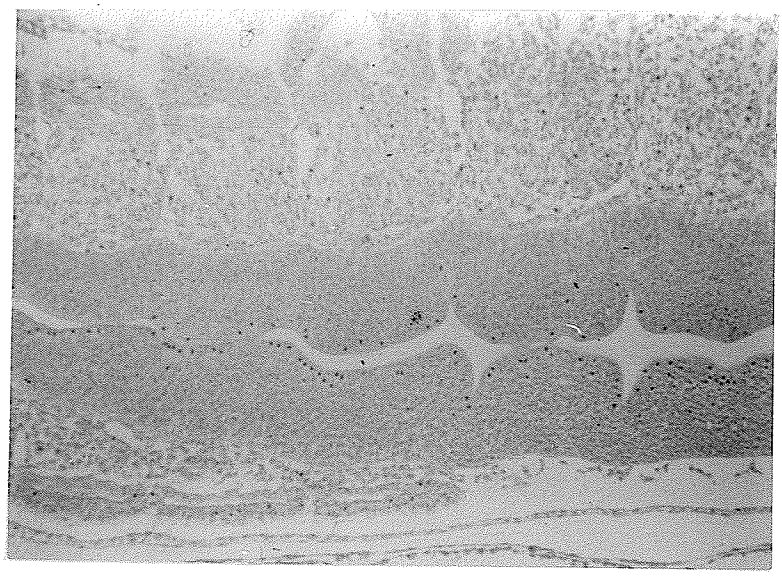


26

FIGURE 27

Histological section through a three day old embryo. Widespread metaphase-arrested cells are seen after colchicine was injected onto the amniotic sac through a "window" in the egg shell. Colchicine action emphasized the inherent neuromery seen in the neural tube. x64



(27)

PART III

STATEMENT

THERE IS MUCH EVIDENCE THAT THE ADHESION MECHANISM OF CELLS HAS IMPORTANT MORPHOGENETIC SIGNIFICANCE. EMPHASIZING THE IMPORTANCE OF THE ADHESION MECHANISM, THESE STUDIES WERE PERFORMED TO:

1) To show that conditions affecting the adhesion mechanism, as exemplified by a transient depletion of divalent ions, can seriously disturb the normal morphogenesis.

2) To characterize the particular neuroepithelial responses under these specified conditions. This will be of interpretive value in further studies with the neuroepithelium.

INTRODUCTORY REVIEW

SIGNIFICANCE OF ADHESIVE MECHANISMS:

During the formative process the adhesion mechanism of cells ensures self-determination of the aggregated cells. The previously random motile cells are localized permanently or transiently depending upon the particular morphogenetic process. An aggregation of cells ensures a homotypic interaction of the cells (Grobstein, 1959) through direct contact between cell surfaces or through the restricted micro-environment they establish (facilitating micro-humoural communication). Another form of intercellular communication is through the junctional portions of the cell surface membranes which form complexes of high permeability. These allowing free flow of metabolites, ions and cellular substances of low molecular weight (up to 10^3 MW.) from cell interior to cell interior (Loewenstein, 1969). Loewenstein has reported an impairment of junctional communication, inferred through electrical interruption between cell interiors, in a variety of abnormal growths. These junctional communications do not only maintain structural integrity but also has possible implications for organized growth control.

Junctional complexes are not confined as epithelial structures but have also been described for mesenchymal cells, even though they may be transient structures in many cases (Hay, 1968). The importance of the special junctional systems in the neuroepithelium now (in view of Loewenstein's studies) acquires some interpretative significance. Because the junctional system has a finite volume as far as intercellular communication of diffusible substances are possible, it has a great potential for regulating size and shape of cell populations.

Diffusional gradients and its rates of exchange can control cell positions in a population. The importance of an intact junctional system in the developing neuroepithelium therefore has functional significance and provides a possible explanation for the orderly detachment of cells from the internal limiting membrane during the normal morphogenesis of the neural tube. Abnormal cell numbers in the neural tube (and subsequently in the developed brain) can be explained in the abnormalities produced in the intact neuroepithelium (Menkes, 1969).

Considering this morphogenetic significance of the adhesion mechanism seen in the neuroepithelium (for junctional communication controlling morphogenetic behaviour), it is very likely that conditions that interrupt the junctional communication (interruption of the adhesions) would result in the abnormal morphogenesis of the neuroepithelium elium. The experimental means for interrupting junctional communications (pH 9, extracellular calcium 10^{-4}M, 1.5x normal tonicity) all deviate well beyond normal safety conditions, but is not invalid in experimental studies since they are representative of pathological deviations (Loewenstein, 1969). Of these factors the local ionized calcium plays a major role and is considered in this study .

DIVALENT IONS AND ADHESIVE MECHANISMS:

Experimental studies on a number of epithelia strongly suggest that divalent cations are essential for the stability of the adhesive mechanisms of the epithelial tissue (the divalent cation bridge hypothesis). Beneditti and Emmelot (1968) have suggested that the cations (calcium) are bound to sialic acid carboxyl groups in the tightly closed junctions. The preincubation of liver and other epithelia in an EDTA

solution was the only procedure which enabled them to selectively stain the acidic groups of the carbohydrate with colloidal iron hydroxide. Furthermore, the electron dense material between the cell surface decreased and the junction appeared to open. The specific removal of divalent ions by EDTA is generally associated with the opening of desmosomes and intermediate junctions in epithelial structures (Manery, 1969). Calcium-free media also produce similar effects in liver cells (Leeson and Kalant, 1961).

Adhesive mechanisms in the form of terminal bars have been described for the neuroepithelium (Duncun, 1957). Furthermore, calcium has been implicated as playing a role in the surface density charge in chick neuroretinal cells. The role these factors play in the structural morphogenetic stability of the neuroepithelium is apparent from the experimental interference with these mechanisms.

EXPERIMENTAL INTERFERENCE OF ADHESIVE MECHANISMS:

The in vitro experimental techniques have largely contributed to the analysis of the adhesive mechanisms between cells and its significance in morphogenetic events. Because the proposed in vitro experiments involve an interference with the adhesive mechanisms by cation depletion procedures, it is relevant to consider the significance of divalent cations at the cell surface.

Role for Divalent Cations at the Cell Surface:

The efficacy of the chelating agent EDTA in disaggregating tissue cells is due primarily to its ability to sequester divalent cations. Cellular reaggregation requires a complete culture medium

containing the divalent cations. The major hypotheses on cell adhesive mechanisms generally emphasize calcium as being the essential cation, but other divalent cations (magnesium, strontium) have also been implicated (Trinkaus, 1969). Studies (Weiss, 1967) on the disaggregated cells indicate that the freed cells have an electronegative surface charge. There is also an established inverse relationship between increased negative charge and cellular adhesiveness (Ambrose and Easty, 1960). The observations were based on electrophoretic studies on normal and tumour cells. The more motile cells (tumour cells) show a greater surface negative charge when compared with the homologous normal cells from which they were derived. Malignant cells are well known to be less adhesive and to have a negative surface charge. Tumour cells are not exceptional in having a low calcium binding capacity since lymphocytes and erythrocytes require even higher concentrations of calcium to effectively bring about a surface charge reversal (Bangham and Pethica, 1960, cited by Weiss, 1967).

These behavioural studies together with the widespread experimental facility for cell separation or detachment in calcium-depleted media largely favour the calcium-bridge hypothesis (or divalent cation-bridge hypothesis). The role of monovalent ions in promoting cell to cell adhesions is much more complicated to interpret since the experimental method used to complex potassium ions, also binds calcium ions (Trinkaus, 1969).

Serious objections to the "calcium bridge hypothesis" lie in its proposed role for specific adhesion. Time-lapse films of aggregating cells show that the initial clustering involves a random

orientation of cells. The cluster of heterologous cell types show no specific assortment during the early stages when the cell surface show maximal deformations. It is likely that these maximal deformations would not facilitate the early specific interaction of cell surfaces. Such an intercellular interaction would occur after greater and closer surface contact. Herein lies a serious objection to the "calcium-bridge hypothesis", because adherent cells would have to lie closer than 10 angstrom (Å) units apart to facilitate the chemical bonds. In the past no support for this hypothesis could be obtained from ultrastructural studies. Electron micrographs show adjacent cell membranes being separated by a 100 - 200 Å gap (Curtis, 1962). Even though this gap has been consistently observed under various conditions of fixation, the possibility that it could be a kind of artifact has not been definitely eliminated. If the gap is real and filled with intercellular maternal (cement) that is electron lucent, it should allow particles of a specified size to penetrate the gap in living tissues as well as after fixation. The observation, that ferritin particles (100 Å diameter) penetrate the 100 - 200 Å gaps of living tissues but do not penetrate the gap after fixation, suggests that fixation either temporarily closes the gap or fills it with viscous material (Trinkaus, 1969).

The possibility that extracellular material (ECM) present on the cell surfaces could reduce the 100 - 200 Å, makes the "calcium-bridge hypothesis" more realistic. This would bring the surface charges closer together, within the limits for calcium-carboxyl bonding. Routine electromicrographs, however, failed to provide any support for this localization of ECM (cell "cements"). It was only after the adaptation of specific staining techniques that this possibility was reconsidered.

The staining of glycoprotein, with periodic acid-silver methenamine, everywhere between cells except at points where cells fuse to form tight junctions, suggested that there is extracellular material localized on the cell surface (Rambourg and Leblond, 1967). Such PASM-stained material was reported to have a uniform width averaging 140 Å, therefore largely filling the 100 - 200 Å gap. A similar localization of cell surface material was reported for chick embryonic tissues fixed in the presence of lanthanum ions (Lesseps, 1967). The electron dense material appeared to vary in thickness (about 50 Å) and could be removed with phospholipase C but not with trypsin, pronase or EDTA. These specific observations suggested that the surface material contained other materials besides glycoproteins.

Apart from the ultrastructural studies on direct observations of cell surface material, many tissue culture studies have focussed attention on the morphogenetic significance of this proposed extracellular material. The cell surface exudates have been considered to be necessary in the promotion of cell to cell adhesions (Moscona, 1962); promotion of motility in contact guidance (Weiss, 1961); and in the ultimate transmission of morphogenetic information between cells during embryonic induction (Grobstein, 1961). Experimental interferences on cell surface material is therefore an important consideration in interpreting responses. The analysis of the degree of interference is difficult since cell surface material is difficult to define in most experiments. Morphogenesis is a dynamic changing process so that it is likely that its cell surface material is constantly modified. It would therefore be expected that the cell surface material would show minute specific variations in chemical behaviour. In vitro studies have the

additional problem of responding in an artificial environment where much of its cell surface material is leached away and has to reform. Lilien (1968) collected the mucoprotein-containing material obtained in the culture media of neuroretinal cells and reported that it specifically promotes the cell aggregation of these cells.

Description of the cell surface material is a prerequisite for a meaningful analysis of the methods of cell adhesion. This is an unsolved problem since the experimental studies involve the artificial separation of cells with the structural observations done on the physicochemical fixed material. Complications in interpretation of cell surface material further arise from the inherent tendency of cells to exude large molecules under normal conditions and also during culture conditions and fixation. The identification of previously incorporated labelled phosphate (p^{32}) in the ECM of cultured cells suggests that nucleotides have been abnormally released from cells (Trinkaus, 1969). The RNA-ase sensitive material left in migratory cell tracts described by Weiss (1967) may also be exudates from cells. Steinberg (1963) has studied the nature and origin of ECM and reported that it contains a highly hydrated deoxyribonucleoprotein gel that tend to form a variety of complexes with molecules in culture media. It was considered to be derived from hydrolysed chromosomes of ruptured cells and explains the abnormal localization of deoxyribonuclease - sensitive material reported in many studies.

The "calcium-bridge hypothesis" depends considerably on the extent of the surface material. The direction of the "bridge" in classical hypothesis is radial, that is between the two cell surfaces. In

view of the extent of the intercellular gap, Weiss (1967) suggested that the "calcium-bridge" would be more tangential to the cell surface and associated with surface groups deeper than 8 Å from the hydrodynamic slip-plane. In these sites it would increase the cohesive strength of the membrane surface. From his quantitative calculations, he considered that the minimum distance for radial links (16 Å) to be ineffective. The tangential cross links on the cell surface would be unstable during calcium-depletion conditions. The characteristic blebbing of cell surfaces seen in calcium depleted media (Dornfeld et al, 1958) strongly suggests such a stabilizing role within the cell surface. The suggestion of the relative importance of tangential over radial "calcium-bridges" introduced the distinction between the cohesive and adhesive forces operative at the cell surface. Cell separation would theoretically occur if either of these bridges are broken (Weiss also considered that cell separation may not be the reverse of cell attachment). In the event that a cohesive bridge is disrupted a considerable amount of cell surface material will be left on the adherent surface. In support of this hypothesis Weiss and Mayhew (1966) reported that cells that has previously incorporated tritiated uridine usually leave behind material ("tracts") which can be removed by ribonuclease. This observation was reported for two types of neoplastic cells but could not be identified for normal rat fibroblasts. The possibility that the material was an exudate of the cell, which was considered by the author,is not the only drawback in this hypothesis. Curtis (1967) considers that measurements of actual strength involved in adhesions under a variety of conditions would aid in evaluating the adhesion mechanism involved.

The role of divalent cations (calcium) in the cellular

adhesive mechanisms has been implicated in a variety of experimental systems. The identification of the exact interference at the cell surface and its consequences is a major difficulty largely because the cell surface material is difficult to define. These difficulties undoubtedly contributed to the establishment of the various hypothesis on cellular adhesive mechanisms. It might eventuate that a number, if not all, of these hypothesis may be partly correct. Calcium may be active in a number of bonds and activities at the cell surface.

METHODS AND MATERIALS

Experimentation was performed in a sterile operating cabinet equiped with an ultra-violet lamp for presterilization of the operating area and instruments. Microdissection was done in a dissecting hood kept sterile by frequent swabbing with 70% ethanol. All cleaned instruments were immersed in 70% ethanol, incubator dried and stored in the ultra-violet cabinet.

CULTURE MEDIA AND SOLUTIONS:

The standard control culture medium ("complete medium") consisted of medium 199 with glutamine. Supplementation was with a 10% chick embryo extract prepared from live embryos. (Preparation of medium and renewal schedule was the same as in Part I, page 7).

Solutions:

The disaggregation medium consisted of ethylenedinetrilo-tetra-acetic acid (F.W. 292.25) dissolved in an alkaline calcium and magnesium depleted Hanks balanced salt solution (EDTA in CMD-HBSS). Buffering of this solution to obtain complete dissolution under alkaline conditions (Dyer and Mellor, 1964) was performed with sterile 10% sodium bicarbonate and with acidified (0.3N HCL CMD-HBSS. A stock solution of a 0.05M EDTA was prepared. Glaeser et al (1968) selected this concentration with their studies on 7 day chick embryos since it had a maximal chelating potential with minimal osmolar perturbation. Since it is well known (Grover, 1963) that younger embryonic tissues disaggregate more readily, a number of more dilute solutions were prepared from the stock solutions (eg. 0.05 M, 0.005 M, 0.001 M EDTA solutions)

Dilution was performed with sterile CMD-HBSS. Each of these solutions were sterilized by passage through separate millipore filter apparatus.

THE EXPLANT AND EXPLANTATION PROCEDURE:

Neuroretinal tissues with associated mesenchyme (choroidal and scleral mesenchyme) were dissected from 4 - 5 day old White Leghorn chick embryos. Aging of embryos was based on the stages described by Hamburger and Hamilton (1951). Dissection of embryonic eyes was facilitated by the appearance of pigmentation, first observable in the intact eye at 64 - 72 hours (Romanof, 1960). The eyes were dissected rapidly and with as little damage or manipulation of the tissues as possible. The retina with associated mesenchyme was excised with single sharp impressions with a scalpel. The special vertical scalpel had a horizontal cutting edge. The explants (1mm - $2mm^2$) were transferred to millipore filters (pore size 0.45 micra) by means of Pasteur pipettes. Frequent renewal of the dissecting medium (HBSS) was observed in order to remove waste tissues, yolk and blood that accumulated during dissection.

The explants on millipore filters were cultured directly on Trowell platforms (Fig. 1a). In some experiments, explants were cultured on gelfoam sponges as described in Part I.

To ensure that all the explants were approximately in the same differentiative stage, the following precautions were taken:

1) Careful aging of embryos as described by Hamburger and Hamilton (1951) was performed. Both developmental age and incubation chronological age were recorded. Pasteur pipettes were prepared with a range of bore-diameters. These were used to transfer the whole eyes to the fresh dissecting medium.

During dissection the eye size (hence developmental age) were rapidly estimated by the particular diameter pipette used for transfer. Easy elimination of abnormal (smaller) or older (larger) eyes can be made with this procedure.

2) Neuroretinal explants were dissected from the equatorial region of the eye because of the small surface available at the developmental age studied (Fig. 28). This procedure was followed since differentiation of the neuroretina (Braekevelt and Hollenberg, 1970) and associated mesenchyme (Romanof, 1960) proceed from the posterior pole (near the optic papilla) and spread peripherally (toward the lens). This developmental sequence occurs also in the chick embryo. Denham's (1967) proliferation study on the two-day old rat retina was performed specifically in a region around the optic papilla to ensure that analysis was performed in the same developmental stage.

Left and right eye explants from single embryos were randomly transferred to control and experimental culture dishes by means of a transfer loop or Pasteur pipette.

THE EDTA METHOD FOR PROMOTING CELLULAR DISAGGREGATION:

Rationale:

The enzymatic methods (trypsin, collagenase) used to complete disaggregation of tissues are generally more appropriately used in tissues with abundant intercellular material eg. cartilage. The proteolytic enzymes are used in a manner so that it could act extracellularly and hopefully not excessively on the significant cell surface material. Since epithelial tissues are to be used in the proposed

studies excessive experimental interference on the cell surface should be avoided. Cellular disaggregation will be obtained by depletion of the extracellular divalent ion concentration. Zwilling (1954) used the chelating agent EDTA (ethylenediaminetetraacetate), which removes di- and tri-valent cations, to effectively disaggregate chick embryo cells.

