THE RELATIONSHIP BETWEEN THE EFFECTS OF TERATOGENS *IN VIVO* AND *IN VITRO*.

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To the memory of my mother.

DECLARATION

I hereby declare that the work presented in this thesis has been performed by me.

CONTENTS

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ABSTRACT

The effects of the selected agents, insulin, diphenylhydantoin, chloroquine sulphate, chlordiazepoxide HCl, meprobamate, cobaltous chloride and lead nitrate, on the development of chick embryos and on the morphological and biochemical differentiation of cells in culture were investigated. The cultures were established from neural retina, limb and kidney fibroblasts derived from 8-day old chick embryos, and lens epithelium from day-old chicks.

In *in vivo* studies, insulin treatment caused limb, beak, tail and eye defects. Chlordiazepoxide HCl caused growth retardation, especially of the limbs. Cobaltous chloride induced lens opacity and thin limbs and lead nitrate caused spinal and brain damage. The incidence and the degree of the defects depended both on the stage of treatment and the amount of the agents administered. In contrast, diphenylhydantoin, chloroquine sulphate and meprobamate did not induce any detectable gross morphological abnormality.

In cell culture studies, insulin treatment of cultured lens epithelial and neural retina cells affected both the synthesis and accumulation of several protein components. The effect of insulin on lens epithelial cells was found to vary with the genotype. The effect of insulin on limb fibroblast cell cultures was dependent on the level of FCS in the culture medium. Chlordiazepoxide HCl treatment of cultured cells affected the synthesis and accumulation of the proteins in neural retina, limb and kidney fibroblast cells. Diphenylhydantoin treatment affected protein synthesis and accumulation in lens epithelial and neural retina cell cultures. Chloroquine sulphate treatment affected protein synthesis and accumulation in lens epithelial, neural retina and brain

cell cultures. Diphenylhydantoin, chloroquine sulphate and chlordiazepoxide HCl also affected the activity of choline acetyl transferase in neural retina cell cultures. Lead nitrate was found to affect protein synthesis and accumulation only in neural retina cell cultures. Finally meprobamate affected protein synthesis and accumulation in limb fibroblast cell cultures.

The data suggest that certain correlations do exist between *in vivo* and *in vitro* teratogenic tests.

SUMMARY

A study was made of the correlation between the effects of specific agents on developing embryos with the effects of these agents on the embryonic cells *in vitro*. The effects of the selected agents, insulin, diphenylhydantoin, chloroquine sulphate, chlordiazepoxide HCl, meprobamate, cobaltous chloride and lead nitrate, on the development of chick embryos and on the morphological and biochemical differentiation of cells in culture were investigated. The cultures were established from neural retina, limb and kidney fibroblasts derived from 8-day old chick embryos, and lens epithelium from day-old chicks. The effects of chloroquine sulphate and lead nitrate were also investigated on brain cell cultures established from 8-day old chick embryos.

In studies of embryonic development, the agents were administered onto the chorioallantoic membrane at 48, 108, and 192 hours of incubation, the embryos were then examined daily, dead ones being discarded, and living embryos examined for gross abnormalities between days 6-18 of incubation. Insulin treatment caused limb, beak, tail and eye defects. Chlordiazepoxide HCl caused growth retardation, especially of the limbs. Cobaltous chloride induced lens opacity and thin limbs and lead nitrate caused spinal and brain damage. The incidence and the degree of the defects depended both on the stage of treatment and the amount of the agents administered. In contrast, diphenylhydantoin, chloroquine sulphate and meprobamate did not induce any detectable gross morphological abnormality, but high levels of these agents were embryo-toxic, with older embryos being most resistant.

In cell culture studies, the effects of the selected agents on the synthesis and accumulation of water soluble proteins was investigated.

Insulin treatment of cultured lens epithelial and neural retina cells affected both the synthesis and accumulation of several protein components. The effect on protein synthesis was found, in the case of lens epithelium, to be mediated via a change in the mRNA population of treated cells. The effect of insulin on lens epithelial cells was found to vary with the genotype from which the cells were originally derived. Insulin treatment of limb fibroblast cell cultures affected protein accumulation but this effect was dependent on the level of FCS used in the culture medium. Chlordiazepoxide HCl treatment of cultured cells affected the synthesis and accumulation of the proteins in neural retina, limb fibroblast and kidney fibroblast cells and in the limb fibroblast cell cultures, it also affected translatable mRNA population. Diphenylhydantoin treatment affected protein synthesis and accumulation in lens epithelial and neural retina cell cultures. Chloroquine sulphate treatment affected protein synthesis and accumulation in lens epithelial, neural retina and brain cell cultures. Diphenylhydantoin, chloroquine sulphate and chlordiazepoxide HCl also affected the activity of choline acetyl transferase in neural retina cell cultures.

Cobaltous chloride affected the synthesis and accumulation of proteins both in lens epithelial and kidney fibroblast cell cultures. Lead nitrate was found to affect protein synthesis and accumulation only in neural retina cell cultures. Finally meprobamate affected protein synthesis and accumulation in limb fibroblast cell cultures. With the exception of chloroquine sulphate, the effect on protein synthesis and accumulation was diminished within 24-48 hours following the last treatment with the teratogens. With chloroquine sulphate the effect was still evident even after 20 days following the single treatment.

The data suggest that certain correlations do exist between *in vivo* and *in vitro* teratogenic tests. The present *in vitro* cell culture technique can demonstrate such phenomena as drug and tissue specificity. The effect of an agent both on the embryo and on the cultured cells is dependent on the amount administered, the duration of treatment, the stage of development and differentiation at the time of treatment, and, in the one case investigated, the genotype of the cells.

1. INTRODUCTION

1.1 The Problem of Teratogenicity

Genetically transmitted conditions comprise the main single contributory factor to human congenital malformations. This, however, accounts for only 20% of all recorded malformations. From the medical reports it has been estimated that 2-3% of all congenital malformations in man are caused by drugs and various other environmental agents (Wilson, 1973). The true figure however is probably much higher when one considers that a high proportion of early still births are unrecorded, and that in many countries accurate medical records are not available. Moreover if amongst the largest group of congenital malformations (65 - 70% of the total) which have no known cause, interactions of unrecognised environmental agencies with susceptible genotypes are important, as is surely implied by studies of gene environment interactions in general and (for example) pharmacogenetics in particular, then many malformations would be preventable if the environmental 'trigger' could be identified and eliminated.

In the past, teratologic studies have generally focused either on the history of developmental defects before birth or on descriptions of the defects after birth, rather than searching for the cause and the mechanism by which these defects occur.

Modern teratology has matured as a science in the last 45-50 years, in the wake of several significant developments. The first of these was a series of experiments which indicated that dietary deficiency in rats could produce predictable proportions of particular malformations in the offspring (Warkany and Nelson, 1940; Warkany and Schraffenberger, 1944). It was also noted that a high proportion of

children born to women infected with rubella virus during the first trimester of pregnancy had various congenital defects (Gregg, 1941). Thus within a short period of time it became evident that mammalian embryos including human embryos, are susceptible to various environmental influences during intrauterine development.

The problem of teratogenicity, however, only attracted widespread attention in the late 1960's following the thalidomide drug incident in humans (Lenz, 1964; McBride, 1961). The fact that this drug affected more than 10,000 children within a two year period highlighted the probability that numerous other agents present in either food, water or the air could also potentiate such an effect.

1.2 The Nature of Teratogens

In general terms a teratogen can be defined as an environmental agent which can adversely effect embryonic development and thus cause malformations and malfunctions in the offspring (Fraser, 1959; Saxen and Rapola, 1969; Wolff, 1975). According to this definition a teratogen may be an element, such as lead, (Palmisano, Sneed and Cassady, 1969), a drug such as thalidomide (Lenz, 1964, 1966), or a virus such as rubella (Sever, 1975).

Several hundred individual agents are now known to affect mammalian development and these have been listed and classified both in review artiles (Fave, 1964; Wilson, 1964b; Karnofsky, 1965a; Kalter and Warkany, 1959; Tuchmann-Duplessis, 1965; Cahen, 1966; Lenz, 1966; Wilson, 1973) and in the "Catalog of Teratogenic Agents", (Shepard, 1973, 1976, 1980). The situation is further complicated by the fact that there are also various agents such as chlorpromazine, sodium salicylate and cortisone which are not highly teratogenic by themselves

but have been shown to promote the effects of known teratogens such as insulin and vitamin A (Zwilling, 1948; Woollam, Millen and Fozzard, 1959; Hartel and Hartel, 1960; Kalter, 1960; Smithberg, 1961; Landauer and Clark, 1963, 1964; Wilson, 1964b; Wilson, Jordan and Schumacher, 1969).

Teratological studies, so far, indicate that substances which are known to have teratogenic effects on the developing human conceptus include certain drugs of therapeutic use, such as anticonvulsants (e.g. diphenylhydantoin, phenobarbital, paramethadone and trimethadone), antimalarial drugs (e.g. Ouinine, Chloroquine), antituberculous drugs (e.g. imipramine), neurotropic-anorexogenic drugs (e.g. dexamethetamine, amphetamine, phenmetrazine), folic acid antagonists (e.g. amphoterin), steroid hormones (e.g. particularly androgens), alkylating agents (e.g. busulfan, chlorambucil, cyclophosphamide, 6-mercaptopurine), and environmental pollutants (e.g. lead) (Wilson, 1972, 1973; Shepard, 1973, 1976; Howard and Hill, 1979; Shepard, 1980).

1.3 The Response to Teratogens with Respect to Developmental Stage and the Duration of Exposure

The degree and the type of abnormalities caused by teratogenic agents varies under different conditions. For a given teratogenic agent the particular type of malformation(s) induced depends on both the duration of exposure and the stage of development at the time of treatment. Embryonic mortality is higher amongst those offspring exposed to an environmental agent during the early stages than those exposed at later stages of development. Embryos at the stage of gametogenesis and blastogenesis being particularly susceptible, (Wilson 1954a; Kalter, 1957; Saxen and Rapola, 1969; Gumpel, Hayes and

Didgeon, 1971; Shepard, Miller and Marois, 1975). It has been suggested by Saxen and Rapola (1969) that during the early stages of embryonic development (prior to any significant differentiation) all the cells would be susceptible to the teratogen and if the cells were affected severely, this would be expected to lead to a complete destruction of the zygote. There is evidence however which suggests that this may not be true for all teratogens. For example, irradiation (Rugh and Wohlfromm, 1962) or such agents as actinomycin D (Wilson, 1966) have been reported to induce local defects in the offspring of animals treated immediately after fertilizations. Such congenital defects could arise if the effects of a particular environmental agent discriminated between the types of cell formed during the stages of early embryonic development. Furthermore, the susceptibility of the various organ systems may also depend on the state of development and differentiation of that organ (Wilson, 1973; Shapard, Miller and Marois, 1975; Jauchu, 1982).

From these studies it is evident that the effect of a given agent can vary according to the stage of differentiation at the time of treatment. This phenomenon may partly be due to the fact that placental function and permeability changes during gestation so that the level of an agent reaching the embryo may vary and also due to the fact that the sensitivity of each cell type in an organ system differs at different stages of development.

Wilson, Roth and Warkany,(1953b,) reported that when a period of vitamin A deficiency in rats is terminated on day 10, the offspring suffers only cardiac defects, whereas when the deficiency is terminated

on day 15, defects of the eyes, aortic arches, diaphragm, lungs and urinogenital organs are also apparent. More recently, it has been reported that the 'Ultra lente' type of insulin (with a long lasting 48 hour hypoglycemic effect) causes microphthalmia and anophthalmia in the offspring of rats treated on days 6-9 of gestation, whereas normal insulin (with a short-term 12 hour effect) has no effect, (Miyamoto, Sakauch and Midorikawa, 1979).

The duration of exposure of the foetus to a potentially teratogenic agent may be affected by genetic variation and the rate of metabolism and elucitation of the agent as determined by genetic variation, non-genetic medical factors or by the synergistic effects of other substances.

Work by Zimmerman and Bowen (1972) has shown that the resistance of a mouse strain to triamcinolone-induced cleft palate is related to the rate of metabolism of the drug by the mother; the offspring of rapid metabolisers being resistant while those of slow metabolisers are more susceptible. Presumably this is the case because the exposure is chronic rather than acute in the latter. This might correspond to the effect observed with different forms of insulin, "ultralente" and normal, as described above.

The human population is extremely heterogeneous, not only genetically, but also in terms of general health. For example, it has been reported that both the metabolism and excretion of diphenylhydantoin is much slower in those individuals suffering from renal failure (Borga et al., 1979). In addition it has been found that the blood levels of diphenylhydantoin can vary according to the weight and size of the conceptus as well as that of the mother (Rane, Hoppel and Hojer, 1979).

Wilson (1973) has reviewed evidence which suggests that mothers with medical conditions including hepatic disease, renal disfunction, congenital hip or heart disease (Wolff, Koehn and Coleman, 1968), or with various pharmacogenetic susceptibilities such as favism (G6PD variant) (Sartori, 1971) will have a different reaction to teratogenic agents from healthy individuals.

Moreover, it is known that the administration of two or more agents together can influence their rate of metabolism and excretion. For example, alcohol is known to effect the rates of metabolism and excretion of chlordiazepoxide in man (Whiting et al., 1979). Further, it has been established that genetic variants exist in man in their ability to metabolise alcohol (Propping, 1978). Since both diphenylhydantoin (Hanson et al., 1976; Mulvihill and Yeager, 1976; Shapiro et al., 1976; Ouellete et al., 1977) and alcohol (Jones et al., 1973; Mulvihill and Yeager, 1976) are teratogenic in man then those factors such as the rate of metabolism of these agents, the blood levels and the rate of elimination from the blood would be expected to influence the incidence of any defects induced by these drugs.

1.4 Genetic Variations

Many studies have shown that there are considerable variations between species, strain, and even between individuals of the same species, in response to a given teratogenic agent. Wilson (1973) reported that mice are more susceptible than hamsters, guinea pigs and rats to the teratogenic effects of cortisone treatment. Similarly, embryos of man and higher primates are more susceptible to thalidomide than are mice or rabbits, but embryos of rats and lower primates are unaffected (Wilson, 1973). This indicates the difficulty of extra-

polation from the results obtained in animal studies to the situation in man.

The incidence of a particular malformation or series of malformations induced by a teratogen also varies between strains of the same species. For example, the incidence of cleft palate formation following either cortisone or galactoflavin treatment of mice depends on the genotype (Fraser and Fainstat, 1951; Kalter and Warkany, 1957), and Gunberg (1958) noted differences in the incidence of mortality and malformations amongst the offspring of three strains of rats treated, during the same period of gestation, with trypan blue. There is also evidence suggesting that there is strain specific variation in teratogenic response to hypervitaminosis A between three strains of rats (Nolen, 1969). Rabbits from different genetic backgrounds also respond differently to thalidomide treatment (Sawin et al., 1965). Studies with chick embryos have shown that the induction of micromelia in different chick strains by 1.3 propanediol varies from 5 to 100% according to the strain of chick (Gebhardt, 1968).

The incidence of malformations may be influenced both by the rate of metabolism of the drug by the mother and by the genotype of the embryo. For example, Kalter (1954) suggested that the differences in the degree of cleft palate induced by cortisone treatment of two strains of mice could be due both to differences in the ability of the mother to metabolise cortisone and to differences in the genotype of the embryos.

1.5 Summary of Section 1.3 and 1.4

It is evident from the earlier studies that a variety of factors such as the genetic constitution, the general health, the stage of development at the time of treatment, the rate of metabolism of the drug, placental permeability and the co-administration of particular drugs may all contribute to the variations observed in the types and the degrees of the defects in the offspring of the treated mothers. Therefore the observations on morphological malformations will not lead to prediction of potential decisively teratogenic agents and their elimination from the market.

For this reason other test systems must be developed to investigate the mechanism by which a teratogen induces a given type of malformation which may provide explanations for the type and the degree of malformations induced in strains and species of animals at different stages of development.

1.6 Available Test Systems for Screening Teratogens

If we consider the large increase in the number of new products produced each year, and the possible hazardous effects of such products on humans, it is now more than ever necessary to devise practical but efficient tests for the screening of large numbers of environmental agents. The test devised should take into account the tremendous variations apparent between individuals as regards to susceptibility to a given agent.

Since for ethical reasons it is not possible to use humans for such tests, other screening techniques such as whole animal testing or *in vitro* culture methods using either animal or human tissues must be employed.

It is necessary to substantiate the results obtained from whole animal tests and their usefulness in extrapolating to the human situation. Similarly, the validity of *in vitro* tests to investigate those components of individual susceptibility to a particular agent which may be due to such factors as drug metabolism, time of treatment, genetic constitution or placental permeability must be examined. Moreover, tests might be devised which may not only permit a classification of the defects but also lead to an understanding of the mechanism by which a particular agent acts.

1.6.1 *In Vivo Tests*

Several animal species have been suggested as suitable for screening of potential human teratogens, these include:- (1) invertebrate embryos, (2) amphibian embryos, (3) avian and reptile embryos, (4) rodent or rabbit embryos, and (5) simian embryos (reviewed by Wilson, 1975).

(1) Invertebrate embryos. These may be obtained and used in large numbers. However, because their embryology and anatomy is so distant from that of man, considerable doubts are raised concerning their validity for teratogenic studies.

(2) Amphibian embryos. The use of amphibian embryos is limited since it is complicated by the fact that they lead a dual existence, in water and on land. This introduces some atypical processes that may be irrelevant or misleading for use in teratologic studies pertinent to man.

(3) Avian and reptile embryos. Avians are available in vast numbers but reptiles are more difficult to obtain. Although they share typical vertebrate developmental processes with mammals, a major drawback in using them for screening of teratogenic agents is that they depend on abundant yolk, and there is no equivalent to the foeto-maternal

relationship of mammals associated with the placenta. Nevertheless an accessible and well-studied non-mammalian embryo such as the chick would appear to be of particular value in the investigation of one fundamental question: the nature of the relationship between the response to drugs of cells in an *in vitro* system to the response to those same drugs of the developing organs *in vivo*. The ease of culture, the accessibility of the tissues in significant amounts, and the known responses of the chick to many teratogens have prompted us to use this system for investigations into this basic problem.

(4) Rodent and rabbit embryos. Mice, rats and rabbits have been the most widely used animal species and have been widely considered as valid models for human development. One major disadvantage, however, is that during early embryogenesis there is a highly permeable yolk-sac placenta and thus rodent embryos lack the protection offered to embryos of higher mammals with a chorio-allantoic placenta.

(5) Simian embryos. Apes and monkeys are no doubt the most valid animal models for possible teratogenicity to man, but their scarcity and cost make their use prohibitive.

Whole-animal *in vivo* tests mainly with rats or rabbits have developed to a relatively standardised form and are utilized world-wide. They suffer, however, from two serious problems: they are expensive and time-consuming to perform and the extrapolation of the results to the human population is confounded by the well known species variation in teratogenic response (Wilson, 1977). Finally although gross defects are recorded, low level effects may be neglected, which could be significant for man.

1.6.2 In Vitro Tests

The disadvantages of whole-animal tests include the slowness and the expense of the tests and also the decreased predictive value for the situation in man due to species and strain differences. Moreover in the last few years experiments with live animals have not met with much public enthusiasm. However, the number of compounds which must be tested for teratogenicity has increased dramatically with the continuous development of such agents as therapeutic drugs, cosmetics, and food additives, agricultural products, pesticides and so on. In addition governmental regulations require more rigorous toxicity testing of those industrial and commercial agents to which the human population could conceivably be exposed. Thus the development of *in vitro* tests, even if only as an adjunct to animal testing as a preliminary screen, for example, is very much required. The potential value of culture techniques for the screening of pharmacological agents was first recognized by Pemeral and Leake (1954). Several investigators have already suggested that many of the processes which involve cell and tissue interactions and which play a key role in the development of multicellular organisms can be made to occur *in vitro*, using controlled culture conditions (Moscona, 1975; Hausman and Moscona, 1976; Kaighn, 1976). Since then a number of *in vitro* test systems including culture of whole-embryos and whole-eggs as well as cell, tissue and organ cultures, have been established to investigate the problem of teratogenicity. Of these the cell, tissue and organ cultures are considered to be valuable tools for the primary screening of potential teratogens, (Moscona, 1975; Clayton, 1976; Kochhar, 1976; Shepard and Pious, 1978; Clayton *et al.*, 1980; Beck, 1982; Clayton and Zehir, 1982).

1.6.2.1 Embryo Culture

Whole embryo culture studies offer certain disadvantages. Embryos do not remain viable for long periods in culture thus one cannot study the long term effect of a potential teratogen on the target tissue(s) of the embryo (Shepard, 1976; Zimmerman, 1976). Moreover, it is possible that embryonic development *in vitro* may not mimic exactly that observed *in vivo*. Further, with these preparations it is not possible to determine the role of the mother which includes such parameters as maternal activation of the agent or altered placental function. In this respect Fantel *et al.*, (1979) recently observed that cyclophosphamide treatment of 10 day old rat embryos in culture at a level of 250 μg/ml had no effect, however even low levels of 6.25 μg/ml induced embryonic defects if administered together with a hepatic microsomal fraction and co-factors for a mono-oxygenase system. Thus the teratogenic effect must be due to a metabolite, and the results suggest that maternal activation plays an important role in the action of this teratogenic agent. Finally, whole embryo cultures are costly to establish and, therefore, are of limited use in the screening of large numbers of potential teratogenic agents.

1.6.2.2 Organ Culture

Organotypic culture, an intermediate system between experiments on the whole animal and experiments on cell culture, is capable of providing valuable information about the teratogenicity and mode of action of particular agents (Wolff and Haften, 1952; Cahen, 1964; Kaighn, 1976). The great majority of organ systems would not, however be appropriate as testing techniques. This is primarily because of the limited development *in vitro* since the organs are explanted at an

advanced stage of differentiation (Brown and Fabro, 1982). Further, despite the fact that organ cultures allow the investigator to study the primary site and the mechanism of action of a potential teratogen, the system suffers from the same limitations as have been outlined for whole-embryo culture.

1.6.2.3 Cell and Tissue Culture

In contrast, to cultures of whole-embryos and of embryonic organs, the use of differentiating cell cultures could be a relatively inexpensive tool for the primary screening of large numbers of teratogenic agents (Moscona, 1975, 1976; Clayton, 1976; Wilk et al., 1980; Beck 1982; Clayton and Zehir, 1982). Cell culture provides a suitable system for the investigation of the effects of potential teratogens on cellular processes such as cell growth, cell movement, cell recognition and selective cell adhesion, all of which are involved in embryonic morphogenesis, tissue differentiation and organ development.

Although cell culture has been used in the past in an attempt to screen for teratogenic agents as yet it has not been firmly established that it can be used in a reliable manner. If cell culture could be proved a reliable tool in the study of teratogenicity, then the use of human tissue would yield valuable information as to the effect of selected agents in man. For this reason further investigations of the cell culture system should be made. Indeed several investigators have suggested that it should be possible, in the future, to predict the teratogenic effects of certain drugs and the mechanism of their action by studying their metabolism in foetal cell cultures, and by quantitating the effect of a drug or its metabolite on the cell culture (Saxen and Rapola, 1969; Nebert, 1973; Clayton, 1980, 1982; Wilk et al., 1980; Beck, 1982; Clayton and Zehir, 1982).

1.7 The Relationship Between the Biochemistry of Cells, Histogenesis of Tissues and Development of Organs

During the process of embryonic development and differentiation, various intracellular biochemical events, cellular interactions and morphogenetic movements of cells and tissues occur; these are controlled by the temporal and spatial expression of genes. Throughout embryonic development cells interact with one another and with their environment to form tissues whose interaction leads to the development of organs and the whole organism.

The fine regulation of cell proliferation, programmed cell loss, cell adhesion and tissue communication is a prerequisite for normal development and for the maintenance of the developed organism. Any agent, which interferes with any of these processes may lead to abnormal development, either functional or structural or both, and act therefore as a teratogen.

It has been suggested that a reduced mitotic rate could contribute significantly to growth retardation independently of cell necrosis (Franz, 1971) - this would be the case if the reduction is overall. Local or specific reductions would lead to such phenomena as malproportions and failures of induction, which may cause abnormal development. Alterations in cell loss may also contribute to abnormal development. Ritter et al., (1972) have postulated that if the rate of cell death exceeds that of the regenerative capacity of the tissue then abnormal development would occur. The important question, therefore, is whether such processes, which are recognised through their effects on the developing system may also be recognised by cell culture tests. Developing a test for any particular process would require first

that any effect on a tissue is primary on the component cells and second, that these effects may be identified in cultured cells.

Evidence from studies using such teratogens as 6-aminonicotinamide, (a dehydrogenase inhibitor) and vitamin A, which affects the cell surface, suggest that alteration in cellular properties or metabolic processes of cells may lead to abnormal development. It has been shown that 6-aminonicotinamide treatment of pregnant mice on the 13th day of gestation leads to the development of cleft palate, due to an interruption of cartilage proliferation and matrix synthesis in the cranial base in the offspring (Goldstein, Pinsky and Fraser, 1963; Long, Larsson and Lohmander, 1973).

Vitamin A treatment of pregnant mice also causes a cleft palate as well as a high incidence of limb abnormalities in the offspring (Kochhar, 1970, 1973). Kochhar (1973) first suggested that these abnormalities could be the result of a disruption of cell movement and cell shape both of which are involved in the spatial organization of mesenchymal cell condensations. Subsequently he showed that mesenchyme cells derived from the limb buds of embryos exposed to vitamin A in vivo do indeed show a reduced rate of cell migration (Kochhar, 1976). Similarly, vitamin A treatment of limb bud fragments in vitro also causes a retardation of cell movement and alterations in cell morphology (Kochhar, 1976). Vitamin A has also been shown to inhibit chondrogenesis in mesenchyme cell cultures of mouse limb buds (Lewis et al., 1978). Finally, it has been postulated by Kwasigroch and Kochhar (1980) that the abnormalities induced by vitamin A treatment are probably the result of alterations in the properties of cells which regulate the internal viscosity of the tissue.

Other examples also link abnormalities in development with a precise change in the synthesis or structure of a specific protein. For instance, β-aminoproprionitrile causes various congenital abnormalities, including cleft palate, in rats and mice, and a close correlation has been established between the induction of cleft palate *in vivo* and the inhibition of collagen cross-linking both *in vivo* and in culture. (Pinnel and Martin, 1968; Pratt and King, 1972). Thus it would appear that the types of change in the synthesis or structure of particular proteins and cellular interactions in developing organ systems, which would be expected to lead to an abnormal development *in vivo*, are also those which may be observed in cell culture. Moreover, the above studies indicate that teratogenic agents act in specific ways to initiate a sequence of abnormal developmental processes which can often be shown to be derived from one or more earlier events and could lead to the same type of malformation (see section 1.2.2).

1.8 The Systems Under Test

1.8.1 Cell Cultures

In the past, most *in vitro* teratogenic tests have been carried out with tissue or organ cultures. More recently with continued improvements in laboratory methods, cell culture tests utilizing tissues from both mammalian and avian species have been established (Kaighn, 1976; Moscona, 1976; Clayton *et al.*, 1980; Beck, 1982; Clayton and Zehir, 1982). The essential conditions to be considered are the culture system, the cell types required to be representative of the embryonic tissues and the system of evaluation of effect. The cells and tissue systems selected should be those for which culture conditions are known and which permit them to express their capacity to undergo

in vitro changes similar to those observed during normal growth and differentiation. Thus deviations from such normal processes, following treatment with a test agent, might be recognized and subjected to further evaluation and analysis.

We have selected a small number of tissues which are representative of a range of embryonic tissue types. They include mesodermal tissues (kidney and limb fibroblasts), neuronal tissues (brain and neural retina) and ectodermal tissues (lens epithelium) all of which are able to behave in tissue specific ways in culture, and to undertake differentiation or cell organization.

1.8.1.1 Limb and Kidney Fibroblasts

As regards mesodermal tissues, cell tissue and organ cultures derived from both mammalian and avian limb and kidney have been used extensively to investigate various aspects of development (Grobstein, 1965; Okada, 1965; Yaffe, 1968; Takahashi and Okada, 1970; Crocker 1973; Abbott et al., 1974; Hauschka, 1974; Croisille, 1976; Konigsberg, 1976). These studies have shown that kindey and limb mesodermal tissue can differentiate in vitro into kindney tubules and muscle fibres respectively and express tissue-specific proteins.

Cultured muscle cells from the hind limbs of chick embryos form fibroblasts and biopolar myoblasts after 24 hours and show the characteristic multinucleated muscle fibre formation on the 4th day of culture(Fujisawa, 1969; Konigsberg, 1976). The studies by Fijisawa (1969) also showed that there is a gradual increase in these cultures in the M/H subunit ratio of the lactate dehydrogenase isozyme similar to that found during normal limb muscle development in vivo.

Differentiation of myoblasts to form multinucleated muscle fibres is also associated with the synthesis of other specific biochemical markers such as myosin (Paterson and Strohman, 1972), and multiple forms of acetyl cholinesterase (Kato et al., 1980). The 19S form of acetyl cholinesterase has also been shown to be synthesized by differentiated muscle cells grown in vitro (Kato et al., 1980). Moreover the 19S form of acetyl cholinesterase is considered to be a marker of synapse formation both in rat and chick skeletal muscle (Vigny et al., 1976).

1.8.1.2 Brain Tissue

Brain cells also differentiate in culture and express tissue specific biochemical markers at levels similar to those synthesised in vivo.

Cell culture studies by Peterson, Webster and Shuster (1973) have shown that those established from 7-day old chick embryos are comprised of two main cell populations: (1) neurone-like cells which develop axonal outgrowths and form synapses with other axons and which express choline acetyltransferase and acetyl cholinesterase activities, (2) flattened cells which express butyryl cholinesterase and a choline independent acetylase activity.

These authors also found that the pattern of increase in the activities of these enzymes in culture was similar to that observed in developing chick brain.

Similar findings have been reported in cultures derived from embryonic mouse brain cells (Seed, 1971; Seed and Vatter, 1971).

1.8.1.3 Neural Retina

The retina, which responds to light, is the most complex structure of the eye. During development the retina is derived from the optic

cup which itself is formed by invagination of the optic vesicle, a lateral outpushing of the brain (Maximow and Bloom, 1948). The invagination follows the induction by the optic cup of the lens placode from the overlying head epidermis. The inner distal layer of the invaginated eye cup forms the neural retina whereas the outer layer proximal to the brain forms the pigmented epithelium.

Neural retina cell cultures derived from 6-, 7- or 9-day old chick embryos differentiate into neuronal and neuroepithelial (glial) cells. Assays of neurotransmitters in these cultures have shown that cholinergic neurones can differentiate *in vitro*, and that the specific activity of choline acetyltransferase (the enzyme involved in the synthesis of acetyl choline) increases at a rate similar to that found during development *in vivo*.(Vogel and Nirenberg, 1976; Vogel *et al.*, 1977; Crisanti-Combes *et al.*, 1977; Crisanti-Combes, Pessac and Calothy, 1978).

The synthesis of glutamate synthetase and carbonic anhydrase which are known to be convenient biochemical markers of differentiation and specialisation in the embryonic neural retina can be induced by hydrocortisone in neural retina cell cultures, where a direct relationship has been established between the degree of histiotypic cell association and the inducibility of the enzymes (Morris and Moscona, 1970, 1971; Morris, 1976; Moscona, 1976). However, both the appearance of these enzymes and the development of axonal connections formed by the neurones occurs in cell aggregates and in short term primary cultures. In long term cultures, neural retina cells transdifferentiate into lentoid bodies and pigmented cells when grown in Eagle's minimal essential medium supplemented with 10% F.C.S. (Itoh *et al.*, 1975; Okada *et al.*, 1975; de Pomerai, Pritchard and Clayton, 1977).

1.8.1.4 Lens Epithelium

The lens of the vertebrate eye develops from the competent head ectoderm which differentiates into the lens vesicle following induction by the eye cup. The anterior part of the lens vesicle becomes the future lens epithelium and the posterior part differentiates into lens fibre cells. The region between the undifferentiated epithelium and the mass of lens fibre cells is the germinal zone of the lens, where the epithelial cells are continously dividing and elongating to become the terminally differentiated fibres (Balinsky, 1970).