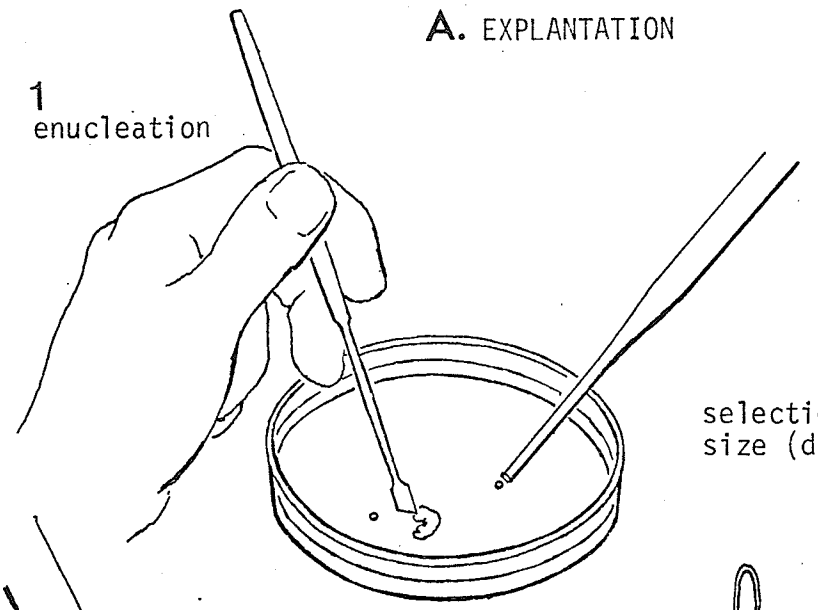
The cells of the explant studied (neuroretinal cells) do bind calcium ions under experimental conditions. Collins (1966 ii) studied the electrophoretic mobility and surface charge densities of "dissociated" seven day chick neuroretinal cells, over a range of extracellular calcium concentrations. From his calculations he deduced that there are anionic sites on the neuroretinal cell surface which bind calcium. These studies also stressed that tryptic "dissociation" may adversely remove cell surface material that bound calcium and that an incubation period would be required before this material could be regenerated. Deleterious effects of tryptic "dissociation" of embryonic chick neuroretinal cells are also possibly indicated in the surface properties of the enzyme separated cells. Barnard et al (1970) measured the electrophoretic mobilities over a 72 hour period and reported an increase in mobility over the first 20 hours, after which time it remained constant. The age of the embryos were also reported to be unrelated to the electrophoretic mobility of the cells at dissociation (even though many studies (Kuroda, 1968) indicate an age-dependent correlation with aggregation ability). Maslow (1970) suggests that in these studies, the enzyme remained at the electrokinetic surface of the cells after treatment and only gradually became desorbed in culture over a 24 hour period. Since aggregation depends on the stereochemical structure of the cell surface, the presence of enzymic action on the cell

FIGURE 28 (a and b)

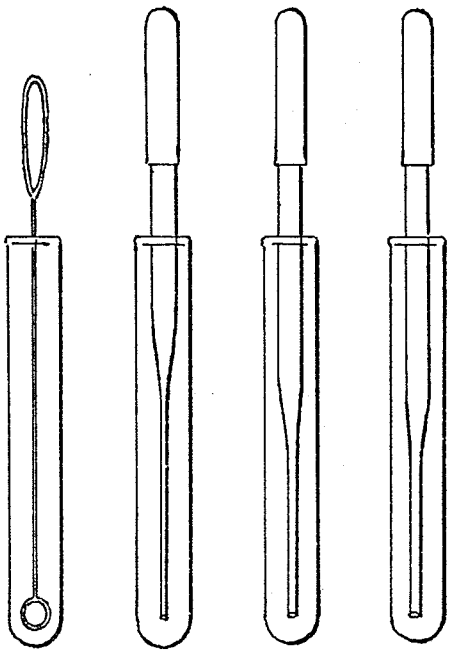
EXPLANTATION AND "WASHING" PROCEDURE

A. EXPLANTATION

1
enucleation

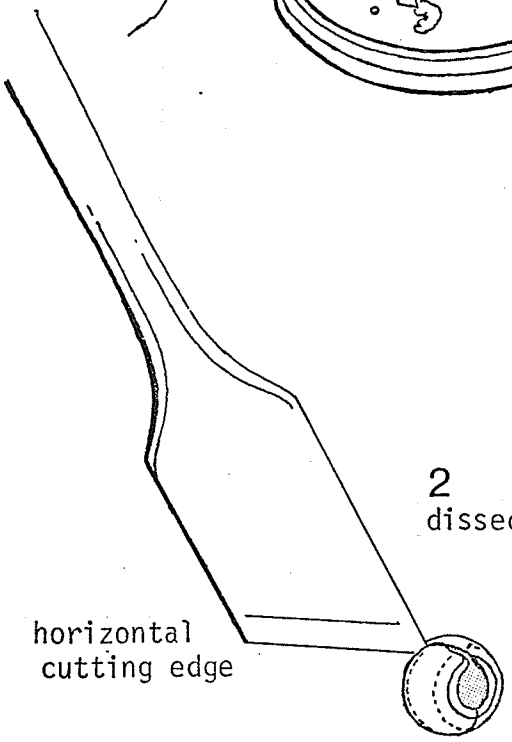


selection of eyes of similar size (differentiative state)

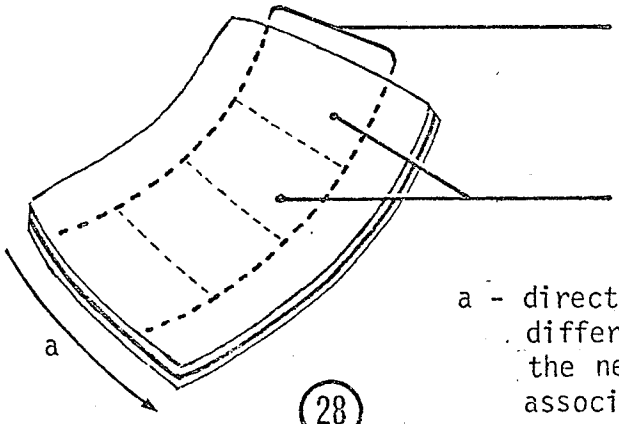


transfer loop and pipettes

2
dissection



horizontal cutting edge



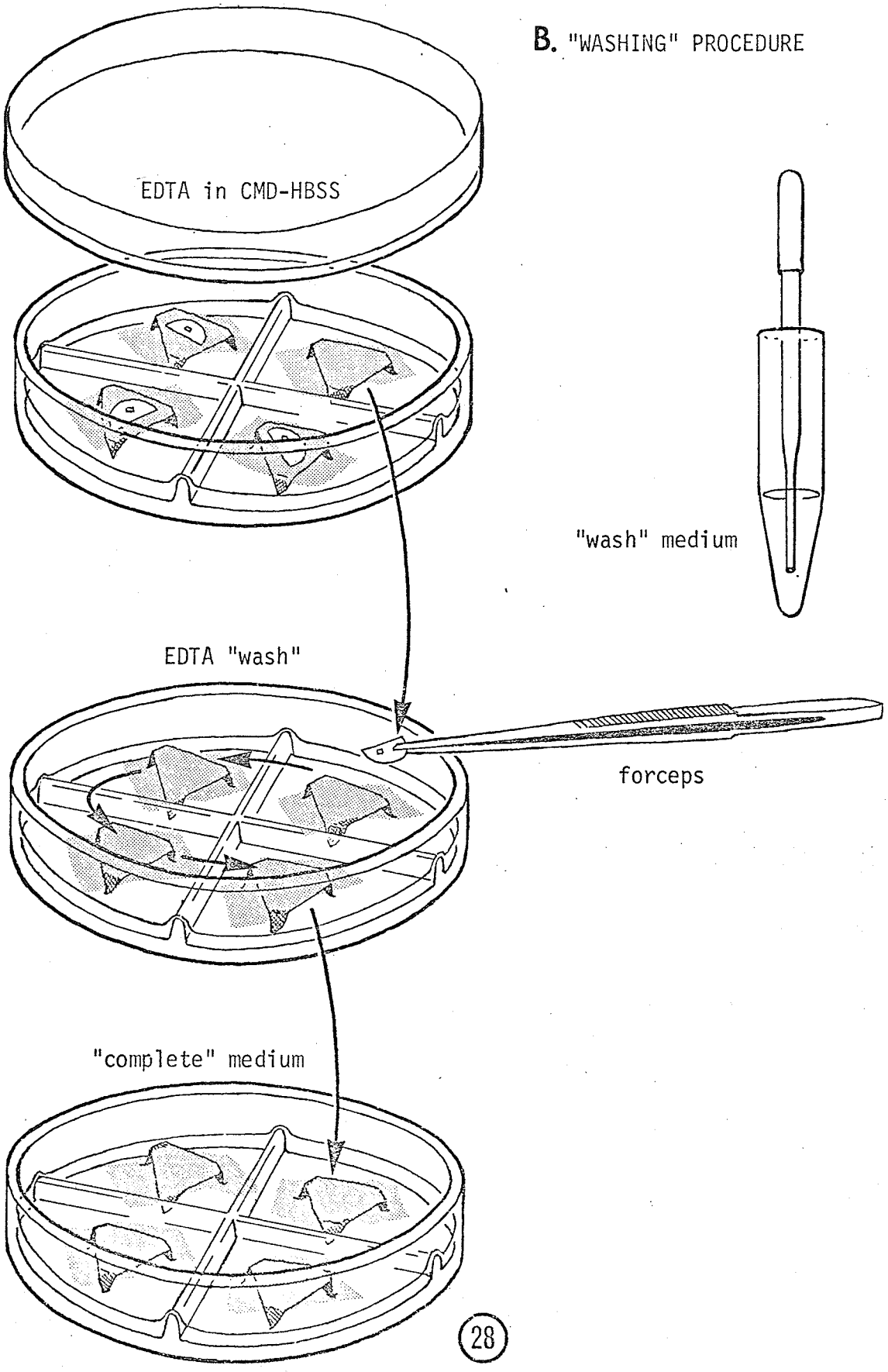
zones of eye dissected (equatorial two-thirds)

3

explants

a - direction of progressive differentiation of the neuroepithelium and associated mesenchyme.

B. "WASHING" PROCEDURE



surface is to be avoided in *in vitro* experiments.

Since it is fairly well established that calcium is essential in the adhesion mechanism of neuroretinal cells, exposure to the chelating agent (EDTA) should affect the cell attachments with a minimum amount of interference with the cell surface structure. Conversely, the return of the disaggregated or loosened epithelium to a complete medium containing calcium would facilitate the reaggregation and attachment of closely aligned cells. The EDTA solution will be dissolved in a calcium and magnesium depleted Hanks balanced salt solution to increase the effectiveness of chelation. The procedure is referred to as a calcium and magnesium depletion procedure (CMD procedure) (Details described under "method of precedure").

These apparently extreme environmental conditions used to disrupt the attachment devices are not considered to be invalid since they are representative of pathological (embryopathological) deviations (Loewenstein, 1969).

METHOD OF PROCEDURE:

The millipore-filtered EDTA solutions were buffered (pH 8.0) and incubated at 37.5 C. Taylor (1962) has studied the chick embryonic cell responses to changes in the pH of the medium. Under relatively alkaline conditions, the cells tend to revert to a rounded shape which incidentally is also the shape at which cells tend to disaggregate or detach more readily. Similar cellular responses were also observed during disaggregation of tissues at higher temperatures (37°C) compared to lower temperatures (Weiss, 1967 b).

Enucleation of embryonic eyes were performed in HBSS. Dissection of explants were carried out in frequently renewed sterile HBSS (see EXPLANT AND EXPLANTATION P. 88). As soon as all the explants were prepared from each embryo, the warm (37.5°C) EDTA solution was placed on the stereoscopic microscope stage under the glass dissecting hood. The explants were then rapidly transferred, by means of a Pasteur pipette, onto the millipore filters on the Trowell platforms. The millipore filters were well wetted with the CMD fluid so that the explants instantly imbibed the CMD fluid on contact with the millipore filter. The sealed dishes were then returned to the incubator (37.5°C) and left there for the appropriate exposure times.

The exposure of the neuroepithelial explants on the millipore filter to the CMD medium, is one where no direct purposive agitation of the explant cells are included. Most other procedures require repeated mechanical pipetting to complete disaggregation. Curtis (1967) considers this aspect of the disaggregating procedure to be a difficult one to assess or quantify and it could be a possible source of much of the variability obtained in these studies.

At the end of the CMD exposure period, the culture dishes were quickly transferred to the prepared sterile cabinet. With the aid of fine forceps, the millipore filter (plus explant) was gently placed on prepared Trowell platforms covered with a continuous film of complete media at room temperature (25°C). This "washing" procedure (Fig.28b) in a complete medium containing 10% chick embryo extract, consisted of a diffusional displacement of the CMD fluids in the explant. The diffusional exchange procedure was repeated in four separate volumes of

complete medium (Fig.28b), to ensure rapid removal of all CMD fluid from the explant. During the successive transfers only the millipore filter was handled with the forceps. The pore size of the millipore filters was 0.45 micra and was one in which rapid diffusional exchange could be obtained without it being too fragile. Gentle agitation of the large volume of medium under the Trowel platform (5 ml) was done to enhance diffusional exchange of fluids. After many preliminary studies, the total duration of the "washing" procedure was limited to 3 - 5 minutes. The complete medium used for the "washing" procedure was previously buffered at pH 7.4 - 7.6 in order to curb further chelating action. Theoretically, chelation is favoured under more alkaline conditions (Dwyer and Mellor, 1964). Chelating action cannot otherwise be immediately stopped. In the enzymatic disaggregation techniques activity of the enzymes can be stopped by addition of serum which serves as an extraneous substrate which maximally occupies enzymic action.

In view of the possibility that supernatants from recently disaggregated cells may specifically enhance the reaggregation of cells (Lilien, 1968; Kuroda, 1968), excessive "washing" of the EDTA-exposed explants were prevented. The routine of "washing" of explants on a millipore filter established, was to promote adequate removal of the EDTA solution without causing mechanical disorganization or "leaching" of the tissues. The effectiveness of this technique was arbitrarily judged from the favourable morphological characteristics (organizations, proliferation) of the subsequently cultured tissues.

The "washed" explants were placed on prepared Trowel platforms covered with a film of complete medium. These culture dishes were pre-incubated at 37.5^{o} C and buffered at pH 7.4 - 7.6. The subsequent

culture responses (reaggregations and other responses) were studied over various durations in the fixed tissues. The adaptation of this procedure of disaggregation and self-reaggregation of neuroretinal cells on a millipore filter theoretically ensures:

i) That there would be an interaction of homotypic cells after disaggregation. Grobstein (1959) considers that "whatever is involved in the minimum aggregate requirement can be produced in the mutual behaviour of cells of like type". Since cells from localized regions of the epithelium will remain close to each other, the probability that two cells will form and maintain an adhesion is favourable. The technique differs markedly from other techniques that involve the pooling of epithelia and the rotation-mediated reaggregation of the disaggregated pooled cells (Moscona, 1957). The techniques involve a considerable amount of experimental manipulation and pooling of cells from different regions of the retinas.

ii) That the initial population of responding (reaggregating) cells would not be in access to what is normal for the epithelium. In this regard, it is more comparable to the possible morphogenetic responses in vivo. Possible changes in cell population number due to cell loss can be followed, so can the possible regulatory ability of the surviving cells. Population cell numbers are important determinants in tissue culture responses. In order to validly simulate in vivo response potentials, the in vitro model should not deviate excessively from the possible situations in vivo. Moscona (1957) has alluded to some modulatory responses of chick neuroretinal cells following specific experimental disruption and organizations in culture. Cultures of reorganized tissues showed different histogenetic properties and developmental pathways when

exposed under overpopulated culture conditions.

EXPERIMENTAL DESIGN

EXPERIMENTS PERFORMED:

To obtain the specific information sought, as outlined in the "STATEMENT" (Page 74), the following series of experiments were performed.

a) Estimation of the histological effects of a generally non-toxic dose and concentration of EDTA solution on developing embryonic tissues *in vivo*.

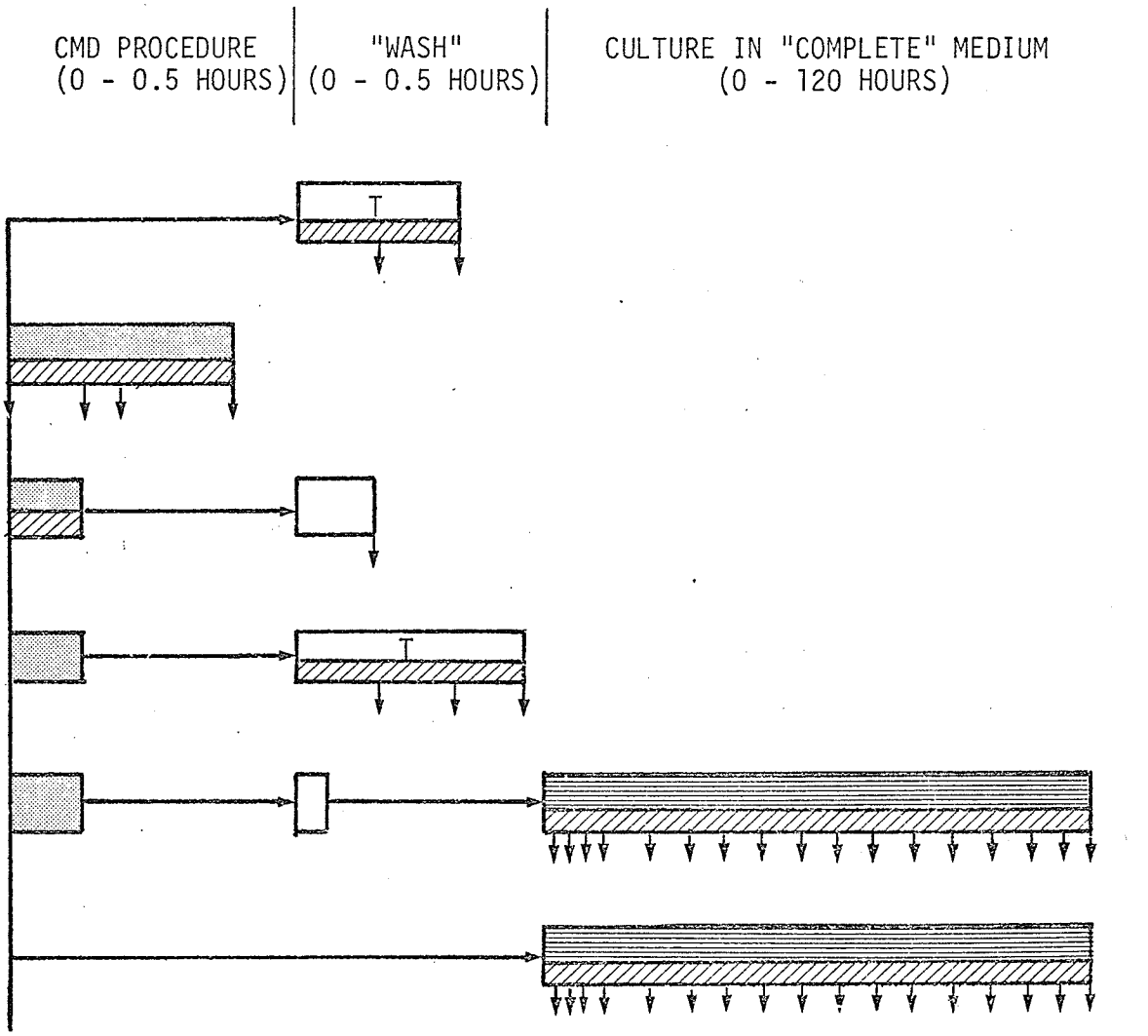
Twelve three-day old embryos were directly exposed to the selected dose, and also to the solvent alone, by means of an *in ovo* "windowing" experiment (Rugh, 1962).

b) Categorization of cultured explant responses after exposure to a decided range of concentrations of EDTA solutions (0.001 M, 0.005 M, 0.05 M, CMD HBSS). Exposure periods to each of these solutions ranged from 5, 10 to 15 minutes, and the subsequent culture periods after each of these exposures ranged from 0 - 120 hours. Explants were fixed at 12 hour intervals and the complete culture media were changed every 24 hours.

Estimation of the most favourable exposure period was based on histological observations showing the least toxicity and maximal disaggregational and reaggregational ability.

c) Detailted study of the selected non-toxic concentration of EDTA solution. The progressive explant responses during and after exposure to this CMD procedure were studied as summarized in figure 29.

FIG.29 SCHEDULE OF EXPERIMENTAL EXPOSURE OF EXPLANTS



0.05 M EDTA in CMD-HBSS, pH. 7.6-8.0, 37.5 C.

"WASH" MEDIUM, pH. 7.4-7.6, 25 C or T = 37.5 C.

TRITIATED THYMIDINE (1 microcurie per ml).

COMPLETE CULTURE MEDIUM, pH. 7.4-7.6, 37.5 C.

FIXATION OF EXPLANTS IN CARNOY II, 4^{o} C.

HISTOLOGICAL PREPARATION OF TISSUES AND ANALYSIS:

A total of 158 explants were prepared for histological study and radioautography. All explants were fixed in Carnoy II at 4C for two hours and subsequently embedded in paraffin. Serial sections were cut at three micra and mounted on alcohol-cleaned slides. Staining was with toluidine blue O (for routine histological study) and with Harris haematoxylin and eosin (for radioautography).

In order to estimate the synthetic ability of cells (DNA synthesis) during exposure to the CMD procedure, "washing" and subsequent culture, tritiated thymidine was added to the appropriate culture media (1 microcurie per millilitre medium, specific activity 5 curies per millimole). The explants were fixed according to the schedule in Fig. 29, embedded in paraffin and prepared for radioautography by coating with Kodak NTB-2 emulsion (Kopriwa and Leblond, 1962). After an exposure period of 5 days at 4C, the sections were developed and mounted according to the adapted procedure described in Part II (see "Radioautographical technique").

With the aid of a hand telly counter, systematic cellular counts on serial sections of the explant were done under oil magnification (eyepiece = 8x, objective = 100x, NA 1.25). To avoid counting the same nuclei in sections, the nuclei observed in every third serial section was counted. With the above magnification the area observed through an eyepiece grid (Whipple graticule) was calculated to be 4225 square micra. Bullough and Johnson (1951) did cellular counts on adult explants along unit lengths of 1 centimetre and took the average value of 6 - 10 observations. Considering the analysis of the embryonic

explants used in these experiments, the smaller surface area of explant tissue available and the gradient of differentiation in the embryonic eye, made such a counting routine unfeasible.

In these studies cellular counts were always done in the area between the internal limiting membrane (ILM) and external limiting membrane (ELM). With the aid of the eyepiece graticule (which covered an area of 4225 sq. micra), the entire area between the ILM and ELM was first counted in a vertical and then horizontal extent. In this manner the cellular density could be recorded. After counting the total number of nuclei within the graticule area, the same area was scanned for mitotic figures, pycnotic cells, the number of normal and disoriented metaphases and anaphases, and the number of detached cells. In this way the cellular density, percentage of disaggregated cells, pycnotic index, mitotic index and karyokinetic index could be calculated.

The values obtained from the cellular density, percentage of disaggregated cells and mitotic index could be indicative of the specific differentiative state of the explant analysed. In vivo, the more differentiative zones of the retina is thicker, has a greater cell number, and have accumulated more detached cells under the external limiting membrane.

1) Histological studies were made on all the stained sections and qualitative descriptions were recorded.

2) For each explant, a number of indices were calculated as follows:

Mitotic Index (MI): the percentage of mitotic cells in the total population of cells counted. A minimum of 1000 - 2000 cells were counted in

each explant.

Pycnotic Index (PI): the percentage of pycnotic cells in the population counted. This was the same population in which the MI was counted.

Karyokinetic Index: represented the percentage distribution of mitotic cells in various stages of the mitotic phase. The cells in each mitotic stage were represented as a percentage of the total number of mitotic figures in the population. With the aid of the karyokinetic histogram, the stathmokinetic responses could be detected.

The percentage disoriented spindles was calculated as follows:

$$\frac{\text{Number of disoriented metaphase and anaphase spindles}}{\text{Total number of metaphase and anaphase spindles}} \times \frac{100\%}{1}$$

Such an index would represent the daughter cells that would lose their attachment to the ILM. Since all the attached cells would complete mitosis adjacent to the ILM, the percentage ectopic mitotic figures were also calculated.

The percentage ectopic mitotic figures was calculated as follows:

$$\frac{\text{Number of ectopic mitotic figures}}{\text{Total number of mitotic figures}} \times \frac{100\%}{1}$$

The significance of the increase or decrease in the above values (especially MI, PI) over the initial periods of exposure, was estimated by the Student t-test where allowances have been made for small sample numbers.

RESULTS AND DISCUSSION

A) *IN OVO* TOXICITY:

The toxicity of a sample of the EDTA solution on embryonic tissues was estimated by means of an *in ovo* "windowing" experiment. Amongst the low concentrations of EDTA solutions used to disaggregate embryonic cells was the 0.001 M solution used by Curtis and Greaves (1965). Complete disaggregation of 5 - 9 day old chick embryos was obtained with this solution of EDTA dissolved in CMD-HBBS and followed by mechanical disaggregation. The effect of a dose of 0.001 M EDTA in CMD-HBSS in 3 - 5 day old chick embryos was studied in a preliminary study.

No histological abnormalities were observed in the three day old embryos that were directly exposed to 0.5 ml of either 0.001 M EDTA in CMD-HBSS or CMD-HBSS for 48 hours. The millipore-filtered solutions were injected through a "window" directly onto the amniotic sac of the embryos to ensure that these solutions will reach the embryonic tissues by diffusion. The injected solutions undoubtedly reached the embryonic tissues since all the tissues showed colchicine-arrested metaphases when colchicine was injected in the same manner, dose volume and route (figs. 27, 30). The range of concentrations of EDTA solutions decided to be further analysed were larger than 0.001 M.

B) SELECTION OF A FAVOURABLE NON-TOXIC CONCENTRATION OF EDTA:

The concentration of EDTA and duration of exposure that produced the least toxicity (lowest pycnotic index) and maximal dissaggregational ability was the 0.05 M solution over a duration of 10 minutes

at 37.5°C. Explants exposed over longer periods or to higher concentrations (0.5 M. up to 15 minutes) showed rapid necrosis (Fig. 31). The lower concentrations (0.001 M EDTA)was ineffective in producing effective disaggregation even though some disaggregated cells were observed in the less differentiated peripheral retina (Fig. 32).

Detailed studies on the selected concentration (0.05 M for 10 minutes) stressed the significance of the adhesive mechanisms in the normal cytokinetic activity of the neuroepithelium. The procedure of fixation of explants during and after each of the culture exposure conditions (summarized in Fig. 29), facilitated a reconstruction of the sequential structural events in the gradual disruption and reconstructive responses. Responses were observed in the following sequence:

C) SEQUENTIAL RESPONSES OF THE NEURORETINA:

(i) RESPONSES AT EXPLANATION:

In view of the importance of the "initial shock phase" of explants in vitro (see discussion on "life history" of explant. Part II), the early responses of explants were monitered over the initial 10, 20 minute period of culture. In order to monitor the progressive responses over this initial period, a number of morphological responses of explants were compared after 10 minutes and 20 minutes culture (summarized in TableIII). The only direct synthetic responses observed over this short period was the incorporation of labelled thymidine. Figure 33 shows the dilute, diffuse labelling throughout the S-zone of the ventricular cells. The qualitative presence of synthetic activity was therefore detected almost immediate on introduction to this in vitro environment.

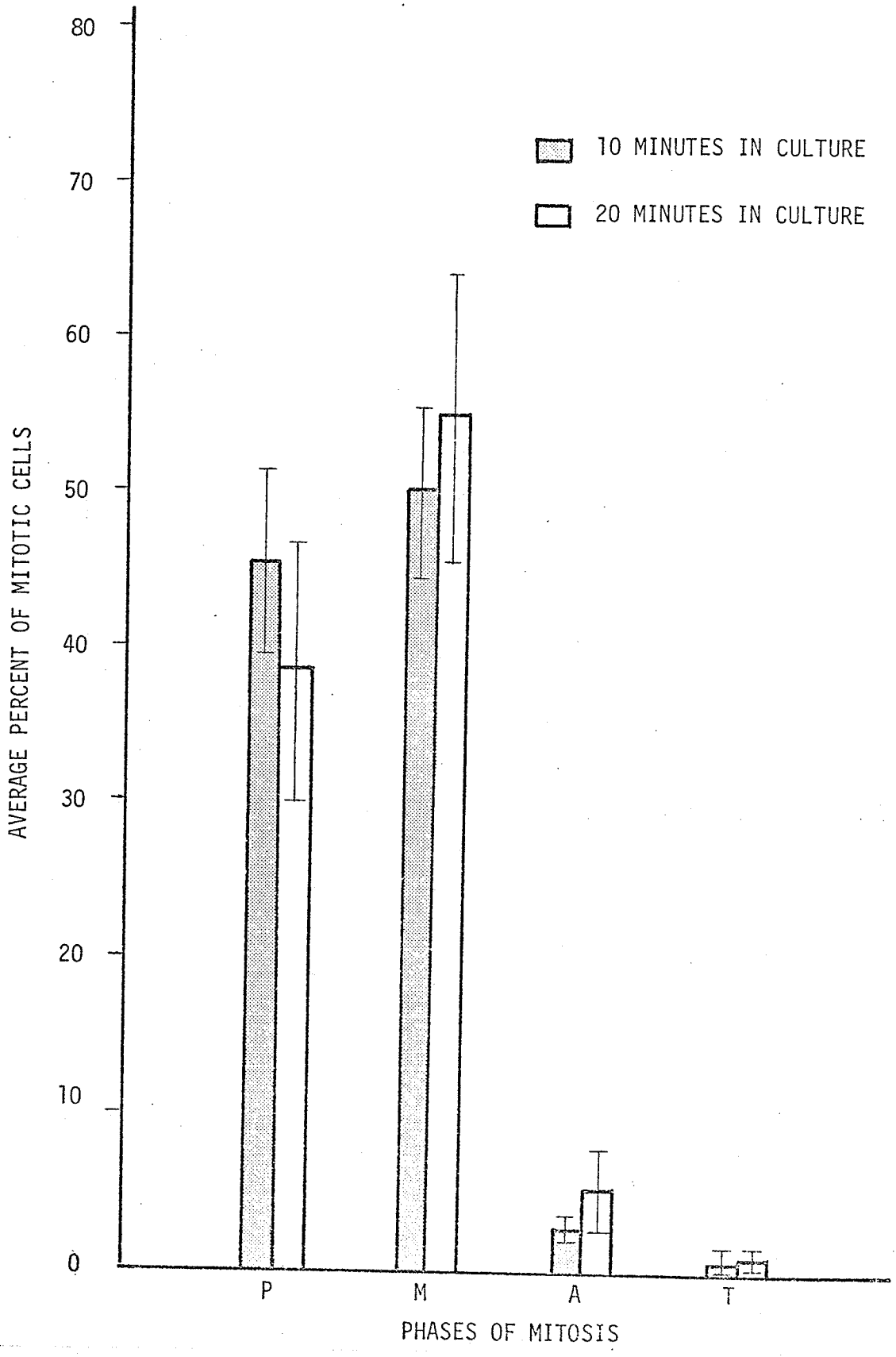
Cell loss from the explants due to cell death and exfoliation was nil. Confinement of all cells between intact ILM and ELM ensured reliable cell population estimations. The interpretation of the other explant responses (TableIII) will therefore not be affected by cell population changes during the initial culture period.

During the initial 20 minute culture period, the mitotic index dropped from 8.7 ± 1.63 to 6.8 ± 0.87 but this reduction was not significantly lowered ($p = 0.05$). Comparisons of the average percentage of distribution of mitotic phases over the same periods indicated a reduction in prophases with increases in metaphases, anaphases and telophases. This could be interpretted as indicating a progressive later stathmokinetic effect (after 20 minutes compared to 10 minutes) even though the increased percentage metaphase cells did not indicate a significantly marked accumulation (Figure 34).

An unequivocal stathmokinetic response cannot therefore be deduced during this brief period in vitro. It might be possible, that over longer periods, this effect might be more marked. It would, however, be difficult to exclude the possibility that these were inherent cellular responses expressed in vitro and not a result of the initial shock phase. Fujita (1962) reported that the neuroepithelium showed a progressive marked inherent increase in mitotic time (and generation time) in ovo.

In conjunction with the increased percentage of metaphase and anaphase cells, there was an increased percentage of disoriented mitotic spindles (metaphase and anaphase spindles) (see Fig. 35). This is of interpretative significance in view of the importance of these

FIG. 34 AVERAGE PERCENTAGE DISTRIBUTION OF MITOTIC PHASES DURING THE EARLY "SHOCK" PHASE OF CULTURE



initial explant responses and its effect on subsequent developmental responses *in vitro* (Saxen, 1969). Under normal conditions of cytokinesis, the orientation of ventricular cell mitotic spindles lie parallel to the ILM, consequently ensuring that both daughter cells are in contact with the ILM. Langman has re-examined the orientation of mitotic spindles *in vivo* and has reported a small percentage of cells that had oblique and perpendicular oriented mitotic spindles. This is considered to be an alternative mechanism for detachment of cells from the ventricular zone. The observation, of an association of increased metaphase and anaphase cells with increased percentage disoriented mitotic spindles, could be of mechanistic significance. Prolongation of mitotic time (especially metaphase and anaphase times) could represent a means for promoting disorientation of mitotic spindles, eventually resulting in detachment (differentiation) from the ILM.

Secondly, the observation of the stathmokinetic effect after 20 minutes (compared to 10 minutes) could be an early structural manifestation of the "metabolic lag period" observed in explants after 8 hours of initial culture (Saxen et al, 1968).

Another possible explanation for the accumulation of turgid metaphase cells could be due to the disturbance in fluid distribution in the explanted tissue *in vitro*. The "shock" phase during explantation is associated with fluid imbalance and it could be that the rounded mitotic cells would be the most susceptible cells. This possibly, also accounts for the turgidity of these cells seen in Fig. 35.

All the explants studied were probably in a similar differentiative state since the percentage disaggregated (detached) cells were similar

in both populations compared.

TABLE III

EXPLANT RESPONSES DURING THE FIRST 20 MINUTES

IN THE CULTURE ENVIRONMENT

	VALUES AFTER 10 MINUTES	VALUES AFTER 20 MINUTES
Explants	5	5
Total Length	6045 micra	6240 micra
Total Cells	3258	5595
Total Pycnosis	2	0
Average Percentage Pycnosis	0.0	0.0
Average Percentage Mitosis	8.76 ± 1.63	6.838 ± 0.87
Percentage Disoriented Spindles	14.48	19.9%
Percentage Disaggregated Cells	1.31	1.64
DNA Synthesis	+	+

TABLE IV

DISTRIBUTION OF MITOTIC PHASES IN EXPLANTS AFTER A 10 MINUTE AND 20 MINUTE EXPOSURE TO THE CULTURE ENVIRONMENT

	AFTER 10 MINUTES	AFTER 20 MINUTES
Explants	5	5
Total Mitosis	293	394
Average MI	8.76 ± 1.63	6.84 ± 0.87
Prophases	144	172
Percentage Prophases	49.14%	43.65
Metaphases	138	198
Percentage Metaphases	47.09%	50.25
Anaphases	7	18
Percentage Anaphases	2.38%	4.56
Telophases	3	6
Percentage Telophases	1.02%	1.52

CONCLUSIONS AND RECOMMENDATIONS:

The "shock phase" at explantation was studied over the initial 20 minute period. Indications that there is a "metabolic rate decline" could not be deduced by the possible stathmokinetic effect observed over this short period since initial fluid imbalance could be responsible. The cell loss during this period was nil.

A stathmokinetic effect is a convenient morphological response to study in responding tissues but the interpretation must be more critical in view of the universality of this response. In the experiments reported, a possible stathmokinetic effect during the initial "shock phase" in culture was related under the untreated normal culture conditions. It further emphasizes the well-known fact that accumulation of cells in the metaphase stage can be produced by a variety of substances/conditions through diverse modes of actions. The complexity of the particular process of stathmometaphase (which is longer than the normal metaphase) has been analyzed by Deysson (1968). This phase is considered to be a susceptible phase in animal cells particularly in the chick embryo (Deysson, 1968; Gelfant, 1963).

In subsequent studies (with the folic acid antogonist, methatrexate) where stathmokinetic effects will also be studied (Part IV), parallel control cultures will be used as standards for comparison. In this way stathmokinetic responses unrelated to the drug studied can be detected and considered.

Previous studies on the stathmokinetic effects of substances

(Gelfant, 1958) have utilized the universal stathmometaphasic agent colchicine as a standard to detect possible stathmokinetic effects of other agents. The premise in these studies, that substances that show stathmokinetic effects could be detected when compared with colchicine, is too simplified. Colchicine specifically impairs spindle formation ensuring metaphase arrest whereas metaphase arrest can be affected by a variety of unspecific substances and conditions. Substances that do show stathmokinetic effects under certain conditions may not show a stathmokinetic effect on comparison with colchicine. It is misleading to relate conditions or actions of substances as having "colchicine-like effects".

The use of parallel control cultures as standards for comparison is more ideal since the complications of in vivo differences are eliminated and more important, the standard selected is one where the cells are studied under normal mitotic conditions.

SEQUENTIAL RESPONSES OF THE NEURORETINA:

II) EVENTS DURING THE GRADUAL DISRUPTION OF THE NEURORETINA:

Explants fixed furing the exposure period to the EDTA solutions and the "washing" period gave indications of the events in the early disruption of the epithelium. Some of the major responses were:

DETACHMENT OF MITOTIC CELLS:

Interruptions in the ILM were first noted in regions where mitotic cells were located (Fig. 36). Mitotic cells are known to be more susceptible to detachment under a variety of culture conditions.

Detachment of cells were, however, probably indiscriminate of cell phase as many of the interphasic ventricular cells had also been detached within the initial 10 minute exposure period. Mitotic cell detachment was more easily noticeable because of its round turgid shape and position adjacent to the ILM. The observation of labelled detached cells, thirty minutes after the initial exposure to the EDTA solutions also suggests that the ventricular interphasic cells were detached early. If these cells were detached only after entering mitosis at the ILM, then the cells would not be seen as freed cells. Earlier studies (Part II) have indicated that the minimum time for the cells to complete DNA synthesis and migrate through the interkinetic zone to the mitotic zone was 90 minutes. Since the thymidine label was detected in the detached cells after 30 minutes, the cells likely became detached while in an interphasic stage (Fig. 39).

FOLDING OF THE NEURORETINA:

Folding of the neuroretina was frequently observed along the length of the explant. Unlike the neuroretina, the associate pigmented epithelium was relatively intact in almost all the explants (Fig. 36).

Folding of epithelial explants was previously observed after exposure to divalent ion depleted solutions (Dodson, 1967), but it has not been explained. The possibility of fluid imbalance during the initial "shock" phase cannot be discounted but there was some structural basis for the folding observed in the present study. The first observation included an early detachment of the neuroretinal epithelium from the pigmented layer to which it previously had a firm attachment. The second consistent observation in this regard, were the large gaps left

by the detached mitotic cells in the otherwise compact epithelium. These gaps were seen as focal points in many folds.

COMPLETELY DISAGGREGATED CELLS:

Complete disaggregation of the neuroepithelium was observed in explants taken from the more peripheral regions of the neuroretina. The freed cells were spherical in shape with many long cell surface projections (Fig. 39). The instantly-fixed material is reminiscent of cells described under in vitro time lapse photography (Dornfeld, 1958). In the reported studies, the spherical fibroblasts were seen to pulsate and send out long pseudopodial-like projections. This type of projection is frequently observed in cells disaggregated by trypsin or EDTA (Trinkaus, 1969 p. 32-33) and has also been observed in situ in the transparent sea urchin blastula. In the blastula it was associated with normal exploratory mechanisms for making contacts and promoting movement of the cell away from the vegetal pole into the blastula.

The nuclei fixed during the exposure to the 0.05 M EDTA solution stained characteristically hyperchromatic (Fig. 39). This could be an indication that there may be intracellular effects due to ionic imbalance during the exposure period. It is relevant to reconsider that Mazia's (1958) studies predict that the structural integrity of chromosomal material would be jeopardy during this period. The hyperchromaticity observed is probably due to the promotion of the Toluidine blue O staining reaction by the formation of new stain-binding sites in the CMD procedure. It is probably related to the CMD procedure since the hyperchromaticity is reversible on transfer of the explants into a complete medium containing divalent ions (Fig. 40).

Furthermore, no chromosomal abnormalities could be noted in any of the cells completing mitosis after "washing" and culture in a complete medium (Fig. 45).

The appearance in disaggregated cells of an irregular cell surface with extensive microprojections is of interpretive significance. It is relevant to consider the suggestion (Weiss,L. 1967) that cell separation may not be the reverse of cell adhesion and that separation may possibly occur by means of "non-lethal microruptures" in the weakest part of the peripheral zone of the cells. These sites would be spacially different from those involved in cell adhesion. The microprojections observed, however, do not necessarily support this possibility since the ultimate separation of the cells would depend on the disruption of the apparently secure adhesion mechanisms. The terminal bar system in the neuroepithelium is not easily disrupted as is evidenced by its intactness even though under considerable disruption pressure by the accumulation by the turgid colchicine-arrested metaphases (Part II, Fig. 26 p. 71). The elongated micro-projections observed is an expected response to calcium deprivation since calcium very likely play a stabalizing role in the molecular structure of the plasma membrane (Manery, 1969). Cells exposed to this medium over longer periods show disruption of their cell surfaces with poor cellular details throughout the explant.

III) EVENTS DURING THE REORGANIZATION OF THE COMPLETELY DISRUPTED NEURORETINA:

Explants that were exposed to 0.05 M EDTA, "washed" in complete media and subsequently cultured showed the following major responses:

1. The hyperchromaticity seen in the stained cells of explants exposed to the EDTA solution, was no longer evident as the cells reverted to the normal staining characteristics (Fig. 39). The cell border microprojections were still evident in many of these cells after 30 minutes in culture but were not as marked as during the direct exposure to the EDTA solution. With prolonged periods of culture the microprojections were no longer evident in the instantly-fixed tissue cultures.

2. Many of the disaggregated cells showed normal mitotic figures including many anaphases. These observations suggest that there was a progression through mitosis. Therefore, if there were stathmokinetic effects during the CMD procedure, these were not irreversible. Dornfeld et al (1958) reported the possibility of there being stathmokinetic effects in EDTA solutions. The EDTA - exposed cells in the present studies were still capable of DNA synthesis since tritiated thymidine label could be detected in these cells 30 minutes after exposure. (Fig. 39).

3. The spherical disaggregated cells eventually assume polarized shapes but with no definite associative orientations (Fig. 40). Mitotic figures are rare.

4. Reaggregation of neuroretinal cells were observed after 24 hours of culture of the disaggregated cells. The previously disoriented polarized cells (Fig. 40) formed aggregates of rosette formations. Mitotic figures were localized in the juxta-lumenal regions of the circular rosettes which showed an internal limiting membrane (See discussion of rosette-formation, p. 117).

Prolonged culture of these reaggregated tissues (up to 120 hours) resulted in necrosis in some aggregates and disaggregated cells. Many of the cells appeared multipolar and many viable rosettes were still evident (Fig. 42).

Structurally, there was no modulation of cell function or transformation into lentoid-like structures as has been observed in cultures of neuroretinal rosettes on "plasma clot". Moscona (1957) had reported a lentoid-like transformation of neuroretinal rosettes when they were cultured as such in the presence of cytolysed cells for several days under conditions of overcrowding. Moscona considered that with disintegration of cells the experimental alteration of intercellular relationships and the ensuing process of repair and reorganization, provided conditions under which cells followed altered developmental pathways. The experimental conditions of reaggregation in this study differs from Moscona's in that it ensured the interaction of homotypic cells in numbers inherent to the particular epithelial explant. No pooling of cells from many retinal explants were required. The present model for disaggregation and reaggregation of the neuroretinal tissue is one where the tissue differentiation is promotion and not overly challenged.

IV) EVENTS DURING THE REORGANIZATION OF THE INCOMPLETELY DISRUPTED NEURORETINA:

These events could be followed in cultured explants that were previously exposed to dilute solutions of EDTA over short exposure periods, or in the more differentiated zones of the retina. The consistent sequential responses include observations of ectopic mitotic figures, proliferation and migration, accentuation of neuroretinal foldings,

pseudorosettes and limited pycnosis.

OBSERVATIONS OF ECTOPIC MITOTIC FIGURES:

The mitotic cells that appeared to be most easily detached during the CMD exposure period could be observed outside the normal mitotic zone in later stages of division. Since cells attached to the ILM, complete division in the restricted mitotic zone, the artificially detached mitotic cells are designated as "ectopic mitotic figures". Kauffman (1969) considered mitotic cells in an ectopic zone of the neuroepithelium as resulting from an interference during the G2 phase of the cell cycle, due to premature mitosis in the interkinetic zone. In the reported in vivo study it was not possible to ascertain from the published photographs whether the ectopic mitotic cell had any attachment to the ILM. It is almost impossible to follow complete cellular outlines in the compact pseudostratified neuroepithelium. The possibility that detached mitotic cells could be located in similar sites is suggested from the present study. This is supported by the observation (Fig. 26) that attached mitotic cells are withdrawn toward the ILM where it completes mitosis. Menkes et al (1969) has also associated the abnormal localization of mitotic figures with destruction of the ILM.