Lens fibre cells as well as lens epithelial cells synthesise crystallins, the structural proteins of the lens, which are comprised of the products of three gene families (see reviews by Clayton, 1970, 1974, 1979). In the chick lens there exist three major classes of crystallin proteins: alpha (α), beta - (β) and delta - (δ) crystallins. α crystallins of the chick lens can be resolved by SDS polyacrylamide gel electrophoresis into two distinct bands with molecular weights of 19K daltons ($\alpha 2$) and 20K daltons ($\alpha 1$) (Thomson et al., 1978). There are at least six major β-crystallin subunits with molecular weights ranging between 20.5K daltons and 38K daltons (Thomson et al., 1978; Ostrer and Piatigorsky, 1980; Ostrer, Beebe and Piatigorsky, 1981). Delta crystallins, the major proteins of the chick lens, can be resolved by SDS polyacrylamide gel electrophoresis into two distinct bands with molecular weights of 50K daltons ($\delta 1$) and 48K daltons ($\delta 2$) (Piatigorsky, 1975; Reszelbach, Shinohara and Piatigorsky, 1977; Thomson et al., 1978). The composition and relative proportion of each crystallin class within a lens cell varies with the state of

development of the embryo and of the differentiation of the fibre cells (Maisel and Langman, 1961; Rabaey, 1962; Genis-Galvez, Maisel and Castro, 1968; Yoshida and Katoh, 1971; Clayton, 1974; McDevitt and Clayton, 1979).

Lens epithelial cells also retain their capacity for normal differentiation in cell culture. Studies with dissociated lens epithelial cells from embryonic and day-old chick have shown that lens epithelial cells differentiate into lentoid bodies composed of lens fibre cells (Okada, Eguchi and Takeichi, 1971; Okada, Eguchi and Takeichi, 1973; de Pomerai, Clayton and Pritchard, 1978). All these studies have shown that lens fibre differentiation *in vitro* and the associated pattern of crystallin synthesis is similar to that observed *in vivo*, where there is an increased synthesis of β2- and δ- as well as that of several β-crystallins during embryonic development. Lens fibre cells obtained from early embryonic lens epithelium synthesise δ predominantly but relatively more β and less δ if taken from older individuals, thus the differentiation of these cells *in vitro* mirrors the differentiation *in vivo*.

The *in vitro* studies have also shown that the quantitative profile of the crystallin content of differentiating lens cell cultures reflects various changes in growth conditions. The crystallin profile may be modified by factors which affect either the mitotic rate, changes in cell mass, cell-cell contact, or cell-substrate adhesion. The profile may also be modified by the presence of metabolic inhibitors and by the genotype of the cells (Eguchi, Clayton and Perry, 1975; Clayton *et al.*, 1976a, 1976b; Creighton, Mousa and Trevithick, 1976; Clayton de Pomerai and Pritchard, 1977; Vermorken *et al.*, 1978). Immuno-

-logical studies of differentiating lens epithelial cell cultures have shown that, in addition to the crystallin proteins, they also contain various cytoskeletal proteins including actin, α-actinin, myosin, tropomyosin, tubulin and vimentin (Ramaekers et al., 1980). It has been suggested that the cytoskeletal structures play a significant role both in cell elongation and the differentiation of lens forming cells (Piatigorsky, Webster and Wallberg, 1972). It is therefore possible that any significant alteration in the conditions governing lens development and differentiation, may be reflected in changes in the crystallin or the cytoskeletal elements.

It is evident from the studies summarized above that the selected cell types, such as lens epithelium, limb fibroblasts, neural retina and brain, do differentiate to some extent under controlled culture conditions in vitro and express various cellular and biochemical processes similar to those expressed in vivo. Thus the effect of teratogenic agents which must operate on these cellular and biochemical processes may be detectable in vitro. Should there prove to be a relationship between the effects on specific cell types in vitro and the effects obtained in vivo then responses in vitro may prove to have some predictive value. A general predictive value would require mainly that there be a correlation between substances known to be teratogenic in vivo with their effects in vitro. A specific predictive value would require that tissue specificity of response are similar in vivo and in vitro.

1.9 The Agents Under Test

1.9.1 Insulin

Insulin, the hormone which regulates carbohydrate metabolism in vivo

is secreted by the beta cells of the islets of langerhans situated in the pancreas of all vertebrates. Insulin preparations are used in the treatment of diabetes.

Some studies have suggested that there may be an increase in the incidence of congenital defects in the babies of diabetic mothers who had been treated with antidiabetic drugs including insulin (Sobel, 1960). However there is as yet no conclusive evidence to indicate that this is the case in man. Studies using laboratory animals have shown that insulin is teratogenic in some mammals, for example rats, rabbits and mice (Chomette,1955; Brinsmade, Buchner and Rubsaamen, 1956; Smithberg, Sanchez and Runner, 1956; Smithberg and Runner, 1963; Miyamoto, Sakauchi and Midorikawa, 1977, 1979; Cole and Trasler, 1980) and avian species (Landauer, 1945; Landauer and Lang, 1946; Landauer, 1947; Moseley, 1947; Zwilling, 1948, 1949). The abnormalities indiced by insulin treatment are mainly confined to the brain and skeleton (see review by Landauer, 1972).

In chick embryos insulin treatment causes such external malformations as caudal deficiencies (rumplessness), limb abnormalities (micromelia), beak abnormalities ("parrot beak") and eye defects (microphthalmia, anophthalmia and buphthalmia) (see review by Landauer, 1972). The kind and the frequency of abnormality induced by insulin treatment depends on the stage of development at the time of treatment, the level of insulin administered and the genotype of the animal (Landauer, 1952, 1953). Insulin injections prior to 72 hours of incubation results mainly in caudal deficiencies, (Landauer and Bliss, 1946; Moseley, 1947; Landauer, 1948; Zwilling 1951; Landauer, 1952). While limb beak and eye defects are predominant in embryos treated at later stages

(Landauer, 1947; 1952; Zwilling, 1948, 1949). It has also been reported that the treatment of 96 hour old chick embryos with 1 iu of insulin caused an incidence of 16.7% micromelia, 9.4% beak and 2.1% eye defects while 2 iu of insulin leads to an incidence of 48.3% micromelia, 33.3% beak and 6.7% eye defects (Landauer and Rhodes, 1952; Landauer, 1953, 1957a). The incidence of rumplessness following insulin treatment has also been shown to depend on the level of insulin administered (Landauer and Bliss, 1946). In both mice and chicks the effect of insulin treatment has been shown to vary with the genotype (Landauer, 1945; Landauer and Bliss, 1946; Smithberg and Runner, 1963; Cole and Trasler 1980).

Insulin causes hypoglycemia in mammals, including man (Smithberg and Runner, 1963; Anand, Ganguli and Sperling, 1980) and in chicks, (Zwilling, 1948, 1949; Langslow et al., 1970). This action of insulin is considered to be the main factor contributing to the teratogenic effects observed in some animals such as rats (Miyamoto, Sakauchi and Midorikawa, 1979), rabbits (Brinsmade, Buchner and Rubsaamen, 1956), and chicks (Zwilling, 1951). However studies in vitro indicate that insulin has various direct effects on a wide range of tissues. For example, insulin inhibits cartilage development in vitro (Chen, 1954; Hay, 1958; Zwilling, 1959) and stimulates mitosis in adipocytes (Armato, Draghi and Andreis, 1978), liver cell (Gerschenson et al., 1972), kidney cells (Kletzien and Day, 1980) chondrocytes (Hajek and Solursh, 1975), fibroblasts (Paul and Pearson, 1960), myoblasts (Kumegawa et al., 1980), lens epithelial cells (Reddan et al., 1972) and in embryonic chick neural retina cell cultures (de Pomerai and Clayton, 1980). Moreover, insulin has been shown to affect microtubule assembly in rat

adipocyte cultures (Soifer, Braun and Hechter, 1971) and microtubule assembly and cell elongation in cultures of embryonic chick lens epithelium (Piatigorsky, Webster and Craig, 1972; Piatigorsky, 1973).

Insulin also affects ribonucleic acid metabolism in rat diaphragm (Wool and Munro, 1963), embryonic chick fibroblasts and lens epithelia, (Baseman, Pailini and Amos, 1974; Milstone and Piatigorsky, 1977). In addition, insulin affects protein synthesis in rat heart, diaphragm and skeletal muscle tissues (Sinex, MacMullen and Hastings, 1952; Wool and Kurihara, 1967; Wool, Stirewalt and Kurihara, 1968; Fahmi and Leader, 1977, 1978), mouse mammary epithelial cells (linebaugh and Rillema, 1979) and chick chondrocytes (Hajek and Solursh, 1975). Insulin affects crystallin protein synthesis and especially by stimulating that of δ-crystallin in both lens and lens epithelial cells from embryonic and day-old chicks (Piatigorsky, 1973; de Pomerai and Clayton, 1978; Clayton et al., 1980; Clayton and Zehir, 1982), and in transdifferentiating cell cultures of embryonic chick neural retina tissue (de Pomerai and Clayton, 1980).

In the lens cells the effect of insulin on crystallin synthesis is modulated by the genotype of the chick from which the cells were originally taken (de Pomerai and Clayton, 1978; Clayton et al., 1980; Clayton and Zehir, 1982).

Evidence suggests that the mechanism of action of insulin at the cellular level involves an initial interaction between the hormone and specific membrane receptors (Landauer, 1972; Butcher et al., 1973; Hinchliffe, 1974; Kahn et al., 1978; Sandra, Leon and Przybylsky, 1979).

1.9.2 Diphenylhydantoin

Diphenylhydantoin or phenytoin sodium (Epanutin), is an anticonvulsant drug, and is chiefly used in the treatment of various types of epilepsy. It has been reported that long term diphenylhydantoin treatment in man leads to cerebellar degeneration, hypoglycemia and lowering of insulin and thyroxine levels (Fariss and Lutcher, 1971, Funicane and Griffiths 1976; Funicane, 1978; McClain and Langhoff, 1980). Chronic treatment of children with this drug interferes with the metabolism of calcium and vitamin D and also with bone development (Dent et al., 1970; Bowden, 1974; Hahn, 1976; Kuntzman, 1969; Weisman et al., 1979). Several investigators have suggested that the latter results both from the induction of hepatic catabolism of vitamin D (important for normal bone development) and by a direct effect on the membrane cation transport system (Hahn, 1976; Weisman et al., 1979).

In the progeny of mothers treated with anticonvulsant drugs, including diphenylhydantoin, a noticeable increase has been observed in the rate of congenital malformations including cleft lip and palate, congenital heart defects and mental deficiency (German, Kowal and Ehlers, 1970; Ajodhia and Hope, 1973; Fedrick, 1973; Monson et al., 1973; Annergers et al., 1974; Hill et al., 1974; Biale, Lewenthal and Aderet, 1975; Yang et al., 1978; Mallow, Herrick and Getman, 1980). However, it must be noted that it is often difficult to assess the effect of an individual anticonvulsant drug on the embryo, because in many instances the mother is in receipt of two or more anticonvulsant drugs over the same period. In studies in which mice, rats and rabbits were treated with diphenylhydantoin the defects observed in the offspring included cleft palate, exencephaly, foetal haemorrhages,

orofacial defects, microphthalmia, open eyes and limb abnormalities, (Becker and Harbison, 1970; Harbison and Becker, 1974; Sullivan and McElhatton, 1975; Fritz 1976; Paulson, Paulson and Treissaty, 1979; Petter, 1979; Sulik et al., 1979; Walker, 1979; Witkowski and Sucheston, 1979; McClain and Langhoff, 1980).

Studies in vitro have shown that diphenylhydantoin inhibits glucose -induced insulin secretion and increases extracellular calcium in pancreatic β-cells (Levin et al., 1970, 1972; Pace and Livingston, 1979). This drug has also been shown to inhibit nucleic acid and protein synthesis and microtubular polymerization in human lymphocytes (MacKinney and Vyas, 1972; MacKinney, Vyas and Lee, 1975; MacKinney Vyas and Walker, 1978), and the phosphorylation of acetylcholine receptors in receptor enriched membranes (Do Lorenzo, 1977; Gordon, Davis and Diamon, 1977; Gordon, Milfay and Diamond, 1979). Diphenylhydantoin has been shown to affect membrane permeability and interfere with the membrane transport of Ca^{++}, Na^{+} and K^{+} ions (Kizer et al., 1970; Pincus and Lee, 1973; Carnay and Grundfest, 1974; Perry, MacKinney and de Weer, 1978; Tuttle and Richelson, 1979). Such action might explain the inhibitory effect of diphenylhydantoin on the synthesis of collagen and its effect of increasing collagen degradation in tissues including chicken embryo tibia and cat palatal mucosa and calvaria in vitro (Bergenholtz and Hamstrom, 1979; Hamstrom, Lerner and Gustafson, 1979; Dietrich and Duffield, 1980).

It has been suggested that the teratogenic activity of anticonvulsant drugs, including diphenylhydantoin is mediated through folic acid antagonism (Druskin, Wallen and Bonagura, 1962; Reynolds, 1972; Netzloff et al., 1979). It is also possible that many of the effects

reported on nucleic acid and protein synthesis and microtubular organisation may be a secondary consequence of the effect on cell membrane permeability. Finally, it has been suggested that the teratogenic effects of these drugs may be modified by both hereditary and environmental factors (Biale, Lewenthal and Aderet, 1975; Borga et al., 1979; Rane, Hoppel and Hojer, 1979).

It is important to note that the results obtained from the in vitro studies support the suggestion by Hahn (1976) and Weisman et al., (1979) that the inhibitory effect of diphenylhydantoin on bone development is partly due to a direct action of the drug on the membrane cation transport system. Further, these studies suggest that in vitro observations may have a predictive value on the mechanism by which a given drug induces developmental abnormalities in vivo.

1.9.3 Chloroquine

Chloroquine sulphate (Nivaquine), an antimalarial drug, is also used for the treatment of rheumatoid arthritis. Various side effects, however, have been observed in individuals on this drug, which may include damage to the central and peripheral nervous system (Torrey, 1968).

Studies with humans, mice, rats, cats, and monkeys have shown conclusively that chloroquine not only accumulates in eye tissues such as the iris, cornea, pigmented epithelium and neural retina but also can induce pathological changes in the retina (Hobbs, Sorsby and Freedman, 1959; Zivaifler, Rubin and Bernstein, 1963; Mier-Ruge, 1965; Hodgkinson and Kolb, 1970; Smith and Berson, 1971; Barnett and Eishorn, 1972; Rosenthal et al., 1978; Brinkley, Dubois and Ryan, 1979; Ogawa et al., 1979; Heckenlively, Martin and Levy, 1980).

In the monkey chloroquine is known to cross the placenta and accumulate in the foetal retina and adrenal cortex (see review by Shepard, 1979). The teratogenic effects of chloroquine treatment in man include damage to the 8th nerve, neural retina (retinopathy) and cochlea, mental retardation, neonatal convulsions and posterior column defects (Hart and Naunton, 1964; Smith, 1966; Matz and Naunton, 1968; review by Howard and Hill, 1979).

Studies in vitro with human skin fibroblasts have shown that chloroquine not only accumulates in lysosomes but that it inhibits lysosomal pinocytosis and enhances the secretion of lysosomal hydrolyses, (Weismann, Didonata and Herschkovitz, 1975; Coetzee, Stein and Stein, 1979; Sando et al., 1979; Gonzeles-Noeriga, 1980). Chloroquine has also been found to inhibit the lysosomal pathway of protein degradation in rat and chick embryo fibroblasts (Wibo and Poole, 1974; Libby, Bursztajn and Goldberg, 1980), and in rat adipocytes (Seglen, Grinde and Solheim, 1979). It has also been shown to inhibit DNA repair and synthesis in both bacterial and mammalian cells (Cleaver and Painter, 1975; Field et al., 1978; Schupbach, 1979).

1.9.4 Minor Tranquillizers Chlordiazepoxide HCl and Meprobamate

The mild tranquillizer drugs, Chlordiazepoxide HCl (Librium) and Meprobamate (Equanil), are used clinically for the relief of anxiety, supression of the convulsive state and relaxation of muscular tension. Chlordiazepoxide HCl is also used for the treatment of acute alcoholism.

Although several studies suggest that chloriazepoxide HCl and Meprobamate treatment of pregnant mothers increases the incidence of congenital defects (Bitnut, 1969; Milcovich and Vandenberg, 1974) their proven teratogenicity in the human is still questionable (Hartz et al., 1975).

Studies with rats suggest that these drugs exert a teratogenic action at the level of the central nervous system since the progeny suffer from an impaired learning ability (Werboff and Kesner, 1963; Hoffeld and Webster, 1975; Harris and Case, 1979).

1.9.5 Cobaltous Chloride

Cobaltous chloride has many commercial uses. It is used in the painting of glass and percelain and also in the preparation of catalysts, fertilizers, synthetic vitamin B12 and various food additives. Cobaltous chloride is also used clinically for the treatment of hematinic anaemia.

Cobaltous chloride has been shown to reduce the levels of haem and cytochrome P450 in treated rats (Tephly and Hibbeln, 1971; Maines and Kappas, 1974, 1975; Nakamura, Yasukochi and Minakami, 1975; De Matties and Gibbs, 1976; Maines et al., 1976; Igarashi, Hayashi and Kikuchi 1978; Sinclair et al., 1979). The same effects were also observed in monolayer cultures of rat hepatocytes (Guzelian and Bissel, 1976). Further in vitro studies using rat hepatocytes showed that cobalt fails to affect haem synthesis in culture conditions but it blocks the formation of cytochrome P450, (Guzelian and Bissell, 1976). These studies suggest that cobalt accelerates the breakdown of newly synthesized hepatic haem rather than inhibition of its synthesis. The injection of cobaltous chloride into rats and rabbits caused a significant rapid increase in hematocrit with a simultaneous decrease in plasma volume, and proteinaemia (Giovannini et al., 1978)

Cobaltous chloride is known to be teratogenic in both mice and chickens, causing cleft palate in the former (Kasirsky, Gautieri and Mann, 1967), and "brain softening", anaemia, and defective eye

development leading to anophthalmia and microphthalmia in the latter (Ridgway and Karnofsky, 1952; Kury and Crossby, 1968). Skeletal abnormalities were also recorded in cobaltous chloride treated chick embryos, the long bones of the hind limb being especially shortened, (Kury and Crossby, 1968). All the abnormalities observed in cobalt-treated chick embryos were reported to be dose-dependent (Kury and Crosby, 1968).

1.9.6 Lead Nitrate

Lead is used in the manufacture of matches, dyes and explosives and in the printing and photographic trades. Lead serves no metabolic function and therefore its presence in the body represents a contamination from various environmental sources such as automobile exhaust fumes (Ferm, 1972).

Lead can cross the placenta from maternal to the foetal circulation (Gershanic and Brooks, 1974; Pearl and Boxt, 1980), and maternal exposure to lead is associated with an increased concentration of lead in the milk (Ryu, Ziegler and Fomon, 1978), and this contamination of the milk has been shown to increase the net transfer of lead to the nursing offspring, in both rats and Guinea pigs (Mykkanen, Dickerson and Lancaster, 1979; Keller and Koherty, 1980). Studies with rats have shown severe haemorrhagic reactions in the cerebellum of the suckling offspring whose mothers were treated with lead (Millar et al., 1970; Ahrens and Vistica, 1977).

In man there is some evidence which indicates that lead increases the incidence of both foetal deaths and mental retardation (Baker, 1960; Engle and McIntyre, 1964; Moore, 1980). Evidence does suggest that in young children high levels of lead in the body can cause mental

retardation and kidney and cardiovascular damage (moore, 1980).

Lead is teratogenic in chicks, hamsters, rats and mice, (Ridgway and Karnofsky, 1952; Ferm and Ferm, 1971; McClain and Becker, 1975; Gale, 1978; Beaudoin and Fisher, 1981). The defects observed include a reduction in foetal growth rate, brain damage and skeletal abnormalities such as delayed ossification, fusion of ribs and urorectocaudal syndrome.

Studies in vitro have shown that lead stimulates RNA synthesis and hexokinase activity in mouse lymphocyte cultures (Gallagher, Matarazzo and Gray, 1979). Lead has been shown to be preferentially accumulated in the mitochondria of capillary endothelial cells (Silbergeld, Wolinsky and Goldstein 1980). These authors suggest that this accumulation could be associated with lead-induced disruptions in intracellular calcium metabolism and the trans-epithelial transport system.

1.10 Aims of this Investigation

Considering the chemical diversity of both known and potential teratogens, the multiplicity of their effect on different strains and species of animals and the lack of detailed knowledge concerning their mechanism of action at the cellular level, it is unlikely that any one test system would be sufficient to act as a reliable screening method for teratogenic agents. The increasing number of new potential teratogens, whether pharmaceutical preparations, agricultural products, food additives or industrial pollutants, together with the increasing cost of whole-animal testing and its time consuming nature, makes the establishment of a rapid and relatively inexpensive in vitro screening test system an urgent requirement.

In order to develop an in vitro cell-based test system for pre-screening of teratogenic agents, it is essential to determine whether

functional correlations can be established between the effects of particular agents *in vivo* and alterations observed in the properties of treated cells *in vitro*. In this respect it is important to determine first whether known teratogenic agents affect specific functions of cultured cells derived from both target and non-target organ systems. For example does a teratogen which induces abnormalities of the eyes but not the limbs only affect cultured cells derived from the former organ system? To investigate these phenomena *in vivo*, chick embryo was chosen as an experimental model since the direct effect of a given teratogenic agent can be easily monitored. Using *in vitro* methods an attempt was made to establish the possible correlations between *in vivo* and *in vitro* experiments. For this, following investigations were made:

(1) Do teratogens exhibit a tissue specific effect *in vitro*, as they do *in vivo*?

(2) Is the effect of a particular teratogenic agent dependent on both the level administered and on the stage of differentiation of a tissue at the time of treatment, as it does *in vivo*?

(3) Is the effect of a teratogen *in vitro* influenced by the genotype of the cells since, *in vivo* it depends, in certain instances, on the genotype of the individual. With respect to the latter phenomenon only the effect of insulin on cultured lens epithelial cells derived from the two genetically different chick strains, hyperplastic (Hy-2) and a normal control (Db) was investigated.

In order to determine the validity of the cell-based assay system for the screening of teratogenic agents, the effects of several selected agents on the gross morphological development of chick embryos

were compared with their effect *in vitro*. The parameters which were measured *in vitro* included cell growth and synthesis and accumulation of water-soluble proteins in cell cultures derived from limb, kidney and ocular tissues (lens epithelium and neural retina). For certain agents, these parameters were also measured in cultures derived from brain tissue. The agents whose effects on the above parameters to be investigated, included insulin, diphenylhydantoin, chloroquine sulphate, chlordiazepoxide HCl, meprobamate, cobaltous chloride and lead nitrate.

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Animals

Day-old chicks of the broiler strain with hyperplastic lens epithelium, Hy-2 and those of a normal egg-laying control strain (Db) were obtained from Sterling Poultry Products Ltd., Newbridge, Midlothian, and H. and N. Pfizer Incorporated, Dunbar, East Lothian, U.K. The Hy-2 strain is an F1 cross between two inbred strains, each of which has been selected for a high growth rate for over 25 years (Clayton 1975).

2.1.2 Eggs

Unincubated fertilized eggs from egg-laying chick strains were obtained from Marshall Ltd., Newbridge, Midlothian, U.K.

2.1.3 Tissue Culture

Nuclon plastic culture dishes were obtained from Gibco Bio-Cult Ltd., (Paisley, U.K.). Eagle's minimal essential medium (EMEM), Earle's Salts, Foetal calf serum (FCS), Penicillin-streptomycin solution (100 iu/ml) and L-glutamine (2mM) were obtained from Gibco Bio-Cult Ltd., Paisley, U.K.

2.1.4 Enzymes

Deoxyribonuclease 1 (DNase-1) from bovine pancreas and trypsin were both obtained from the Sigma Chemical Co., Ltd., London.

2.1.5 Radiochemicals

A tritrated (^{3}H) amino acid mixture and L-(^{35}S) Methionine were purchased from the Radiochemical Centre, Bucks., U.K.

2.1.6 Immunological Reagents

Specific antisera against the chick lens crystallins (α, β and

δ) were prepared according to Campbell, Clayton and Truman (1968).

Sheep red blood cells in Alsever solution was purchased from Oxoid Ltd., U.K.

2.1.7 Polyacrylamide Gel Electrophoresis

Acrylamide, ammonium persulphate, NN¹-Methylene bis acrylamide, Sodium dodecylsulphate (SDS) and N, N, N¹, N¹-tetramethylene diamine (Temed) were purchased from the British Drug Houses (BDH), U.K. Tris hydroxymethyllaminomethane was obtained from Sigma Chemical Co., Ltd., London.

Coomassie brilliant blue was obtained from GT. Gurr, Bucks., U.K.

The molecular weight markers aldolase, α-phosphorylase, chymotrypsinogen and Cytochrome-C were obtained from Boehringer Corporation, (London) Ltd., Actin (G-actin, lyophilized powder form, from chick leg muscle) and Ribonuclease A were purchased from Sigma Chemical Co., Ltd., London. Bovine serum albumin was from BDH, U.K.

2.1.8 Scintillation Chemicals

2,5-diphenyloxazole (PPO), Dimethyl POPOP (1, 4-bis- -2 (4-methyl-S-phenyloxolyl) benzene, Toluene and Triton X-100 were supplied by Koch-Light Laboratories, Bucks, U.K.

The Triton/toluene scintillator used contained 25g PPO and 1.5g dimethyl POPOP in 3.5L toluene and 1:5L Triton X-100.

2.1.9 Selected Agents

Insulin (In) (bovine crystalline) was obtained from BDH Chemicals Ltd., U.K., as a crystalline powder (10mg = 230iu).

Diphenylhydantoin (DPH) (phenytoin sodium) marketed as Epanutin was supplied by Park, Davis and Co., Pontypool, U.K. as a solution (50mg/ml) for clinical use.

Chloroquine (CQ) (Chloroquine sulphate) marketed as Navaquine was obtained from May & Baker, Ltd., Dagenham, U.K. as a solution (40mg /ml) for clinical use.

Meprobamate (MB) (Equanil) was purchased as a crystallin powder form from Wyeth Laboratories, Maidenhead, U.K.

Chlordiazepoxide (CDP) hydrochloride marketed as Librium was obtained in powder form together with ampoules of solvent for the use of intramuscular injection from Roche Products Ltd., Welwyn, U.K.

Cobaltous Chloride ($CoCl_2$) as a crystallin powder was purchased from BDH Laboratory Chemicals, Ltd., UK.

Lead Nitrate (LN) was obtained from BDH Laboratory Chemicals Ltd., U.K.

2.1.10 General

Bouin's Fixative was purchased from Searle Co., Essex, U.K. Ethanolamine (pure) and 2-mercaptoethanol were obtained from Koch-Light Laboratories Ltd., Bucks, U.K. Magnesium chloride was from Fisons Analytreal Reagent, U.K. Sodium chloride, Sodium acetate and boric acid were obtained from Sigma Chemical Co., Ltd., U.K. Sodium thiocyanate and calcium chloride came from BDH Laboratory Chemicals Ltd., U.K. CNBr-activated Sepharose 4B gel powder was manufactured by Pharmacia Fine Chemicals AB, S-75104 Upsala 1, Sweden.

2.2 METHODS

2.2.1 Treatment of Developing Chick Embryos

Fertile eggs from egg-laying chicks were incubated at 38°C. The developing chick embryos were treated at various stages with one single injection (unless otherwise stated) of the agents under test by

injecting onto the choiroallantoic membrane (CAM).

Cobaltous chloride, Lead nitrate and Meprobamate powders were dissolved in saline (0.9% NaCl). Librium was prepared in the solvent supplied by the manufacturers. Diphenylhydantoin and Chloroquine was diluted in saline to the required concentrations used. The agents that were dissolved in saline were sterilized by passage through a 0.22μm millipore filter. The pH of the solutions was adjusted to within the range of 6 to 8 using either 0.1M HCl or 0.1 N sodium hydroxide. The total volume of each agent injected ranged from 0.05ml to 0.2ml. The eggs were positioned with the blunt end uppermost for two hours before treatment in order to allow the embryo to appear underneath the air space. After sterilizing the shell with 70% ethanol, a hole was made into the air space at the blunt end of the egg. A small piece of the second shell-membrane was removed from over the embryo, and the eggs treated under sterile conditions by injection onto the chorio-allantoic membrane. In all experiments the eggs were divided into four groups, those injected at 48 hours, 108 hours and 192 hours stages of incubation and finally control eggs injected with 0.9% NaCl at these three stages. Following treatment the hole was sealed with transparent sellotape. Both the treated and control eggs were incubated until harvesting in a humid atmosphere at 38°C. From the 6th day the viability of both the treated and control embryos was monitored, and all embryos dying prior to day 18 were examined for any external or internal gross malformations.

In all <u>in vivo</u> embryo studies in order to establish a dose-response test, in most cases three concentrations of each agent were administered. These being (a) non-toxic level, (b) a concentration

with an LD50, and (c) a high level with an LD 70%.

2.2.2. Staining of the Intact Chick-Embryo Skeleton In Vitro

The staining method used was essentially a modification of those methods described by Burdi (1965) and Burdi and Fecker (1968). The 17-20 day old embryos were fixed in 95% ethanol, then the skin, fat, viscera and brain were removed. The remains were then soaked in acetone for 48 hours to extract the remaining fat.

To stain the cartilage the specimens were washed with tap water for 30 minutes then fixed in FAA (Formalin, 1; acetic acid, 1; 70% ethanol, 8) for 1 hour. After washing in tap water for 30 minutes the specimens were stained for 48 hours in a 0.12% toluidine blue solution in 70% ethanol. The soft tissues were then gradually destained for periods of 24 hours in solutions of increasing percentages of ethyl alcohol (35%; 50% and 70%).

Toluidine blue stained specimens were counter-stained in 30% freshly-prepared aqueous potassium hydroxide containing 0.001% alizarin red. The specimens were transferred daily to a fresh solution of stain until the bones were stained to the desired intensity and the soft tissues had destained completely.

The stained skeletons were stored in glycerin.

2.2.3 Cell Cultures

All tissue culture work was done in a sterile horizontal Laminar Flow-Hood (Slee Co., Ltd., London).

2.2.3.1 Lens Epithelium (LE)

In order to establish lens epithelial cell cultures, eyes were removed from day-old chicks and sterilized in 70% ethanol for 30 seconds. The eyes were then washed three times with sterile saline

(0.9 NaCl), once in phosphate buffered saline (PBS) and then left in PBS. After cutting the eyeball into two parts LE tissue was dissected, using a microscope, from the anterior lens containing part in a calcium and magnesium free Hank's saline (CMF) solution.

The lens epithelial cells were dissociated in a 0.05% solution of trypsin in CMF by incubating for 30 minutes, at 37°C. The cells were then washed three times in PBS supplemented with 10% Foetal calf serum (FCS), and the cell number was estimated using a Rosenthal haemocytometer.

The cells were seeded at a concentration of 0.75×10^5 cells per ml and grown in 58mm culture dishes containing 4ml of medium (Earle's minimal essential medium) supplemented with 6% FCS according to Okada, Eguchi and Takeichi (1971). The cultures were maintained at 37°C in a humidified atmosphere of 95% air and 5% Co_2 for periods of up to 10 weeks and the medium renewed every 2-3 days.

2.2.3.2 Neural Retina (NR)

Neural retina cell cultures were established from 8 day old embryonic tissue. Chick embryo heads were washed and sterilized as described previously (section 2.2.3.1). The vitreous and lens were removed from the eyes and that part of the eyeball containing the NR tissue was dissected out in CMF. The remaining procedure was as described previously (section 2.2.3.1).

The dissociated NR cells were seeded at the concentration of 1.25×10^6 cells/ml in 58mm culture dishes containing 4ml of medium (EMEM) supplemented with 10% FCS according to Okada et al., (1975). The medium was renewed every 2-3 days and the cultures grown for periods of up to seven weeks.

2.2.3.3 Kidney and Limb Fibroblast Cell Cultures.

Kidney and limb fibroblasts were dissected from 8.5 day old chick embryos and treated by the same procedures as used for NR cultures. The dissociated cells were seeded at a concentration of 1.25×10^5 cells/ml and grown for periods up to 3 weeks under the same conditions as for NR.

2.2.3.4 Limb Bud Cell Cultures

Cell cultures were established from whole limb buds of 4.5 day old chick embryos, as described in section 2.2.3.2. The cultures were grown for up to 3 weeks, and treated from day four until the last day, when they were terminated.