ACCENTUATION OF NEURORETINAL FOLDING:

The early interruptions in the ILM, seen during the exposure to the EDTA solutions, were highly accentuated during subsequent culture periods in a complete medium. The interruptions in the region of the ILM formed structural focal points for subsequent foldings of the neuroepithelium. The accentuation of the folds was associated with high

proliferative activity of attached and detached neuroepithelial cells and the migration and exfoliation of detached cells (Fig.). The excessive exfoliation of interphasic and mitotic cells through interruptions in the ILM (Fig. 43) have two important consequences in the neuroretinal reorganization and separation. First, it is a means of depleting the initial cell population of the neuroepithelium. Neuroepithelial hypoplasia is a characteristic teratological basis for CNS malformations. The hypoplastic nervous system may not only be due to retarded proliferative activity but also as a result of a reduction in the initial cell population due to early cell loss. All loss in these explants were due chiefly to exfoliation since pycnosis was rare. Figure 46 represents an epithelium that has been exposed to the CMD procedure and subsequently cultured for 60 minutes in a complete medium. The epithelium is definitely hypoplastic since outlines of cells can be followed from the ILM to the ELM. Usually under light microscopy, the compact pseudostratified epithelium does not facilitate such observations. It is likely that cell loss due to exfoliation is more frequently detected if there are many, large interruptions in the limiting membranes. Cell loss due to pycnosis only is usually more easily detectably since the cells accumulate under the ELM (Fig. 21).

The importance of the regulatory ability of such an epithelium can be followed in such <u>in vitro</u> studies. It is possible,that depending on the action (duration and concentration) of the experimental agent, such an initial hypoplastic epithelium can possibly be able to restore the lost cells by the process of regulation of the proliferative cells. Impending malformations could be removed by such a mechanism and likely accounts for many negative results with known teratogenic agents in some

species. The conditions of drug exposure and regulative possibilities are major factors in determining the final development.

PSEUDOROSETTE FORMATIONS:

These were observed and were associated with highly accentuated retinal folds that had reestablished continuity of interrupted ends (discussed in "Distinction between true rosettes and pseudorosettes", p. 121).

INTERPRETATIVE SIGNIFICANCE OF THE ORGANIZATIONAL RESPONSES OBSERVED

SIGNIFICANCE OF THE HISTOGENESIS OF ROSETTE-FORMATIONS:

The observation of rosette-formations under specified conditions is of significance because it has also been observed in both neoplastic and normal neuroepithelial tissues exposed to a number of other conditions. Previous descriptions of rosette-formations were based on the use of gyratory shakers to promote reaggregation of "dissociated" cells. The suspension of discrete cells were obtained by treatment of the pooled retinal epithelia with cation-deficient solutions, followed by trypsinization and mechanical pipetting (Moscona, 1957; Sheffield and Moscona, 1970). The observations reported in this dissertation are based on reconstruction of consistent events followed in individual epithelial explants. Degrees of disaggregation were obtained by pretreatment only with a dilute EDTA solution. This direct in situ observation (under less complicated experimental procedures) is more valid in interpreting the rosette-formations observed under teratological conditions in vivo.

The reported observations of rosette-formations under the variety

of teratological conditions and in neoplasia have largely consisted of descriptions of the formed structure. The mechanisms of formation will be discussed in relation to the results obtained in this study.

MECHANISMS INVOLVED IN ROSETTE FORMATIONS:

The clustering tendency shown by neuroepithelial cells of the neural tube or neuroretina after being freed by a variety of experimental conditions indicate a strong polarization of these cells. The re-establishment of adhesive mechanisms at one end of the cells can be deduced by the mitotic activity seen in the rosettes. In figure 10 the mitotic figures are all restricted to the lumenal side of the rosettes, also the spindle orientation is parallel to the lumenal surface. This cytokinetic activity is similar to the neural tube activity where the mitotic cells are strongly held to the lumenal surface by terminal bars. According to Fawcett (1961) little is known about the reestablishment of adhesive mechanisms after separation but that unmatched halves of desmosomes are rarely, if ever, seen on surfaces of contact between like cell types. One therefore assumes that the formation of the complete desmosome or terminal bar involves the simultaneous and complementary specialization of corresponding areas of the two opposing surfaces. Histochemically, the attachment structures appear to consist of protein only (Puchtler and Leblond, 1958). Protein synthesis, an expression of differentiation, would therefore be necessary in the completion or reconstruction of these attachment devices. The bonding forces within these terminal bars probably involve divalent ions and have been discussed elsewhere (section III,P. 76). The time of this protein synthesus during the cell cycle is not known.

Overton (1962) made some important observation on the re-establishment of adhesions during the reaggregation of chick area pellucidal cells. She reported the formation of new desmosomes but also that there was evidence for the persistence and realignment of half desmosomes. Although these were reported for desmosomes the distinction between desmosomes and terminal bars is not clearly defined (discussed by Watterson, 1965).

Apart from the consideration of protein synthesis in the reestablishment of attachment devices, the long range chemical forces are likely to play an important role of adhesion at the sites of these surface specializations. This is deduced from the observation that no formed elements can be seen in the intercellular space in desmosomes or terminal bars (Fawcett, 1961). The mechanism, however, is quite tenacious as can be observed by its intactness after the accumulation of many turgid colchicine-arrested metaphases (Fig. 26). During the normal cytokinesis in vivo, the restitution of the proteinacious terminal bar material and reestablishment of the chemical bonding forces are critical events in the mitotic zone of the neuroepithelium. Experimental intervention in these events could result in the premature or abnormal release of cells from the internal limiting membrane. Exposure of the neuroretina to the calcium and magnesium depletion procedure indicated that the mitotic cells in the m-zone were the cells that were more easily released from the internal limiting membrane under various exposure conditions of durational extension and concentration changes (Figs. 36, 37).

ROSETTE FORMATION UNDER EXPERIMENTAL CONDITIONS

Neuroepithelial cells seem to have an inherent capacity to form

strong bonding forces between its cells that are in a similar differentiative state.

The formation of "rosettes" (or pseudorosettes) was reported in the in vivo and in vitro studies of Tansley (1933) with neonatal rat retinas. This study could also be considered as the first experimental study to refer to the relationship of rosette formation and the differentiative state of the explants. Although Tansley had reported that rosettes could not be obtained in the hanging drop cultures of retinas from 15 day old rats, Hild and Callas (1967) reported rosette formation in the outer nuclear layer of rat retina. These two studies are not conflicting since rosette formation is related to differentiative state of neuroepithelial cells as referred to in the whole embryo or to specific zones in the neuroepithelial wall. The sequential release of neuroepithelial cells during normal morphogenesis of the neural tube result in the formation of specific zones of neuroblasts. As a result of the temporal difference in origin from the neuroepithelium, these zones develop distinct differentiative properties. Langman and Welch (1967) have observed that in the fetal rat cortex, the more superficial cortical cellular layers are the more recently differentiated (in that they are the last to be released from the neuroepithelial layer). The possibility that observations of rosette formations depend more on the differentiative state of detached cells is further supported by the observation of rosette formations in a distinct cerebral cortical zone of cultured explant previously exposed to a CMD procedure (Fig. 47).

Tansley had also made relevant observations concerning the possible mechanisms of rosette formation. The absence of mitosis in the

observed rosettes in cultures of three-day old rat retinas suggested to her that overgrowth of the neuroepithelium was not the primary cause in the production of the rosettes. The author did not elaborate on this observation. Rosettes were also reported in the folded neuroepithelium in vivo after trephening of the undeveloped eyes of post natal rats. The author related rosette-formations with changes in intraocular pressure even though she observed that "sometimes the eye may appear slightly collapsed but mostly the pressure is good and the site of the trephine hole is often very difficult to find". Even though the site of trephening trauma and rosette formations were not clearly distinguished in these early studies, it has been more accurately defined in studies by Coulombre (1956) and Hicks (1957). Coulombre interpreted the foldings observed in the developing chick retina as a result of the unrestrained neuroretinal growth after there has been a reduced intraocular pressure on this epithelium. Hild and Callas (1967) considers the same explanation for retinal folds observed in congenital retinal dysplasia. Hicks' many studies on the regeneration and malformation of the nervous system following irradiation injury enabled him to explain the mechanism of rosette formation that is consistent with the mechanism of formation under a variety of experimental conditions. Normal growth of the neuroepithelium layer is maintained in continuity over a broad surface by several balancing factors. There is a continual changing equilibrium between the inner luminal pressure (ventricular or ocular) and the expanding tissue pressure. In vivo a factor that disturbs the dynamic balance between the tissue pressure and luminal pressure would result in an irregular neuroepithelial surface, resulting in folds and rosettes. It is noteworthy that the cavities of many of the rosettes

seen in malformed embryos were continuous with the main neural cavity. The in vivo studies mentioned above considers the importance of relative growth pressures of associative tissues in the production of these malformations. In the reported in vitro studies, it is the inherent tendency of the neuroepithelium to respond in isolation that is analysed. The results indicate that the neuroepithelium can be solely responsible in the production of these malformations (foldings and rosettes) since they were reproduced in the isolated tissue in vitro where the relative pressure effects are eliminated.

DISTINCTION BETWEEN THE ROSETTES AND PSEUDOROSETTES:

The distinction (in an organizational basis) between developmental rosettes and the rosettes sometimes observed in neoplasia (neuroblastoma, retinoblastoma) should be made to avoid misinterpretations. The view (Mann, 1937) that the resemblance is superficial still require elaboration on the formative process, even though in the more recent literature reference is often made to "pseudorosettes" and "rosettes". The clusters in neoplasia are structurally more uniform in that they are small with a circular lumen seen in every plane of section, so that they are really small hollow spheres. In contrast, most of the congenitally observed rosettes (x-ray aborted human fetuses discussed by Mann, microphthalmic eyes) all originate as branchings or foldings of the tube at some time during development. Hicks (1954, 1957) has presented many instances of direct connections of these rosette structures to the main neural canal. Foldings of neuroretinal explants in three-dimensional cultures to form similar rosettes have been frequently observed (Fig. 10). This type of cluster is better referred to as a pseudorosette.

The distinction of the two types of rosettes seem to be based on observed organizational criteria. This depends on the initial degree of cellular attachment of the composite cells in the cluster. Another difference (also noted in Hicks' 1954 study) is that these clusters usually contain a core of highly prolific neuroepithelial cells (Figs. 44,45). The numerous mitotic cells are localized along the intermediate intact internal limiting membrane and not in the region of recent reestablishment of cellular connections (Fig. 45). Mitotic figures in general are rare, if they ever do occur, in the true rosettes of neoplasia. The observed absence of mitotic figures in the cultured neonatal rat rosettes as reported by Tansley (1933) is not a generalization as it does occur under certain culture conditions (Fig. 47) and it also depends on the mitotic pattern inherent in the explanted tissue. It is a well known fact that the older the explanted tissue, and the longer the culture duration, the less frequent are the observed mitotic figures.

Highly malignant tissues are characteristically non-adhesive and more motile and are consequently more likely to be observed as disorganised individual cells. Under conditions of reaggregation, as would be in slowly-growing tumours that theoretically may tend to differentiate, the clustering of these cells would result in the formation of small hollow rosettes. The mechanism of formation of small hollow rosettes would depend on the self-recognition of individual cells that would be in the similar state of differentiation. It is conceivable that in highly prolific cells this self-recognition would not be temporarily facilitated, consequently in the highly undifferentiated neuroblastomas, rosette formation is not facilitated. If the individuality of cells is a prerequisite for the formation of small hollow rosettes then it

should also be seen in the disaggregated normal embryonic neuroepithelium. In figures 36 - 45 the sequential stages in the complete disaggregation and reaggregation of the embryonic neuroretinal cells is seen. In cultures where there has been incomplete disaggregation of the epithelium, attempts to reconstruct a complete epithelium during the reaggregation period resulted in the formation of "pseudorosettes" (Fig. 44). The clusters formed from the reaggregation of completely disaggregated neuralretinal cells were small and had circular lumena seen in all planes of section. Many of these are seen in the reaggregation of cells (Fig. 41). Furthermore, the above expressed view that the reaggregation of individual cells also depend on the differentiative state of the cells is generally borne out by the formation of true rosettes in the less differentiated zone of the neuroretina and in the distinct differentiative zone in cultures of cerebrum (Fig. 47). The whole explant shown in the figure has been exposed to the same disaggregation and reaggregation procedures as the neuroretinal explants. A feature of the rosettes in these normal embryonic tissues is the presence of mitotic figures. The proliferative activity of rosettes in neoplasia still remains to be studied in detail so that comparisons cannot be made. Furthermore, the correlations between rosette formation, differentiation with reduced malignancy has not been studied in detail and many of these correlations are based on independent descriptive histological studies.

Whatever the significance of rosettes, however, they are of continued interest for they do appear to represent an attempt at differentiation. The clustering tendency, reports of formations of adhesive mechanisms (Introductions) reduced mitotic activity all point to a differentiative state. Clinically there is a suggestion that tumours

with rosettes have a better prognosis (Stowens, 1966). The inherent growth rate of the aggregation of cells is a relevant question to this topic. According to Robbins (1968) the dedifferentiation observed in the highly malignant neuroblastoma would represent a direct result of the growth rate. Better differentiation can be induced in these tumours by the administration of vitamin B12 or x-ray therapy. The tumours undergo maturation forming rosettes, or ganglioneuroma with slower growth rates and are more amenable to successful extirpation.

EFFECTS OF EDTA ON THESE ORGANIZATIONAL RESPONSES:

The mechanism of detachment of ventricular cells from the ILM during exposure to the CMD procedure is probably chiefly a surface phenomenon. Some cells could also have been detached as a consequence of the stathmokinetic response observed during the initial period of explantation (discussed on page 107). Earlier observations have indicated that this initial response during the "shock" phase of explantation predispose to disorientation of mitotic spindles resulting in separation of daughter cells from the ILM.

It is necessary to consider the possible deleterious explant responses during, or as a result of, exposure to the EDTA solution. The theoretical fact that the relatively hydrophilic EDTA would not tend to enter cells (Albert, 1968) does not eliminate its possible intracellular effects due to cation depletion. Whitefield et al (1969) has reported that rat thymocytes could not be stimulated to enter mitosis in "calcium-free media". These observations have implicated divalent ions as an intermediary in the stimulation of mitosis. A series of studies by that author (Science Dimension, 1970) have suggested that the

formation of adenosine 3,5 monophosphate (cyclic AMP), which promotes DNA synthesis, is governed by several hormones and divalent ions in the extracellular environment. The observation of labelled nuclei in explants exposed to EDTA solution does not convey any information in this regard, other than the qualitative identification of DNA synthesis. It is not absolute proof of DNA replication since cells have been reported to incorporate tritiated thymidine under conditions of repair and renewal of chromatin material (Pelc, 1965).

It is therefore relevant to consider Mazia's studies (1954) with EDTA on the relationship of divalent cations and the structural integrity of chromosomes. He observed that chromosomes could be dispersed without splitting of peptide bonds if there was a divalent ion depletion in a medium of sufficiently low ionic strength. The prediction from these studies is that the intergenic phenomena (chromosome breakage, rearrangements and crossing over) will be sensitive to the ionic environment of chromosomes. To circumvent this possible abnormal situation, particular precaution was maintained during preparation of the EDTA solution in a balanced salt solvent (CMD-HBSS). This ensured a sufficiently normal ionic strength of the solution and should not facilitate the chromosomal dissolution which was only obtained in divalent-depleted solutions of "sufficiently low ionic strengths". All explants studied showed high proliferative responses during (Fig. 39) and after (Fig. 44) exposure to the EDTA solutions. The many mitotic figures observed were normal in structure and well preserved (Fig. 45). Dornfeld (1958) had earlier directly observed (timelapse studies) the appearance and completion of mitosis of fibroblasts exposed to dilute EDTA solutions. Many of these EDTA solutions were made up in "calcium-free salt

solvents.

The following responses indicate that the effects of the EDTA exposure produced major responses at the cell surface:

(i) The deformation of the cell surface during exposure to the CMD procedure was reversible on culture in a complete medium.

(ii) Cells in the less differentiated zones (peripheral retina) were more easily disaggregated. The more differentiated zones, with more stable adhesive mechanisms,remained intact and viable.

(iii) The possibility of direct or indirect intracellular effects of the EDTA solution were not supported. The hyperchromaticity observed in stained sections during exposure was immediately reversible when exposed to a complete medium. Furthermore, synthetic activity (DNA synthesis) was detected during and soon after exposure. All the mitotic cells showed normal chromosomal arrangements and cells completed mitosis during and after the "washing" and culture in complete media.

CONCLUSIONS AND RECOMMENDATIONS

1. A transient depletion of divalent ions by the EDTA method *in vitro* seriously disturbed the adhesive mechanisms and the subsequent morphogenesis of the neuroepithelia. The culture method facilitated the direct observation of these responses. Apart from the responses observed after the complete disaggregation of the intact epithelium, there were observations of degrees of depletion of the neuroepithelial population and the formation of a number of malformations (foldings, rosettes). In view of the observation that the subsequent proliferative activity of the exposed explants could be followed in intact cell-sparce epithelia, the estimation of the regulatory ability of these early ventricular cells as a compensatory mechanism is of significance. Restoration of reduced cell populations, during this period can only be confirmed in these sequential culture studies.

The results obtained from a transient depletion of cations included many ectopic cellular aggregates and abnormal cellular densities. These may not be detected as structural malformations but it may eventually manifest itself in abnormal behavior in later life. A decrease in the production of "neurblasts" and the presence of abnormal cells in the cerebral cortex, as a result of excess vitamin A, is considered a possible cause for the abnormal behaviour of newborn mice (Langman, 1969).

The immediate environment of the ventricular neuroepithelial cell population play a regulatory role on the ultimate brain cell number and distribution by influencing the complex proliferation and orderly deadhesion and migration process. Agents and environmental conditions that affect these processes could have serious subsequent effects because

ventricular cells form the basic source of all neurons and glia (Boulder Committee, 1970). These cells are highly susceptible (Menkes et al, 1970). The effects of abnormal brain cell number, observed in offspring of undernourished rats, manifest itself in impaired learning and behavioural responses in psychological water maze tests (Zamenhof, 1969). Although information in this regard on human fetuses is still sketchy (Winick, 1969), it has important public health implications. Undernutrition, or other environmental influences during this critical period of CNS proliferation and migration may produce permanent changes in the CNS.

These studies facilitate detail direct analysis of the response potential of the neuroepithelium during this critical period in development. The direct in vitro study also facilitates a study of the regulatory/regenerative response of the epithelium to be followed after transient exposures of agents.

2. The observations of the specific sequential responses of the neuroepithelium, after exposure to the EDTA solution, would aid in the delineation of direct modes of action of agents.

Some of the observations (ectopic mitotic figures, foldings of the neuroretina and rosette formations) observed during the progress of response of the neuroepithelium have been specifically reported to be associated with malformations produced in vivo with a variety of unrelated experimental conditions. In a study of the mouse neural tube, Kauffman (1969) reported that urethane interfered with the migration of some premitotic nuclei to the "germinal zone" resulting in ectopic mitotic figures. Retinal foldings have been observed in congenital retinal dysplasia and have been associated with reduced intraocular pressure

(Hild and Callas, 1967). Rosette formations have been produced under a variety of conditions including nitrogen mustard, irradiations (Hicks, 1954), and it has also been observed in teratological studies with methylazoxymethanol acetate (Shimada and Langman, 1970).

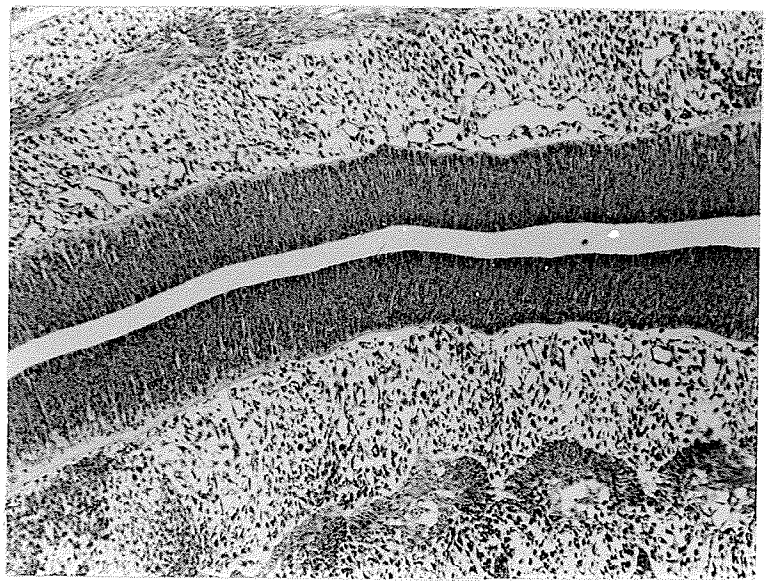
Most of these reports are based on in vivo studies in which the direct association with the experimental substance cannot be confidently made. Furthermore, many factors may produce a single type of response in a particular tissue. It does not necessarily follow that they have the same or similar actions. It might simply mean that the reacting tissues have a restricted response pathway. It is therefore necessary to combine in vivo with in vitro studies in order to confidently predict modes of action. A study of the response potential of a particular tissue can be performed in the isolated epithelia under specified conditions. In the present study, the importance of the adhesive mechanisms in the morphogenetic responses of the neuroepithelium has been established. The characteristic responses observed during and after exposure to the cation-depletion procedure should aid in the delination of the direct mode of action of various conditions or agents.

3. The standard malformative responses under a specific environmental condition observed will serve as one standardized response for ensuing studies on the neuroepithelium (Part IV).

FIGURE 30 Histological section of an embryo exposed to 0.5 ml of a 0.001 M EDTA in CMD-HBSS for 48 hours. No histological malformations are evident. Compare with figure 27. x64

FIGURE 31 Histological section through a neuroretinal explant exposed to 0.5 M EDTA in CMD-HBSS for 15 minutes and subsequently cultured. The tissue consists of necrotic disaggregated cells. x64

FIGURE 32 Histological section through a neuroretinal explant exposed to 0.001 M EDTA in CMD-HBSS for 15 minutes and subsequently cultured. The viable explant shows few disaggregated cells near the peripheral retinal region. x64



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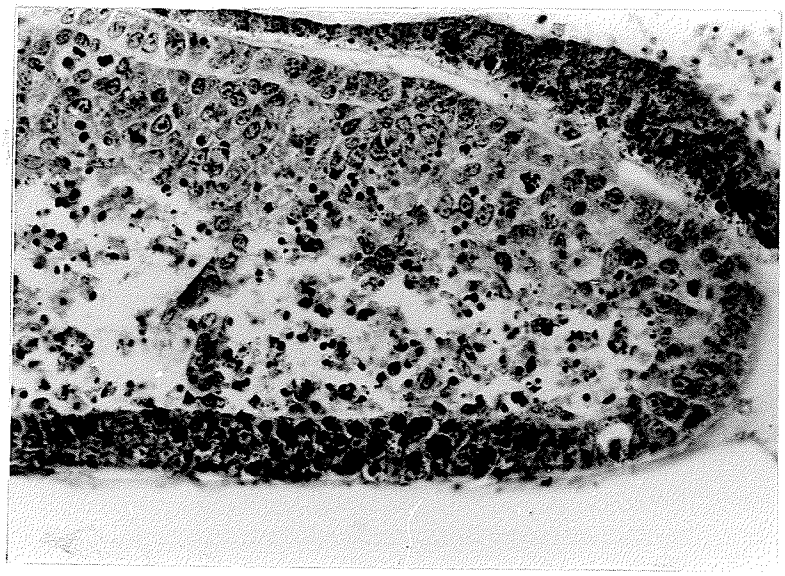

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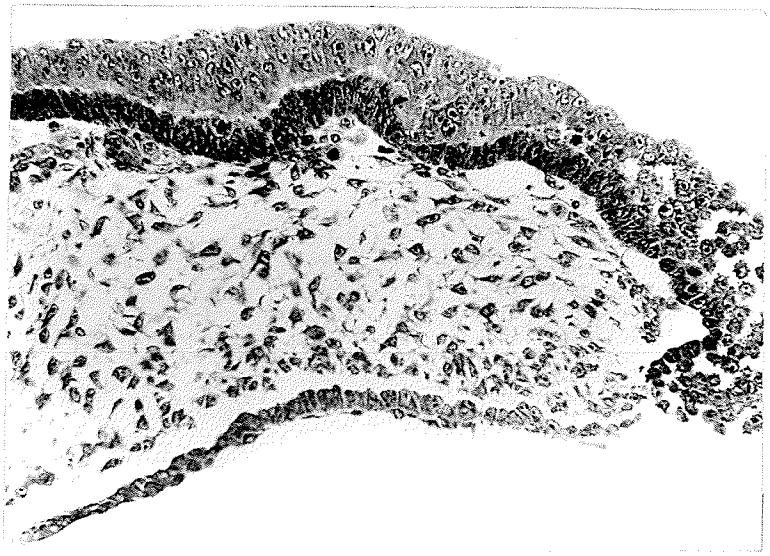

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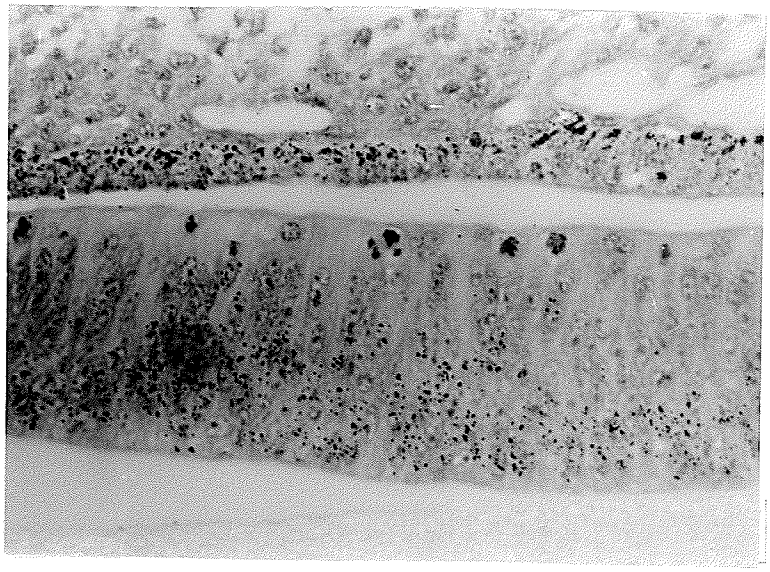
FIGURE 33

Radioautograph of an explant exposed for 10 minutes in the culture environment. Dilute labelling is restricted to the DNA synthetic zone of the neuroretina. x100

FIGURE 35

Histological section of a neuroretinal explant cultured for 20 minutes. A large percentage of the mitotic spindles are disoriented, that is, oblique or perpendicular to the internal limiting membrane (ILM). x100



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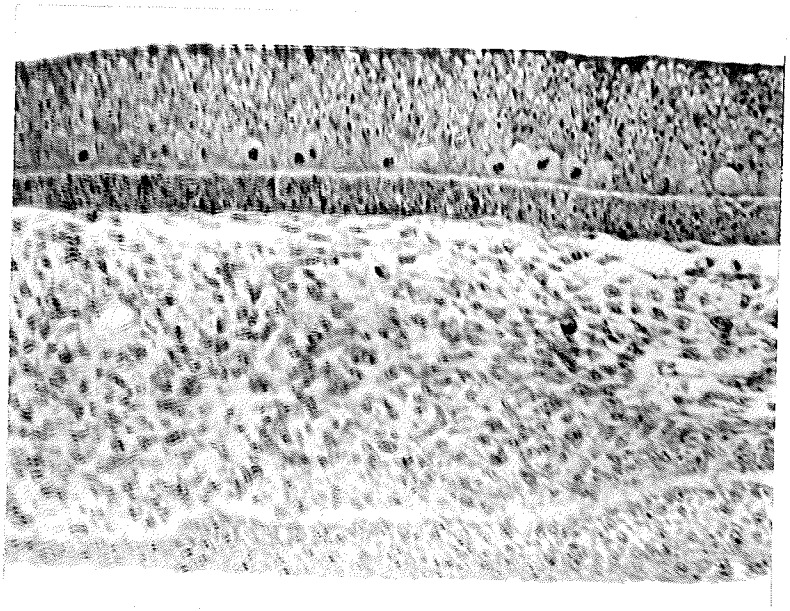

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FIGURES 36-45

Represent histological sections taken from explants fixed at various stages during the gradual disruption of the adhesive mechanisms and during the reconstruction of the epithelium after the washing and subsequent culture in a complete medium.

FIGURES 36-39

REPRESENT EVENTS DURING THE DISRUPTION OF THE NEURORETINA.

FIGURE 36

Interruptions in the internal limiting membrane (ILM) are first seen in regions with mitotic cells. x100

FIGURES 37-38

Degrees of disruption of the neuroepithelium can be seen along its length. Regions of almost complete disaggregation of the epithelium are seen along the edges. Ectopic mitotic figures (em) are seen in the more intact regions. x100, 160

36

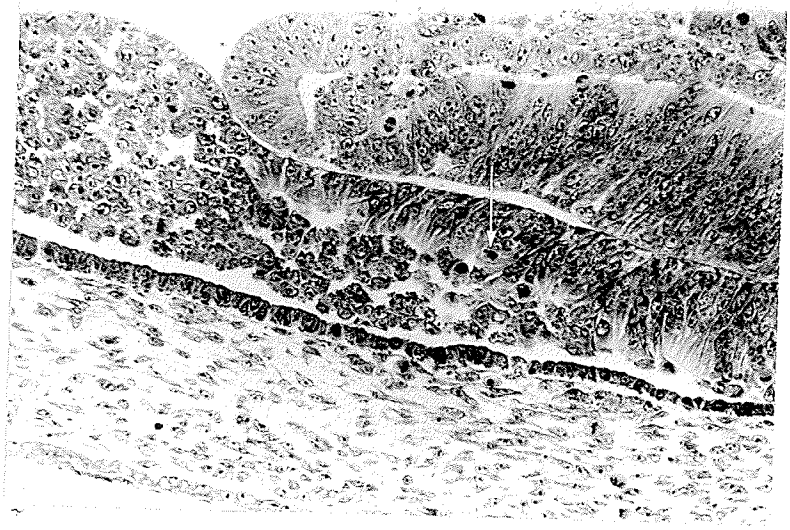

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FIGURE 39

Radioautograph of completely disaggregated cells. The freed cells have irregular cell borders and are hyperchromatic. The explant was placed in a "wash" medium containing tritiated thymidine for 30 minutes. Many of the disaggregated cells had incorporated the label within 30 minutes after disaggregation. x160

FIGURES 40-41

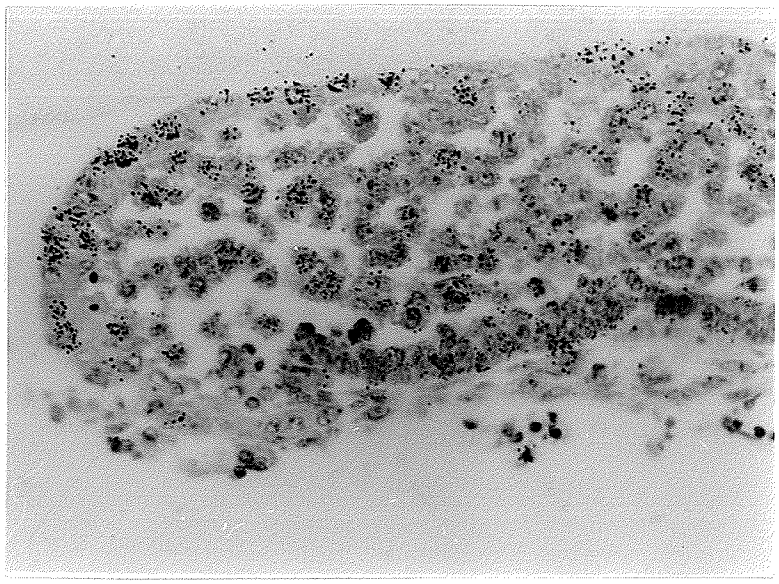
Represent histological sections during the reorganization of the completely disrupted epithelium

FIGURE 40

Disaggregated neuroretinal cells after "washing" and exposure in a complete medium. The cells stain normally and form an aggregation of polarized cells. x160

FIGURE 41

Rosette-formations develop from the aggregation of polarized cells. x128



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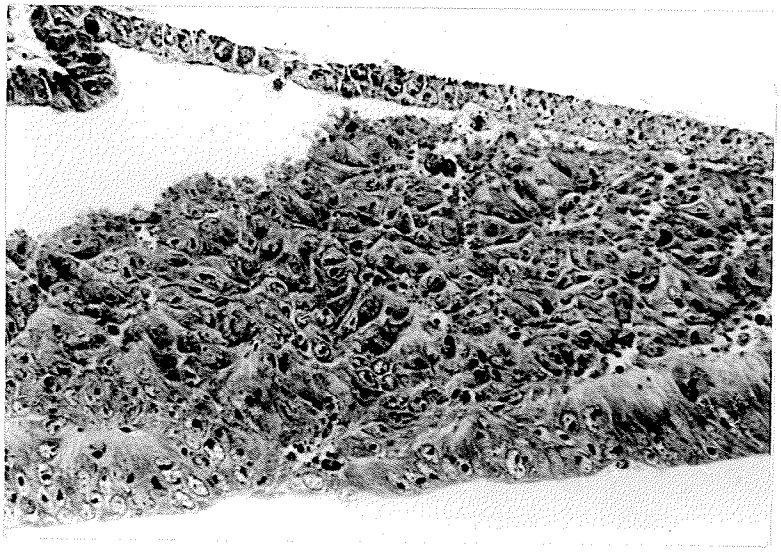

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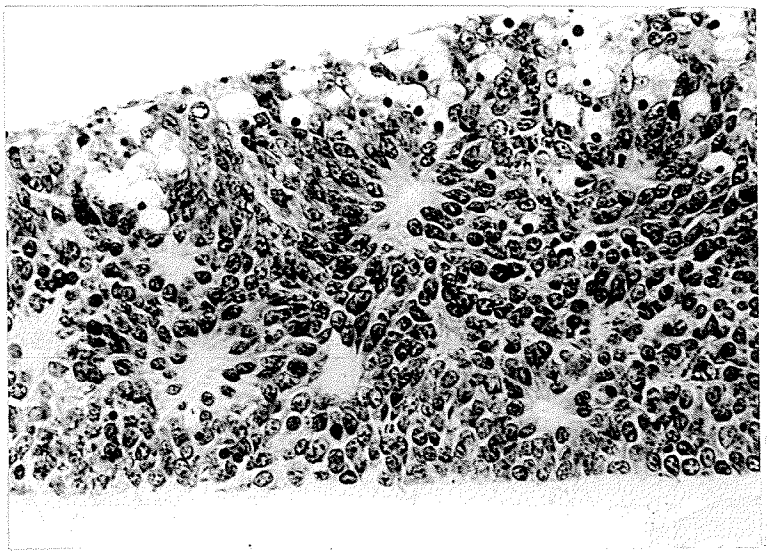

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FIGURE 42

With prolonged culture periods, the cells that do not form rosettes eventually degenerate. x64

FIGURES 43-45

REPRESENT EVENTS DURING THE REORGANIZATION OF THE INCOMPLETELY DISRUPTED NEUROEPITHELIUM.

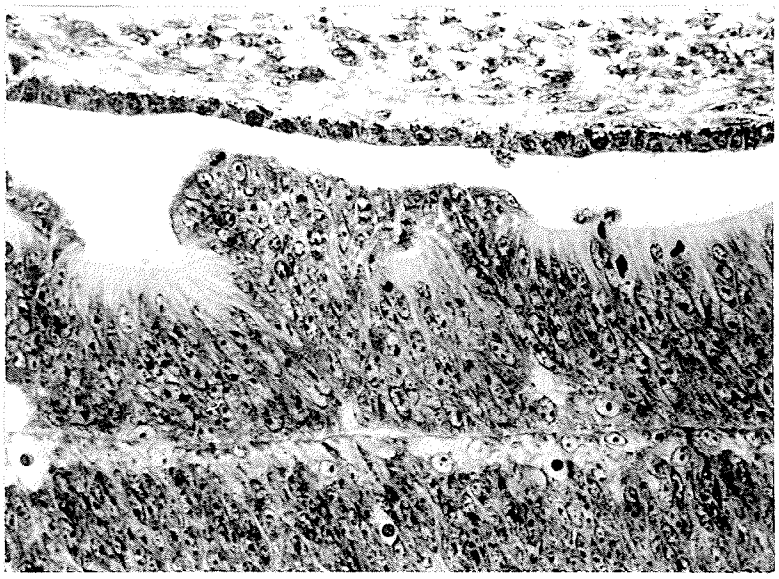
FIGURE 43

Exfoliation of mitotic cells and groups of interphasic cells through interruptions in the internal limiting membrane. The explant has been exposed to the chelating agent for a brief period (ten minutes), "washed", then cultured in a complete medium. x160

FIGURE 44

A similar culture to that observed in figure 43, but with reattachment of the interrupted portions of the neuroepithelium. A number of mitotic figures can be seen in such an explant resulting in highly accentuated folds or in "pseudorosettes". x64

42



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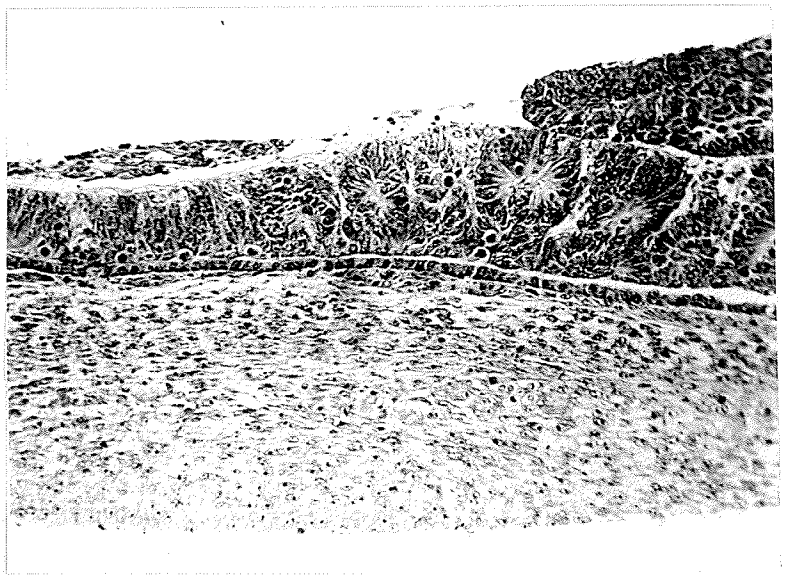

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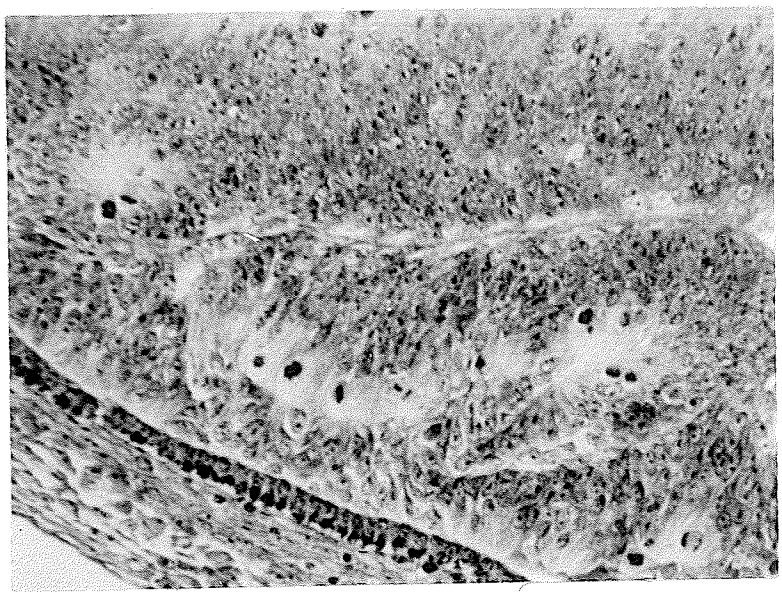
FIGURE 45 A high power magnification of a region of a neuroretinal fold. Many mitotic figures can be seen in the intact portions. x256

FIGURE 46 Histological section of a neuroretinal explant. The epithelium shows many gaps and is sparsely populated. x81.9

FIGURE 47 Histological section of a cerebral cortex explant. The formation of rosettes (with mitotic figures) in a distinct differentiative zone of a cultured cerebral cortex previously exposed to a disaggregation (CMD) procedure. x100



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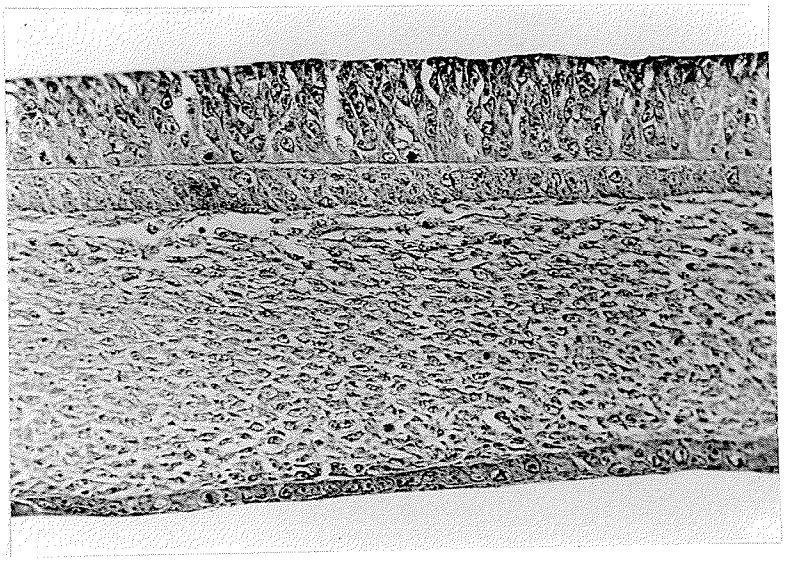

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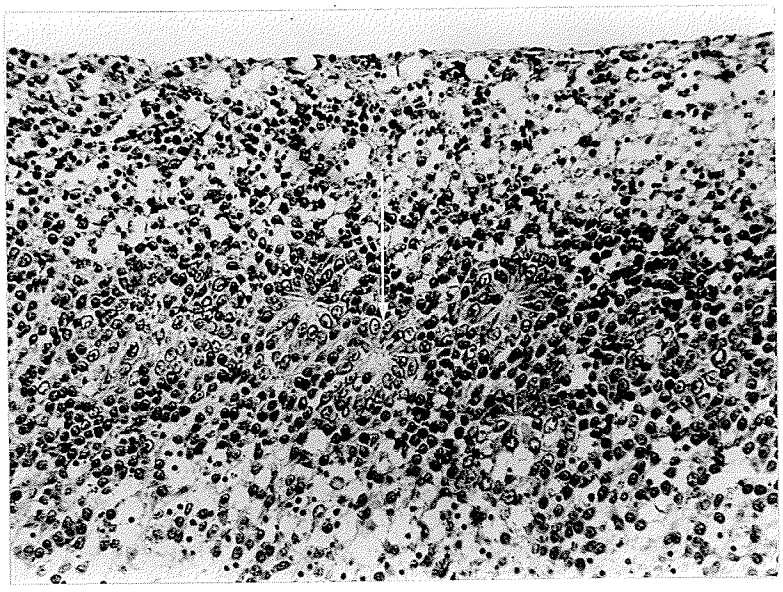

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PART IV

STATEMENT:

It is evident from the preliminary studies (PARTS I, II, and III) that the neuroretinal explants provide an asynchronous population of highly proliferating cells. The ventricular cells are characteristically distributed in the pseudostratified epithelium according to its phase in the cell cycle. These responses are comparable to responses observed throughout the early neural tube and depend on intact adhesive mechanisms in the epithelium. Changes in the shape of the cell, as the cell enters or leaves mitosis results in the movement of the nucleus through specific zones of the epithelium.