2.2.3.5 Brain Cell Cultures (BR)

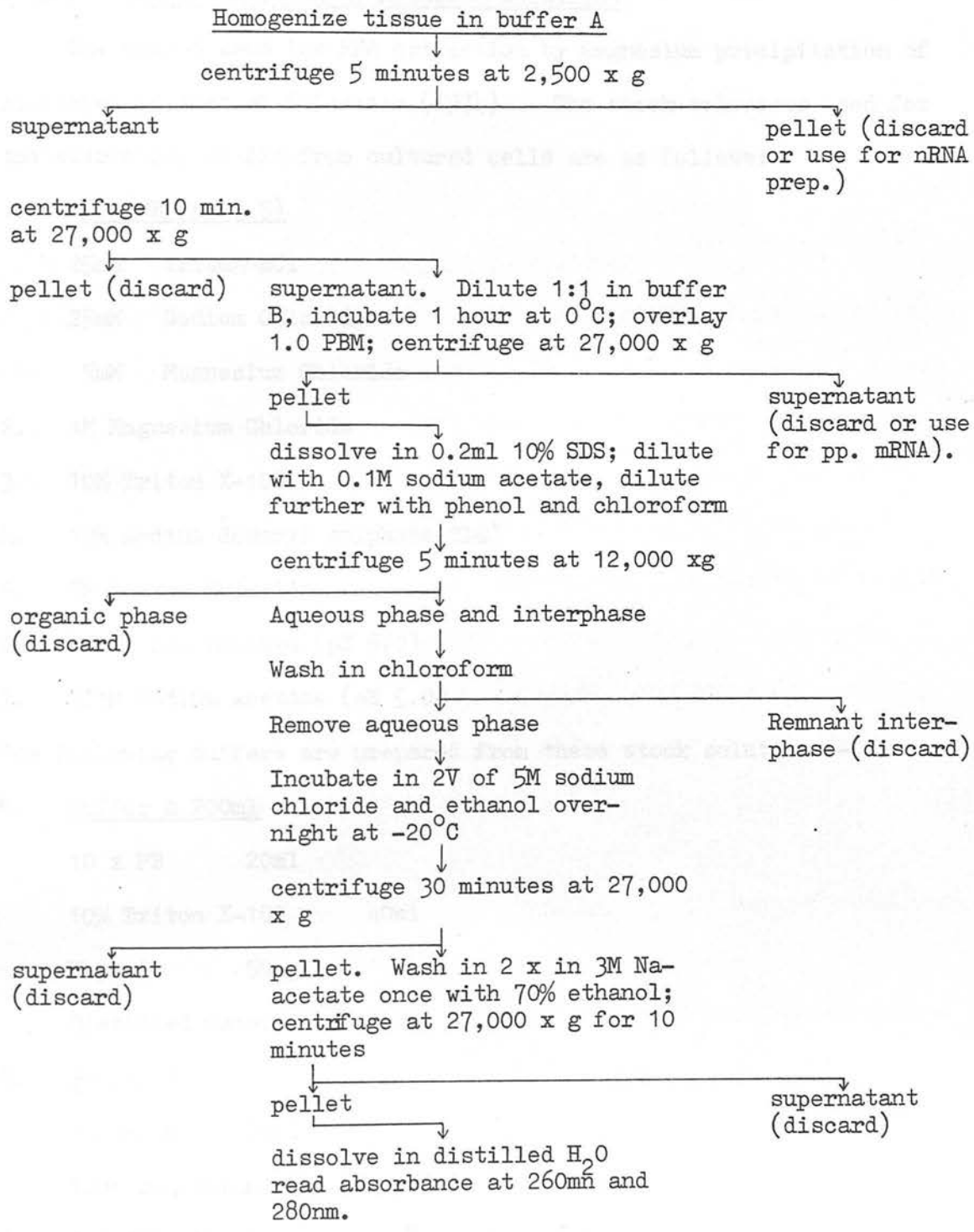
Brain cell cultures were established from 8 day old chick embryo brain. The whole brain was dissected out in CMF following sterilization of the heads.

The dissociation and seeding of the brain cells was identical to that described for NR cell cultures. The medium was renewed every 2-3 days and the cultures grown for periods up to 5 weeks. The cultures were treated from day 4 until the last day every 2-3 days.

2.2.4 Treatment of Cell Cultures

With studies involving cell cultures, all the selected agents investigated were freshly prepared at the desired concentrations in culture medium. The level of administration of a particular agent used was that which was subtoxic to the cultured cells.

FLOW SHEET FOR PREPARATION OF mRNA



Scheme 1: mRNA extraction procedure.

2.2.5 The Extraction of RNA and the Translation of mRNA in a Reticulocyte Lysate Cell-free System.

2.2.5.1 The Preparation of Required Solutions

The method used for RNA extraction by magnesium precipitation of ribosomes is that of Palmitter (1974). The stock solutions used for the extraction of RNA from cultured cells are as follows:

1. 10 x PB (pH 7.5)

   25mM Trizma-HCl

   25mM Sodium Chloride

   5mM Magnesium Chloride

2. 1M Magnesium Chloride
3. 10% Triton X-100
4. 10% Sodium dodecyl sulphate(SDS)
5. 5M Sodium Chloride
6. 3M Sodium acetate (pH 6.0)
7. 0.1M Sodium acetate (pH 5.0)

The following buffers are prepared from these stock solutions:-

1. Buffer A 200ml

   10 x PB 20ml

   10% Triton X-100 40ml

   Heparin 50μg

   Distilled water 140ml

2. Buffer B 100ml

   Buffer A 80ml

   1.0M magnesium chloride 20ml

3. 1.0 PBM 100ml

   sucrose 34.23g (1M)

   10 x PB 10.0 ml

   1.0M magnesium chloride 10.0ml

   distilled water 40.0ml

These buffers were treated with diethylpyrocarbonate (50μ/100ml) to destroy any RNAases. To inactivate any remaining diethylpyrocarbonate the buffer solutions were autoclaved for 15 minutes. All solutions were stored at 2-4°C, except sodium dodecylsulphate which was stored at room temperature.

2.2.5.2 Homogenization and Extraction Procedure

Tissues (0.3g) from cultured cells were homogenized in 3ml of buffer A in a Dounce homogenizer (Kontes glass Co.), by 25 strokes with a tight pestle. The homogenate was centrifuged for 10 minutes at 27,000g at 2°C, the supernatant decanted into a corex tube and an equal volume of buffer B was added. Following incubation in an ice bath for at least one hour, the supernatant was layered over 2.4ml pad of 1M sucrose solution in a 15ml corex tube. This was centifuged at 27,000g for 10 minutes at 2°C. From the pellet polysomal mRNA was extracted and from the supernatant the post-polysomal mRNA was extracted. The extraction procedure is outlined below.

2.2.5.3 Extraction of mRNA

To keep sodiumdodecyl sufate in solution, all subsequent steps were performed at room temperature, unless otherwise stated.

For the extraction of polysomal mRNA, the pellet was dissolved in 0.2ml of 10 SDS, then 4ml of 0.1M sodium acetate was added and the mixture agitated on a Vortex mixer for 10-20 seconds. To precipitate any protein, Liquified phenol (4ml) was added and the tube shaken, then 4mls of chloroform added and the resulting mixture shaken again. This was then centrifuged at 12,000g for 5 minutes at 20°C. Three separate phases were identifiable, these being a clear upper aqueous phase (containing the RNA), an interphase and lower chloroform/phenol organic

phase (containing any protein). The latter phase was aspirated with a Pasteur pipette and then discarded. Chloroform (8ml) was added to the remaining mixture, agitated, vortex mixed and centrifuged as above. The organic phase was again discarded. This procedure was repeated several times until the interphase either disappeared or no longer changed. The upper aqueous phase was removed and mixed with 4 volumes of 5M sodium chloride and 2 volumes of ethanol and the RNA precipitated overnight at -20°C.

To precipitate the RNA the mixture was centrifuged at 27,000g for 30 minutes at 2°C. The pellet was washed twice in 3M sodium acetate then several times with 70% ethanol each time centrifuging at 27,000g for 2 minutes at 2°C.

The pellet was dissolved in 100µl of distilled water and spectrophotometer absorbance readings made at 260nm and 280nm. The reading at 280nm should be less than that at 260nm. If not the results indicate that protein is still present, and therefore further purification of the RNA is required. Quantification of the RNA was based on the 260nm reading and was calculated according to the formula: 1OD unit equals 35µg RNA/ml.

2.2.5.4 Reticulocyte Lysate Cell-free Protein Synthesis

The messanger RNA-dependent reticulocyte lysate translation procedure of Pelham and Jackson (1976) was used for the cell-free protein synthesis. Rabbit reticulocyte lysate was purchased from the Radiochemical Centre, Amersham, U.K.

An "assay mix" comprising 10µl rabbit reticulocyte lysate and 25µl (^{35}S) methionine (5µCi/ml) was prepared. For one assay, 20µl of the

assay mix was added to 2µl total RNA in an 0.7ml plastic tube, (Walter, Sarstedt, W. Germany), then vortexed and incubated at 37°C for 30 minutes. A control tube containing no RNA was treated identically. Following incubation, the reaction was terminated by placing the samples in an ice bath.

2.2.5 Protein Assay

Aliquotes (2µl) from each sample were taken before incubation at zero time and again after 30 minutes of incubation. These were placed on separate glass filter discs (Whatman GF/C), and the proteins precipitated by boiling the filters in 10%-trichloroacetic acid for 10 minutes. The filters were then washed twice with water, twice with 100% ethanol then twice with acetone.

After drying, the filters were transferred to scintillation vials and 5ml of Triton/toluene scintillation fluid was added. The level of radioactivity in each vial was counted in an Isocap/300 liquid scintillation counter (Dunley Instruments Ltd., Sussex, U.K.). The difference between the counts of the samples obtained at zero-time and 30 minutes of incubation was calculated, this being the level of translation.

2.2.6 Preparation and Analysis of Water-Soluble Proteins

Following harvesting, tissues from chick embryos and cultured cells were washed in saline containing 10mM 2-mercaptoethanol then centrifuged at 1500rpm. The pellets were stored in liquid nitrogen until analysis. After thawing, the pellets were homogenized in an equal volume of saline containing 10mM 2-mercaptoethanol then centrifuged at 10,000g for 30 minutes at 4°C in an Ependorf bench centrifuge.

The protein concentration of the supernatant was measured by the

method of Bradford (1976). Bovine serum albumin (1mg/ml) was used as a protein standard and the concentration of protein estimated following spectrophotometer absorbancy readings at 595nm. The proteins were analysed by SDS polyacrylamide gel electrophoresis and some by haemagglutination inhibition assay.

2.2.7 Gel Electrophoresis

Proteins were analysed by a modification of the SDS polyacrylamide slab gel electrophoresis described by Studier (1973) and Laemmli (1970).

Stock Solutions

1. Acrylamide Solution

30% acrylamide

0.8% bis-methylene acrylamide

2. 2 x Separating (lower) gel Buffer (pH 8.8)

0.75M Tris-HCl

3. 5 x Stacking (Upper) Gel Buffer (pH 6.8)

0.25M Tris-HCl

4. 10% Sodium dodecyl sulfate (SDS)

5. 10% ammonium persulphate (always freshly prepared).

All solutions were stored at room temperature except the acrylamide solution which was stored at 2-4°C.

2.2.7.1 Preparation of Polyacrylamide Gel

The lower separating gel (11% acrylamide) was prepared by mixing the following solutions.

acrylamide solution	20ml
2 x buffer	25ml
10% SDS	0.5ml
distilled water	9.0ml
10% ammonium persulphate	0.2ml

Following mixing and degassing 20µl of polymerising catalyst N,N, N', N'-tetramethylene diamine (Temed) was added and the gel immediately poured. This running gel was then overlaid with distilled water and allowed to set for 1 hour. The water was then replaced by a 2cm deep stacking gel.

The 3% acrylamide gel was prepared by mixing the following solutions:

acrylamide solution	2.5ml
5 x buffer	5.0ml
10% SDS	0.25ml
distilled water	17.0ml

Polymerisation was achieved by the addition of 0.1ml of 70% ammonium persulphate and 10µl of temed.

To make sample loading wells, a perspex comb was placed into the stacking gel before polymerization. Following polymerization the comb was removed.

2.2.7.2 Application of Samples

Protein samples from both cultured cells (50 - 100µg) and from the cell-free translation system (20,000-40,000 CPM) were mixed with an equal volume of sample buffer (0.12 M tris, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol and 0.002 - 0.004% Bromophenol blue) and boiled for 2 minutes, to denature all the protein. The samples were then loaded. Samples of day-old chick lens water-soluble proteins were used as standards. The electrode buffer (pH 8.3) used contained 0.025 M tris, 0.192 M glycerine, and 0.1% sodium dodecyl sulfate.

Electrophoresis was carried out on a constant current (10mA) until the bromophenol blue dye marker entered the separating gel, then it was set to 30mA. Electrophoresis was complete after 4-6 hours, once

the marker dye reached the bottom of the gel.

2.2.7.3 Fix-Staining of the Proteins

The electrophoresed proteins were fixed and stained for 30 minutes at 65°C in 50% trichloroacetic acid solution containing 0.25% Coomassie brilliant blue G. The gels were then destained in 8% glacial acetic acid at 65°C and stored in 7% glacial acetic acid.

2.2.7.4 Photography of Stained Gels

The stained gels were photographed on Ilford Pan F films (ASA50) using a yellow filter. From the negatives, prints and positive transparencies were made, to be used for scanning the gels.

2.2.7.5 Fluorography

Protein bands labelled with tritiated (^{3}H) amino acids and (^{35}S) methionine were identified by fluorography carried out at -70°C, (Bonner and Laskey, 1974; Laskey and Mills, 1975), using a pre-exposed Kodak X-Omat RP film.

Developed fluorographs were photographed as above (2.2.7.4) and positive transparencies made.

2.2.7.6 Scanning of Gels

Positive transparencies of gels and fluorographs were scanned with a Kipp and Zonen/Skalar integrating densitometer (Model K53 micro-densiscan, FT Scientific Instruments, Ltd., Yateley, U.K.) with a Bryans XY/t recorder (Model 25000, Bryans Southern Instruments Ltd., Surrey, U.K.) equipped for automatic integration of peak areas.

Scanning was performed through a 0.1mm slit-width at a sensitivity of 10MV/CM at 1cm/sec.

2.2.8 Molecular Weight Estimation

The molecular weights of the electrophoresed proteins were estimated using α-phosphorylase (m.w. 95K daltons), bovine serum albumin (m.w. 66K daltons), aldolase (m.w. 43K daltons), actin (m.w. 43K daltons), chymotypsinogen (m.w. 25.7K daltons), ribonuclease A (m.w. 13.9K daltons) and Cytochrome C (12.5K daltons) as molecular weight standards.

2.2.9 Haemagglutination-Inhibition Assay

The method used was that described by Evans, Stell and Arthur (1974) and further modified by de Pomerai, Pritchard and Clayton (1977).

2.2.9.1 Coating of Sheep Erythrocytes with Day-old and Adult Chick Lens Antigens

Sheep red blood cells were washed three times in PBS and centrifugation was carried out at 3,000rpm for 5 minutes. The cells (0.5ml) were resuspended in PBS (9ml) and a mixture of day-old and adult lens antigens (8-10mg) added and the mixture while being gently stirred at room temperature, 0.25% gluteraldehyde (1ml) was slowly added over a 15 minute period. After mixing for 1 hour, the coated cells were washed three times with PBS as described above.

The antigen coated erythrocytes were stored as a 2% suspension in PBS at 4°C.

2.2.9.2 Haemagglutination Test

In order to calculate the optimum dilution of the α, β and δ antisera to use in haemagglutination-inhibition test, an antibody dilution test was done. A three-fold serial dilution of each antibody (4μl) was incubated with the coated erythrocytes (8μl) (the indicator cells) for 1 hour at room temperature. The end point of haemagglutination was the well in which the inhibition of agglutination was evident.

The dilution of the antibody used for haemagglutination-inhibition test was one third of the observed end-point dilution for haemagglutination test.

2.2.9.3 Haemagglutination-Inhibition Test

A two-fold serial dilution of each antigen test sample in duplicate (8µl) and antiserum (8µl) were incubated for 45 minutes at room temperature. The indicator cells (8µl) were then added and the mixture was incubated for 2-4 hours. The end-point dilution at which haemagglutination was no longer inhibited by the test antigen was marked and compared with that from parallel assays on serial dilutions of a standard containing total day-old chick lens antigens.

The concentration of a given antigen (Ag) in the test sample was quantified to the same antigen concentration in the day-old chick lens by the aquation below:-

$$Ag = \frac{AgS \times EPt \times 1000}{EPtS \times AgT}$$

Ag: Antigen under test

AgS: Concentration of standard antigen

EPt: End point of antigen under test

EPtS: End point of standard

AgT: Concentration of the test antigen in the standard sample

The calculations were based on the values previously calculated by de Pomerai, Clayton and Pritchard (1978) based on the finding that the soluble proteins of day-old chick lens are comprised of approximately 60% δ crystallin, 25% β crystallin and 15% α crystallin.

2.2.10 CNBr-Activated Sepharose 4B Column

The column was prepared using lyophilized powder of CNBr-activated sepharose 4B.

The following solutions were used:

1. 10^{-3}M hydrochloric acid
2. Coupling buffer (pH 8.3)
   - 0.1M borate
   - 0.5M sodium chloride
3. Ethanolamine buffer (pH 8.5)
   - 1.0M Ethanolamine
   - 0.1M borate
   - 0.5M sodium chloride
4. Sodium acetate buffer (pH 4.0)
   - 0.1M sodium acetate
   - 1.0M sodium chloride
5. Borate buffer (pH 8.5)
   - 0.1M borate
   - 1.0M sodium chloride
6. Buffer A (pH 7.2)
   - 0.1M tris-hydrochloride
   - 0.5M sodium chloride
7. Buffer B (pH 9.0)
   - 0.2M tris-hydrochloride
   - 0.5M sodium chloride
   - 1.0M sodium thiocyamide

CNBr-activated sepharose 4B (0.8g) was swollen with hydrochloric acid (10^{-3}M) on a glass filter then washed with the same solution (200ml) for 15 to 20 minutes.

DNase-1 (10mg) was dissolved in the coupling buffer then mixed with the washed gel in a corex-tube. To couple the DNAse-1 to the gel, the mixture was incubated at 2 - 4°C for 18 hours. Following coupling any excess enzyme was washed from the gel with coupling buffer. To get rid of any active groups the mixture was treated with 1M ethanolamine buffer for 2 hours at room temperature. Then to remove any non-covalently absorbed material from the gel, the gel mixture was washed alternatively, four times, with sodium acetate and borate buffers.

Using a 5ml syringe the gel mixture was poured between two Whatman-glass microfiber papers (GF/A, 2.1cm). Once set up, the column could either be used immediately or stored for several months in the buffer A.

Prior to sample loading, the column was washed several times with the buffer A. Water-soluble proteins (1mg) extracted from the lens epithelial cell cultures were then loaded onto the column. The column was then eluted with the buffer A to remove any non-specifically bound proteins. The eluate was collected (sample No.1). The column was then eluted with the buffer B and the eluate collected (sample No.2). To precipitate the proteins, the samples were treated for 30 minutes at 4°C with 10% trichloroacetic acid and then centrifuged at 5000rpm. In order to remove the acid, the pellets were washed three times with 100% ethanol and then dissolved in distilled water. The resulting proteins were analysed by SDS polyacrylamide gel electrophoresis.

3. RESULTS

3.1 In Vitro Differentiation of Chick Tissues.

3.1.1 Lens Epithelium

Cultures of dissociated lens epithelial cells were established from day-old chicks. The cultures established from Db (Dunbar) and Hy-2 (Hyperplastic epithelium) strains reached confluence between 12 and 14 days, respectively, and lentoid body differentiation was apparent at these stages. The number and size of lentoid bodies increased between 12 and 25 days of the culture period (Plate 1A, B and C).

Using SDS polyacrylamide gel electrophoresis, a comparison was made of both the accumulated and the newly synthesized protein components, in the water-soluble extracts of cultured lens epithelial cells from the Db strain after 12, 20 and 25 days of inoculation and these were compared with the components obtained by cell-free translation of mRNAs from these cultures. The results indicate that when lentoids are present the crystallins were found to be increased in importance becoming the major components of the cultured cells. The staining intensity and quantitative densitometric traces of the soluble proteins synthesised in cells indicated that the greatest changes involved the δ_1-crystallin subunit and some of the β-crystallin subunits (β_2 and β_3) which increased significantly in the later stages of culture, over the period of 20 to 25 days (Plate 2B, 2C and 2D).

However, in contrast to the above findings, analysis of the products obtained by cell-free translation of mRNAs from these cultures indicated no change in the ratio of the two δ-crystallin subunits in the later stages of culture nor an increase in β-crystallin subunits (Plate 2E, F and G). Furthermore this data indicated that the relative proportion

of actin mRNA in the total mRNA population from 25-day culture as judged by cell-free translation was decreased significantly compared to the mRNA from either the 12 or the 20 day cultures, whereas the relative amounts of both the accumulated and newly synthesized actin in the cells did not change over the culture period.

Taken together, these data indicate, firstly, that in cells differentiating into fibre cells crystallins are the major products synthesized as judged by analyses both of the protein products in cells and in a cell-free system, secondly, not all the crystallin subunits are increased at the same rate, thus the increase in crystallin polypeptides is differential, and finally, that these proteins accumulate in the cells during terminal differentiation. The quantitative profile of the products obtained by cell-free translation with the profiles synthesized in the cells shows that the available translatable mRNAs are not used with equal efficiency *in vivo*, suggesting that translational regulation is important during lentoid differentiation.

3.1.1.1 Actin in Cultures of Chick Lens Epithelial Cells.

Actin was identified by its capacity for binding to DNase 1. Water-soluble proteins from cells labelled with (^{3}H) amino acids were allowed to flow through a sepharose 4B column to which DNAse 1 was bound as described in section 2.2(Page 52). All proteins non specifically bound to the column were removed by washing. The actin was released by a final wash with the pH 7.5 buffer (Fig. 3) and electrophoresed in SDS polyacrylamide gel. This material was identified in a fluorograph and only one band was found, with a molecular weight of 43 K daltons (Plate 3B). The molecular weight of this band was calculated using both standards of known molecular weight markers (Fig. 2) and

PLATE 1. Cultured Lens Epithelial Cells from Day-old Post Hatch Chick Lenses.

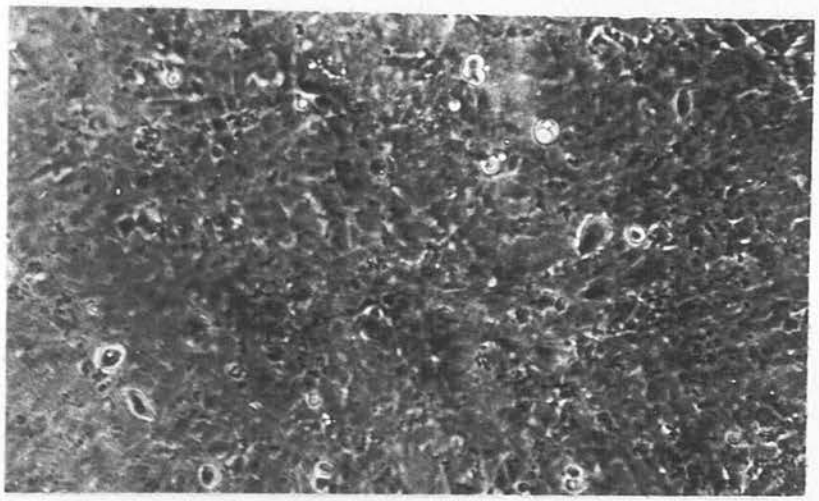
Photographs of various stages of culture (phase contrast 100 x magnification).

A. 12-day culture showing the confluent sheet of epithelial cells.

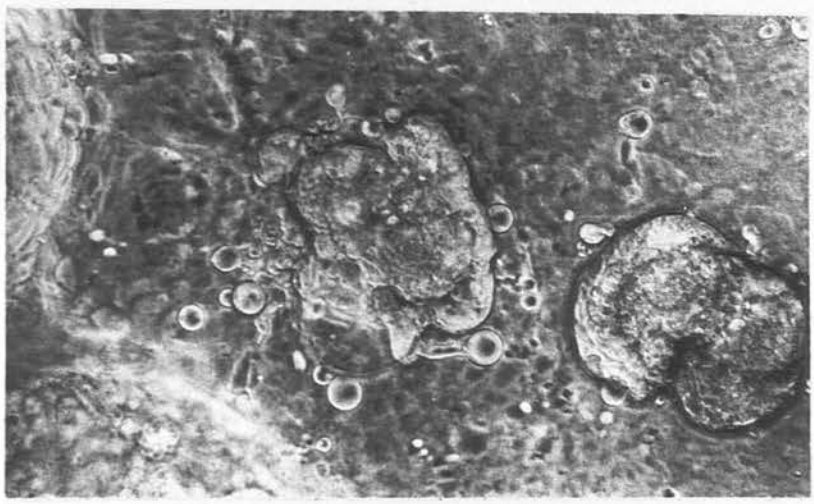
B. 20-day culture showing the appearance of small lentoids with a number of bottle cells around the lentoids.

C. 25-day culture showing large lentoids with a few bottle cells around the periphery.

A



B



C

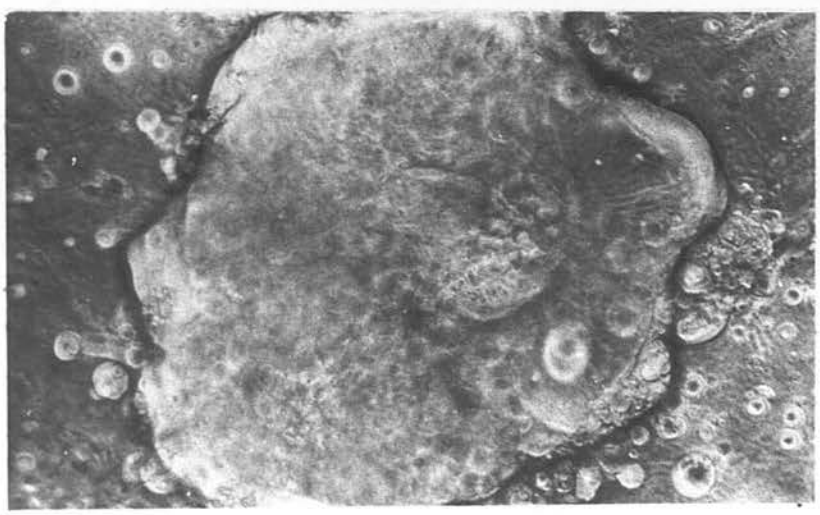


PLATE 1

PLATE 2. S.D.S. Polyacrylamide Slab Gel Electrophoresis (as in Laemmli, 1970) of Accumulated Water Soluble Proteins of the Cultured Lens Epithelial Cells from the Stages in Plate 1.

A. Day-old chick lens proteins as a marker.

B. 12-day culture.

C. 20-day culture.

D. 25-day culture.

E. 12-day culture.
F. 20-day culture.
G. 25-day culture.
} products synthesized in the cell-free translation systems from the mRNA of B, C and D.

The relative proportions of δ1, β2 and β3-crystallin bands is increased as the cultures grow older while the relative proportion of actin is decreased (see also Fig. 1). E. F. G., fluorographs of the proteins synthesised in the cell-free translation system.

Arrows identify the protein bands which show changes.

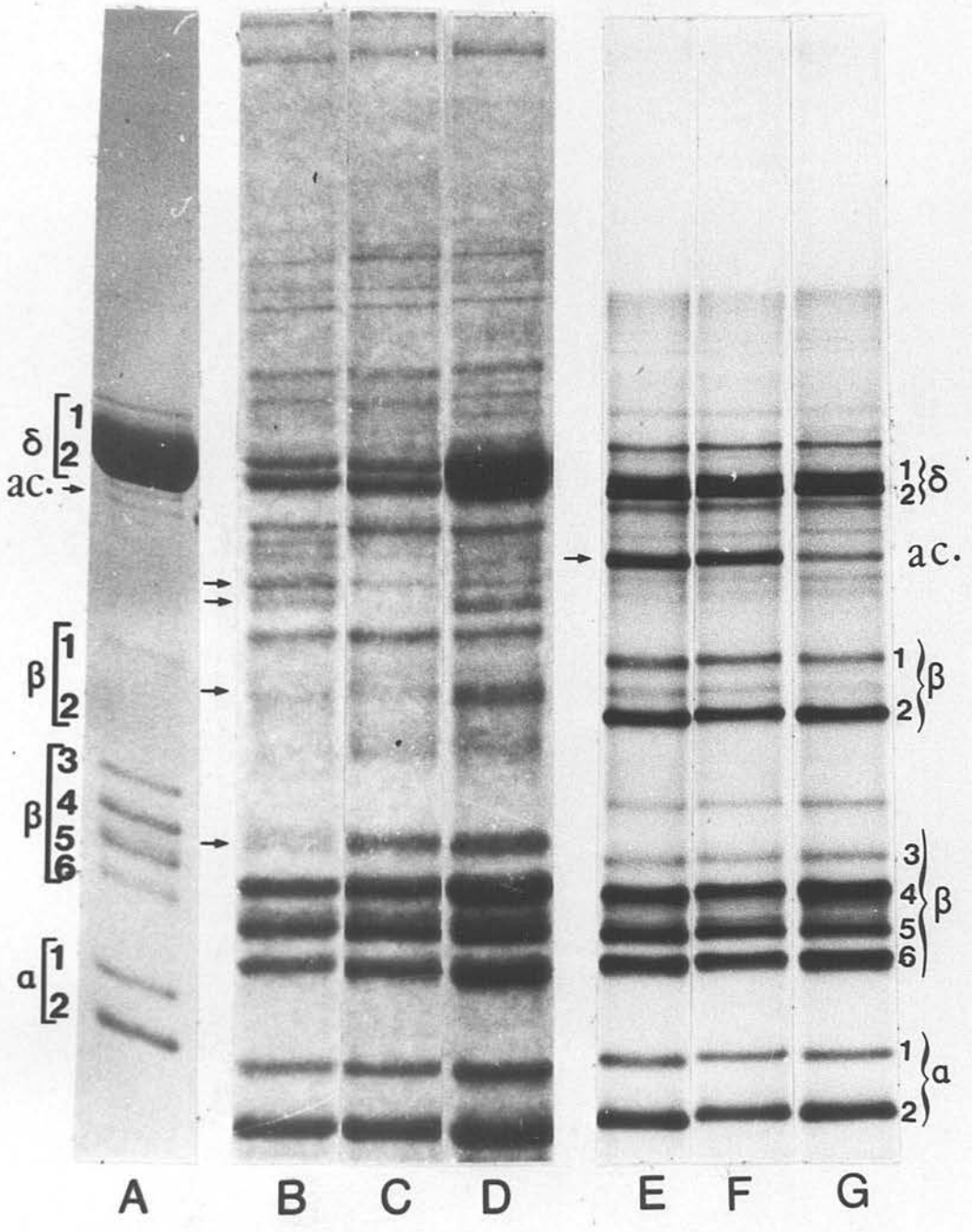


PLATE **2**

FIGURE 1. Traces of plate 2B (a), 2C (b) and 2D (c) respectively. The traces were obtained by an integrating densitometer from positive transparencies of the gels.

Arrows indicate the peaks which show changes in the corresponding protein bands from successive stages of culture.

In all the figures the letters in ( ) represent the traces of the proteins analysed by SDS polyacrylamide gel electrophoresis.

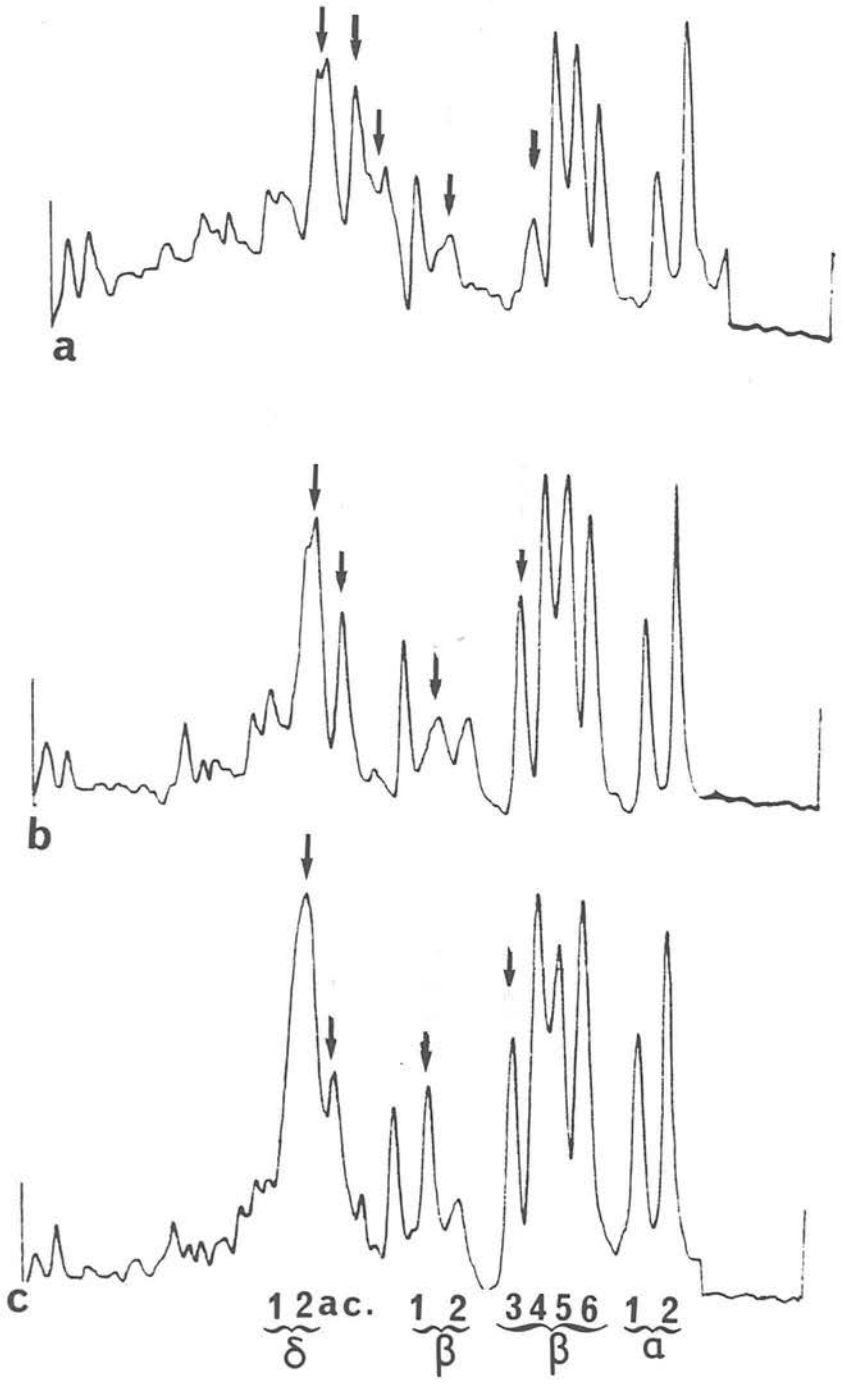


FIG 1

FIGURE 2. Diagramatic Representation of Molecular Weights of Chick Lens Crystallins using Standards of Known Molecular Weights. The log of the molecular weights of the standards was plotted against their migration distance on SDS polyacrylamide slab gel electrophoresis as in plate 2.

MW: Molecular weight.

K: x 10^3.

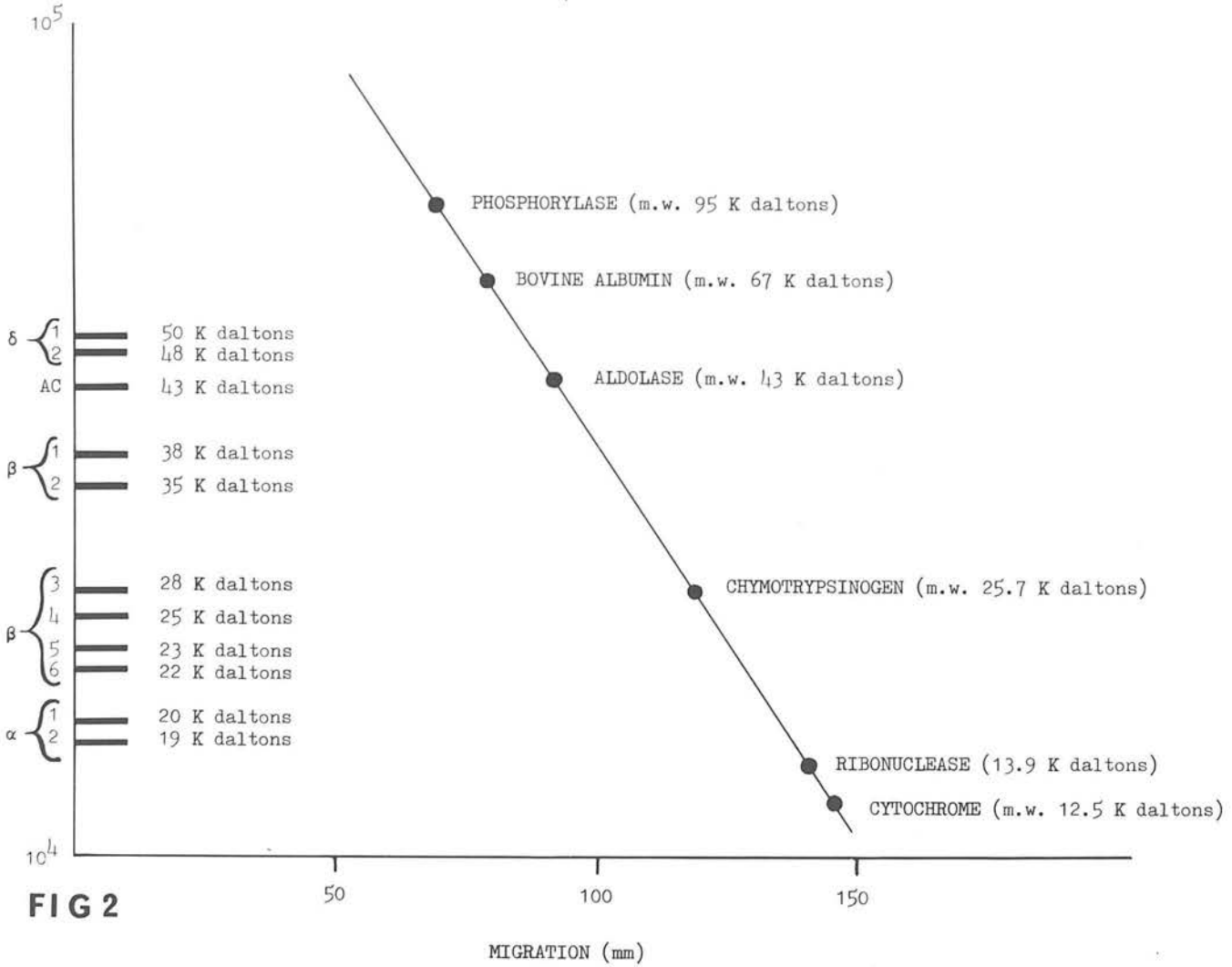

10^5
δ 1 50 K daltons
2 48 K daltons
AC 43 K daltons
β 1 38 K daltons
2 35 K daltons
β 3 28 K daltons
4 25 K daltons
5 23 K daltons
6 22 K daltons
α 1 20 K daltons
2 19 K daltons
10^4
PHOSPHORYLASE (m.w. 95 K daltons)
BOVINE ALBUMIN (m.w. 67 K daltons)
ALDOLASE (m.w. 43 K daltons)
CHYMOTRYPSINOGEN (m.w. 25.7 K daltons)
RIBONUCLEASE (13.9 K daltons)
CYTOCHROME (m.w. 12.5 K daltons)
50
100
150
MIGRATION (mm)

FIG 2

FIGURE 3. Diagramatic representation of the procedure for the purification of Actin on a DNase-sepharose 4B column.
The actin was obtained from the soluble fraction of 15-day cell cultures of chick lens epithelium. The soluble fraction (1mg protein in 0.5ml physiological saline) was diluted with an equal volume of 0.2M Tris, 0.5 NaCl (buffer A, pH 7.2), and applied to the column (1.2 ml containing 10 mg of DN ase-1) packed in 2 ml plastic disposable syringe. The proteins were eluted stepwise from the column with 20ml each of the two buffers shown below and fractions were collected in 2 ml plastic tubes: (1) buffer A. (2) buffer A plus 1.0 M NaSCN (buffer B, pH 9.0). The peak fractions were pooled, TCA precipitated and analysed in polyacrylamide SDS gel electrophoresis as described in Materials and Methods. Peak 1 corresponds to non-specifically bound proteins and peak 2 actin.
The dotted line represents the absorbancy reading for the buffers used.

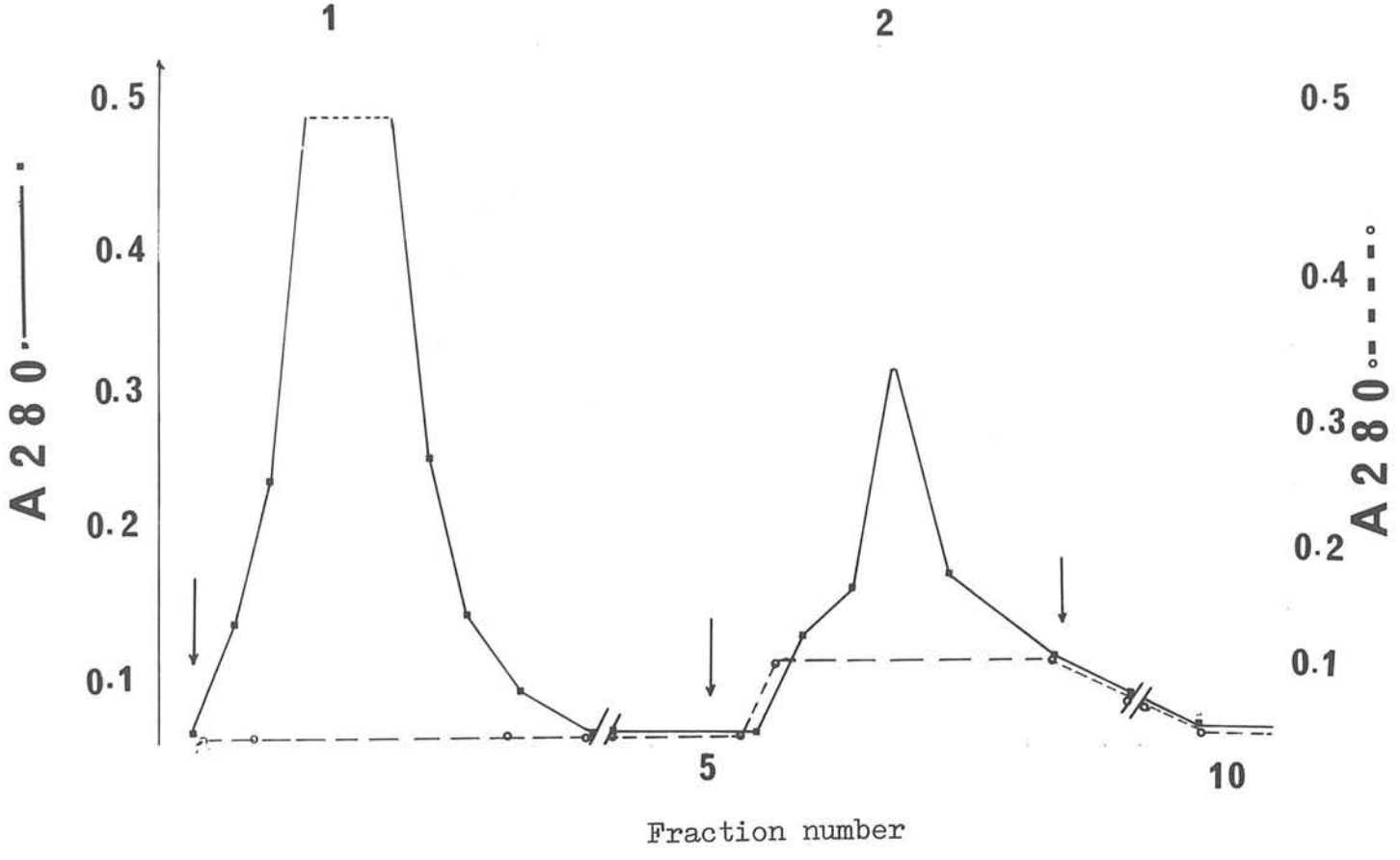


FIG 3

∘ ·· ·· ·· ∘ Absorbance reading for buffers

▪ —— ▪ Absorbance reading for protein sample.

PLATE 3. Actin Band on SDS Polyacrylamide Slab Gel.

The densitometer trace, showing for actin isolated as shown in Fig. 3. (A) Upper (solid line), trace of the water soluble extract of 15-day lens epithelial cell cultures (C). Lower, (broken line), trace of the actin (B) released from the column and run in SDS polyacrylamide slab gel. (D) shows the position of actin on the gel, using commercial preparation of actin from chick leg muscle.

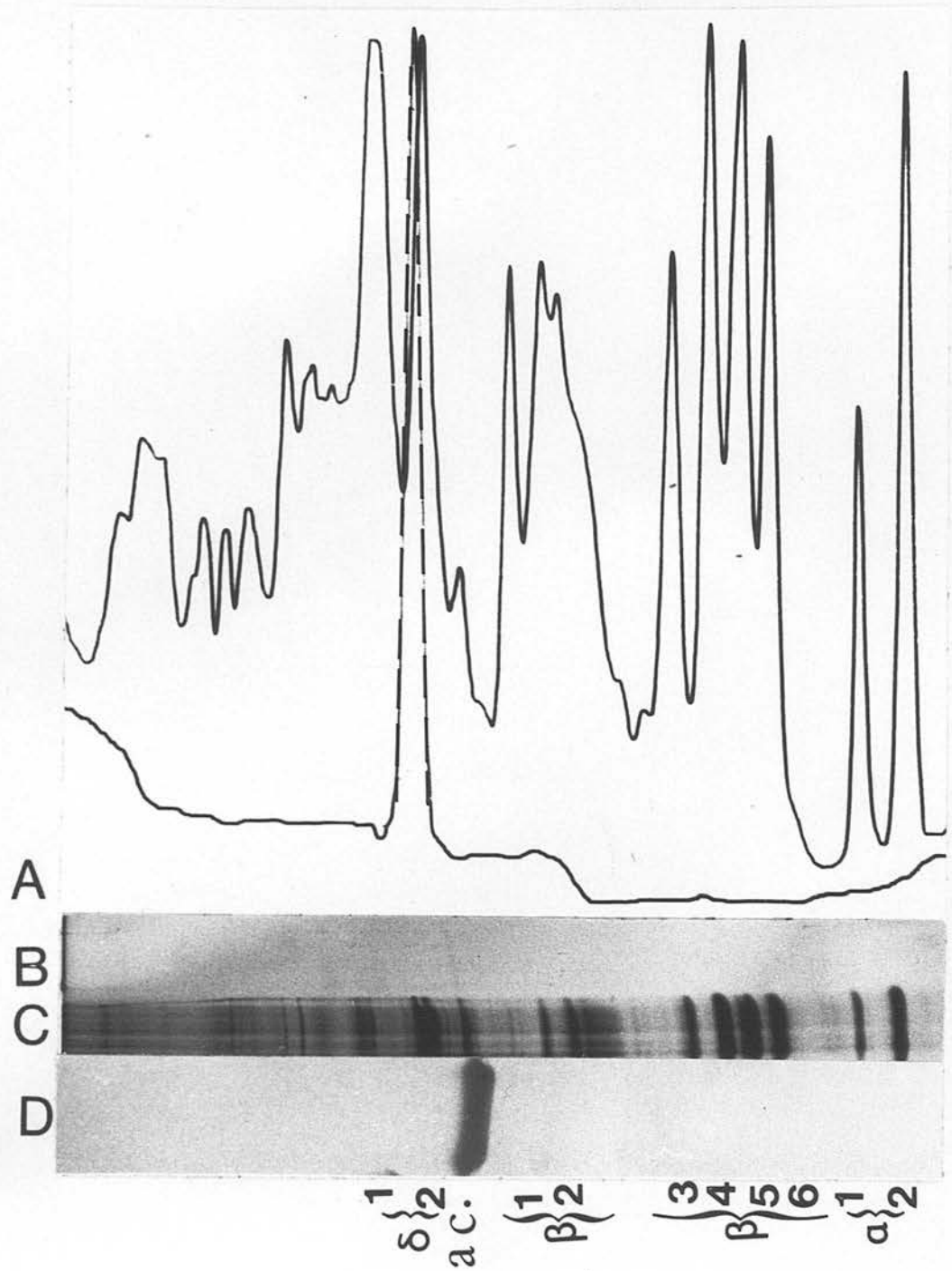


PLATE 3

PLATE 4. SDS Polyacrylamide Slab Gel of the Water Soluble Extracts of Cultured Cells from Various Tissues, to Illustrate the Relative Proportion of Actin during the Stage of experimental cell growth in the culture.

A. Day-old chick lens antigens as a marker.

B. Purified actin obtained commercially from chick leg muscle.

C. Extract of 15-day cultures of lens epithelial cells.

D. Extract of 20-day neural retina culture.

E. Extract of 8-day limb fibroblast cell cultures.

F. Extract of 10-day brain cell cultures. All the samples were analysed as in plate 2.

Actin was identified only in lens epithelial cell cultures, in the other cultures the protein bands having the same M.W. as actin are not as yet immunologically identified in the present study.

Note: β_1 and β_2 are marked with a pen on Lane A to show their migration points.

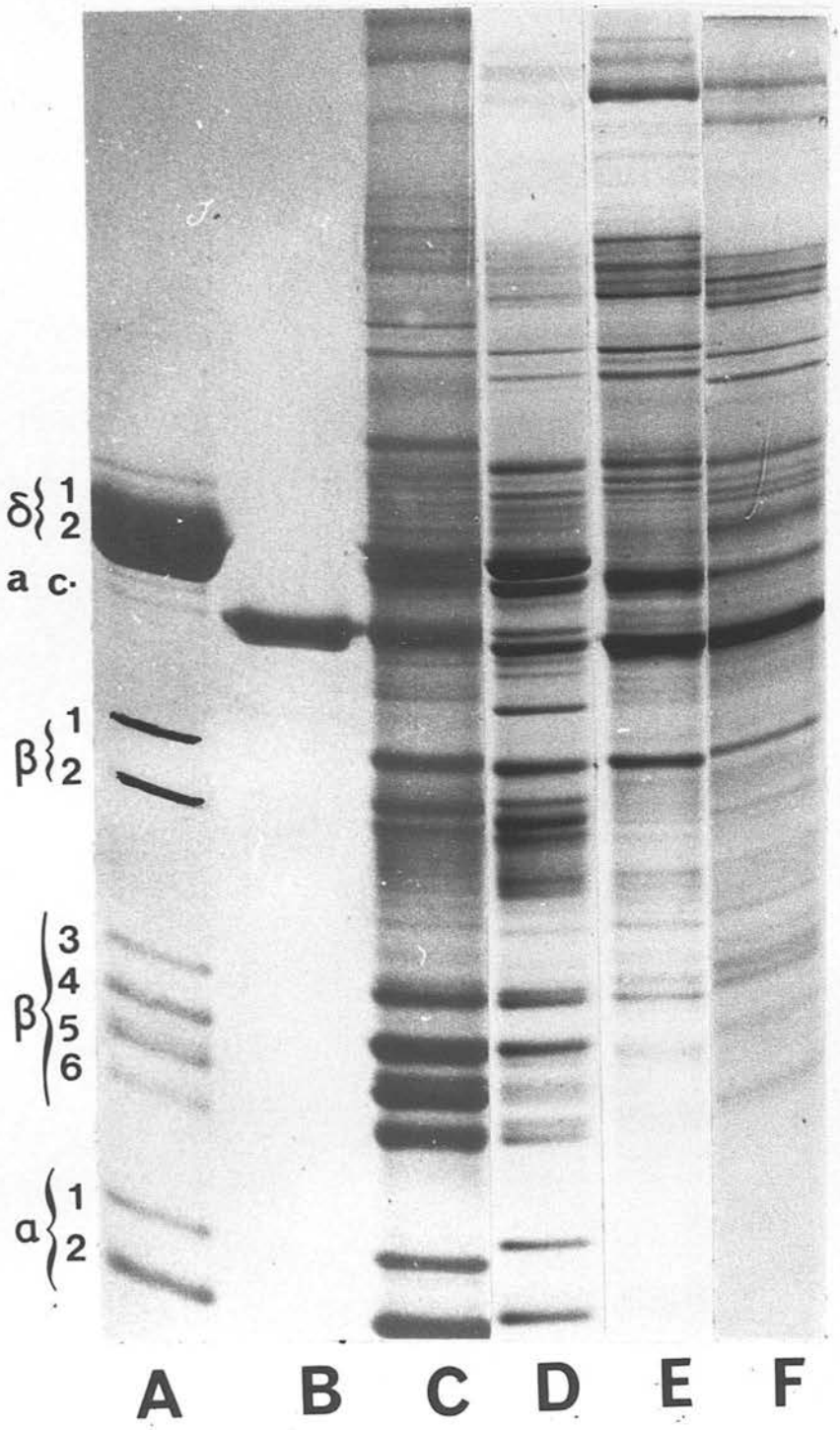


PLATE 4

commercially available pure actin.

Analysis of the water-soluble proteins synthesized and accumulated both in culture and by the cell-free translation system of extracted mRNA(s) indicated that actin is synthesised by lens epithelial cells in culture. Actin was calculated to be 3% of the total water-soluble protein content of the eight-day old chick embryo lens whereas only 0.8 - 1.0% in that of the day-old chick lens. When the water-soluble protein profile of differentiated lens epithelial cells is compared to that of the lens from day-old chicks, it is apparent that actin is a relatively unimportant component in the latter (Plate 2A, B, C and D, and Plate 4A,B and C).

The results indicate that both in differentiated cultures of lens epithelial cells and in the intact lens cells the relative amount of actin declines with increasing differentiation. This is most probably due to the fact that differentiated fibre cells synthesise mostly crystallins.

3.1.2 Neural Retina

Cultures of dissociated neural retina cells were established from 8-day old chick embryos. After 4-5 days neuroblast-like cells with axon-like processes appeared adhering to the established neuroepithelial sheet. From about day 16 most of these neuroblast-like cells were lost from the cultures and a number of transparent swollen cells appeared (pre-lentoids). Fully differentiated lentoid bodies with bottle cells were present from about day 22 (Plate 5A, B, and C).

The analysis of the water-soluble proteins extracted from cells prior to differentiation (days 10-16) when both neuroblast-like and neuro-epithelial (glial) cells are present indicates the presence of 25-35

separate polypeptides. Of these the most abundant was a protein with a molecular weight of 43K daltons (Plate 5A). By day 18 several proteins were apparent with molecular weights similar to those of some of the β crystallins, 25 - 38 K daltons and α crystallins, 18 - 20 K daltons (Plate 5B), and after day 25 following lentoid body differentiation several further major proteins are apparent with molecular weights similar to that of δ crystallins 48 and 50K daltons (Plate 5B and C).

Altogether between days 10-40 of culture a total of 35-40 different molecular weight polypeptides were resolved by SDS polyacrylamide gel elctrophoresis.

3.1.3 Brain Tissue

In cultures of dissociated brain cells from 8-day old chick embryos two major populations of cells were distinguishable by day 3. These consisted of neurone-like cells with long axon-like processes which formed aggregates on an underlying flat neuro epithelial cell sheet (Plate 6A) By day 15 these axon-like processes were lost and the cultures were comprised mainly of the large flat neuro epithelial cells and very few neuron-like cells and by day 30 no neurone-like cell was noted as judged by morphological appearance (plate 6B).

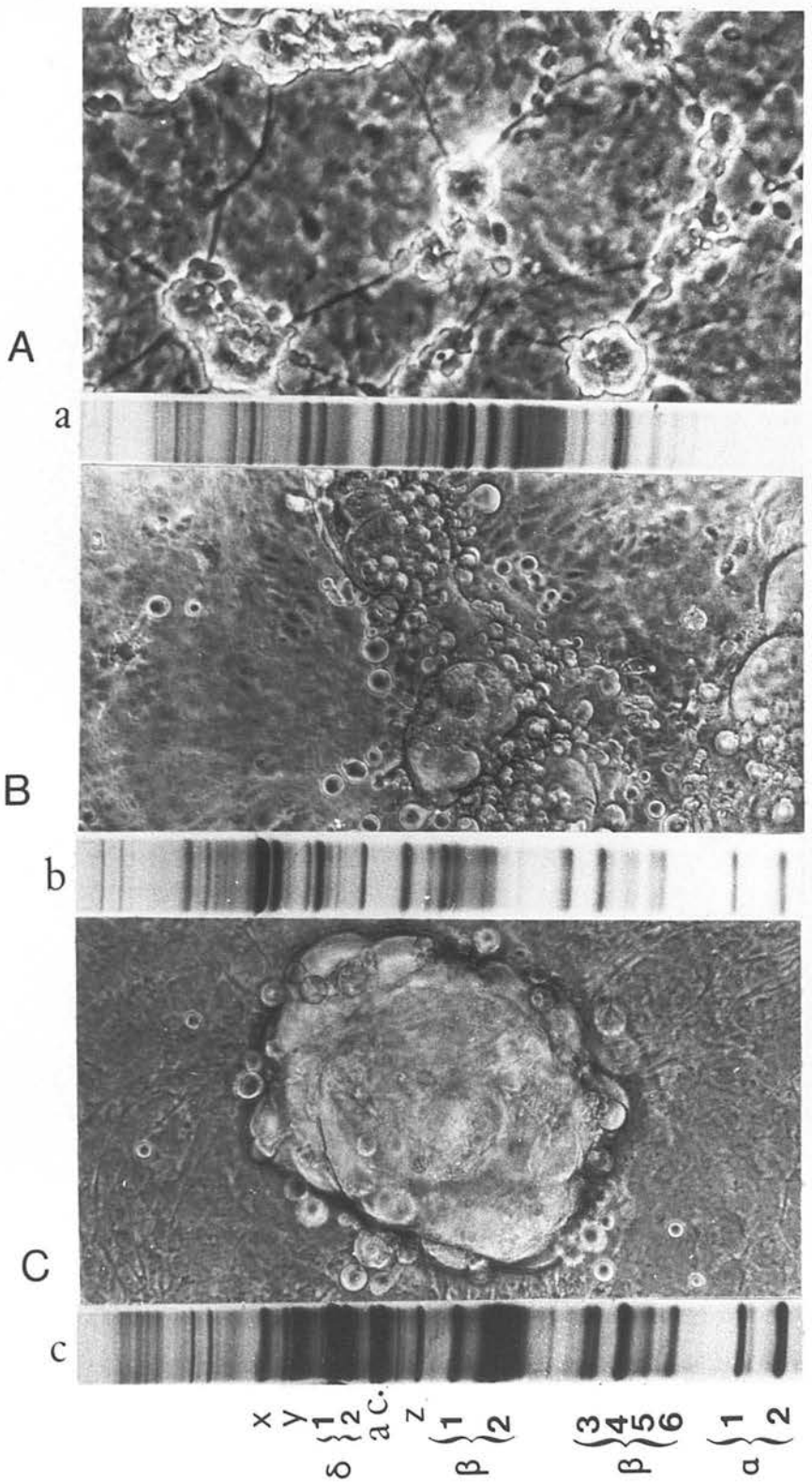
The analysis of water-soluble proteins extracted from 10-day old cultures by SDS polyacrylamide gel electrophoresis revealed the presence of about 18 to 25 distinct polypeptides, one of which, with a molecular weight of 43K daltons was the most abundant (Plate 6A). In 30-day old cultures the proportions of some of the 10-day old culture minor proteins with molecular weights of the range 20-62K daltons increased significantly and a total of 25-35 separate polypeptides were distinguishable (Plate 6B).

PLATE 5. Neural Retina Cell Cultures at Various Stages of Inoculation (Phase contrast 100 x magnification), Established from 8-day Old chick Embryos.

A. 15-day culture showing the neuronal cell aggregates with cytoplasmic processes and neuroepithelial sheet, at the spreading phase.

B. 25-day culture, at the transdifferentiating phase, showing the developed small lentoids with a number of bottle cells around their periphery.

C. 35-day culture, showing a large lentoid with a few bottle cells around the periphery. a, b and c show the SDS polyacrylamide slab gels of the water soluble extracts from A, B and C, respectively.



PL AT E 5

PLATE 6. Brain Cell Cultures at Various Stages of Inoculation. (Phase contrast 100 x magnification).

The cultures were established from 8-day old chick embryos.

A. 10-day culture showing the neurone-like cells with long axon-like processes which form aggregates on an underlying flat neuro-epithelial cell sheet.

B. 30-day culture composed of neuroepithelial cells. a and b show the SDS polyacrylamide slab gels of the water soluble extracts from A and B respectively. The sample was analysed as in Plate 2.

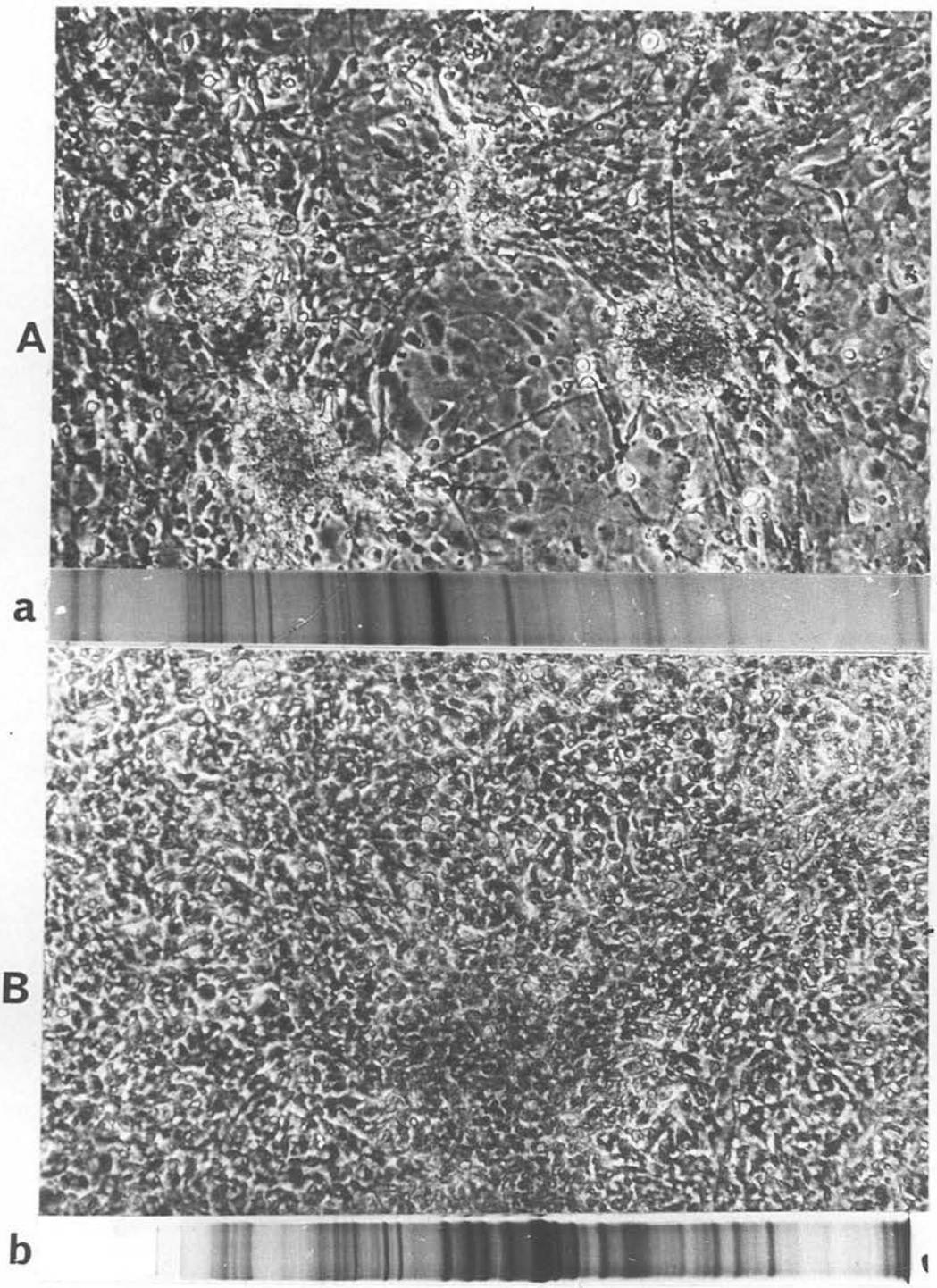


PLATE 6

3.1.4 Limb Fibroblasts

Two major cell populations were evident by the 3rd day of culture of dissociated limb fibroblast cells from the hind limbs of 8-day old chick embryos: these being (1) fibroblasts and (2) biopolar myoblasts (Plate 7A). The latter have differentiated by day 10 into multi-nucleated long muscle fibres, the number of which increased as the culture matured (Plate 7B). The number of fibroblasts also increased and the cultures reached confluence by day 10 to 12.