The objective of the study is to evaluate the use of this model embryonic tissue by studying its responses to methotrexate, a teratogen and chemotheropeutic agent.

In view of the specific effect of methotrexate in the production of a thymidine-deficient state in cells, the synthetic (DNA synthesis) as well as morphological responses (mitosis, adhesions) will be followed over prolonged periods of exposure to a non-toxic concentration of methotrexate. A number of responses of embryonic tissues to methotrexate is still unresolved namely; the relationship of effects of DNA synthesis and mitosis, organizational changes and its ability for regulation under the teratological exposure conditions.

INTRODUCTORY REVIEW

The folic acid antagonist, methotrexate (amethopterin) was selected for this study on the evaluation of this *in vitro* teratological model because of its proposed effects on both the DNA synthesis and mitosis. Both these effects could be studied simultaneously in the neuroepithelium. Furthermore, deficiency in folic acid has serious consequences during pregnancy but is usually not easily studied *in vivo* since the demonstrable folate deficiency often reflects a more general nutritional inadequacy. Folic acid deficiency could, however, be mimicked by administration of folic acid antagonists and its direct effects could be conveniently studied. Many *in vivo* studies with folic acid antagonists have resulted in CNS malformations in experimental animals but the direct events in these responses have not been clarified. The use of the folic acid antagonist methotrexate, should be useful in following the morphogenetic responses of the neuroepithelium under controlled conditions.

METHOTREXATE:

This folic acid antagonist is currently used therapeutically for the treatment of acute leukemia, choriocarcinoma and hydatiform mole (Bagshawa, 1969). It is an antimetabolite which interferes with the conversion of folic acid (pteroylglutamic acid) to folinic acid (tetrahydrogolic acid). The tetrahydrofolic acid can then receive an extra carbon atom as a formyl group for example on the N^{10} position. This newly combined carbon atom is then available for transfer from the tetrahydrofolic acid on to a metabolite requiring methylation (Boesen and Davis, 1969). Folic acid is reduced to dihydrofolic acid and

subsequently to the tetrahydrofolic acid by the action of folic reductases. Methotrexate, the most effective antifolic is structurally similar to folic acid and inhibits both the folic acid reductase enzymes by binding so strongly with them that folic acid cannot displace them. Consequently, tetrahydrofolic acid or its active forms necessary for many biosynthesis (nucleic acids) is no longer formed. At low doses of methotrexate, the reaction most affected in tumour cells (Conners, 1969) is the formation of thymidylic acid by methylation of deoxyuridylic acid. The reduction in thymidylic acid available results in inhibition of DNA synthesis. This mechanism explains its effects on proliferation during the synthetic stages and is reversible if thymidine is made available.

Apart from these well known synthetic functions of folic acid in the production of thymidine, its specific function during the so-called non-synthetic phase (Jacobson, 1964) is unresolved (Gelfant, 1963; Deysson, 1968). This refers to the mitotic change from metaphase to anaphase. Although the antifolic acid interference in the purine synthesis in both nucleic acids, its preferential effects on DNA synthesis has produced more consistent results. Grant (1960) reported inhibition of DNA synthesis by amethopterin but no changes in the amount or incorporation of RNA or its precursors. The mitotic-arresting effects of the folic acid antagonists are unresolved. Apart from the important differences in the technical details in the various conflicting reports on the mitotic-arresting effects of the folic acid antagonists, two other factors probably contribute to the failure in resolving this question. Methotrexate appears to have little influence on protein synthesis as opposed to nucleic acid synthesis (Bagshawe,

1969). The effects on RNA synthesis is more intricate and has not been resolved (Grant, 1960). Secondly, the factors regulating the mitotic stages have not been clearly defined. The possible importance of RNA in the step from metaphase to anaphase is a relevant consideration in normal mitosis and is a consideration on the effects of methotrexate on RNA synthesis and stathmokinesis. Jacobson (1964) suggested that metaphase chromosomes contain in addition to deoxyribonucleoprotein, a layer of ribonucleoprotein (RNP), some of which is transferred to the cytoplasm during the anaphase movements. It is hypothesized that since high concentrations of folic acid antagonists prevent the shedding of chromosomal RNP into the cytoplasm, folinic acid acts in some way in disrupting chromosomal RNP hydrogen bonds thus initiating its shedding and the parting of the two chromosomes. In this way folic acid is important in the initiation of the step from metaphase to anaphase. Biesele (1962) also discusses the accumulation of mitotic cells in prophase after treatment with ribonuclease. Love (1964) has considered the importance of RNA in metaphasic spindles and suggested that it initially accumulates in the prophase nucleus and acts as a template for the assembly of spindle proteins. Support for the metaphase-arresting properties of the folic acid antagonists is reported by Jacobson and Cathie (Jacobson, 1964) based on their 15 minute exposure experiments with chick embryonic fibroblasts and osteoblasts. In opposition, Gelfants in vitro studies with adult male mice ear epidermis were unable to prove metaphase-arresting properties as compared to colchicine over a four hour exposure period. Though a "partial blocking" at metaphase has been observed by Deysson (1968), the author stresses caution in interpreting the positive results since consideration must be placed on

the short period of exposure and also on the sensitivity of the chick cell metaphase in vitro. However, even though these are important technical differences to consider the single criterion of mitotic inhibition should be critically reconsidered. The above comparisons were made on the assumption of a universal mitotic-arresting effect, such as was observed with colchicine (Discussed in Part III). This could be an oversimplification since the arrested mitotic cell is more correctly considered as only a symptom (Biesele, 1962) of a more complex reaction. Therefore, one must re-emphasize the importance of the experimental and technical differences (tissue, age, culture medium constituents and duration of study) as major variables. More controlled environmental conditions are prerequisites for detailed studies on the effects of the folic acid antagonists.

Among the above mentioned models used in studying the direct mode of action of methotrexate (or related folic acid antagonists) are those used in studying the mitotic phase only. Investigations with aminopterin and methotrexate have been reported with cultured adult mouse ear epidermis (Gelfant, 1958), chick embryonic osteoblasts (Jacobson, 1964) and also with the onion root tests (Deysson, 1968). Most of these studies are not directly comparable. The attractive aspect of the mouse ear epidermis culture technique described by Bullough (1954) is that it uses a strictly defined saline medium in an oxygen gas phase. DNA synthesis, however, does not occur in this sytem (Gelfant, 1958). The simplified medium derives its basic energy requirements from the supplemented glucose or lactate. This technique is ideal for studying effects on the normal progression of cells entering mitosis and has been used to study the comparative effects of three compounds

(aminopterin, nitrogen mustard and colchicine) on mitosis (Gelfant, 1958). It must be realized that, although this model precludes the use of unknown factors prevalent in serums or embryo extracts, it is restricted to the analysis of the mitotically-prepared cells. The effects of the folic acid antagonists have not been resolved with these techniques nor with the other technically-distinct models mentioned above. Information on the action of methotrexate seem to be lacking as a result of the limited interpretation possible with the techniques used.

A study of the interrelationships of the phases of the cell cycle is facilitated by the presence of distinct zones of DNA synthesis and mitosis in the neuroepithelium and could be studied in early neuroretinal explants. Observations of DNA synthetic activity could be made by pulse labelling with tritiated thymidine. Cells which accumulate at the G1/S phase of the cell cycle due to methotrexate inhibition (Jasinska et al, 1970) could be released from this block by pulse labelling with thymidine (a thymidine rescue procedure). Blocks applied at the G1/S point were maintained for longer periods and permitted studies on the kinetics of the initiation of the proliferative responses. The effect of the aminopterin pretreatment (10^{-6}M up to 18 hours) on the ability of HeLa cell cultures to incorporate thymidine-2-C14 was studied over a four hour period of exposure to the label. A synchronized wave of DNA synthesis followed by a similar wave of cell divisions was observed after 16 hours pretreatment with aminopterin. This indicated that under these specified experimental conditions it took 16 hours to produce an effective thymidine deficiency as can be deduced by the readiness to incorporate the exogenous labelled thymidine. The wave of

cell divisions observed took place only after the cell's complete complement of DNA has been synthesized.

In these thymidine rescue procedures, the maximal rate of DNA synthesis was observed only after two hours of exposure to the exogenous labelled thymidine (Mueller et al, 1962).

Considering these experiments performed with neoplastic cells, the thymidine rescue procedure to be adapted with embryonic tissues will be over four hours. In the standard culture conditions used for the neuroretinal cultures, the earliest observation of labelled mitotic figures was at about 90 minutes. This would represent the minimum time for the G_2 phase. In order to relate DNA synthesis with mitosis, the appropriate counts will be made before and after the thymidine rescue procedure of four hours.

FOLIC ACID DEFICIENCY:

Considering the role of folic acid in nucleic acid synthesis, it is essential for cellular reproduction and growth. The demands for these substances during pregnancy is considerable. The recommended "daily intakes of 'free' folates" (World Health Organization, 1970), is 200 micrograms per day for anyone 13 years and over. Folic acid deficiency is most likely to occur during pregnancy during which time the normal requirements is doubled. The exceptional demand is also indicated by the observation that there is a five-fold increase in the incidence of folate depletion in women carrying twins (Hibbard and Hibbard, 1970). Folic acid content is also treatened if there is a rapid succession of pregnancies, especially in the nutritionally deprived

women. Folate depletion may affect the development of the foetus directly or by an impairment of the placental and decidual growth. Teratological studies on the effects of transitory maternal pteroylglutamic acid deficiency (folic acid deficiency) in the Long-Evans rats, did not indicate any effects on the placentas of the folic acid deficient embryos (Johnson et al, 1963). The first placental changes of folic acid-deficient embryos were those observed after destruction of the embryo on the twelfth day of pregnancy suggesting that the placenta was affected secondarily. Thiersch and Phillips (1950) used aminopterin to induce folic acid deficiency and also assumed a direct toxic-action on the fetus since dicidual and placental tissue appeared normal in most cases. The authors reported that the histological site of action to be the embryonic mesenchyme. The primary site of the effects of folic acid deficiency is unresolved. The major malformations are observed in the neuroepithelially-derived structures. Malformations, in general, are reported to be twice as common in the infants of women with defective folate metabolism as compared with normal women (Hibbard and Hibbard, 1970). Abortion is one of the main manifestations of a high folic acid deprivation and has been therapeutically induced by the use of folic acid antagonists (eg. aminopterin). With degrees of folic acid deprivation, malformations are usually observed. Experience with the teratological effects of methotrexate is limited (Boesen, 1969), and an isolated observation of treatment of a pregnant woman during the second trimester indicated that normal twins were subsequently born.

METHODS AND MATERIALS

1. CULTURE PROCEDURE:

The culture method and nutrient environment was described in Part I and adapted for Part II. Medium 199 and fresh chick embryo extract (10%) was again used. A total of 160 neuroretinal explants were dissected from 4 - 5 day old White Leghorn chick embryos (staged according to Hamburger and Hamilton, 1951) and cultured on sterile millipore filters (pore size 0.45 micra). The methotrexate (Lederic, 4-amino-N10-methyl pteroylglutamic acid) was diluted with HBSS and buffered before millipore sterilization. Explants were dissected in HBSS and precultured in a complete medium for about half an hour before commencing the experimental exposures. Bullough (1954) and Gelfant (1958) followed this routine of a period of preincubation in their in vitro studies with adult ear explants. This period allowed all mitosis originally present in the explant to pass beyond the metaphase stage. In the embryonic tissues used in these studies, the mitotic activity is much higher. The brief stabilization period used was one of experimental convenience and also to allow for stabilization of the initial adaptation responses.

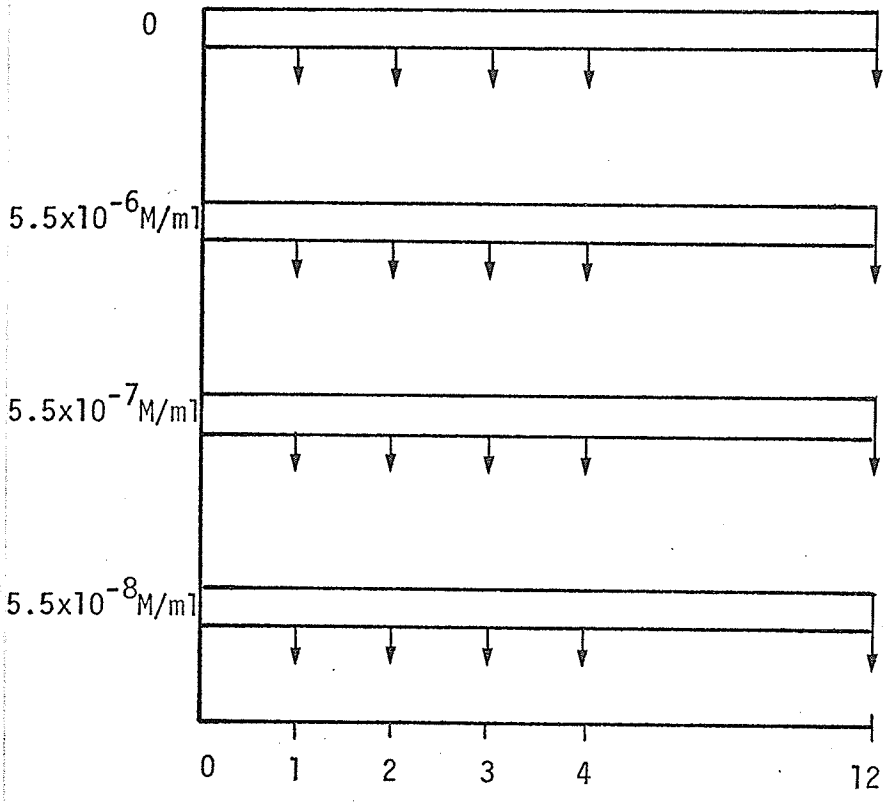
2. EXPERIMENTAL DESIGNS AND ANALYSIS:

The recommendations obtained from preliminary studies (Part I, II and III) aided in the design of the following experiments:

(1) The selection of a non-toxic concentration and duration of exposure of methotrexate (Fig. 48).

(2) Detail study of the neuroretinal responses to prolonged exposure

FIG.48 SCHEDULE FOR THE SELECTION OF A NON-TOXIC CONCENTRATION OF METHOTREXATE* AND DURATION OF EXPOSURE.



↓ FIXATION IN CARNOY II AT 4°C.

* 1 PART METHOTREXATE = 9 PARTS MEDIUM 199

FIG.49a SCHEDULE OF EXPOSURE CONDITIONS FOR NEURORETINAL EXPLANTS (CONTROLS)

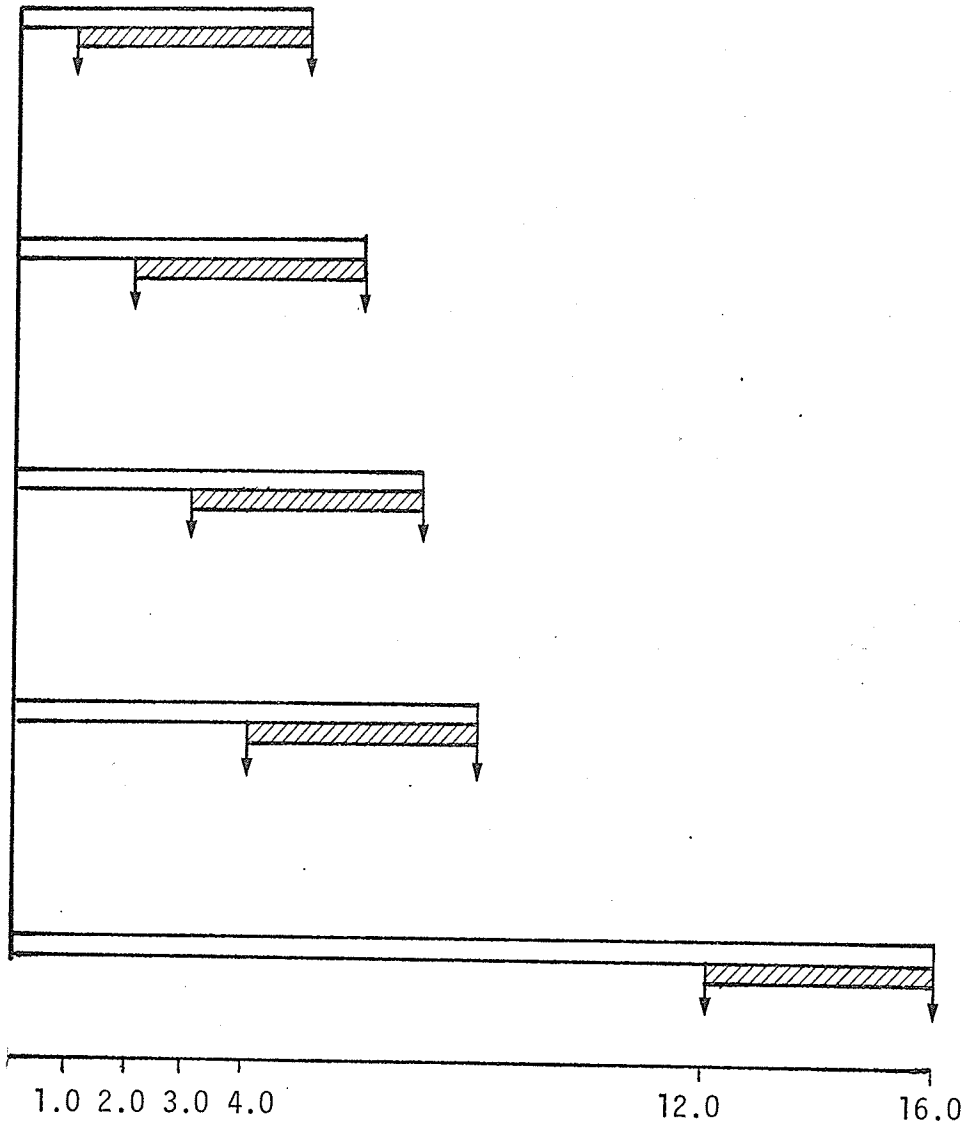
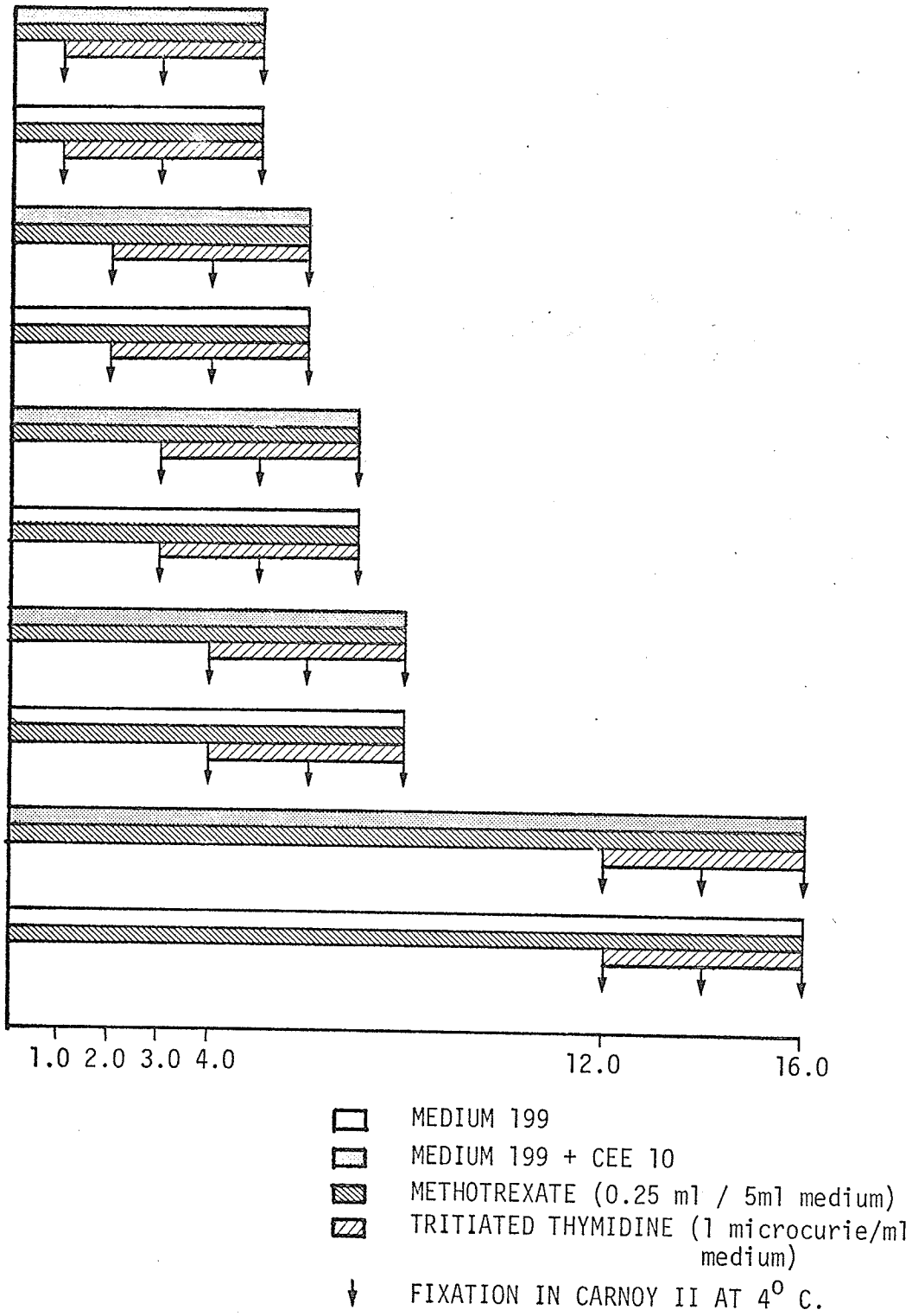


FIG.49b SCHEDULE OF EXPOSURE CONDITIONS FOR NEURORETINAL EXPLANTS (EXPERIMENTAL)



periods to the selected non-toxic concentration of methotrexate (Fig. 49).