Analysis of water-soluble proteins by SDS polyacrylamide gel electrophoresis indicated that there were 25 to 30 distinct polypeptides detectable throughout the 21 day culture period, the most abundant of which had molecular weights of 38K, 43K and 48K daltons.

3.1.5 Kidney Fibroblasts

On the fourth day of culture of dissociated kidney cells from 8-day old chick embryos, large areas of monolayer sheets were established, comprised of both polygonal and spindle-shaped cells. By day 7 to 10 the spindle cells formed a cytoplasmic network while the polygonal cells formed a mosaic pattern among the network of spindle cells (Plate 8A, B and C).

Analysis of water-soluble proteins by SDS polyacrylamide gel electrophoresis revealed that throughout the 21 day culture period a total of 20 to 30 distinct polypeptides were present, the two most abundant proteins were those with molecular weights of 38K and 43 K daltons.

PLATE 7. Limb Fibroblast Cell Cultures at Various Stages of Inoculation. (Phase contrast 100 x magnification).

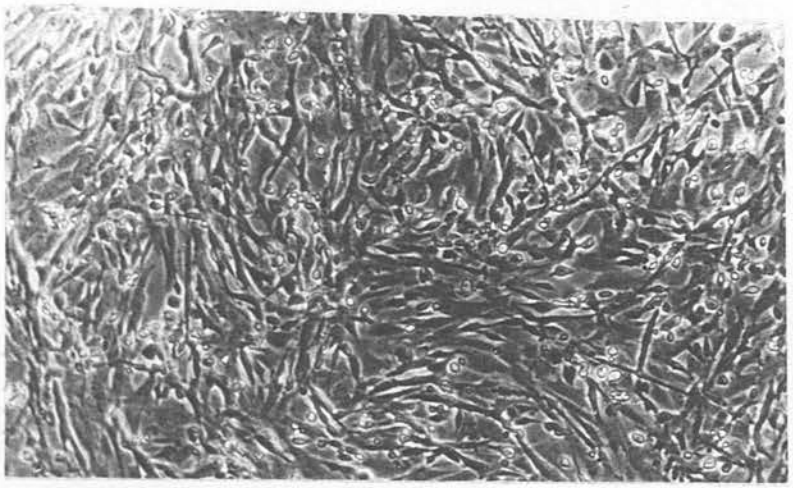
The cultures were established from 8-day old chick embryos.

A. 3-day culture showing the fibroblast cells and bipolar myoblasts.

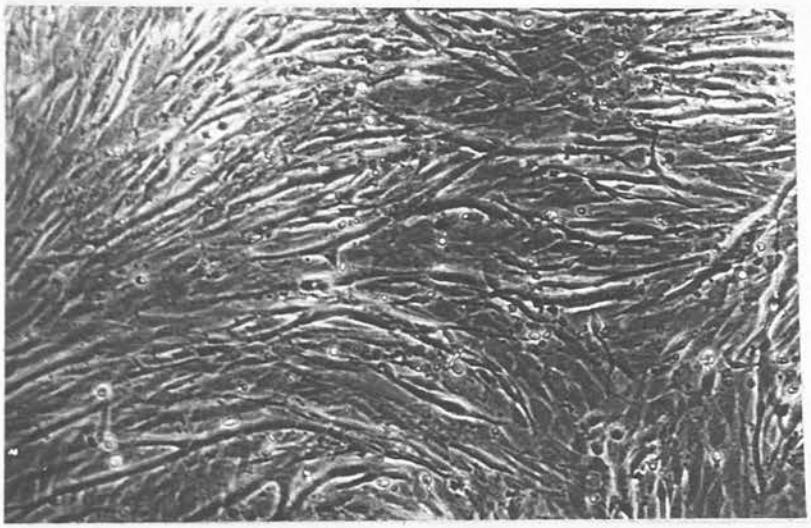
B. 10-day culture showing the multinucleated long muscle fibres.

C. 20-day culture. Note the change in the morphology of the muscle fibres as the cultures grow older.

A



B



C

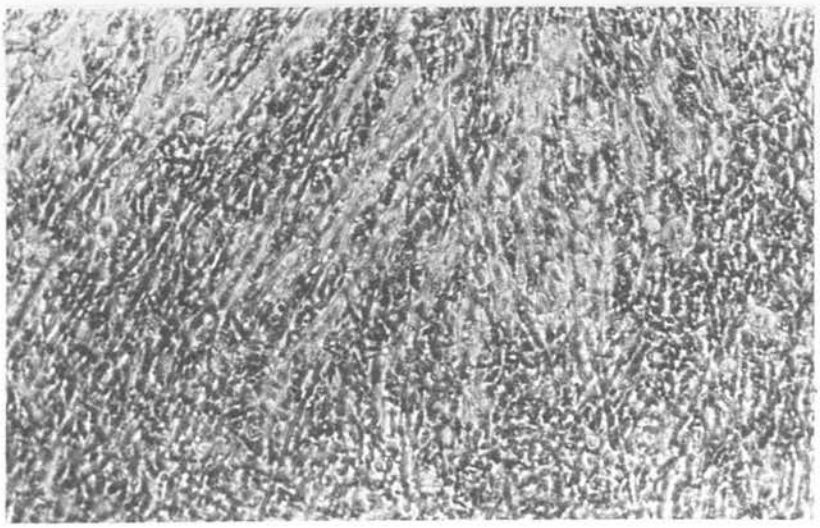


PLATE 7

PLATE 8. Kidney Fibroblast Cell Cultures at Various Stages of Inoculation (Phase contrast 100 x magnification).

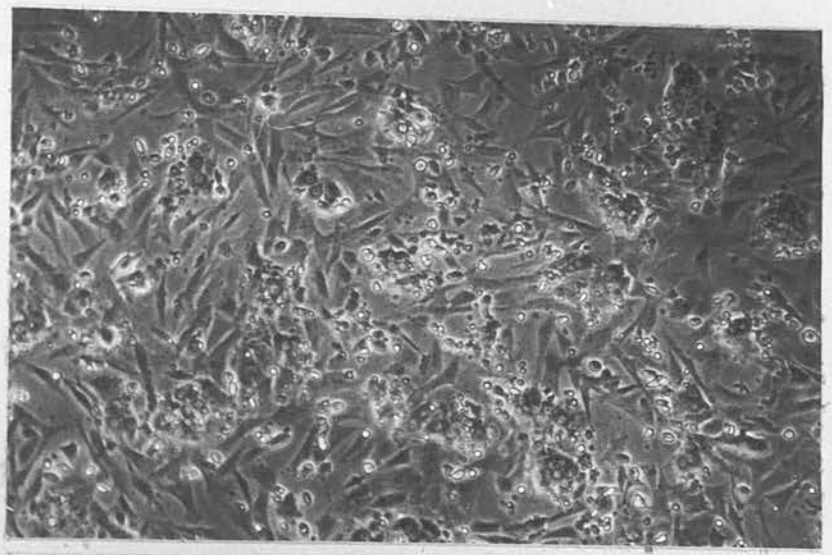
The cultures were established from 8-day old chick embryos.

A. 4-day culture showing both the polygonal and spindle-shaped cells.

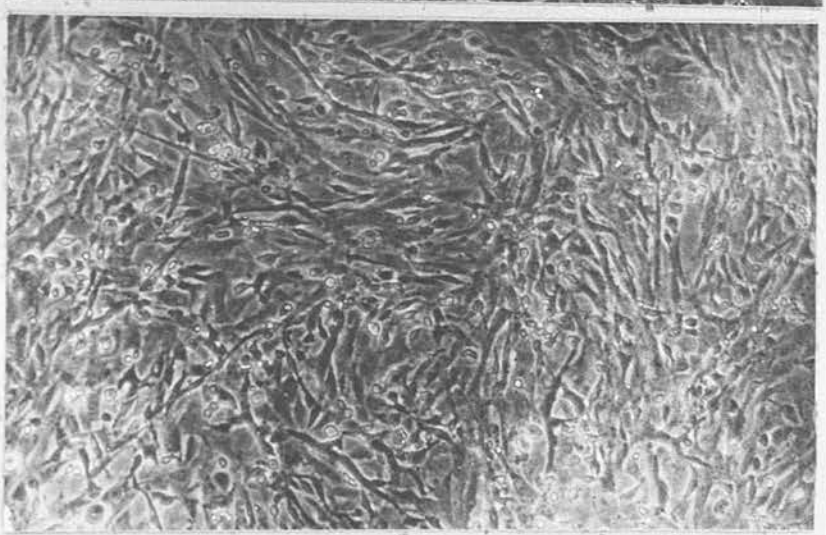
B. 10-day culture.

C. 20-day culture.

A



B



C

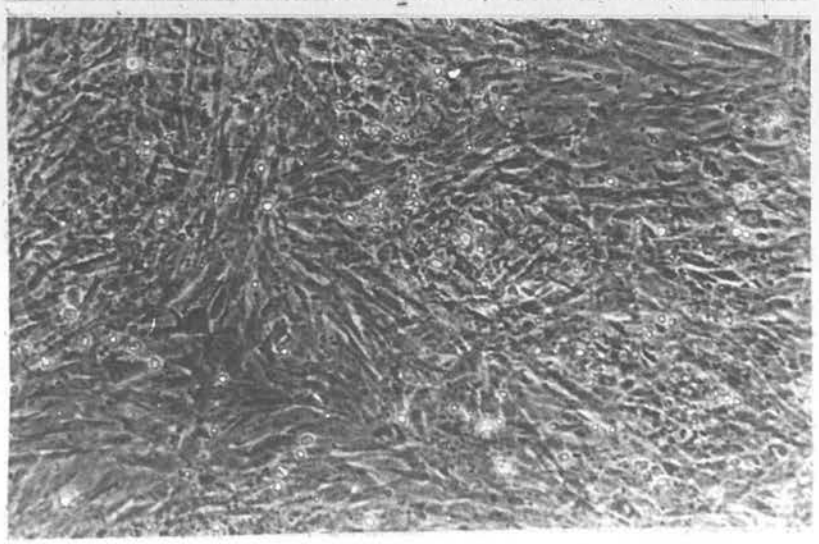


PLATE 8

3.2 The Effect of Selected Agents on the Development of Chick Embryos and on the Differentiation of Cultured Cells.

3.2.1 Studies with Insulin

3.2.1.1 The Teratogenic Effect of Insulin on Developing Chick Embryos

The treatment of chick embryos with either 1 or 2 iu insulin by injection onto the chorioallantoic membrane at either 48, 108 or 192 hours of incubation resulted in various gross malformations between days 6-17. The abnormalities observed were of the tail, beak, limbs and eyes (Table 1).

The most frequent abnormality observed in embryos treated at the 48 hours of incubation was of the tail, which was absent in the majority. In the other embryos, in which complete rumplessness was not observed, the tail was shortened. With embryos treated at the later stages of incubation no tail abnormalities were observed.

Shortening of the limbs due to reduction in the length of the long bones (micromelia), opacity of the eye lens (cataract) and shortening of beak (especially that of the upper beak) were among the most frequent abnormalities observed in those embryos treated at the 108 or 192 hours of incubation (Plates 9A, B and C, and 10A and B). Oedema, confined to the lower abdominal region, was also apparent in those embryos severely affected with the above abnormalities. The legs were proportionately more shortened than the wings. In most instances the abnormality of the beak and limbs (and to a lesser extent those of the eyes) were found in the same embryos. The opaque, cataractous lens induced by insulin treatment was characterized by disorganized and swollen cells in the lens nucleus (Plate 10). The analysis of the water-soluble crystallin proteins from such lenses by haemagglutination

inhibition assay showed a significant increase in both α and β but 25% decrease in δ.

In embryos treated with the higher level of insulin (2iu) the gross abnormalities observed were more severe and the mortality rate was higher (Table 1).

3.2.1.2 The Effect of Insulin on Cultured Cells

3.2.1.2.1 Lens Epithelium

Initial experiments showed that insulin had the greatest stimulatory effect on the mitosis of lens epithelial cells, cultured in medium supplemented with 6% FCS, when applied at a level of 10 μg/ml of medium. Cell counts every two to three days showed that continued insulin treatment, with medium changes every two to three days, from 4 to 25 inclusive, led to a stimulatory effect on mitosis, which was greatest during the stage of exponential cell proliferation (days 7-14) (Fig. 7B). Over this period the treated lens epithelial cell cultures established from the Db strain showed a rate of mitosis about 40% higher than that of control cultures. However, after day 20 when the cultures contained many differentiated lentoid bodies, insulin treatment no longer had any discernible effect on cell proliferation.

When lens epithelial cell cultures from the Db strain were treated with insulin every two to three days through days 4 to 24 inclusive and the water-soluble proteins from 12-day cultures were analysed by SDS polyacrylamide gel electrophoresis, it was found that the insulin treatment had stimulated both the synthesis and the accumulation of actin and the α- and δ-crystallins, but had differentially reduced the synthesis and accumulation of the β-crystallin subunits (β1-3) (Plate 11A, B; Fig. 4 and 5; and Table 2). However in cultures

harvested on day 25, it was found that insulin had no effect on the synthesis and accumulation of water-soluble proteins, except for two minor protein components with molecular weights of between 39-40 K daltons, the accumulation and rate of synthesis of which was reduced by insulin (Plate 12A).

The results from the in vitro cell-free translation of polysomal mRNAs extracted from the 12-day old lens epithelial cell cultures described above also indicated that insulin treatment increased the synthesis of actin and δ_1-crystallin (50K daltons) but reduced that of the β-crystallin subunits (β_1-3) (Plate 11C). This indicates that the effect of insulin is to modify selectively, the transcription or the processing of several mRNA species.

It had been shown previously that the day-old chick lens post-polysomal mRNA population differed from that of polysomal mRNA of the same age, mainly in being greatly enriched in β-crystallins and deficient in α- and δ-crystallins (Thomson et al., 1978). Since these β-crystallins become increased significantly as the cells differentiate the effect of insulin on the post-polysomal translatable mRNA was also investigated.

The effect of insulin on the translation of post-polysomal mRNA was found to be dependent on the duration of treatment. Post-polysomal mRNA from lens epithelial cell cultures treated continously with insulin through days 4 to 24 failed to synthesize any detectable proteins on either days 10 or 21 of culture whereas the control cultures harvested on day 10 synthesized β_6-crystallin and those harvested on day 21 synthesized actin, δ_1 and β_2, 4, 5, 6 and α_1 and α_2 crystallins. By day 25, however, the translation of postpolysomal mRNA from both

the control and insulin treated cultures showed similar levels of synthesis of all the major crystallins, with one exception. The δ_2 (48K daltons) was not detected in the treated cultures but was synthesized in control cultures (Fig. 6).

In summary the results indicate that the terminally differentiated fibre cells are less reponsive to insulin treatment as judged by mitosis and by the anslysis of proteins synthesized in cells and in vitro cell-free translation system. The partition of mRNA species between the polysomal and the post-polysomal compartments is presumably affected by the availability of ribosomes for newly transcribed or processed mRNAs and by differential mRNA stabilities. The effect of insulin would appear to affect one or more of these processes non-coordinately, but the data does not permit the identification of the mode of action.

Both the effect of insulin on mitosis and on the response of specific polypeptides are genotype dependent. When the effect of insulin on mitosis and protein synthesis and accumulation of cultured lens epithelial cells was compared between the two genetically distinct chick strains (Db and Hy-2), a genotype specific response was apparent.

The rate of mitosis of cultured Hy-2 cells is 7% higher than that of Db cells, but the effect of insulin on mitotic rate of Db cells is greater than the effect on Hy-2 cells. The insulin treated Db cells have a mitotic rate 34% higher than that of treated Hy-2 cells (Fig. 7A and 7B). Thus although the mitotic rate of lens epithelial cells of both strains are responsive to insulin, Db cells appear to be more sensitive to this effect.

The effect of insulin on protein synthesis and accumulation also appears to be influenced by the genotype of the chick from which the cells were originally taken. Analysis by SDS polyacrylamide gel

electrophoresis of proteins synthesized by cell-free translation of mRNAs from 14-day old lens epithelial cell cultures which had been treated with insulin every two to three days, through days 4 to 13 inclusive, showed that insulin affected the synthesis of actin and α_1, δ_1, and β-crystallin subunits (β_1 - 5) and this effect was two to three times greater in the cultures from the Db as compared to that from the Hy-2 strain (Plate 14B, C; Fig. 8, 9; and Table 3). The analysis of accumulated crystallins from the 14-day old cultures described above, using haemagglutination inhibition assay, showed that the stimulatory effect of insulin on β- and δ-crystallin accumulation is about two fold higher in cells from the Db than from the Hy-2 strain (Table 3).

These results, firstly, indicate that at all levels, ranging from mitosis, to protein synthesis and accumulation, and to the proportions of translatable mRNA in both the polysomal and the post-polysomal RNA population, the response of lens epithelial cells from the Db strain was much greater than the response of lens epithelial cells from the Hy-2 strain. However the data does not indicate whether these genetic differences reside in receptor sites on the cells or in effector processes at various levels in the cell. The changes in the mRNA populations however, point to an important effect on transcription, processing, or both.

3.2.1.2.2 Neural Retina

Neural retina cell cultures were treated with insulin at 10μg/ml, through days 6-39, every 2-3 days when medium was replaced.

Analysis of water-soluble proteins, by SDS polyacrylamide gel electrophoresis, from 30-day cultures indicated that insulin treatment

caused a significant reduction both in the synthesis and accumulation of a 50K dalton protein (Plate 15B, C and D; Fig. 10 and Table 4). This effect however was not seen in those cultures treated between days 6-39 and harvested on day 40 (Plate 15E and F).

Insulin treatment after the differentiation of lentoid bodies, between days 28-39, did also not have a discernible effect on either the accumulation or the synthesis of proteins in 40-day neural retina cell cultures.

The results suggest as with the lens epithelial cell cultures that the terminally differentiated fibre cells are not affected by insulin treatment.

3.2.1.2.3 Limb and Kidney Fibroblasts

Limb and kidney fibroblast cell cultures were treated with 10μg/ml insulin through days 4-10 in medium containing the following levels of FCS: 2%, 4%, 6% and 10%, the medium being renewed every 2-3 days between days 4 and 10 of culture. The cultures were also grown in the absence of FCS, for 24 hours on day 10 of culture and then harvested. Under all the above conditions insulin treatment failed to affect the accumulation of water-soluble proteins in 11-day kidney fibroblast cell cultures.

The effect of insulin on the accumulation of water-soluble proteins by limb fibroblast cell cultures was however found to be dependent on the level of FCS present in the medium, in that the addition of insulin to medium containing 2% or 4% of FCS stimulated the accumulation of a 48 K daltons protein whereas there was no discernible effect following insulin treatment, in medium containing either 6% or 10% FCS (Plate 16). Insulin also had no discernible effect on the accumulation of water-soluble proteins in the absence of FCS.

TABLE 1. THE EFFECTS OF THE INJECTION OF INSULIN ON DEVELOPING CHICK EMBRYOS AFTER 48, 108, AND 192 HOURS OF INCUBATION.

Hours of incubation prior to injection	Insulin Units (IU)	No. of embryos injected	No. of survivors	Mortality at 6-17 days (%)	Abnormalities					
					Short Tail (%)	Rumpless (%)	Short upper beak (%)	Micromelia (%)	Opaque Lens (%)	Oedema (%)
48	1	80	50	37.5	3(6.0)	17(34.0)	0	0	0	0
	2	26	11	57.7	2(7.7)	5(19.2)	0	0	0	0
	C	74	68	8.1	0	0	0	0	0	0
108	1	60	40	33.3	0	0	0	10(25.0)	12(30.0)	0
	2	72	30	58.3	0	0	5(16.6)	20(66.6)	21(70.0)	2(6.6)
	C	47	40	14.9	0	0	0	0	0	0
192	1	48	48	0	0	0	16(33.3)	32(66.6)	20(41.6)	0
	2	90	45	50.0	0	0	12(26.6)	22(48.8)	26(57.7)	2(4.4)
	C	95	92	4.7	0	0	0	0	0	0

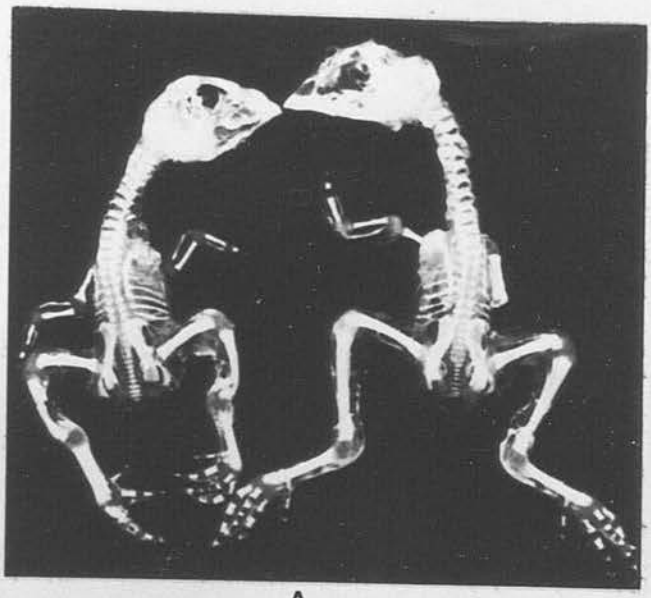
C: Controls injected with the same volume of 0.9% NaCl.

PLATE 9. Skeletons of Chick Embryo showing Insulin-Induced Abnormalities.

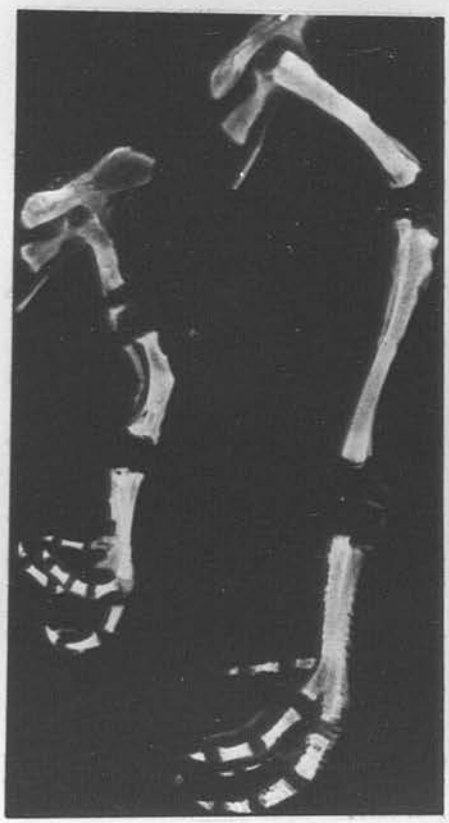
A. 17-day embryos cleared, preparation: stained with alizarin red. Right, control embryo injected with the same volume of 0.9% NaCl; left, treated wmbryo injected with 2iu insulin (0.1mg) per egg at 4.5 days of incubation.

B. Right, hind limbs of the stained skeletons. Right, leg from the control embryo; left, leg from the treated embryo. Note the reduction in length of the long-bones and a deformity of the tibia in the leg of insulin injected embryo.

C. Defective upper beak. Bottom, appearance of a normal beak from the control embryo. Top, shortened upper beak of insulin-treated embryos.



A



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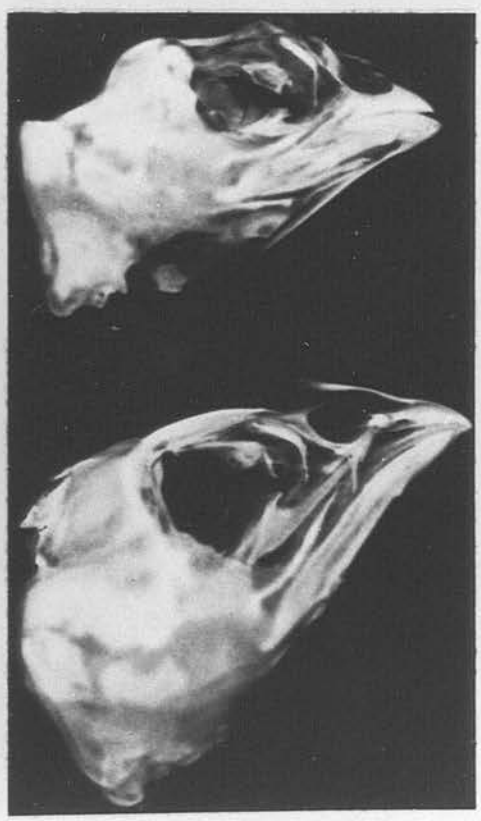


C

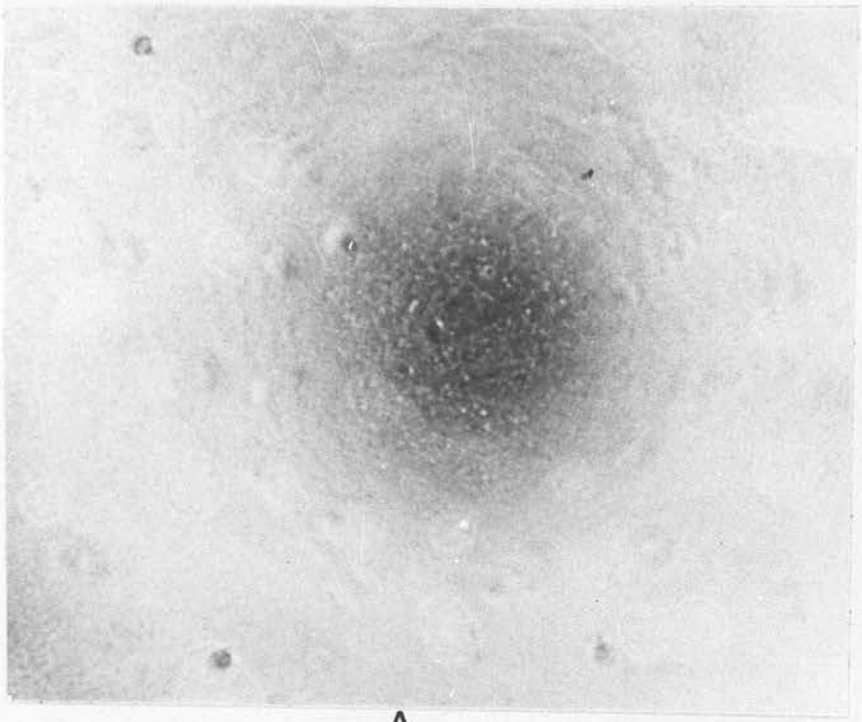
PLATE 9

PLATE 10. The Effect of Insulin on the Chick Lens *In Vivo.*

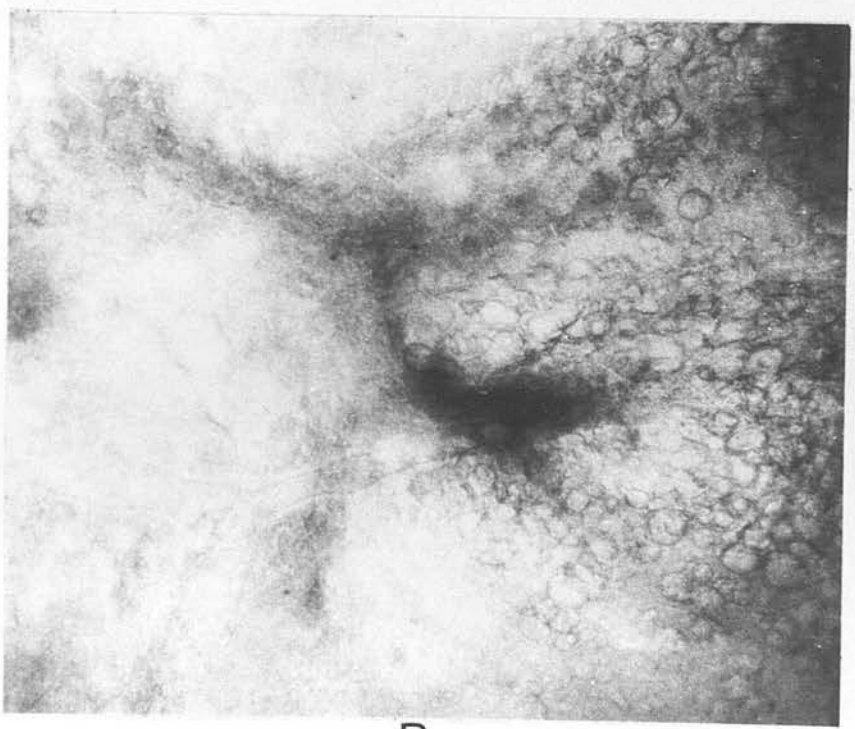
The centre area of the whole lenses of 17-day embryos is seen from the anterior surface, with the focus just below the anterior epithelium (phase contrast 200 x magnification).

A. Lens of a normal control embryo injected with 0.9% NaCl (in 0.1ml). The fibre cells are seen in orderly array.

B. Lens from the embryo injected at 4.5 days of incubation with 2iu of insulin, the fibre cells are disorganised and swollen.



A



B

PLATE 10

PLATE 11. The Effect of Insulin on Cell Growth and Protein Synthesis of Lens Epithelial Cells *In Vitro*.

A. The effect of insulin on the cell growth of 6-day cultures of lens epithelial cells, established from day-old chicks. (a) Control culture grown in standard 6% FCS medium; (b) Epithelial cells in culture after a single treatment on day 4 after inoculation with insulin at 10μg/ml in standard 6% FCS medium. Note the large number of dividing cells and their aggregates in the treated culture.

B. SDS polyacrylamide slab gel of accumulated water soluble proteins in 12-day cultures of lens epithelial cells grown as described above. (a) Control; (b) treated with 10μg/ml insulin as above, every 2-3 days.

C. Fluorographs of (^{3}H) amino acid labelled proteins of the gel B. (a) Control; (b) insulin treated.

D. Fluorographs of products of cell-free translation using mRNA from 12-day lens epithelial cell cultures grown as described above (B). (a) Control; (b) insulin treated.

Arrows identify the components affected.

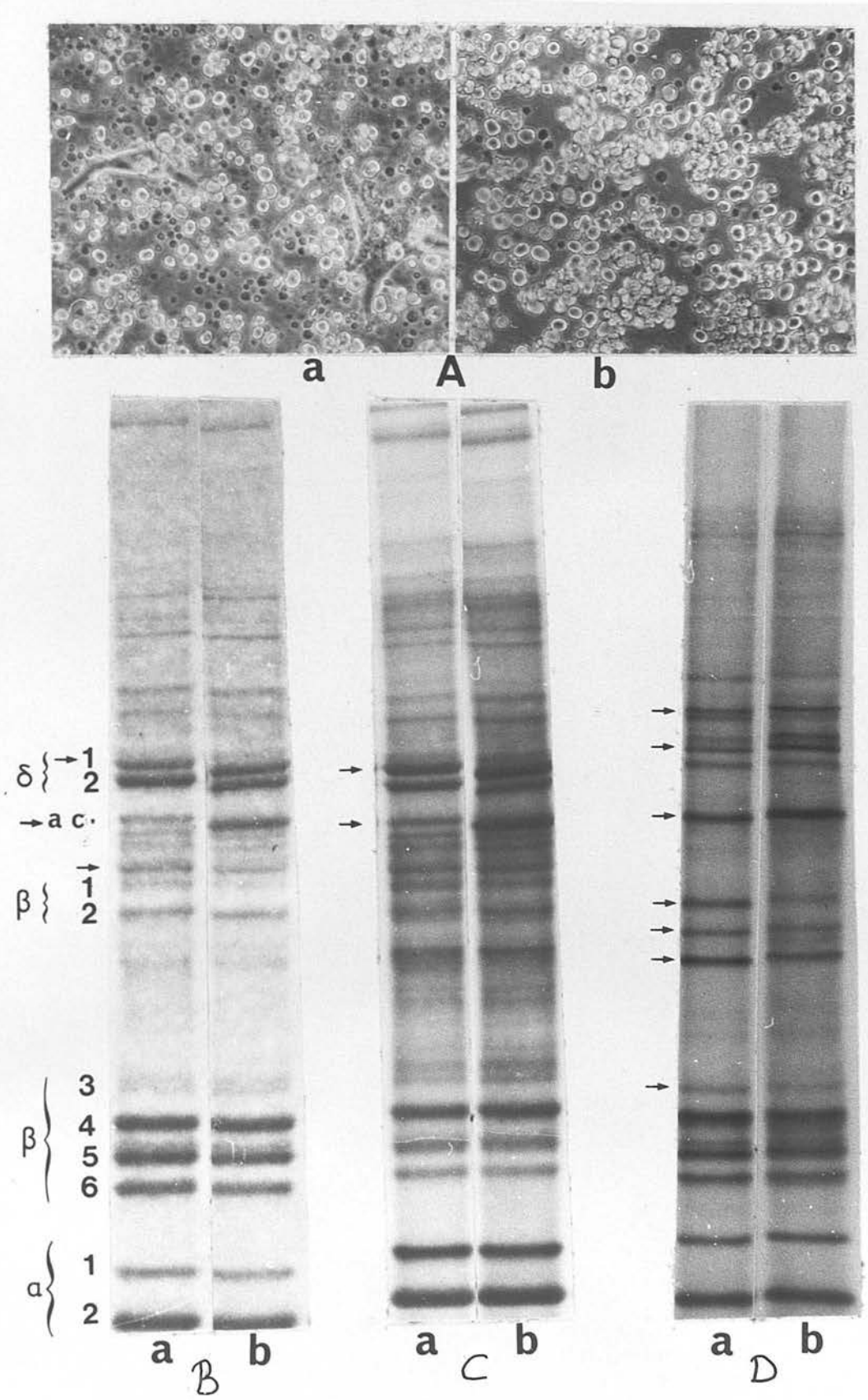


PLATE 11

FIGURE 4. Traces of Fluorgraphs in Plate 11 Ca (a) and 11 Cb (b). Arrows mark the peaks which show changes in the corresponding newly synthesised protein bands in response to insulin treatment of the cultured lens epithelial cells.

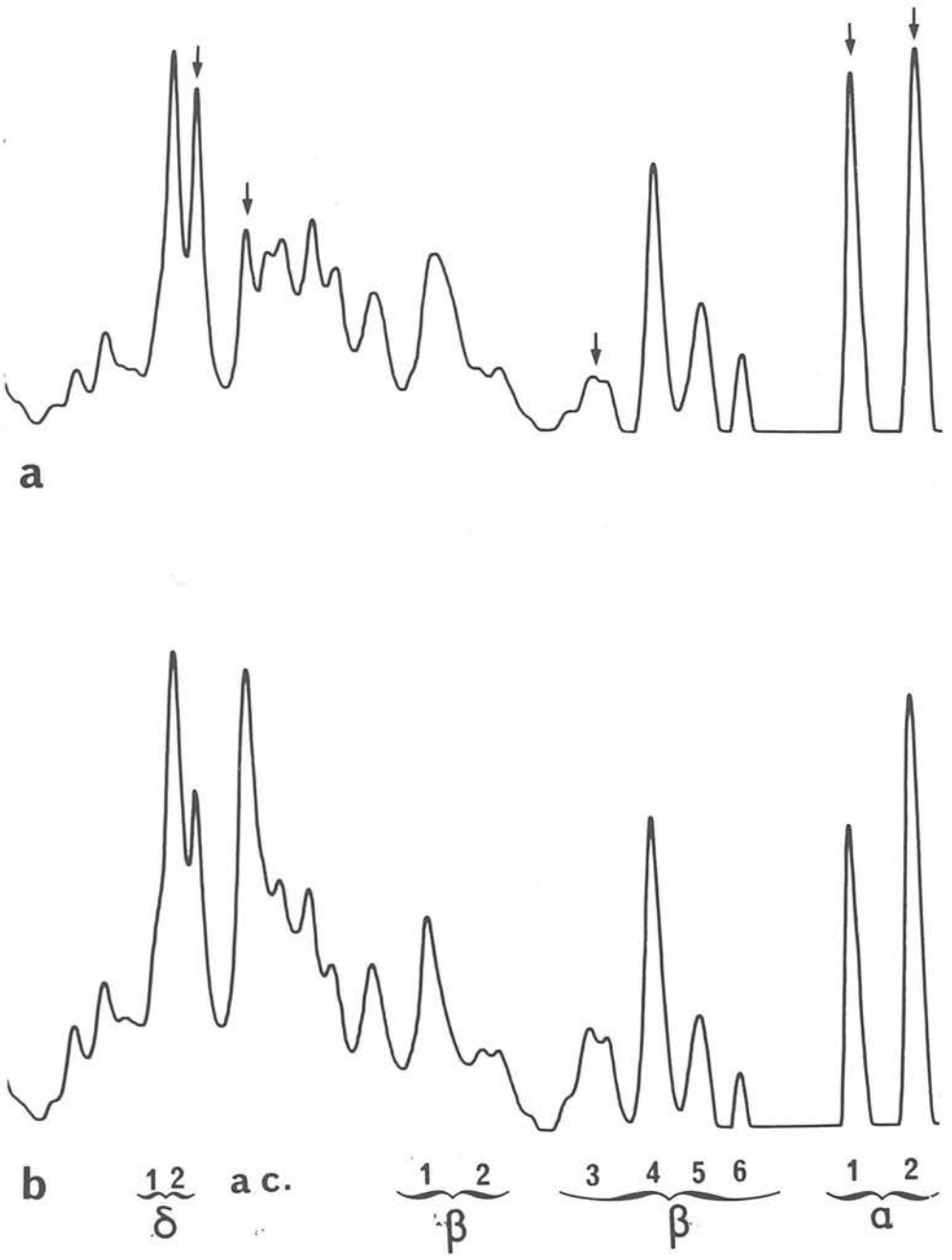


FIG 4

FIGURE 5. Traces of Fluorographs in Plate 11Da (a) and 11Db (b). Arrows mark the peaks which show changes in the corresponding protein bands synthesized in a cell-free system using mRNA from the control (Da) and treated (Db) 12-day lens epithelial cell cultures.

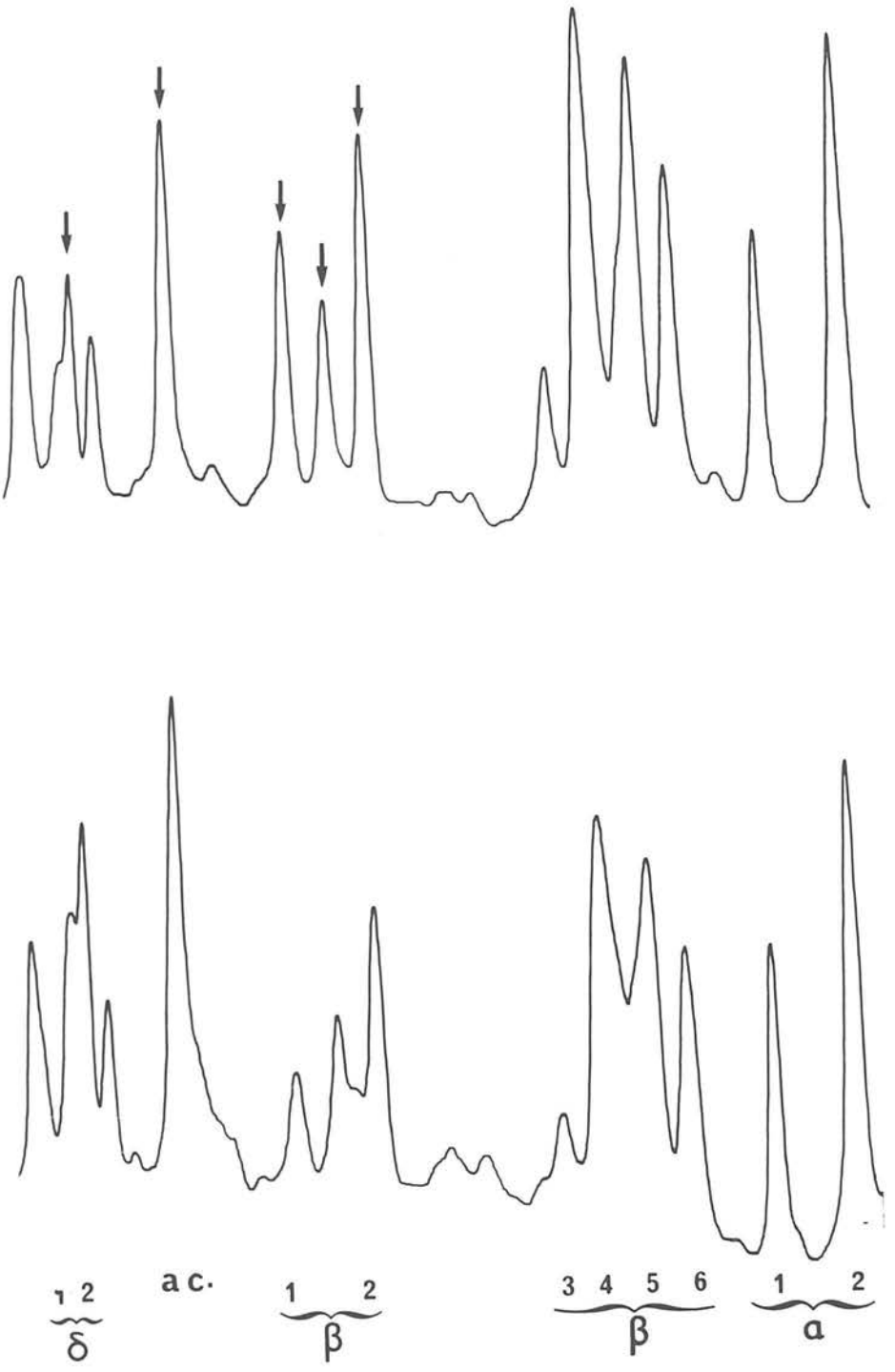


FIG 5

TABLE 2: Quantitative Densitometric Readings of Drug Treated and Control Cultures. The area of each peak representing the numbered bands from plate 11B, C and D, expressed as a percentage of the total.

ELECTROPHORETIC SEPARATION								
		Accumulated Proteins (% of total)		R.a.a. labelled P. (% of total)		Translation in a cell-free system.		
Crystallin	Peak No.	Insulin	Control	Insulin	Control	Insulin	Control	**
δ1	1	6.3	4.7	18.9	14.2	9.4	6.4	(±0.4)
δ2	2	6.2	6.5	7.2	8.8	3.6	3.4	(±1.8)
AC	3	9.0	2.5	18.4	5.2	13.7	8.8	(±0.6)
β1	4	3.0	3.4	4.7	9.7	3.3	5.8	(±0.5)
β2	5	3.4	4.5			3.1	4.0	(±0.6)
β3	6	3.4	2.9			6.1	6.2	(±1.2)
β4	7	7.7	7.5	7.6	8.3	13.1	15.3	(±0.6)
β5	8	7.4	8.6			10.1	13.6	(±1.6)
β6	9	5.4	6.8	1.5	3.5	6.4	8.0	(±0.5)
α1	10	3.9	4.9	5.3	9.1	4.4	5.3	(±0.7)
α2	11	9.2	9.3	10.5	12.6	7.2	10.4	(±1.7)

AC: actin

**: Mean variation between duplicate samples.

PLATE 12. The Effect of Insulin on Protein Synthesis in Ageing Cultures of Lens Epithelial Cells.

The proteins were analysed as described in Plate 2. Treatment of cultures with insulin (at 10μg/ml of standard 6% FCS medium) began on day 4 of inoculation and was repeated every 2-3 days, when the medium was replaced.

A. 12-day cultures; (a) Insulin-treated; (b) Control.

B. 20-day cultures; (a) Insulin-treated; (b) Control.

C. 25-day culture; (a) Insulin-treated; (b) control.

Arrows identify the components which show changes.

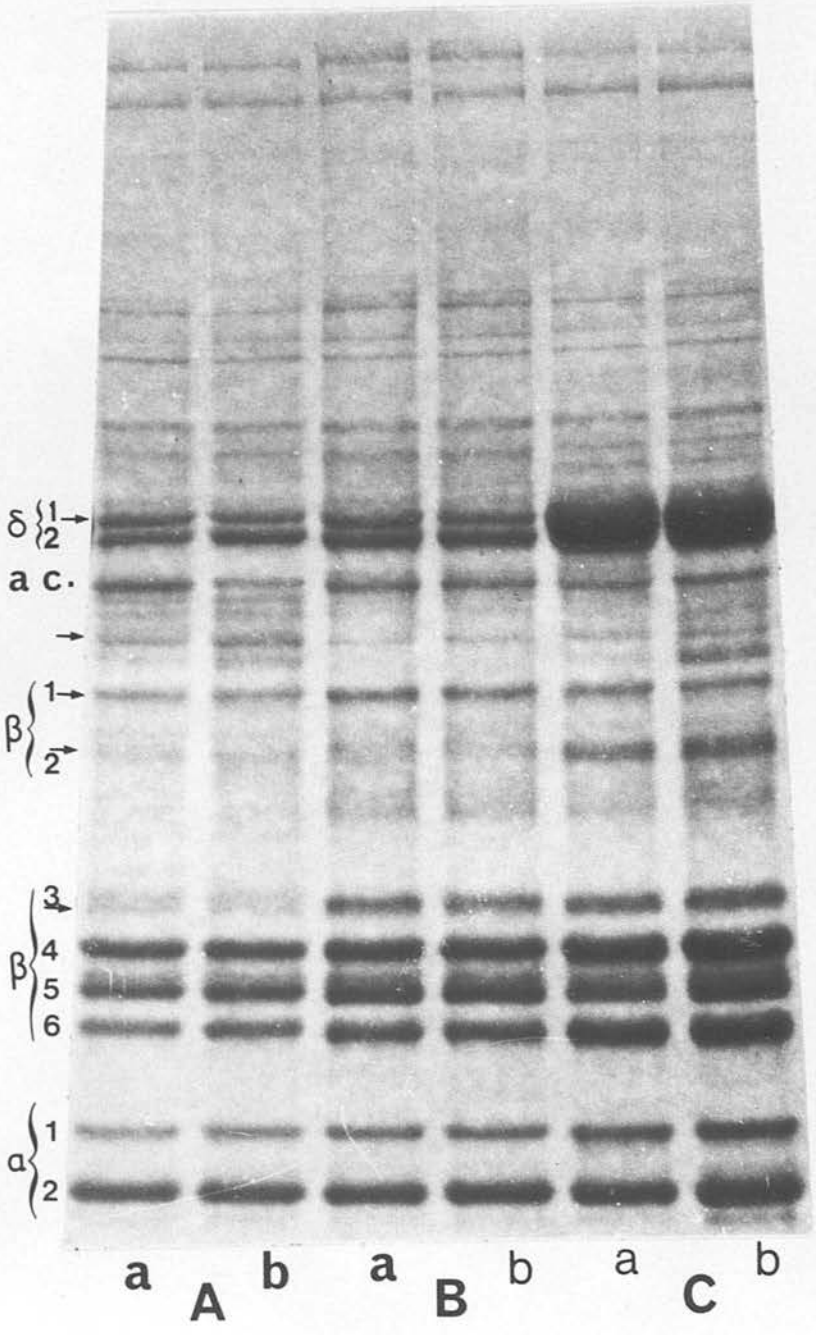


PLATE 12

FIGURE 6. Diagramatic Representation of Protein Bands on SDS Polyacrylamide Slab Gel.

The protein samples were from postpolysomal mRNA translated in cell-free translation system. The mRNA was extracted from insulin treated and control lens epithelial cell cultures at 10, 21 and 25 days of inoculation.

A. 10-day culture. (a) Insulin (b) Control.

B. 21-day culture. (a) Insulin (b) Control.

C. 25-day culture (a) Insulin. (b) Control.

X. Unidentified protein.

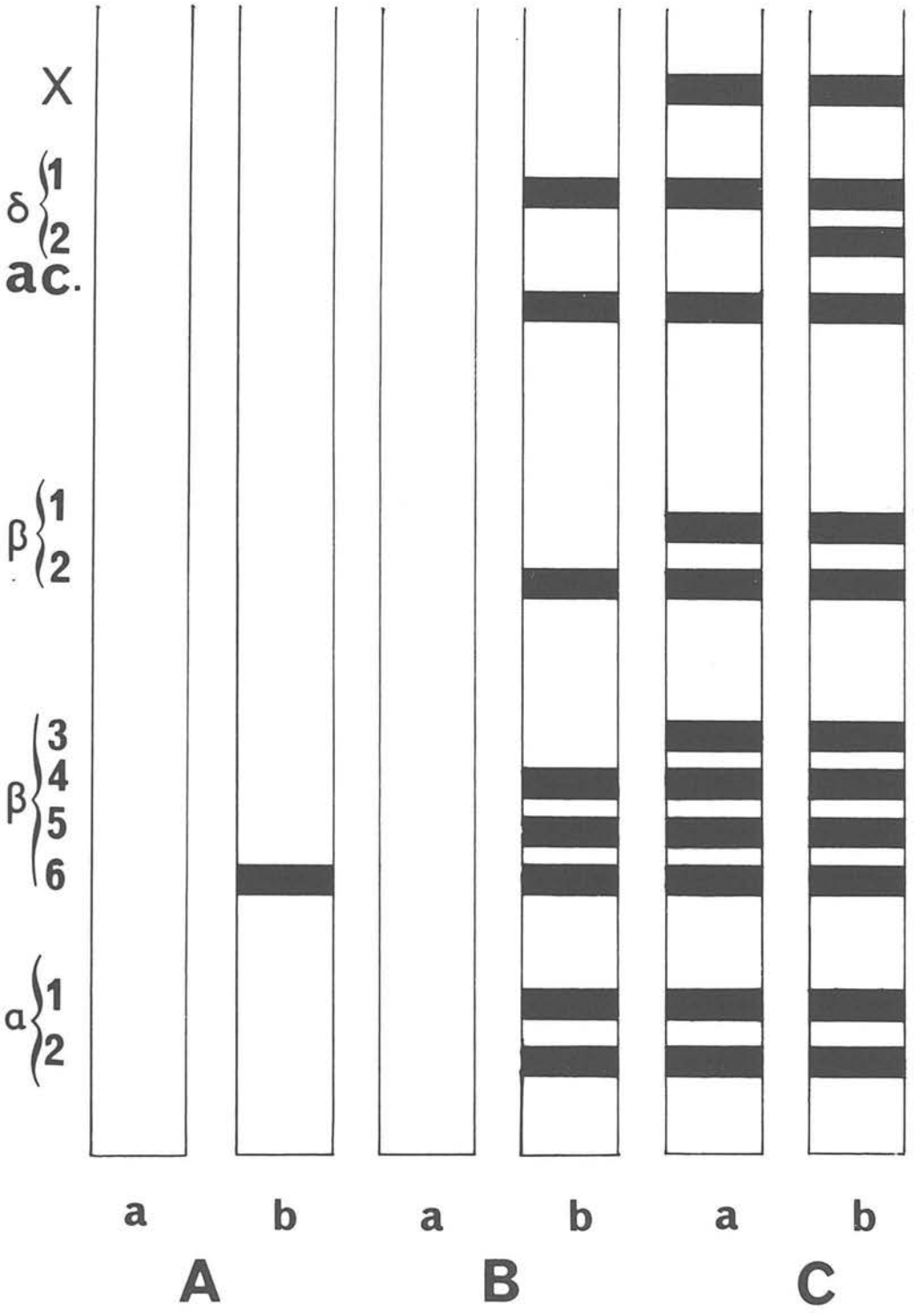


FIG 6

FIGURE 7. The Effect of Insulin on Cell Growth *In Vitro*.

Growth curves of cultured lens epithelial cells established from day-old chicks. Dotted lines, control cultures grown in standard 6% FCS medium; solid lines, treated cultures grown in standard 6% FCS medium plus 10 μg/ml insulin, which was replaced every 2-3 days.

A. Hy-2 strain.

B. Db strain.

Note that the stimulation of cell growth by insulin-treatment is more marked in the Db genotype than that in the Hy-2 genotype. Lentoids first appeared at the stages of culture indicated by arrows.

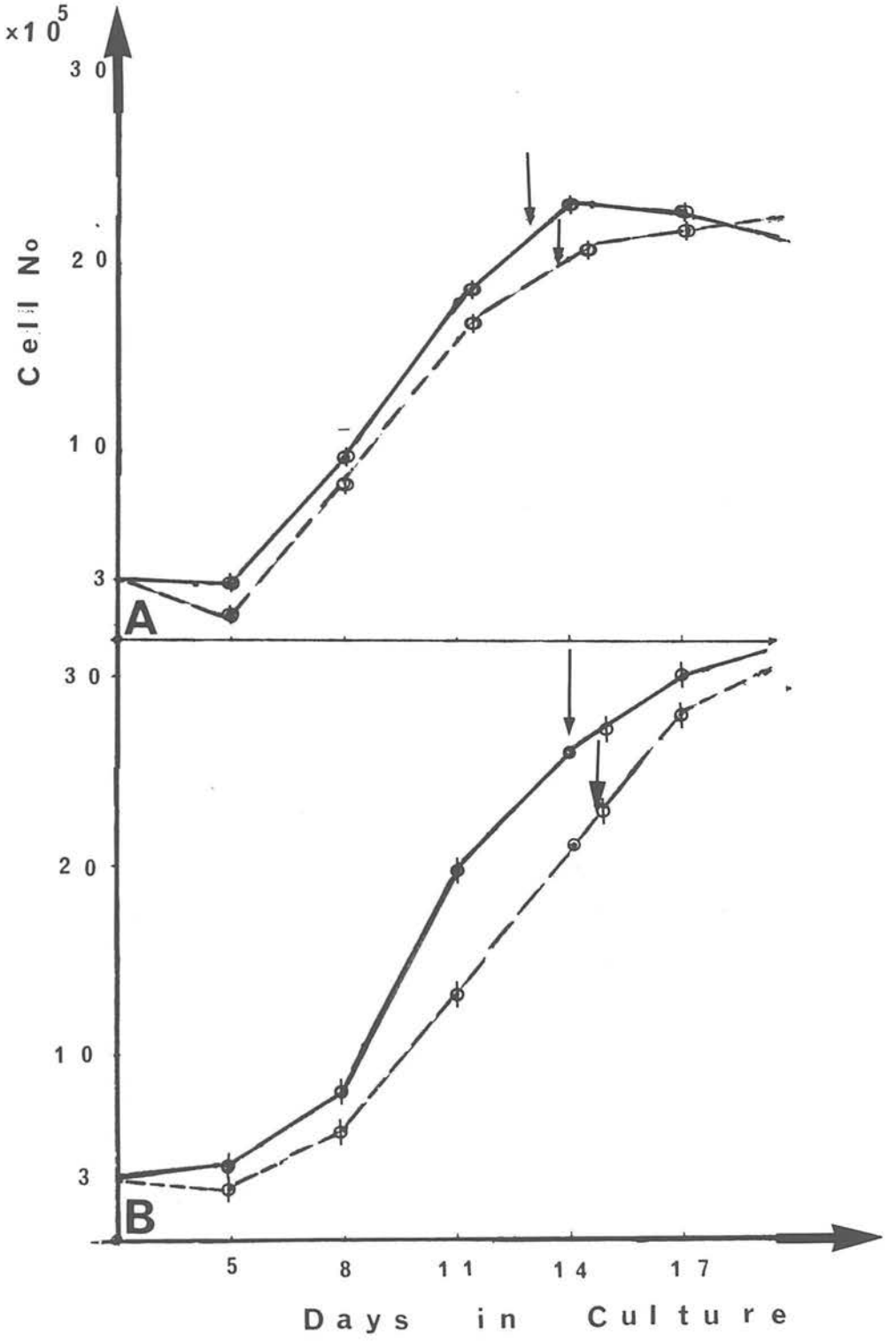


FIG. 7

PLATE 13. Haemagglutination Inhibition Tests for Chick Delta Crystallin from 14-Day Lens Epithelial Cell Cultures, Established as in Plate 11.

A - D. Illustrates the end point for the test antigen from the water soluble extract of the cells grown in standard 6% FCS medium plus 10μg/ml insulin. The cultures were treated from the 4th day every 2-3 days, when the medium was replaced.

E - H. Illustrates the end point for the test antigen from the extract of the control cultures grown in standard 6% FCS medium.

It is evident that insulin treated cultures appear to have twice the amount of δ-crystallin than that of controls, since the end point of treated sample is one well and therefore one doubling dilution further than the control sample.

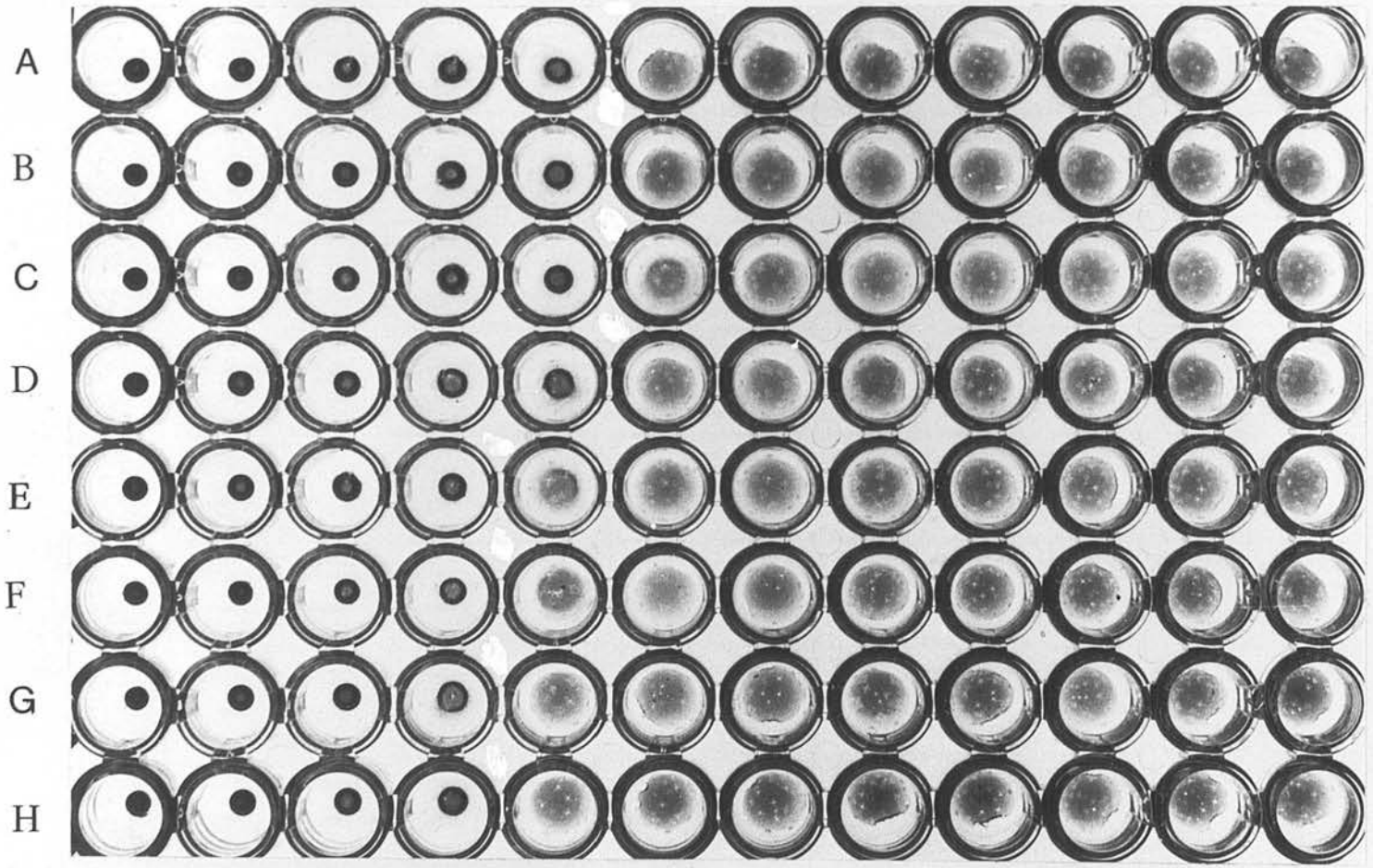


PLATE 13

PLATE 14. Fluorographs of SDS Polyacrylamide Slab Gel Electrophoresis of Products Translated in a Cell-Free System from mRNA Obtained from 14-day Cell Cultures of Day-old Chick Lens Epithelium.

The cultures were grown as for Plate 11, and the proteins analysed as described in Plate 2.

A. Db genotype. (a) Control. (b) Insulin-treated, 10 μg total RNA was used for each translation.

B. Db genotype. (a) Control and (b) Insulin-treated, 5 μg total RNA was used for Ba and Bb translation.

C. Hy-2 genotype, 5 μg total RNA was used for each translation. (a) Control. (b) Insulin-treated. The response of Db genotype is more marked than that of Hy-2 genotype. Arrows identify the components affected.

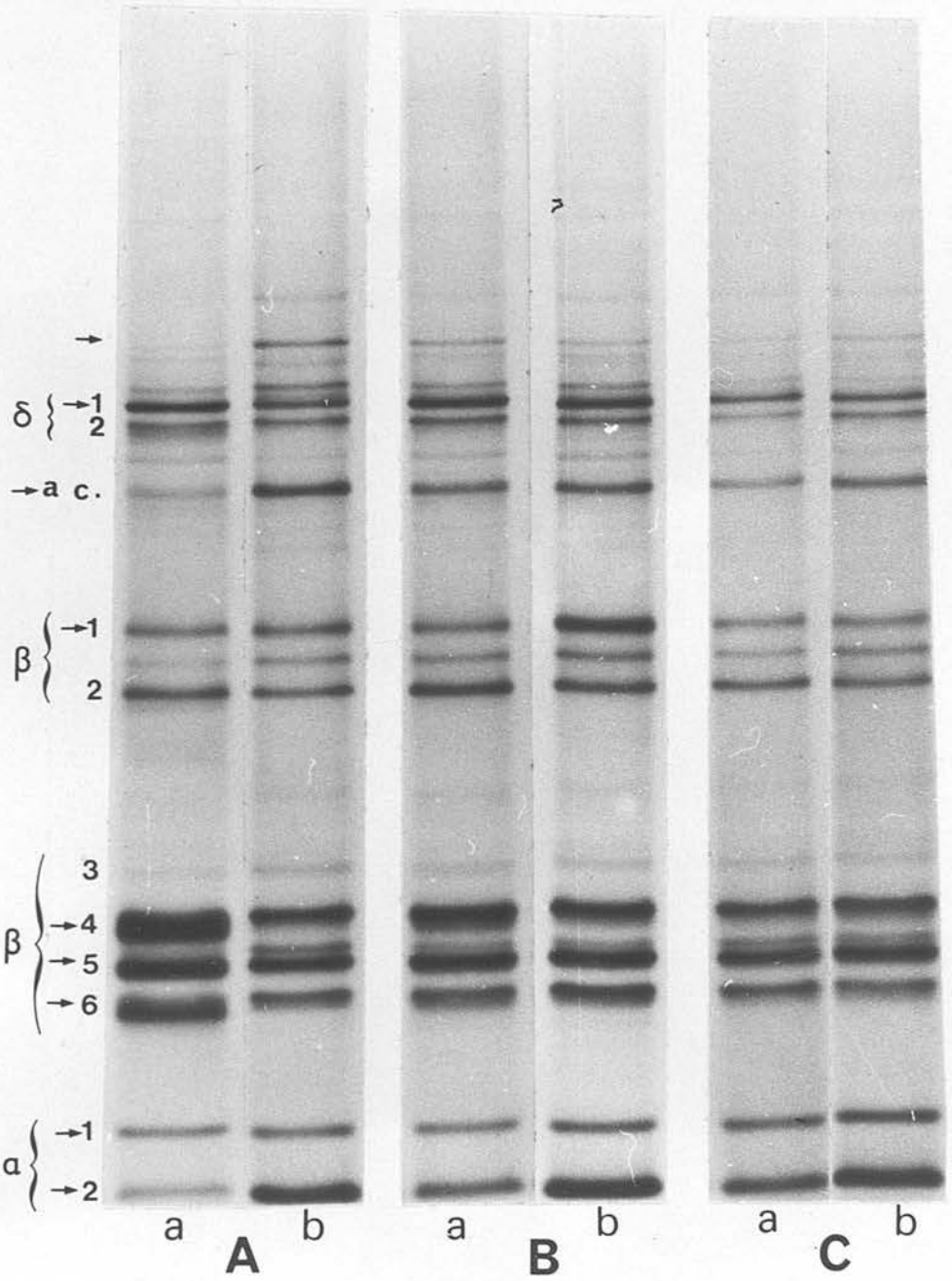


PLATE 14

FIGURE 8. Traces of Fluorographs in Plate 14 Ba (a) and 14 Bb (b) Control and Insulin treated, respectively.