The concentrations of methotrexate used (5.5×10^{-6} M, 5.5×10^{-7}M and 5.5×10^{-8}M per ml medium) were sterilized through separate millipore filters. These concentrations ranged from those likely to cause rapid lethality to a concentration which would be the highest ineffective one. A three day exposure of Hela cells to methotrexate indicated that there was a mitodepressive effect at 7.5×10^{-12} moles per ml and a lethal effect at 10^{-6} moles per ml (Deysson, 1968). In the present studies, the selection of the favourable concentration of methotrexate was based on morphological observations of histological sections. The characteristics studied included, distribution of cell death, mitostatic effects and general organization of the epithelium.

The selected non-toxic concentration of methotrexate was added to experimental culture dishes as indicated in figure 49. The experiment was designed as to follow the proposed action of methotrexate, that is, its ability to produce a thymidine-deficient state in cells. Throughout the culture exposure period a number of responses were evaluated in explants fixed in Carnoy II at 8^{o} C. and prepared for histological study. The paraffin-embedded explants were cut at 3 micra and prepared for routine histological study and radioautography (described in Part II). For each explant, a number of indices were calculated, namely, the mitotic index, pycnotic index, karyokinetic index where possible, mitotic spindle orientation and also the labelling index. The manner of calculating these indices were described in

Part II and Part III (pages

Analytic studies with folic acid antagonists are usually complicated by the unknown concentration of folic acid coenzymes present in the experimental tissue (Grant, 1960). Furthermore, the use of an embryo extract to promote proliferation may introduce unknown factors or cause a bioinactivation of the methotrexate in the culture system. To alleviate these possible problems, the experimental design includes:

(i) Prolonged periods of exposure to methotrexate.

(ii) Cultures exposed to medium 199 plus methotrexate.

(iii) Cultures exposed to medium 199, chick embryo extract plus methotrexate.

(iv) Parallel control cultures (medium 199 plus chick embryo extract) for each experimental culture.

RESULTS AND DISCUSSION

1. SELECTION OF A NON-TOXIC CONCENTRATION OF METHOTREXATE:

The exposure of explants to various concentrations of methotrexate (5.5×10^{-6} M, 5.5×10^{-7} M and 5.5×10^{-8} M per ml medium) over various culture durations (0 to 12 hours) resulted in a number of consistent observations. There was a definite sequence of onset and distribution of responses, namely:

(a) Isolated pycnotic cells first appeared in the DNA synthetic zone (S-zone) of the neuroretina while all other zones and tissues (associated mesenchyme and pigmented epithelium) were viable (see Fig. 50).

(b) The pycnotic cells progressively accumulated in the S-zone. Clones of adjacent cells in the same zone were structurally still viable. These cells were more conspicuous as more pycnotic cells accumulated (Fig. 51).

The above observations appear to simulate the asynchronous distribution of cells in the pseudostratified epithelium. It likely reflects the differential sensitivity of cells as it enters the specific DNA synthetic phase of the cell cycle. The thymidine-deficient cells would eventually degenerate resulting in the observed pycnosis in the G1/S zones of the epithelium. In the same explants mitotic figures were less frequent. The sparcity of mitotic figures is probably a consequence of the reduction in DNA synthesis and was studied in detail in the following experiments.

(c) With the accumulation of pycnotic cells under the external limiting

membrane many cells exfoliated through interruptions in the external limiting membrane, leaving large gaps in the neuroretina (Fig. 51). The result of this loss of cells (pycnosis, detachment and exfoliation) is a cell-deficient or cell-sparce epithelium (Fig. 51).

Designation of such an epithelium as "hypoplastic" is incorrect, since this observation does not describe the proliferative activity or potential of the epithelium. All that is observed is the survival of a fraction of the initial population of cells. The possibility exists that these surviving cells may have a high regulatory capacity and could possible restore the initial population of cells.

(d) Only after the pycnosis extended into the interphasic (G_1 and G_2) and mitotic zone, was there an appearance of pycnotic cells in the associated mesenchyme (Fig. 52). The pigmented epithelium, which had the lowest DNA synthetic activity, was the least affected and was well maintained even after extensive accumulation of pycnosis in the neuroepithelium and mesenchyme. The distribution and onset of pycnosis parallel the DNA synthetic activity of the tissues of the explant.

(e) The progressive decline in the mitotic cells was associated with the reduction in the number of viable cells and was always seen with all the concentrations studied over 1, 2, 3, 4 and 12 hours of exposure. A feature of this observation was the appearance of anaphases and telophases. There was therefore no complete stathmometaphasic effect. These responses were studied in more detail (with DNA synthetic and karyokinetic indices) with a selected concentration of methotrexate.

(f) Exposures to the 10^{-6} M/ml medium concentration of methotrexate

resulted in the earliest onset of pycnosis (3 - 4 hours), whereas with more dilute concentrations, the pycnosis appeared after longer periods of exposure. With the 10^{-7} M/ml medium concentration of methotrexate pycnosis appeared after 12 hours of exposure and was confined to the DNA synthetic zone of the epithelium as in figure 50.

The concentration of methotrexate selected to be studied in detail was the 5.5×10^{-7} M/ml medium solution since the 10^{-6} M solution resulted in the more rapid lethality and the 10^{-8} M solution appeared to be the least ineffective solution studied. Detail studies were performed with the selected concentration over 16 hours of exposure.

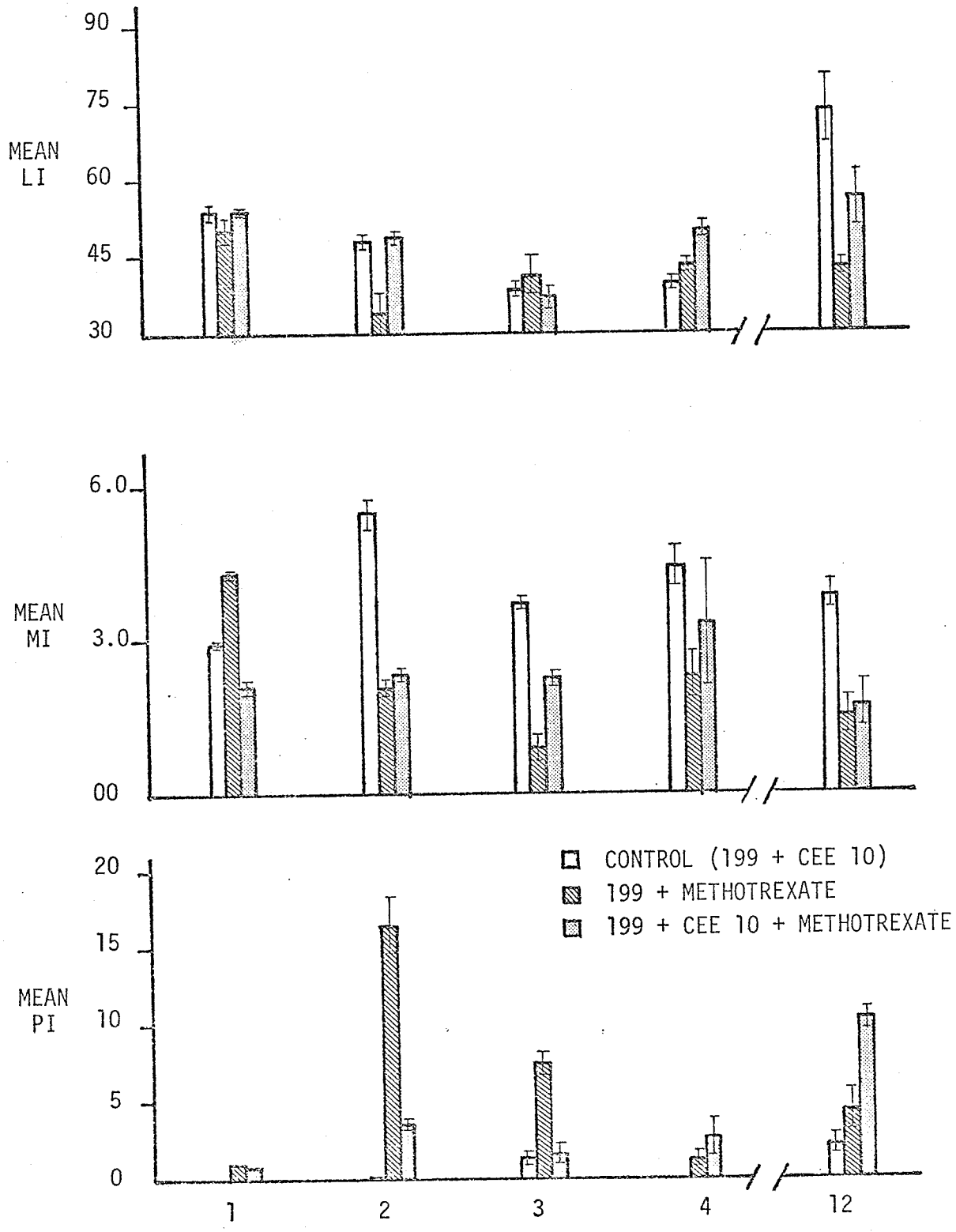
2. NEURORETINAL RESPONSES TO EXPOSURE TO 5.5×10^{-7} M/ml MEDIUM METHOTREXATE.

The neuroretinal explant responses to prolonged exposure periods to 5.5×10^{-7} M/ml medium methotrexate are presented in tables V to IX . In the viable cultures the organization of the neuroepithelium was generally well maintained. The adhesive mechanisms inherent in the neuroretina was not directly affected in any way as can be seen in the figures presented in this section. This qualitative observation was supported by the well maintained cytokinetic activity also observed. In only rare exceptions, which also occur in control explants, were there any disoriented mitotic spindles adjacent to the internal limiting membrane. All the mitotic spindles were usually oriented parallel to the internal limiting membrane, therefore ensuring the normal cytokinesis and "elevator-like" movements of the ventricular cells (see Fig. 53). These normal organizational responses observed

facilitated the counting procedures for the labelling index, mitotic index and pycnotic index.

After the first five hours of exposure to methotrexate, the observed mean labelling index in explants was similar (50.97 ± 5.4, 53.42 ± 1.4) compared to control explants (53.13 ± 4.9, table V). It was only after a prolonged exposure (7 and 8 hours) did the mean labelling index exceed the value for control cultures (Fig. 54). After 8 hours of culture, the explants in the presence of medium 199 plus CEE plus methotrexate had a significantly ($P < 0.05$) higher mean labelling index than the controls. All the cells were very heavily labelled after only a four hour pulse labelling procedure (Fig. 55). Similar intense labelling of nuclei was only previously observed after prolonged continuous labelling (48 hours) in a standardization study (see figure 23, Part II). It therefore took approximately 8 hours to produce an observable thymidine-deficient state in the neuroretinal cells. The accumulation of cells in the G1/S phase resulted in the rapid synchronized DNA synthesis. After twelve hours of exposure the mean labelling index of methotrexate-exposed cells was lower than that of control values (56.35 ± 14.3 compared to 73.45 ± 13.5). The explants exposed to medium 199 plus methotrexate without chick embryo extract had a lower labelling index with prolonged exposure periods, compared to similar explants cultured in the presence of chick embryo extract (Fig. 54). This was associated with the increased pycnosis and reduced mitosis seen in the same explants (Fig. 56). The gradual increase in mean percent pycnotic cells resulted in a gradual depletion of the neuroretina (Fig. 57), but the surviving cells were able to incorporate the exogenous thymidine (Fig. 58). Many of the explants

FIG. 54 NEURORETINAL EXPLANT RESPONSES FOLLOWING EXPOSURE TO 5.5 X 10^{-7} M/ML METHOTREXATE



cultured over such long periods had accumulated labelled pycnotic cells. The mean mitotic index of the methotrexate-exposed explants was always lower than the control values except after the initial five hour period when the explants, exposed in the presence of methotrexate alone, had a higher value (4.25 ± 0.2, Fig. 54). Since the mean labelling index was similar to control values (Table V), the increased mitosis could not be associated with the synchronized appearance of thymidine rescued cells. Very few of the mitosis were labelled and furthermore, the mitotic index at 4 hours was reduced compared to the value seen at two hours (6.81 ± 0.4 compared to 4.25 ± 0.2). The possibility of a direct stathmokinetic effect was also not supported by the observation in the explants of all stages of mitosis including anaphases and telophases (Fig. 59).

The observation of a gradual decline in the mean percent mitosis was associated with two factors; namely, the gradual increase in the mean labelling index after prolonged exposure periods to methotrexate and also with the accumulation of pycnosis. With prolonged exposure periods to methotrexate the thymidine rescued cells were unable to complete normal DNA synthesis and progress to mitosis,since many labelled pycnotic cells accumulated. The pycnotic cells in the explant showed a definite distribution in the S-zone indicative of differential sensitivity (Fig. 58) and not due to effects inherent in the culture system (discussed in Part II). Diffusional limitations in the culture system, only seen in highly folded explants, was evident in a zone of early necrotic cells (Fig. 60). This definite gradient of early cell death in the zone was only seen in explants exposed to methotrexate. The same area in the centre of the folded

epithelium was characteristically distal to both the medium and air phase. Control explants in similar nutritionally-deprived zones did not show this response so that it is associated with the added deleterious effects of methotrexate ie. the produced folic acid deficiency.

The combined analysis of the mitotic index, labelling index and pycnotic index of explants exposed to methotrexate in the presence or absence of chick embryo extract (CEE) resulted in a number of consistent responses.

(a) As observed earlier, there was a differential sensitivity of tissue types in the explant. The most sensitive tissue, the neuroretina, was the tissue with the highest DNA synthetic activity. The associated mesenchyme was relatively well maintained. The pigment layer, the most differentiated tissue, was the least affected and pycnosis was rare even after 16 hours of exposure to methotrexate. With prolonged exposure periods, the well maintained pigment layer was frequently and heavily labelled after thymidine rescue. It is possible that the synchronizing effect of methotrexate is indicated in this response since the labelling of control cultures was rare (Fig. 61).

(b) Over longer periods of exposure to methotrexate, a synchronizing effect was evident by the increased mean labelling index (after 8 hours exposure). This is indicative of the production of a definite thymidine-deficient state in cells. The possibility that the thymidine-rescued cells could restore the depleted original population of cells in the epithelium was not supported by the observation of increasing numbers of labelled pycnotic cells. Over shorter exposure periods, when labelled pycnotic cells were not evident, no definite conclusions

on the regulatory ability of the surviving cells could be made since their labelling was only followed over a four hour period.

(c) Direct effects on mitosis (stathmokinesis) could not be supported since there was a progressive decline in mitosis and all mitotic stages were always seen. This decline in mitosis could be associated with the progressive increase in thymidine-rescued cells indicating that there was a progressive synchronization of thymidine-deficient cells. These observations do not support the reports of direct stathokinetic effects of folic acid antagonists (Jacobson, 1964) on mitosis and is consistent with the observation of Gelfant (1958) who, using a different technique, did not observe any direct arrest of mitosis.

(d) In explants exposed to methotrexate in the presence of CEE, the mean pycnotic indices were lower except after prolonged exposure periods (after 8 and 16 hours). The mitotic and labelling indices were always higher than those exposed to methotrexate without CEE. The importance of this constituent in the experimental medium is stressed especially since many studies (Jacobson, 1964; Kieler and Kieler, 1954) on the stathmokinetic effects of folic acid antagonists were performed in media containing the undefined embryo extracts. The extracts could provide a source of ATP, thymidine and other constituents that may influence the study of the agent. The medium 199 used as a basal medium in these studies, do contain some folic acid but was used because of the highly deleterious responses generally observed in complete folic acid-deficient media.

The effects of the methotrexate on RNA synthesis were not studied so that the influence on protein synthesis on these observations

are not known. Folic acid deficiency is known to affect the RNA content of adult neurons (Haltia, 1970) but no studies have been reported for embryonic tissues.

TABLE V

NEURORETINAL EXPLANT RESPONSES FOLLOWING A ONE HOUR EXPOSURE TO METHOTREXATE (TOTAL CULTURE PERIOD = 5 HOURS*)

CULTURE MEDIUM	CONTROL (199 + CEE_{10})		METHOTREXATE + 199			METHOTREXATE + 199 + CEE_{10}		
TRITIATED THYMIDINE (HOURS)	0	4	0	2	4	0	2	4
MEAN LABELLING INDEX	-	53.13 ± 4.9	-	49.38 ± 4.0	50.97 ± 5.4	-	-	53.42 ± 1.4
MEAN MITOTIC INDEX	3.66 ± 0.1	2.99 ± 0.1	6.81 ± 0.4	2.29 ± 0.2	4.25 ± 0.2	6.84 ± 0.5	-	2.1 ± 0.4
MEAN PYCNOTIC INDEX	0.0	0.2 ± 0.0	0.0	0.8 ± 0.0	1.0 ± 0.0	0.0	-	0.8 ± 0.0

* DURING THE FINAL FOUR HOURS THE EXPERIMENTAL EXPLANTS WERE EXPOSED TO BOTH METHOTREXATE AND TRITIATED THYMIDINE.

± STANDARD DEVIATION OF THE MEAN OF FOUR EXPLANTS.

TABLE VI

NEURORETINAL EXPLANT RESPONSES FOLLOWING A TWO HOUR EXPOSURE TO METHOTREXATE (TOTAL CULTURE PERIOD = 6 HOURS*).

CULTURE MEDIUM	CONTROL (199 + CEE_{10})		METHOTREXATE + 199			METHOTREXATE + 199 + CEE_{10}		
TRITIATED THYMIDINE (HOURS)	0	4	0	2	4	0	2	4
MEAN LABELLING INDEX	-	48.08 ± 3.0	-		34.13 ± 10.3	-	49.61 ± 5.4	49.87 ± 2.6
MEAN MITOTIC INDEX	4.45 ± 0.1	5.47 ± 0.7	0.9 ± 0.5	1.2 ± 0.9	2.08 ± 0.2	0.0	3.59 ± 0.9	2.30 ± 0.3
MEAN PYCNOTIC INDEX	0.0	0.0	0.0	3.83 ± 2.6	16.0 ± 3.7	0.0	3.96 ± 1.0	3.3 ± 0.6

* DURING THE FINAL FOUR HOURS THE EXPERIMENTAL EXPLANTS WERE EXPOSED TO BOTH METHOTREXATE AND TRITIATED THYMIDINE.