Arrows mark those peaks which show changes in the corresponding protein bands in response to insulin-treatment of the cultured cells.

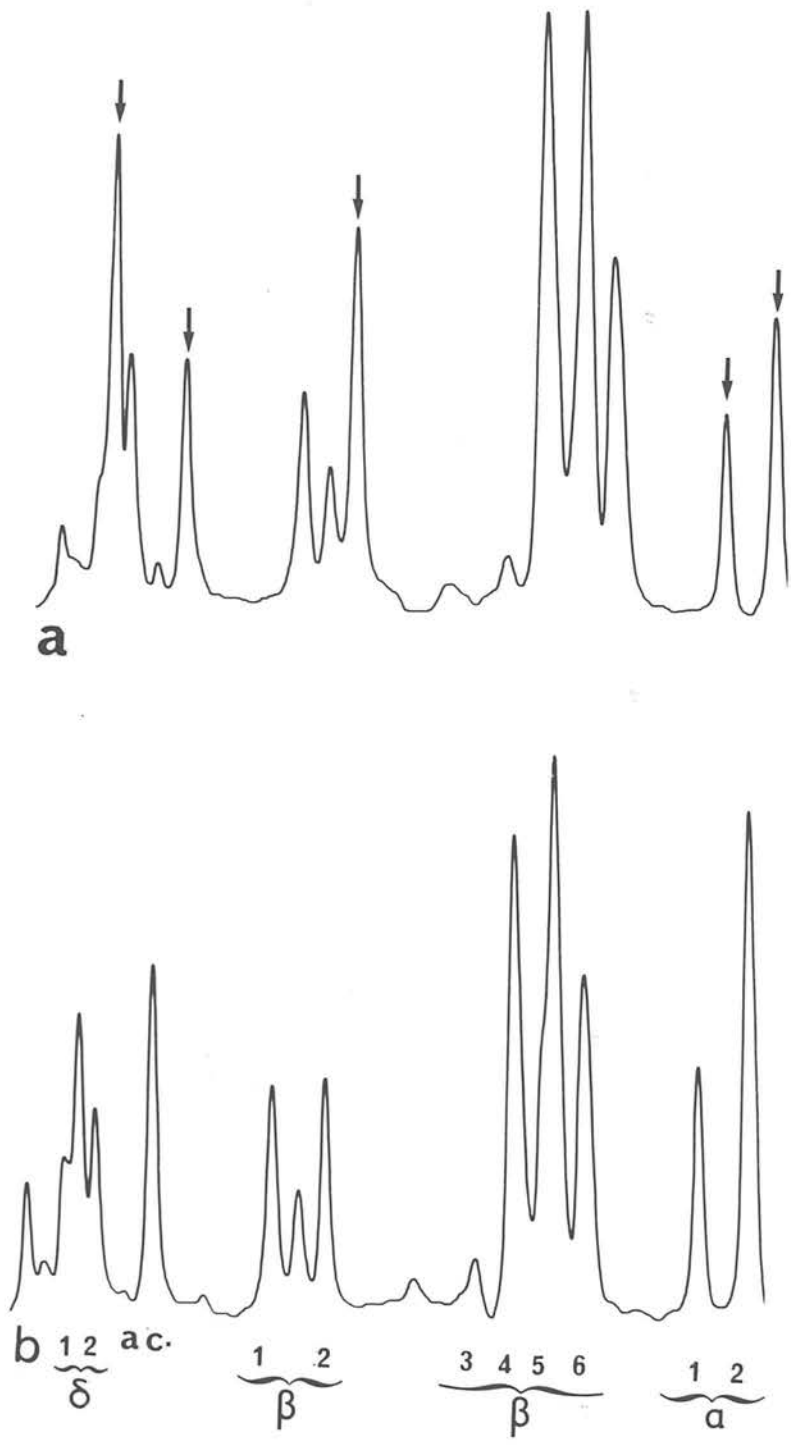


FIG 8

FIGURE 9. Traces of Fluorograph C in Plate 14 Ca (a) Control and 14 C b (b) Insulin-treated, respectively.

Arrows identify the components most affected.

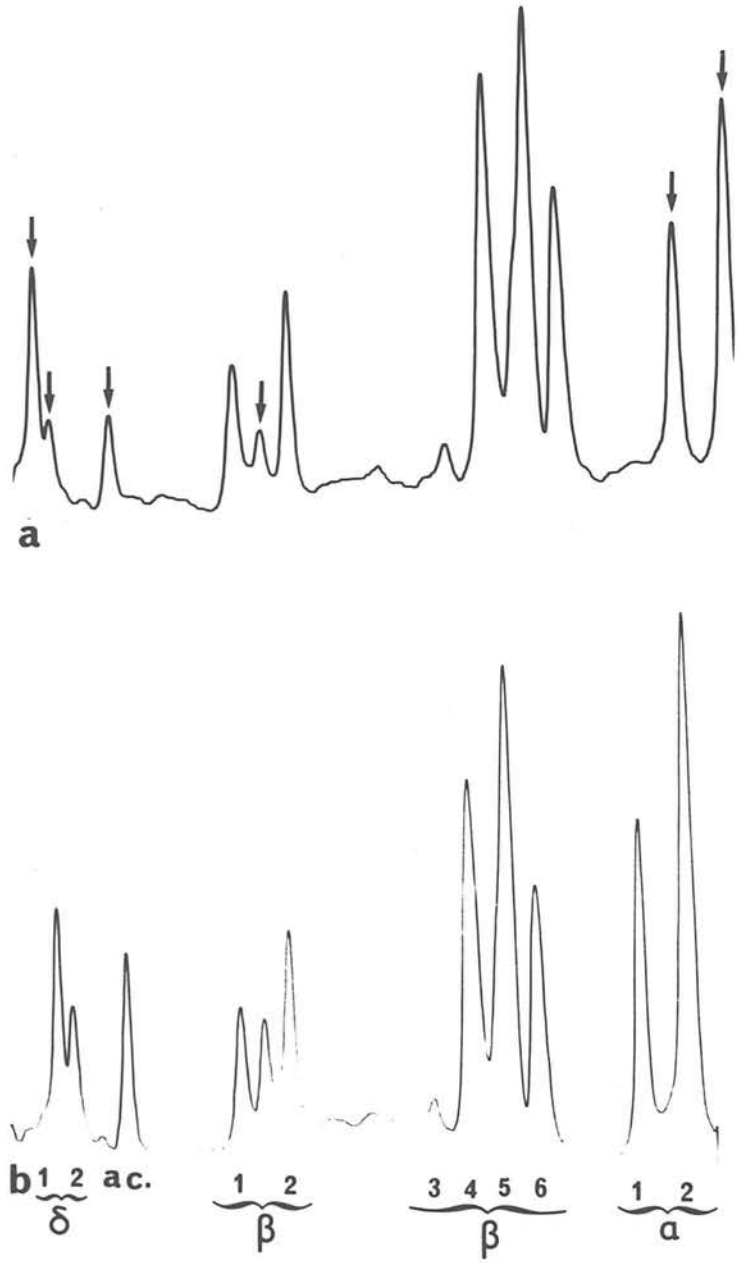


FIG 9

PLATE 15. The Effect of Insulin on Protein Synthesis of Neural Retina Cells in Culture.

SDS polyacrylamide slab gel of the water soluble extracts from 30- and 40-day cultures established from 8-day old embryos.

A. Day-old chick lens antigens as a marker.

B. Control culture of day 30, grown in standard 10% FCS medium.

C and D. The cultures grown in standard 10% FCS medium plus insulin at 10 μg/ml from day 4 onwards.

E. 40-day control culture.

F. 40-day insulin-treated (as above) culture.

Arrows identify the components affected.

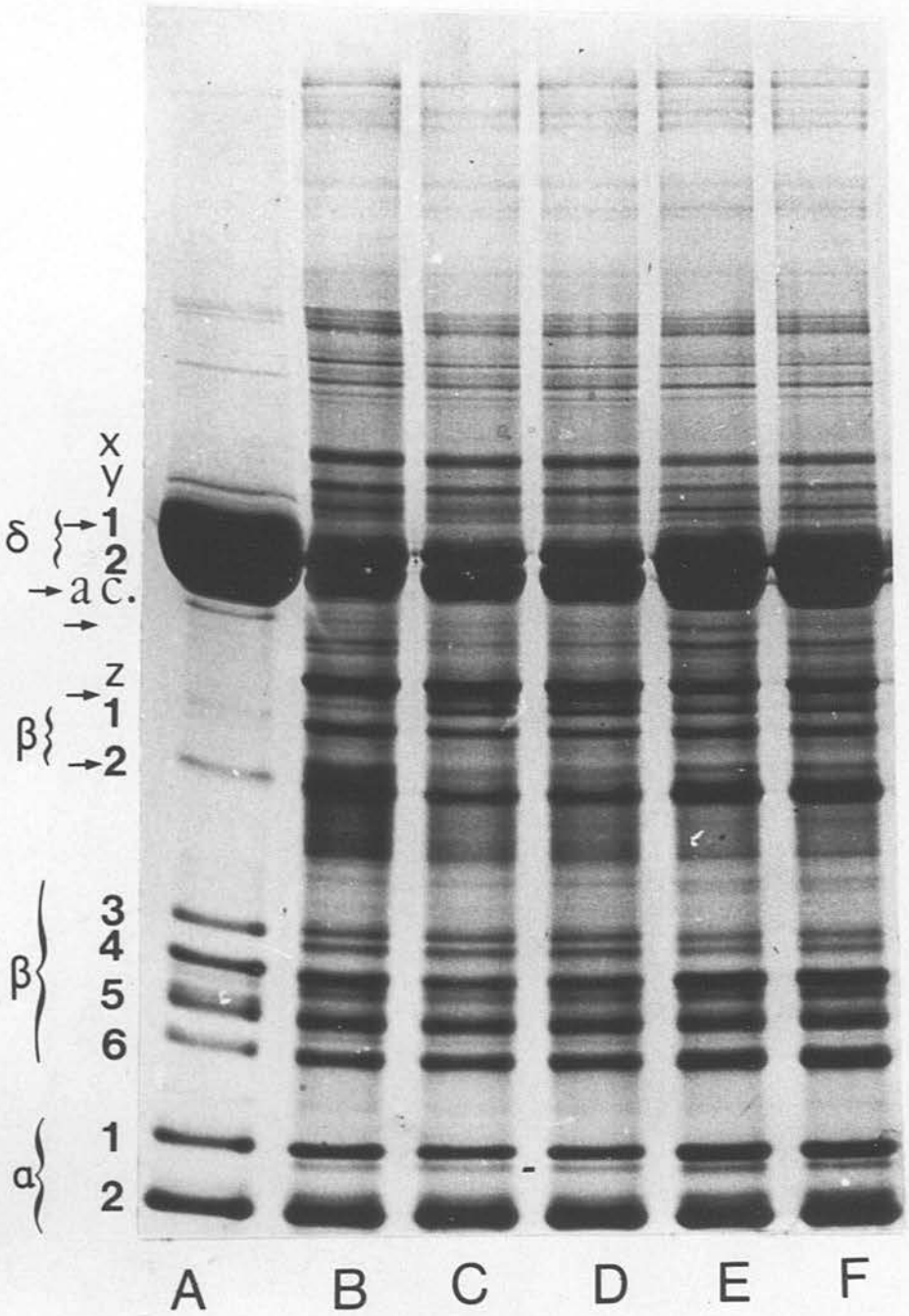


PLATE 15

FIGURE 10. Traces of Fluorographs from Plate 15 B (a) and 15 C (b) respectively.

Arrows identify those peaks which show changes in the corresponding protein bands in response to insulin-treatment of the cultured neural retina cells.

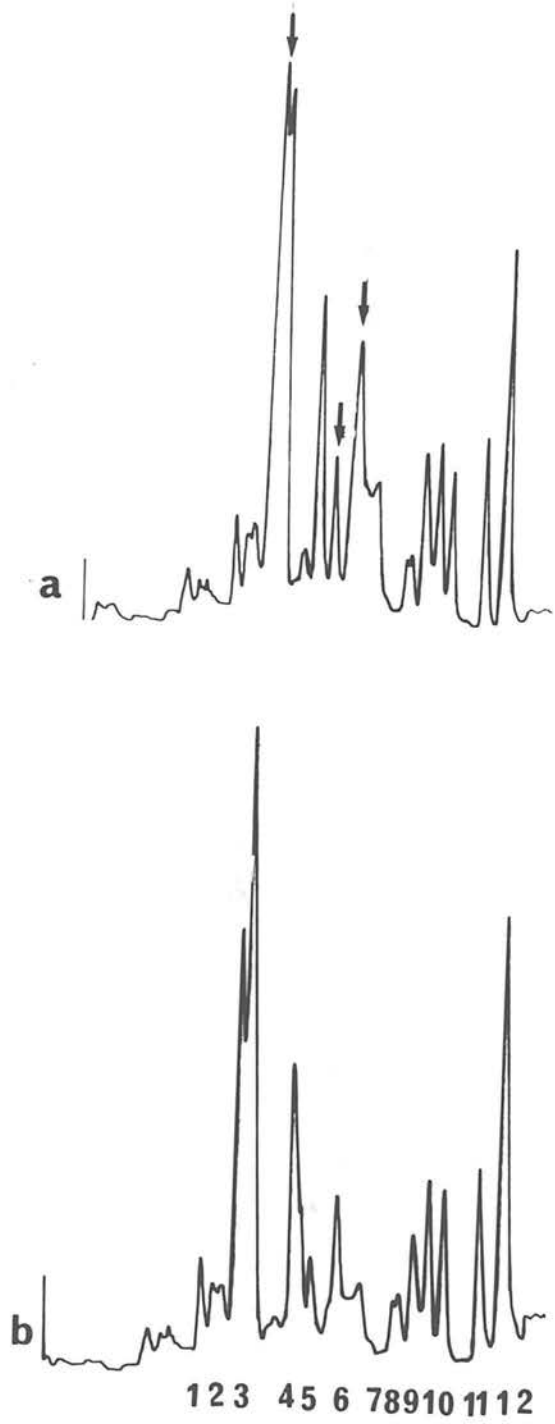


FIG 10

PLATE 16. The Effect of Insulin on Protein Synthesis of Limb Fibroblast Cells in Culture.

S.D.S. polyacrylamide slab gel of the water soluble extracts from 11-day cultures. Samples were analysed as in Plate 2. Control cultures were grown in standard 10% FCS medium. The treated cultures grown in standard medium supplemented with 2%, 4%, 6% and 10% FCS plus insulin at 10 μg/ml from day 4 onwards.

A. 10% FCS control.

B. 10% FCS plus insulin.

C. 6% FCS control.

D. 6% FCS plus insulin.

E. 4% FCS control.

F. 4% FCS plus insulin.

G. 2% FCS control

H. 2% FCS plus insulin.

Although there seems to be differences between control and 10% FCS plus insulin treated cultures, this appears to be an artifact since it was not observed in the duplicate samples.

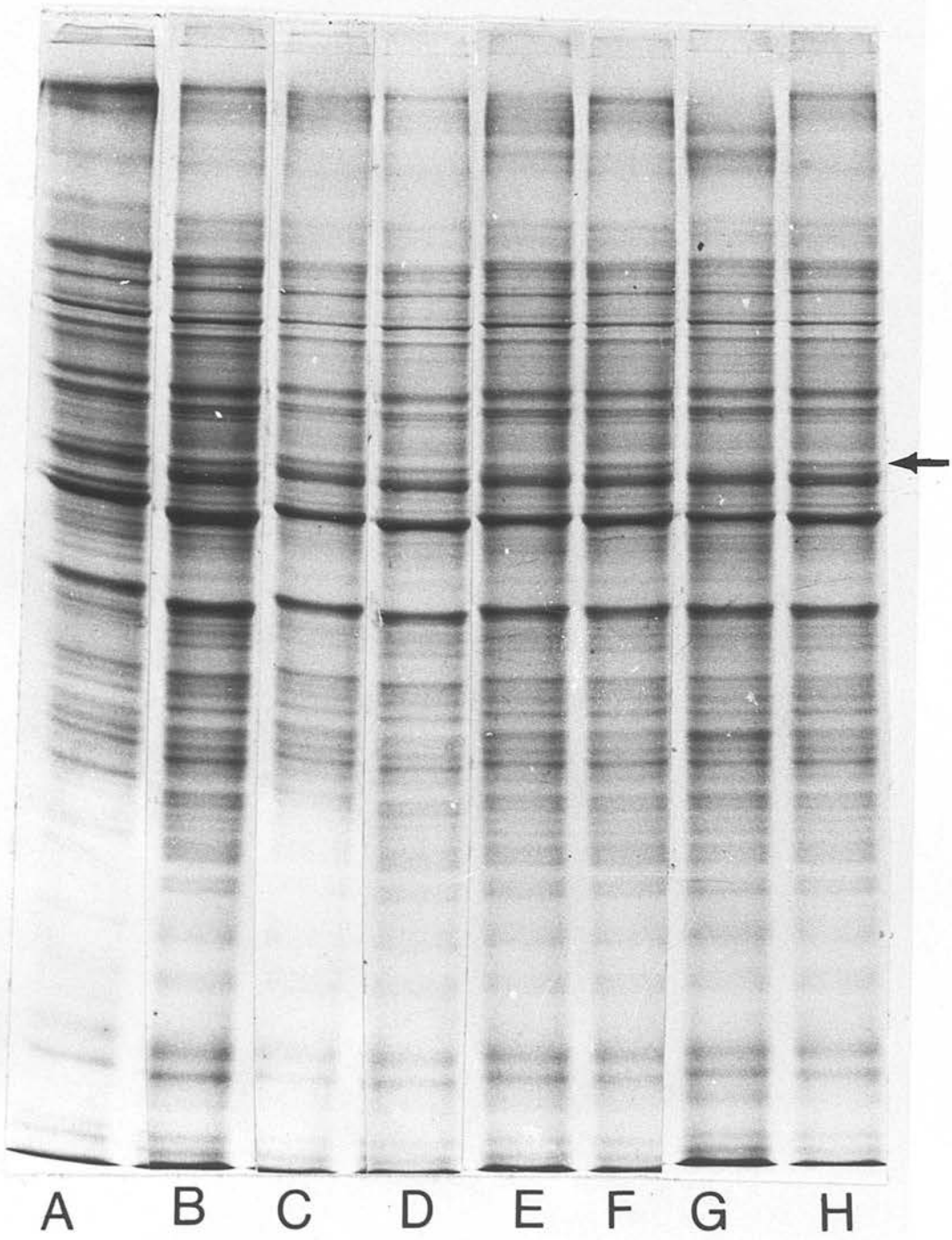


PLATE 16

These results suggest that the effect of insulin on protein accumulation is tissue-specific since it strongly affects LE cells and NR cells under all conditions before the period of lentoid formation, whereas it had no effect on kidney fibroblasts under the same conditions as those used for limb fibroblasts. Further, the higher levels of FCS (6% and 10%) appear to have an inhibitory affect on the action of insulin but this was observed only in limb fibroblast and not in kidney fibroblast cultures.

3.2.2 Studies with Diphenylhydantoin

3.2.2.1 The Effect of Diphenylhydantoin on Developing Chick Embryos

Diphenylhydantoin treatment (0.2 - 1.0 mg/egg) of chick embryos at the 48, 108 or 192 hours of incubation led to a reduced body-size in some cases, but no gross malformations were observed amongst those surviving up to the 17th day. Treatment with levels of 0.5 and 1.0 mg/egg were highly toxic when administered at the 108 or 192 hours of incubation (Table 5). No malformation were seen.

3.2.2.2 The Effect of Diphenylhydantoin on Cultured Cells.

3.2.2.2.1 Lens Epithelium.

Lens epithelial cells were treated with diphenylhydantoin every 2-3 days from day 4-15 then harvested on day 16. Dose levels of less than 40μg/ml had no effect on protein synthesis but levels higher than 60μg/ml were cytotoxic. However treatment with 50μg/ml reduced both the synthesis and the accumulation of the 50K daltons δ_1 and of actin. (Plate 17Ab; Fig. 11 and Table 6). This effect of diphenylhydantoin was transient, in that it had disappeared by 2-3 days following the treatment.

TABLE 5. THE EFFECTS OF THE INJECTION OF DIPHENYLHYDANTOIN ON DEVELOPING CHICK EMBRYOS AFTER 48, 108, AND 192 HOURS OF INCUBATION.

Hours of incubation prior to injection	Dosage mg/egg	No. of embryos injected	No. of survivors	Mortality at 6 - 17 days (%)	Embryos with reduced size (%)
48	0.20	47	36	23.4	0
	C	47	45	4.2	0
108	0.20	20	19	5.0	0
	0.50	22	13	40.9	2(15.4)
	C	22	21	4.5	0
192	0.50	45	32	28.9	2(6.2)
	1.00	29	9	69.0	2(22.2)
	C	40	38	5.0	0

C: Controls injected with the same Volume of 0.9% NaCl.

3.2.2.2.2 Neural Retina

Ten day old neural retina cell cultures were treated with diphenylhydantoin at levels of 10, 20, 40 and 50 μg/ml of medium. On day 11, the activity of choline acetyl transferase was assayed and it was found that lower doses of diphenylhydantoin (10-40 μg/ml) affected a significant increase whereas a dose of 50 μg/ml reduced CAT activity (Fig. 14). The latter treatment also reduced the synthesis and accumulation of two polypeptides with molecular weights of about 38K and 41K daltons (Plate 17B and Table 7). Further treatment every 2-3 days up to day 29 had no effect by day 30 on either the synthesis or the accumulation of the water-soluble proteins analysed.

3.2.2.2.3 Limb and Kidney Fibroblasts

Treatment of limb fibroblast or kidney fibroblast cultures with 50 μg/ml diphenylhydantoin had no effect on either the synthesis or the accumulation of the soluble proteins.

3.2.3 Studies with Chloroquine

3.2.3.1 The effect of Chloroquine Sulphate on Developing Chick Embryos

Treatment of chick embryos at various stages of incubation with chloroquine sulphate (0.20 - 1.0 mg/egg) caused a high incidence of mortality especially with the higher levels administered. The older embryos were more resistant to the toxic effects of high levels of the drug (Table 8). The treatment induced no gross malformations but in some instances an overall reduction in body-size was evident.

3.2.3.2 The Effect of Chloroquine Sulphate on Cultured Cells.

3.2.3.2.1 Lens Epithelium

Chloroquine sulphate treatment of lens epithelial cell cultures at a concentration of 2 μg/ml had no effect on protein synthesis whereas

levels of 5 μg/ml or higher were cytotoxic. However, treatment with 4μg/ml once only on day 3, reduced both the accumulation and synthesis of two minor polypeptides with molecular weights of about 38K and 41K daltons (Plate 18C and Table 9) by day 16. The treatment however did not have any effect on lentoid body differentiation in these cultures.

3.2.3.2.2 Neural Retina

Treatment of neural retina cell cultures on day 15 with chloroquine sulphate (4 μg/ml) caused, within 2 hours, an intense vacuolization of some of the neuroepithelial cells (Plate 18A). The great majority of the vacuoles disappeared after 2-3 days but a few remained for at least 20 days. The cultures continued to grow and transdifferentiation (as defined by the appearance of lentoid bodies) occurred at the normal time. By day 30 the treated cells showed a reduced synthesis and accumulation of two polypeptides with molecular weights of about 38K and 41K daltons (Plate 18B, Fig. 12 and Table 9).

Neural retina cell cultures treated with 5μg/ml chloroquine sulphate on day 10 and harvested after 24 hours showed an increased synthesis of two polypeptide bands with molecular weights of 43K and 94K daltons (Plate 17B and Table 7). This effect was not observed when the cultures were treated with 2.5 μg/ml chloroquine sulphate. However, both treatments reduced the level of CAT activity in these cultures, (Fig. 14).

3.2.3.2.3 Brain Tissue

Treatment of brain cell cultures with chloroquine sulphate (4 μg/ml) on day 6 had no effect on either the accumulation or the synthesis of water-soluble proteins by day 10 of culture. Treatment on day 10 had a delayed effect, reducing both the synthesis and accumulation of two polypeptides with molecular weights of about 52K and 55K daltons

(Plate 18D), by day 30. Treatment on day 29 reduced both the synthesis and the accumulation of two polypeptides with molecular weights of 38K and 41K daltons within 24 hours (Plate 18D and Table 9).

3.2.3.2.4 Limb and Kidney Fibroblasts

The treatment of limb fibroblast or kidney fibroblast cultures with chloroquine sulphate (2 or 4 μg/ml) had no effect on either the synthesis or the accumulation of the water-soluble proteins analysed.

3.2.4 Studies with Chlordiazepoxide

3.2.4.1 The Effect of Chlordiazepoxide HCl on Developing Chick Embryos

Chick embryos were treated with chlordiazepoxide HCl at various stages of incubation, at dosage levels in the range of 0.12 - 2.50 mg/egg (Table 10). It was found that the mortality rate was higher in those embryos treated (1.25 mg/egg) at the 48 hours stage than later stages (108 and 192 hours). No gross malformation was observed in surviving embryos treated at the 48 hour stage. However, with those treated with chlordiazepoxide HCl (0.25 and 1.25 mg/egg) at the 108 hours stage a reduction in overall body-size (in 7.7% and 16.7%, respectively,) was observed by day 17. A similar result was found with embryos treated with either 1.25 or 2.50 mg/egg at the 192 hours stage of incubation. A characteristic of those embryos with overall reduced body-size was an acute shortening of the long bones of the hind limbs where the lengths of femur, fibula and tibia were reduced by almost half (Plate 19A).

3.2.4.2 The Effect of Chlordiazepoxide HCl on Cultured Cells.

3.2.4.2.1 Lens Epithelium

Treatment of lens epithelial cell cultures with subtoxic levels of chlordiazepoxide HCl (25 and 50 μg/ml) failed to affect the accumulation or synthesis of the water-soluble proteins. Treatment with a higher but slightly toxic level (75 μg/ml) as judged by the dead cells floating

in the medium, reduced both the synthesis and accumulation of the polypeptides with molecular weights higher than 30K daltons including some of the β-crystallins and that of actin.

3.2.4.2.2 Neural Retina

Neural retina cell cultures were treated with Chlordiazepoxide HCl (25 or 50 μg/ml) from the 4th up to the 10th day of inoculation. The high level of chlordiazepoxide HCl (50 μg/ml) reduced the accumulation and synthesis of several polypeptides with molecular weights of the range 35K and 43K daltons, whereas the lower level (25 μg/ml) had no effect either on the accumulation or synthesis of the water-soluble proteins, (Plate 17B). However if the treatment took place over days 15 to 25 the effect was not apparent.

When neural retina cells were treated with various levels of Chlordiazepoxide HCl (10, 20, 40 and 50 μg/ml of medium) on the 9th day of inoculation, and then harvested 24 hours later, a dose-related effect on the activity of cholineacetyl transferase (CAT) was observed. Low levels(from 10, 20 and 40 μg/ml) showed a dose related stimulation. However the highest level (50 μg/ml) inhibited the activity of CAT. (Fig. 14).

3.2.4.2.3 Limb Fibroblasts

Cell cultures established from 8-day old chick embryo hind limbs were treated with chlordiazepoxide HCl (25 or 50 μg/ml) on the 4th, 7th and 10th days, and then harvested 24 hours later. The low level of Chlordiazepoxide HCl (25 μg/ml) had no effect whereas the highest level used (50 μg/ml) reduced both the synthesis and accumulation of a protein with a molecular weight of about 38K daltons (Plate 19A, 19B, Fig. 13, and Table 11). This effect of the drug on protein synthesis is

transitory and disappears 48 hours following treatment.

Results from the in vitro translation of mRNA extracted from control and treated cultures also showed a reduced synthesis of a protein with a molecular weight of about 38 K daltons (Plate 19C and Table 11).

3.2.4.2.4 Kidney Fibroblasts

Kidney cell cultures from 8-day old chick embryos were treated with chloriazepoxide HCl (25 or 50 µg/ml of medium) every 2-3 days, from day 4 to 10 and harvested 24 hours later. Chlordiazepoxide HCl treatment at 25 or 50 µg/ml but not with any lower dose reduced both the synthesis and the accumulation of actin (Plate 19D and Table 11). Treatment with the higher level (50 µg/ml) also reduced that of a protein with molecular weight of about 38K daltons (Plate 19D).

3.2.5 Studies with Meprobamate

3.2.5.1 The Effect of Meprobamate on Developing Chick Embryos.

Chicken embryos at 48, 108, and 192 hours of incubation were treated with meprobamate at levels ranging from 0.08 - 0.80 mg/egg. These treatments induced no gross malformation in those embryos which survived up to the 18th day stage. However, a dose- and stage-dependent toxic effect of meprobamate was observed. (Table 12). Meprobamate treatment of chick embryos at 108 hours of incubation at levels of 0.25, 0.50 and 0.80 mg/egg induced mortalities in 10.0%, 17.4% and 30.0% of the embryos respectively. Treatment with 0.8mg/egg at the 192 hours of incubation induced only a 10% mortality (Table 12).

3.2.5.2 The Effect of Meprobamate on Cultured Cells.

Meprobamate at concentrations of 10-50 µg/ml of medium had no effect on either the synthesis or accumulation of proteins of lens epithelial, neural retina, and kidney cell cultures, but at 50 µg/ml it increased

both the synthesis and accumulation of a polypeptide with m.w. 28 K daltons in limb fibroblast cell cultures (Plate 19E).

3.2.6 Studies with Cobalt

3.2.6.1 The Effect of Cobaltous Chloride on Developing Chick Embryos

Chick embryos at the 48, 108 and 192 hours stages of incubation were treated with cobaltous chloride (0.05 - 0.5 mg/egg). The mortality rate was lowest with those embryos treated at the 192 hours stage while the treatment with the same level at either the 48 or 108 hours stages lead to a higher incidence of mortality (Table 13).

In addition to a reduction in overall body-size the most common malformation induced by this treatment, especially with embryos of the 192 hour stage, was an opacity of the lens often associated with microphthalmia (Plate 20).

3.2.6.2 The Effect of Cobaltous Chloride on Cultured Cells

3.2.6.2.1 Lens Epithelium

The treatment of lens epithelial cell cultures every 2-3 days with cobaltous chloride from day 4 until day 14 had an effect detectable by day 15; both the synthesis and accumulation of a polypeptide with a molecular weight of about 41K daltons was increased (Plate 21). Cobaltous chloride treatment at levels greater than 5 μg/ml were cytotoxic whereas those less than 2 μg/ml had no effect on protein synthesis.

3.2.6.2.2 Neural Retina and Limb Fibroblasts.

Treatment of either neural retina or limb fibroblast cell cultures with cobaltous chloride (2 or 4 μg/ml) did not have any effect on the water-soluble proteins synthesised by either tissue.

3.2.6.2.3 Kidney Fibroblasts

Kidney fibroblast cultures treated with cobaltous chloride (4 μg/ml) every 2-3 days from day 4 to 10 reduced by day 11, both the synthesis and the accumulation of a protein with a molecular weight of 43K daltons (Plate 21B, and Fig. 15). Treatment with 2 μg/ml had no effect.

3.2.7 Studies with Lead

3.2.7.1 The Effect of Lead Nitrate on Developing Chick Embryos

Chick embryos at the 48, 108 and 192 hours of incubation were treated with lead nitrate (0.02 - 0.2 mg/egg). It was found that the mortality rate was highest with embryos treated at the earlier stages and with those treated with the higher levels of lead nitrate (Table 14). Treatment at the 48 hours of incubation with 0.1 mg lead nitrate caused a reduced overall body size in 26% of the surviving embryos examined on the 17th day of incubation. This treatment also caused spinal injury at the caudal region and such embryos did not survive beyond 10 days of incubation.

Treatment at the 108 hours of incubation with lead nitrate (0.05, 0.10 and 0.20 mg/egg) induced a high incidence of brain haemorrhage, spinal injury, and especially of hydrocephalus, by the 10th day. Some embryos with hydrocephalus or brain haemorrhages survived until the 17th day (Plate 22).