± STANDARD DEVIATION OF THE MEAN OF FOUR EXPLANTS.

TABLE VII

NEURORETINAL EXPLANT RESPONSES FOLLOWING A THREE HOUR EXPOSURE TO METHOTREXATE (TOTAL CULTURE PERIOD = 7 HOURS*).

CULTURE MEDIUM	CONTROL (199 + CEE_{10})		METHOTREXATE + 199			METHOTREXATE + 199 + CEE_{10}		
TRITIATED THYMIDINE (HOURS)	0	4	0	2	4	0	2	4
MEAN LABELLING INDEX	-	38.8 ± 2.8	-	43.18 ± 11.3	40.40 ± 10.7	-	39.91 ± 3.2	37.10 ± 6.9
MEAN MITOTIC INDEX	4.5 ± 2.0	3.75 ± 0.4	2.85 ± 0.2	1.87 ± 0.6	0.87 ± 0.6	2.59 ± 0.6	1.68 ± 0.7	2.25 ± 0.5
MEAN PYCNOTIC INDEX	1.3 ± 0.9	1.25 ± 0.9	1.03 ± 0.8	6.99 ± 7.6	7.42 ± 1.3	0.0	0.33 ± 0.5	1.38 ± 1.8

* DURING THE FINAL FOUR HOURS THE EXPERIMENTAL EXPLANTS WERE EXPOSED TO BOTH METHOTREXATE AND TRITIATED THYMIDINE.

± STANDARD DEVIATION OF THE MEAN OF FOUR EXPLANTS.

TABLE VIII

NEURORETINAL EXPLANT RESPONSES FOLLOWING A FOUR HOUR EXPOSURE TO METHOTREXATE (TOTAL CULTURE PERIOD = 8 HOURS*).

CULTURE MEDIUM	CONTROL (199 + CEE_{10})		METHOTREXATE + 199			METHOTREXATE + 199 + CEE_{10}		
TRITIATED THYMIDINE (HOURS)	0	4	0	2	4	0	2	4
MEAN LABELLING INDEX	-	40.07 ± 2.6	-	47.78 ± 5.0	43.63 ± 2.4	-	52.07 ± 1.5	50.18 ± 2.7
MEAN MITOTIC INDEX	5.25 ± 0.5	4.47 ± 2.6	0.83 ± 0.3	3.48 ± 0.5	2.35 ± 0.9	2.25 ± 0.6		3.33 ± 2.5
MEAN PYCNOTIC INDEX	0.0	0.0	2.1 ± 1.5		1.28 ± 0.8	1.15 ± 0.9		2.53 ± 2.3

* DURING THE FINAL FOUR HOURS, THE EXPERIMENTAL EXPLANTS WERE EXPOSED TO BOTH METHOTREXATE AND TRITIATED THYMIDINE.

± STANDARD DEVIATION OF THE MEAN OF FOUR EXPLANTS.

TABLE IX

NEURORETINAL EXPLANT RESPONSES FOLLOWING A TWELVE HOUR EXPOSURE TO METHOTREXATE (TOTAL CULTURE PERIOD = 16 HOURS*).

CULTURE MEDIUM	CONTROL (199 + CEE_{10})		METHOTREXATE + 199			METHOTREXATE + 199 + CEE_{10}		
TRITIATED THYMIDINE (HOURS)	0	4	0	2	4	0	2	4
MEAN LABELLING INDEX	-	73.45 ± 13.5	-	-	41.73 ± 3.3	-	-	56.35 ± 14.3
MEAN MITOTIC INDEX	5.00 ± 1.6	3.88 ± 0.6	0.30 ± 0.1	-	1.50 ± 0.8	0.0	-	1.75 ± 1.1
MEAN PYCNOTIC INDEX	3.13 ± 0.2	2.03 ± 1.4	22.10 ± 2.7	-	4.30 ± 2.6	4.0 ± 1.0	-	10.30 ± 1.8

* DURING THE FINAL FOUR HOURS, THE EXPERIMENTAL EXPLANTS WERE EXPOSED TO BOTH METHOTREXATE AND TRITIATED THYMIDINE.

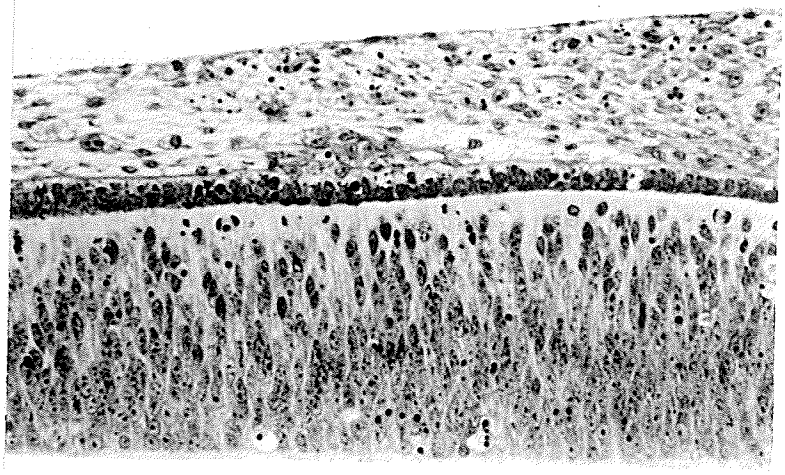
± STANDARD DEVIATION OF THE MEAN OF FOUR EXPLANTS.

FIGURES 50 and 51

Histological sections of neuroretinal explants exposed to Methotrexate over prolonged periods. Pycnotic cells accumulate in the S and G zones, many of which exfoliate. Associated mesenchyme is normal. Mitotic figures, including anaphases (arrow) are seen. x160

FIGURE 52

Histological section of a neuroretinal explant with extensive pycnosis throughout the epithelium and associated mesenchyme. x160



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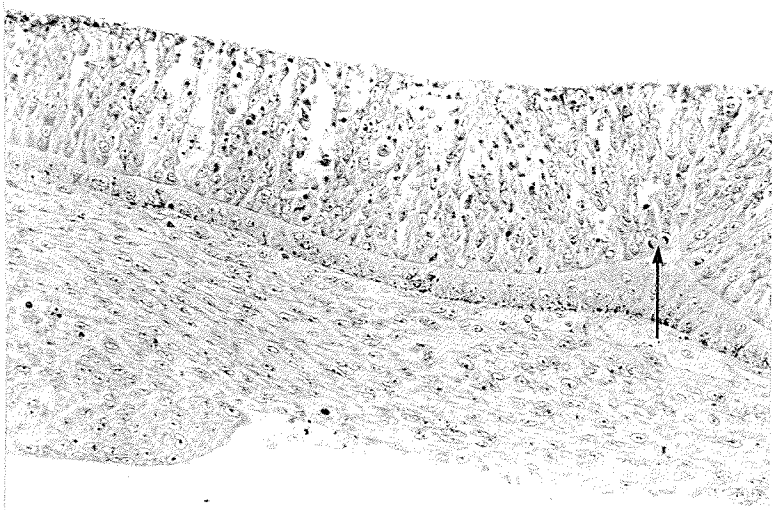

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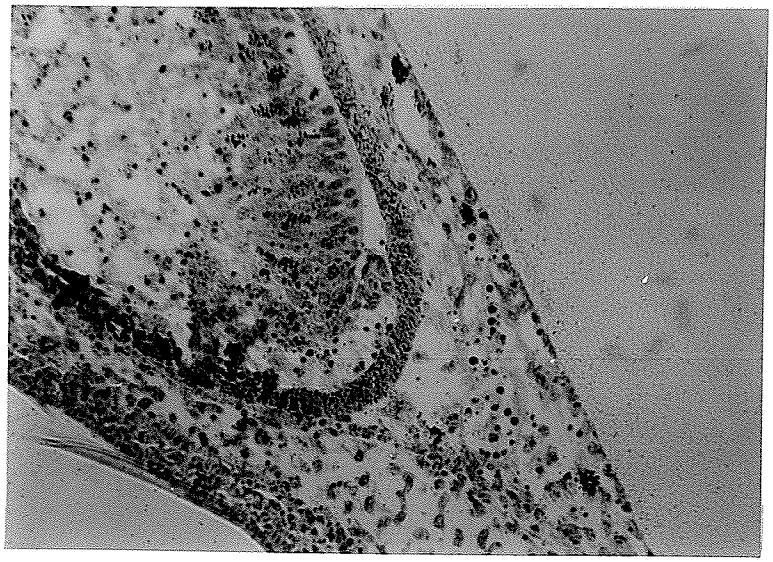

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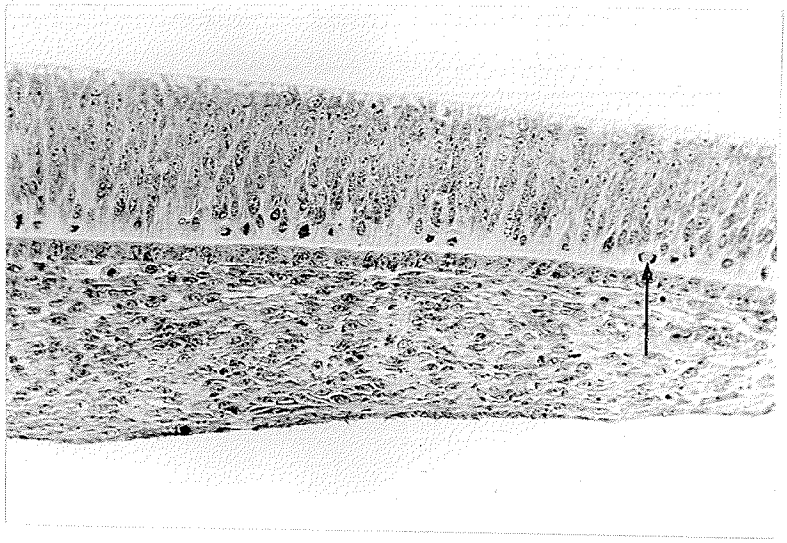
FIGURE 53 Histological section of a typical control neuroretinal explant. All mitotic stages in the epithelium. x100

FIGURE 55 Radioautograph of a neuroretinal explant exposed to methotrexate for eight hours. Heavy labelling in neuroretina and pigmented epithelium proximal to the source of medium. Few pycnotic cells are seen in the mesenchyme. x100

FIGURE 56 Radioautograph of a neuroretinal explant exposed to methotrexate in the absence of chick embryo extract. Mitotic cells are rare and pycnotic cells accumulate some of which are labelled (arrow). x128



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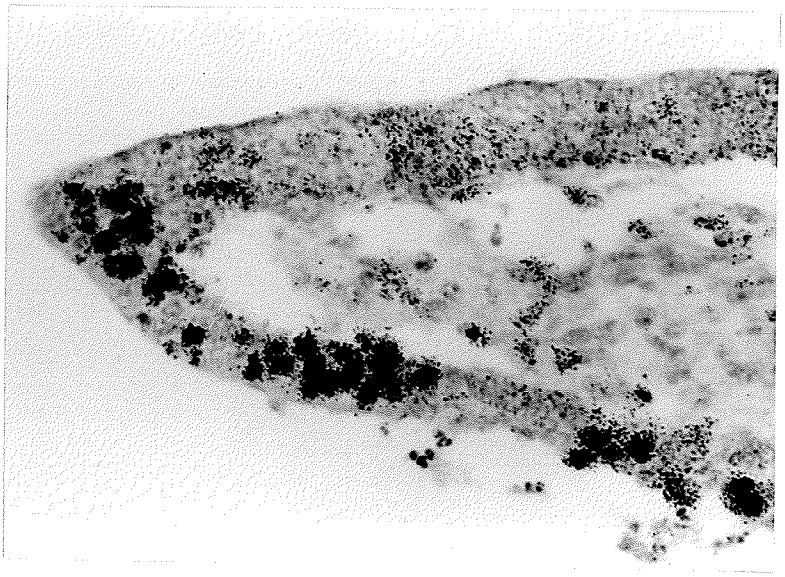

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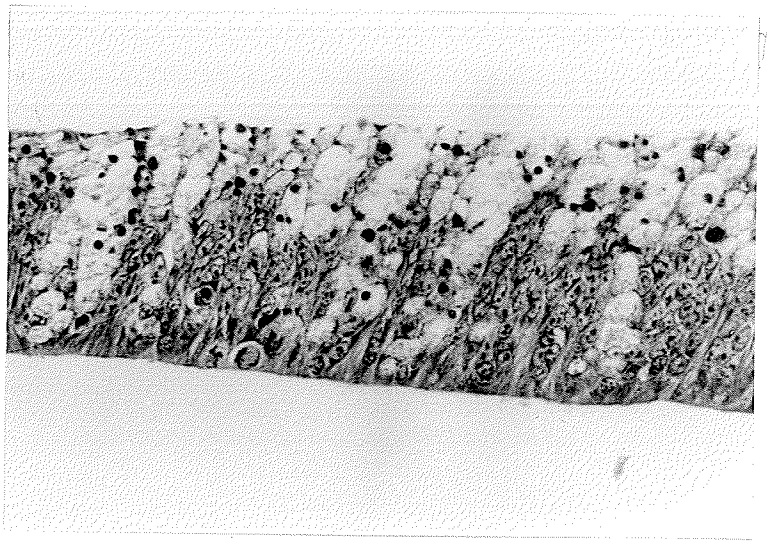
FIGURE 57 Histological section of a neuroretinal explant with extensive pycnosis and cellular depletion. x128

FIGURE 58 Radioautograph of a neuroretinal explant after 16 hours of exposure to methotrexate. The few surviving cells are labelled, mitosis figures are rare. x128

FIGURE 59 Histological section of a neuroretinal explant exposed to Methotrexate and Medium 199 for 5 hours. All mitotic figures are present. x128



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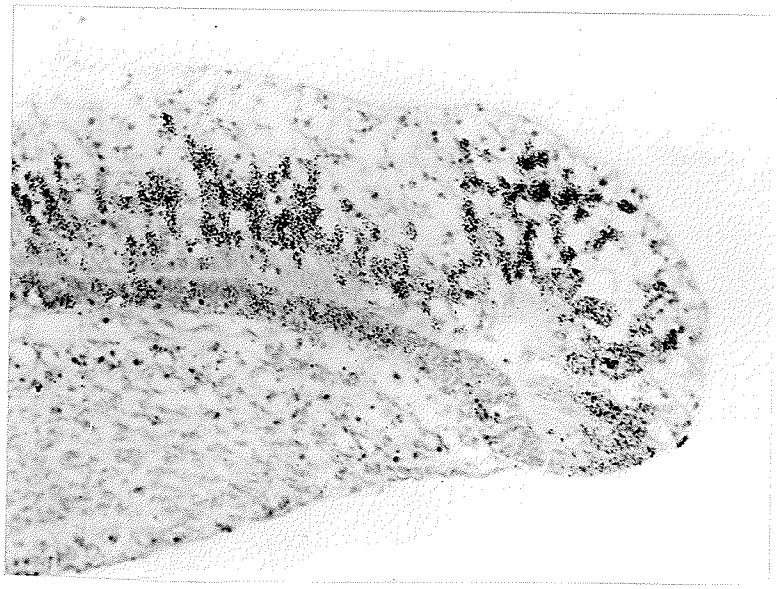

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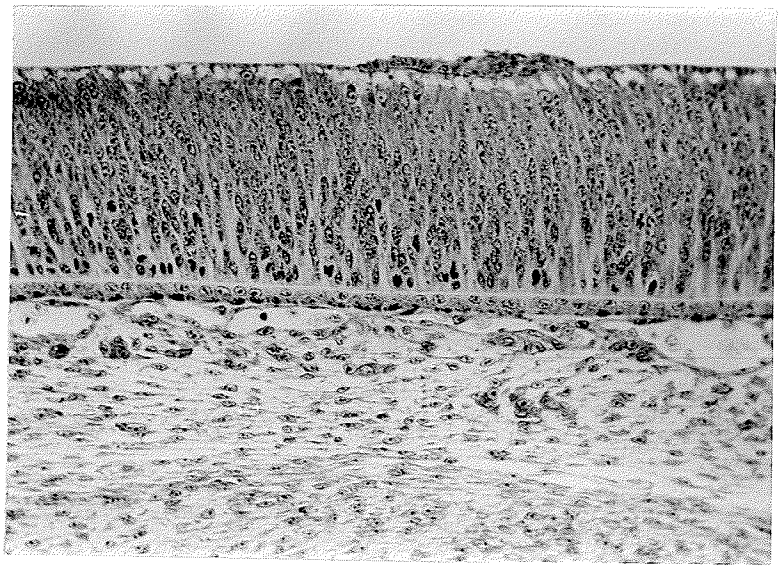

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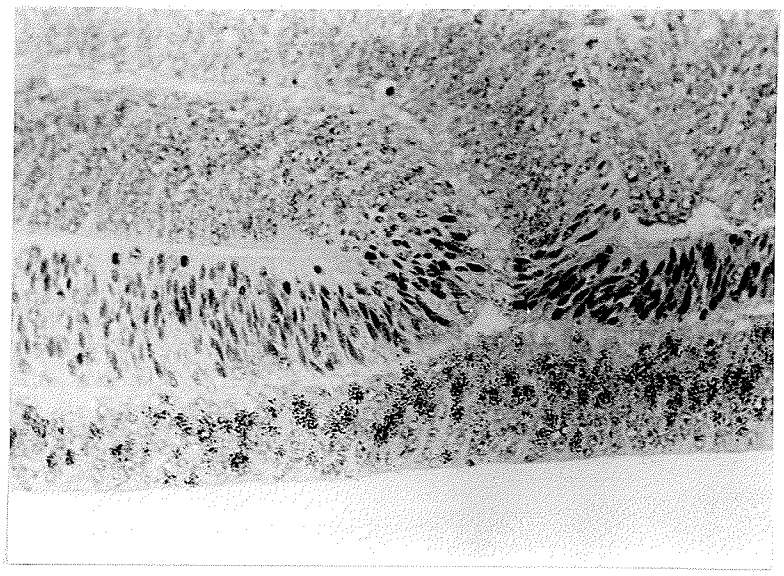
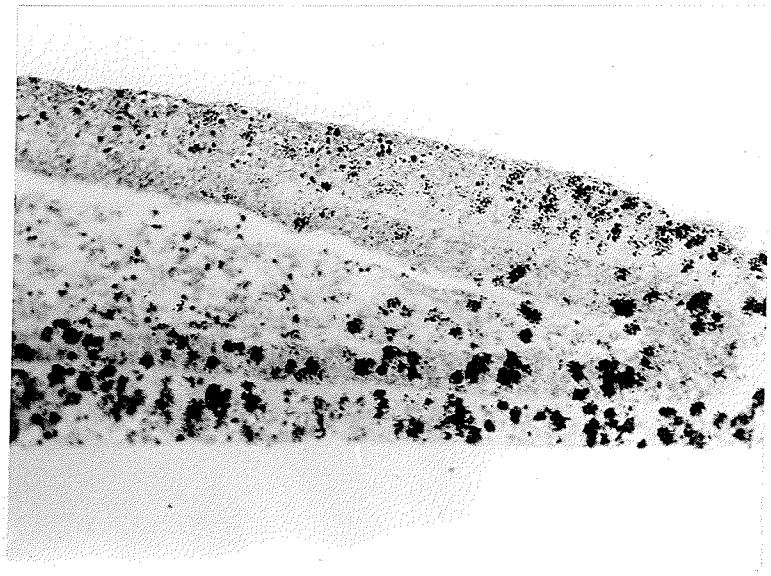
FIGURE 60 Radioautograph of a folded neuroretinal explant exposed to methotrexate. A uniform zone of cell death is seen in the mid-region of the explant. x100

FIGURE 61 Radioautograph of a neuroretinal explant exposed to methotrexate for eight hours. The pigmented epithelium is heavily labelled. x100



60



61

SUMMARY

1. The DNA synthetic zone of the neuroretina was the most sensitive to methotrexate exposure. The effects on the associated mesenchyme was only evident after prolonged exposure. A gradual accumulation of pycnosis was greater in methotrexate-exposed explants compared to controls. These deleterious effects were less marked in explants exposed in the presence of chick embryo extract.

2. With prolonged exposure to methotrexate,many of the pycnotic cells observed after a thymidine rescue procedure, were labelled. This is an indication that the thymidine-deficient cells had entered DNA synthesis but had degenerated rapidly. With prolonged exposure to methotrexate, the possibility of "abortive" DNA synthesis after the thymidine-rescue is suggested. The effect of the induced thymidine-deficiency on the cells' ability to progress through mitosis could not be followed since very few labelled mitosis were observed over the four hour thymidine rescue procedure.

The synchronization of cells in the thymidine-deficient state could also have morphogenetic consequences since many of the cells accumulate in the G1/S zone. With the reduction of mitosis, there would be a redistribution of nuclei in the epithelium.

3. No direct mitotic effect could be observed. The progressive decline in mitosis was associated with the production of thymidine-deficient cells. All mitotic figures were always seen. Analysis of the stathmokinetic effects can only be confidently done if the synthetic properties (DNA synthesis) of the cells and viability is followed over a

progressive period of exposure.

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