Embryos treated with lead nitrate (0.1 and 0.2 mg/egg) at 192 hours of incubation (stage) had a higher incidence of brain defects than embryos treated at other stages, but other effects were not seen. (Table 14).

In the embryos developed brain haemorrhages or hydrocephalus, the brain was swollen overall. Necrosis of brain tissue was detected

by the 18th day of incubation. In such embryos there was only a thin layer of nervous tissue and the brain lumen was filled with haemorrhagic fluid (Plate 22).

3.2.7.2 The Effect of Lead Nitrate on Cultured Cells.

Cell cultures of lens epithelium, brain, neural retina, limb fibroblasts and kidney fibroblasts were treated with lead nitrate at levels of 5 μg/ml and 7.5 μg/ml of medium.

Continuous treatment with 5 μg/ml over the period of 4 -15 days of culture failed to affect either the profiles of accumulated proteins or the relative rates of synthesis of these proteins by lens epithelium, limb fibroblasts, kidney fibroblasts and brain cell cultures. Levels above 7.5 μg/ml were cytotoxic.

However, the continuous treatment of neural retina cell cultures with lead nitrate (5 μg/ml) through days 6-29, showed that on day 30 the treated cultures contained significantly reduced levels of two proteins, 38K daltons and a 41K daltons (Plate 21C).

Lead nitrate treatment (2.5 μg/ml and 5 μg/ml) of neural retina cell cultures on day 9 failed to affect choline acetyl transferase activity by day 10. However, the effect of continuous lead nitrate treatment at these levels on choline acetyl transferase activity was not investigated because such treatments are cytotoxic to neuronal cells.

PLATE 17A. The Effect of Diphenylhydantoin and Meprobamate on Protein Synthesis of 16-Day Cultures of Lens Epithelium from Day-Old Chicks.

The cultures were transferred to medium containing the drugs on day 6, with medium changes every 2-3 days and harvested on day 16. The cultures were treated with the drugs every time the medium was changed.

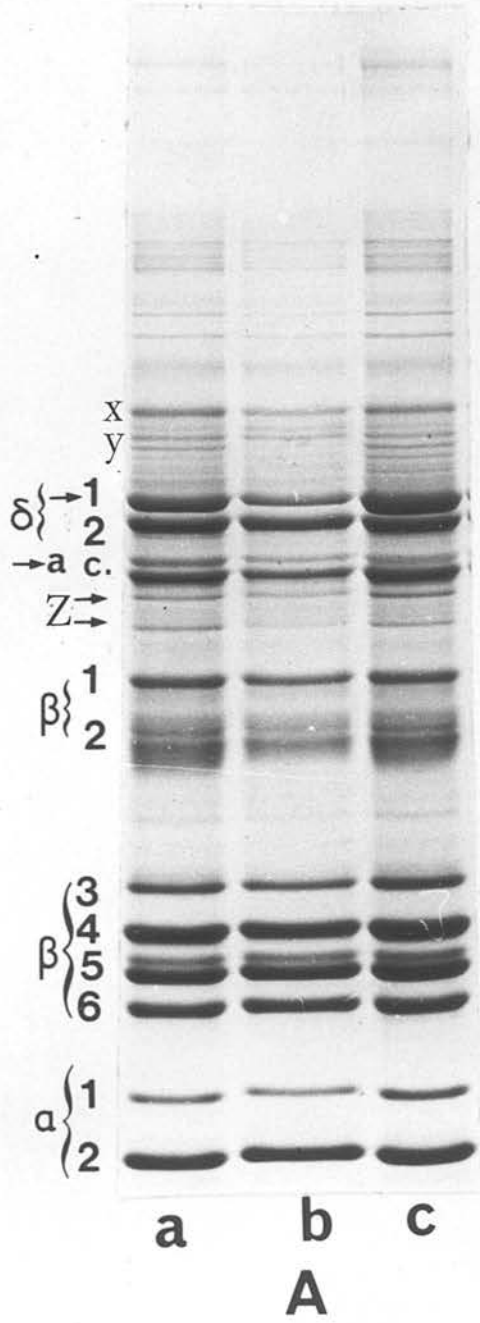
A. Culture 1. (a) Control; (b) 50 μg/ml Epanutin; (c) 50 μg/ml Meprobamate. Note that Meprobamate did not affect the protein profile.

PLATE 17B. The Effect of Several Substances on the Protein Synthesis in 10-Day Neural Retina Cell Cultures, established as described in Plate 15.

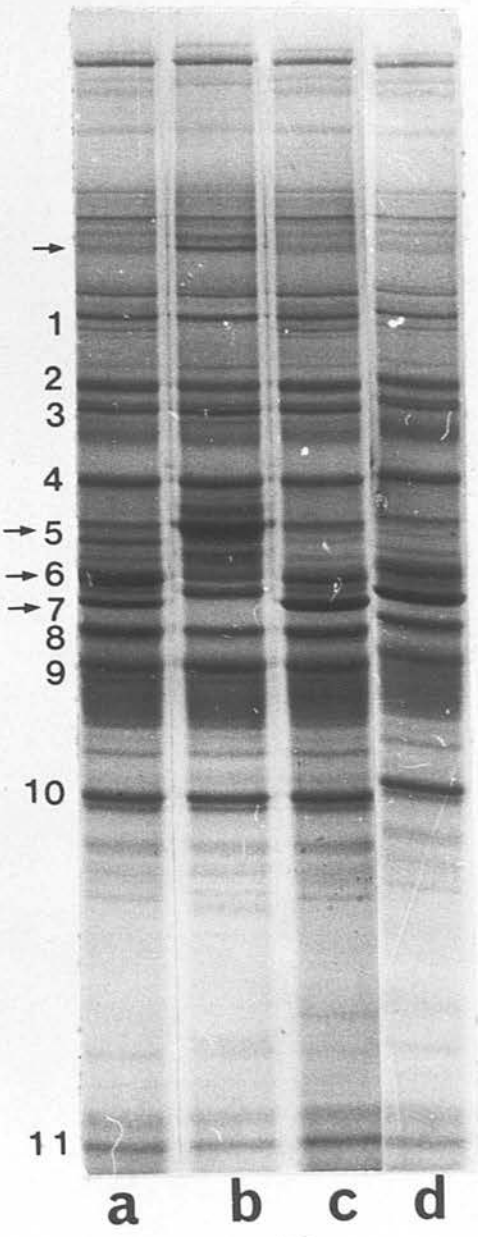
Both neuroepithelial and neuronal cells are present in 8-day old embryo neural retina cell cultures, at this stage. The cells were treated with a single dose of each substance on day 8 of inoculation in standard 10% FCS medium.

Control culture grown in standard 10% FCS (a) medium, (b) chloroquine sulphate (Nivaquine) 4 μg/ml, (c) chlordiazepoxide HCl (Librium), 50 μg/ml; (d) diphenylhydantoin or phenytoin sulphate (Epanutin), 50 μg/ml, the cultures were treated on day 9 of inoculation in the the presence of 10% FCS.

Arrows indicate the components most affected.



A



B

PLATE 17

FIGURE 11. Traces of Plate 17 Aa (a) and 17 Ab (b) respectively. Arrows mark the peaks which show the components most affected.

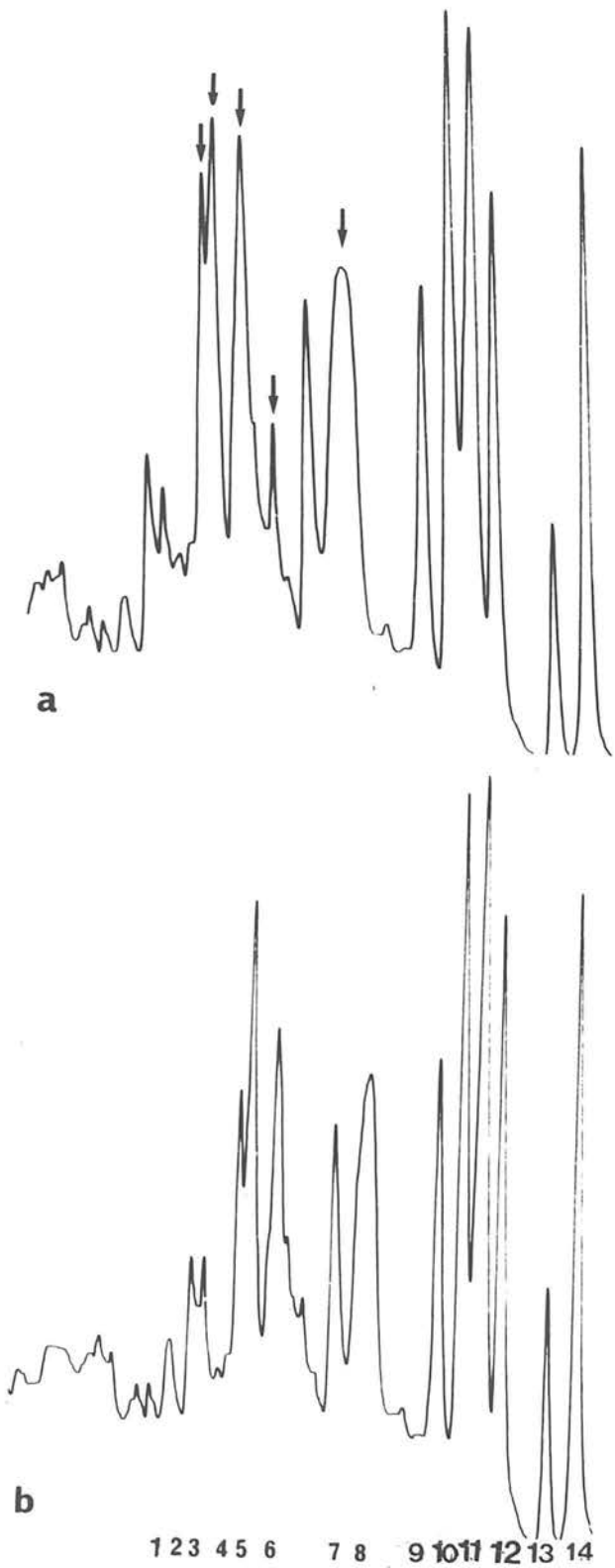


FIG 11

TABLE 6: Quantitative Densitometric Readings of Drug-Treated and Control Cultures.

The area of each peak representing the numbered protein bands from plate 17A (a,b), expressed as a percentage of the total.

Electrophoretic Separation of Accumulated Proteins				
Crystallins	Peak No.	Control	D.P.H.	**
X	1	3.3	2.9	(±0.5)
Y	2	2.4	2.6	(±0.8)
δ1	3	7.5	5.9	(±0.4)
δ2	4	6.0	6.4	(±1.8)
AC	5	9.2	8.3	(±0.5)
Z	6	4.2	4.2	(±0.7)
β1	7	5.3	5.2	(±0.5)
β2	8	12.3	10.3	(±0.6)
β3	9	4.8	5.1	(±0.2)
β4	10	6.0	6.6	(±0.6)
β5	11	6.8	8.1	(±1.6)
β6	12	4.5	5.2	(±0.5)
α1	13	1.5	1.5	(±0.7)
α2	14	3.9	4.4	(±1.7)

X,Y.Z: Unidentified Proteins

D.P.H.: Diphenylhydantoin

**: Mean variation between duplicate samples.

TABLE 7: Quantitative Densitometric Readings of Drug Treated and Control Cultures

The area of each peak, representing the numbered protein bands from plate 17Ba, Bb Bc and Bd, expressed as a percentage of the total.

Electrophoretic Separation Accumulated Proteins					
Peak No.	Control	C.Q.	D.P.H.	C.D.P.	**
1	6.3	5.0	5.0	5.4	(±0.8)
2	8.4	6.3	7.3	8.7	(±0.7)
3	8.1	6.2	8.2	7.2	(±0.9)
4	10.2	7.1	11.2	9.5	(±1.2)
5	5.1	15.0	8.6	8.2	(±1.4)
6	5.7	4.6	6.2	5.3	(±0.3)
7	8.7	18.0	4.1	3.8	(±1.6)
8	4.4	3.3	4.0	4.4	(±1.3)
9	14.4	12.3	14.6	14.5	(±0.8)
10	5.6	4.8	5.9	5.6	(±0.4)
11	5.7	3.2	5.3	6.0	(±0.3)

C.D.P.: Chlordiazepoxide hycrochloride

D.P.H.: Diphenyl hydantoin

C.Q.: Chloroquine sulphate

**: Mean variation between duplicate samples

TABLE 8. THE EFFECTS OF THE INJECTION OF CHLOROQUINE SULPHATE ON DEVELOPING CHICK EMBRYOS AFTER 48, 108, AND 192 HOURS OF INCUBATION.

Hours of incubation prior to injection	Dose mg/egg	No. of embryos injected	No. of survivors	Mortality at 6-17 days (%)	Embryos with reduced size (%)
48	0.20	31	8	74.2	1(12.5)
	C	20	18	10.0	0
108	0.20	50	30	40.0	0
	0.40	42	14	66.7	3(21.4)
	C	30	30	0	0
192	0.40	53	38	28.3	5(13.1)
	1.00	21	5	76.2	3(60.0)
	C	40	40	0	0

C: Controls injected with the same volume of 0.9% NaCl.

PLATE 18. The Effect of Chloroquine Sulphate (Nivaquine) on Cellular Morphology and Protein Synthesis of Cultured Cells.

A. 16-Day neural retina(NR) Culture established from 8-day old embryos (phase contrast 100 x magnification), and grown in standard 10% FCS. (a) control. (b) treated once with nivaquine (4 μg/ml) in the standard medium containing 10% FCS on day 14. Cells are abnormal and nuclei are swollen following nivaquine treatment.

B. Water soluble fractions of 30-day NR culture. (a) Control. (b) Treated once with nivaquine as in A above, on day 29. (c and d) Treated with nivaquine as in A above on day 15.

C. Soluble fractions of 16-day lens epithelial cell cultures, established from day-old chicks, grown in standard 6% FCS. (a) Control. (b) Treated once with nivaquine as in A above on day 6.

D. Fractions from 30-day brain cell cultures, established from 8-day old embryos, grown in standard 10% FCS. (a) Control. (b) Treated once with nivaquine as in A above, on day 29. (c) Treated once with nivaquine on day 15.

Proteins were analysed as in Plate 2. Arrows identify those components that are most affected.

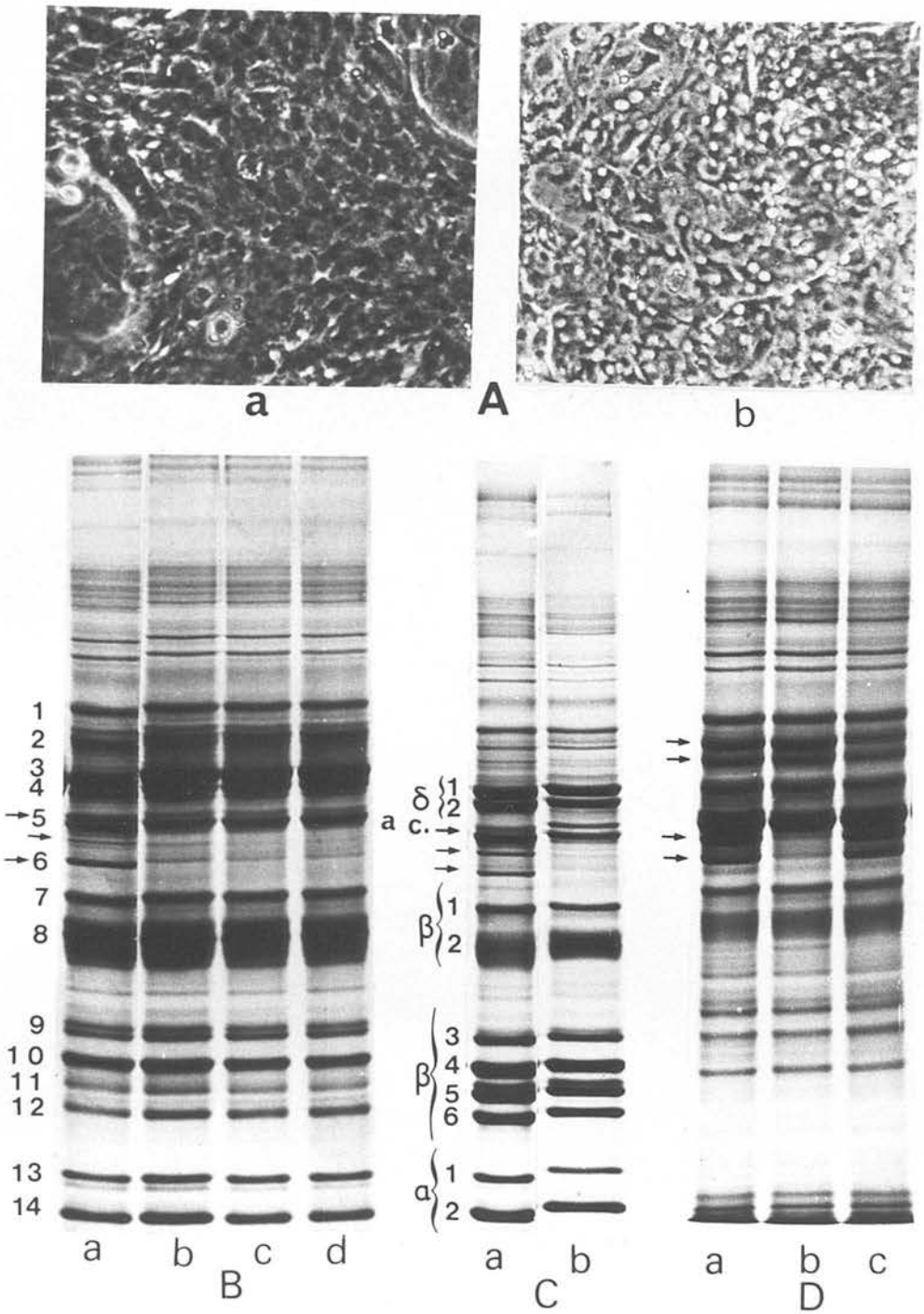

a
A
b
1
2
3
4
5
6
7
8
9
10
11
12
13
14
a b c d
B
a
δ 1 2
c.
β 1 2
β 3 4 5 6
α 1 2
a b
C
a b c
D

PLATE 18

FIGURE 12. Traces of Plate 18 Ba (a) and 18 Bb (b) respectively. Arrows mark the peaks which show changes in the corresponding protein bands in response to chloroquine sulphate treatment of the cultured neural retina cells.

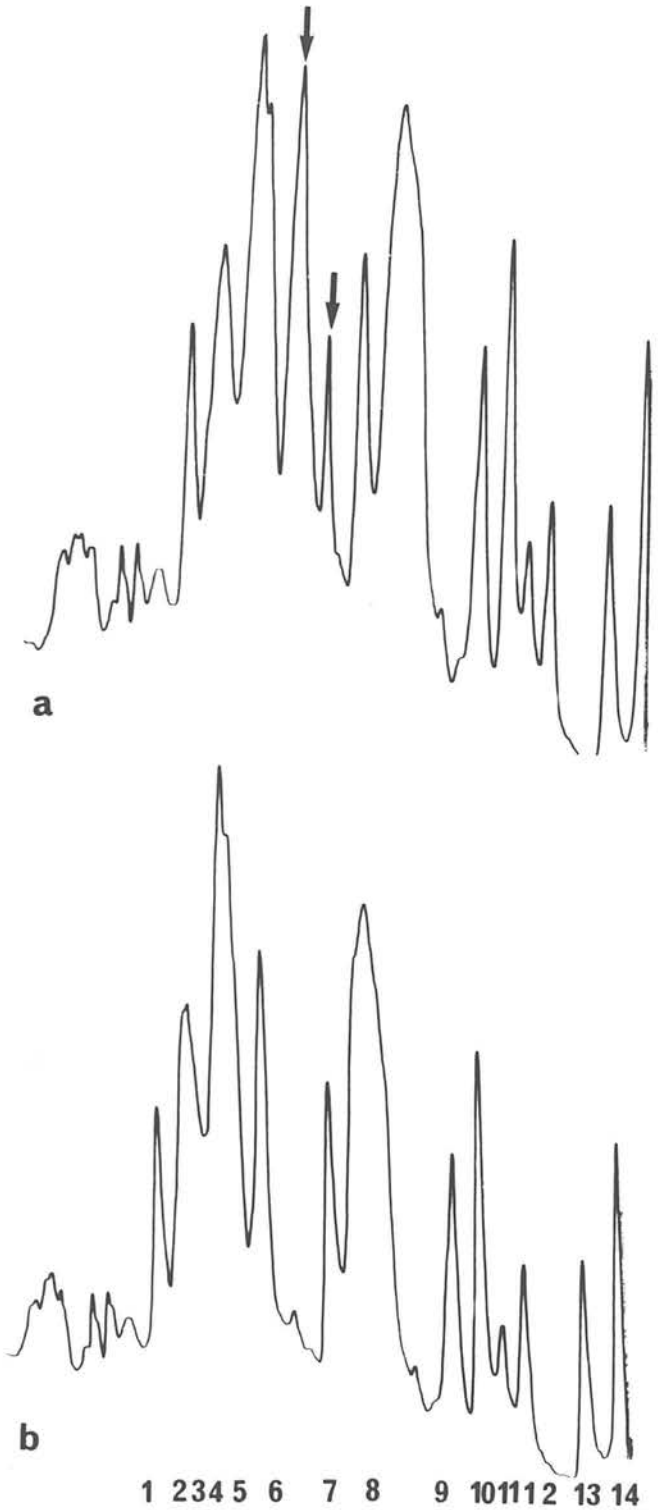


FIG 12

TABLE 9 : Quantitative Densitometric Readings of Drug Treated and Control Cultures.

The area of each peak representing the numbered protein bands from Plate 18Ba,Bb, Ca, Cb, Da and Db, expressed as a percentage of the total.

Electrophoretic Separation of Accumulated Proteins.

		Tissues Tested						
		Neural Retina		Lens Epithelium		Brain Cells		
Crysallin	Peak No.	Control	C.Q.	Control	C.Q.	Control	C.Q.	**
X	1	1.5	1.7	1.3	1.2	2.1	2.1	(±0.5)
Y	2	1.2	1.4	1.5	1.6	1.4	1.6	(±0.8)
δ1	3	6.7	5.5	5.7	7.0	7.0	7.0	(±0.4)
δ2	4	2.6	2.4	6.4	6.4	3.0	3.2	(±1.8)
AC	5	1.8	1.2	10.1	6.8	1.5	1.7	(±0.5)
Z	6	5.6	5.2	2.1	1.7	5.0	5.2	(±0.7)
β1	7	11.4	11.0	4.5	4.6	11.6	9.0	(±0.5)
β2	8	13.3	14.5	11.4	13.1	8.2	7.2	(±0.6)
β3	9	10.3	8.8	4.8	4.5	12.1	10.3	(±1.2)
β4	10	3.2	3.4	6.6	6.1	4.8	4.5	(±0.6)
β5	11	5.3	5.1	7.8	7.1	5.4	5.2	(±1.6)
β6	12	20.0	19.5	3.9	3.8	4.5	4.6	(±0.5)
α1	13	3.3	4.5	1.0	1.2	9.2	9.3	(±0.7)
α2	14	4.8	4.1	3.7	3.5	5.1	5.5	(±1.7)

X. Y, Z: Unidentified proteins.

C.Q.: Chloroquine sulphate.

**: Mean variation between the duplicate samples.

Note: Crystallins representing the proteins only in Lens Epithelium.

TABLE 10. THE EFFECTS OF THE INJECTION OF CHLORDIAZEPOXIDE HCl ON DEVELOPING CHICK EMBRYOS AFTER 48, 108 AND 192 HOURS OF INCUBATION.

Hours of incubation prior to injection	Dose mg/egg	No. of embryos injected	No. of survivors	Mortality at 6-17 days (%)	Reduced size (especially legs) (%)
48	0.12	16	14	12.5	0
	0.25	20	16	20.0	0
	1.25	20	4	80.0	0
	C	20	18	10.0	0
108	0.12	24	24	0	0
	0.25	26	26	0	2(7.7)
	1.25	20	6	70.0	1(16.7)
	C	16	16	0	0
192	1.25	41	39	4.9	2(5.1)
	2.50	110	45	59.1	3(6.7)
	C	56	54	3.4	0

C: Controls injected with the same volume of 0.9% NaCl

PLATE 19. The Effect of Chlordiazepoxide HCl (Librium) on Chick Embryos and the Effect of Meprobamate on Cultured Cells.

A. 17-day chick embryo skeletons stained as in Plate 1A. Left, untreated; right, injected with 2mg Libruum per egg at 8-day of incubation.

B. Limb fibroblast culture from 8-day old chick embryos, transferred to medium containing Librium on day 4, with medium changes every 2-3 days, and harvested on day 12. Water soluble proteins were analysed as in Plate 2. (a) Control. (b) 25 μg/ml Librium. (c) 50 μg/ml Librium. (d) 4 μg/ml Cobaltous chloride.

C. Fluorographs of translation products of mRNA from 12-day cultures as in B above. (a) Control. (b) 50 μg/ml Librium. Here the affected protein (3) has run to a much lower point on the gel than the accumulated protein (3) in B above. This is because the two samples were run at different times.

D. Kidney fibroblasts from 8-day old chick embryos. Treated with Librium as in B above, and harvested on day 12. (a) Control. (b) 25 μg/ml Librium. (c) 50 μg/ml Librium.

E. Limb fibroblasts established as in B above, treated with Meprobamate as in B above and harvested on day 12. (a) Control. (b) 50 μg/ml meprobamate.

Arrows identify the components most affected.

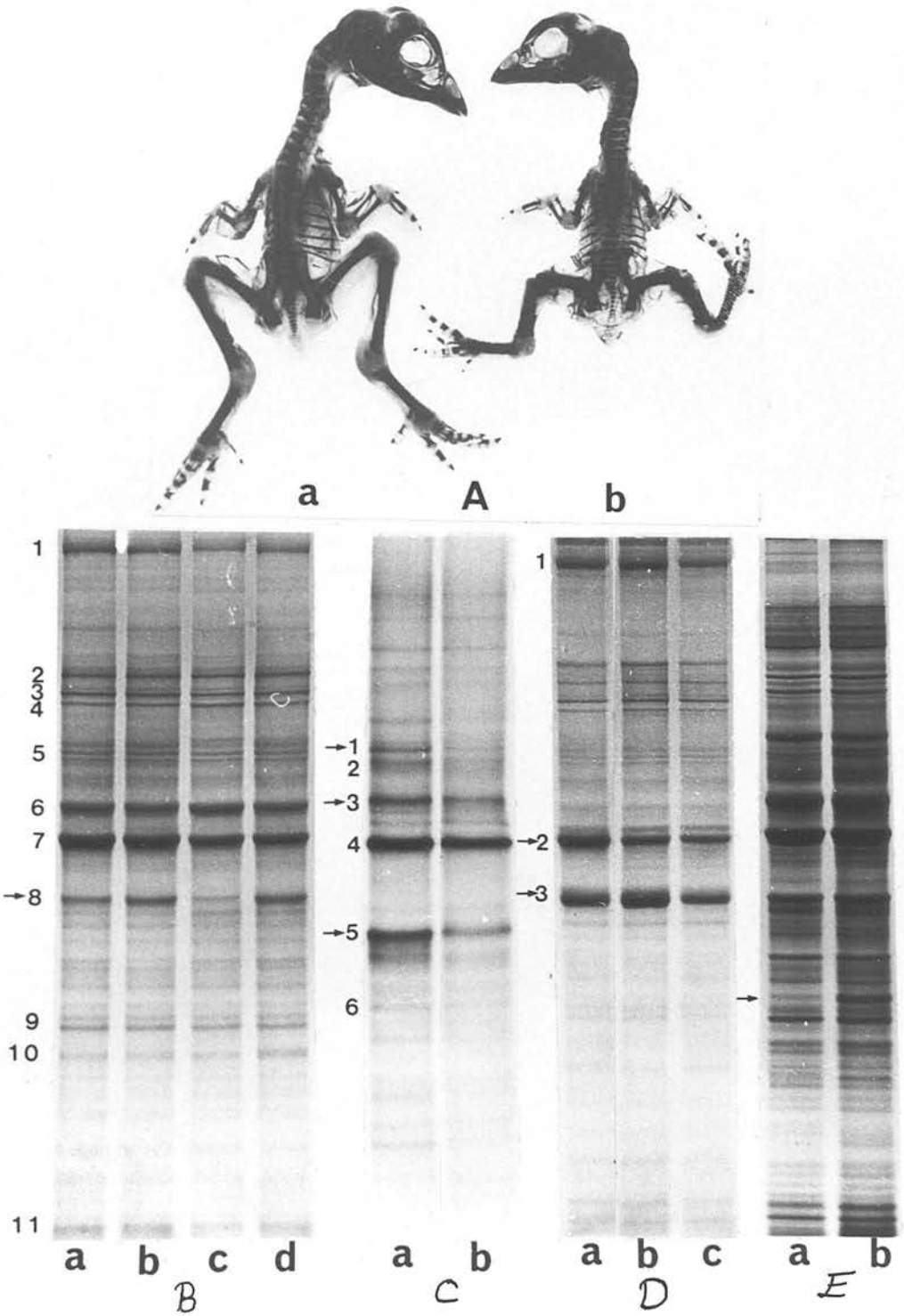


PLATE 19

FIGURE 13. Traces of Plate 19 Bb (a) and 19 Bc (c) respectively. Arrows identify the peaks which show the components most affected.

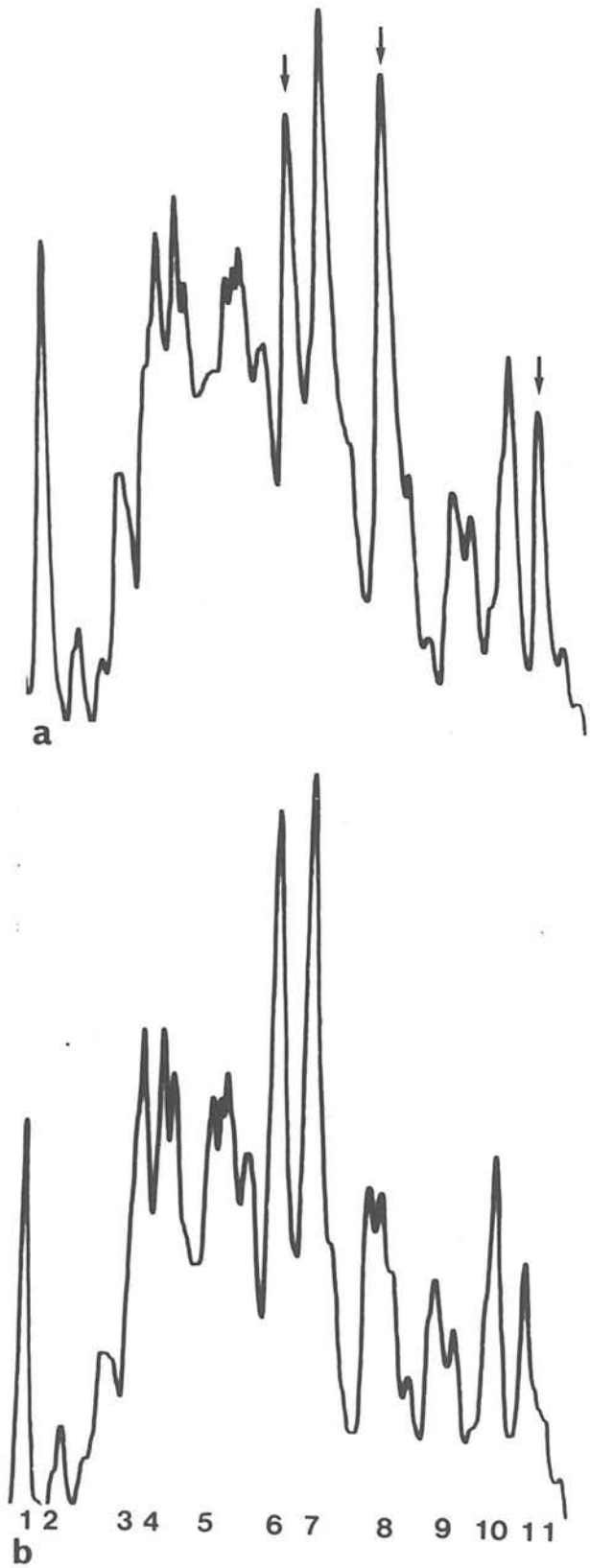


FIG 13

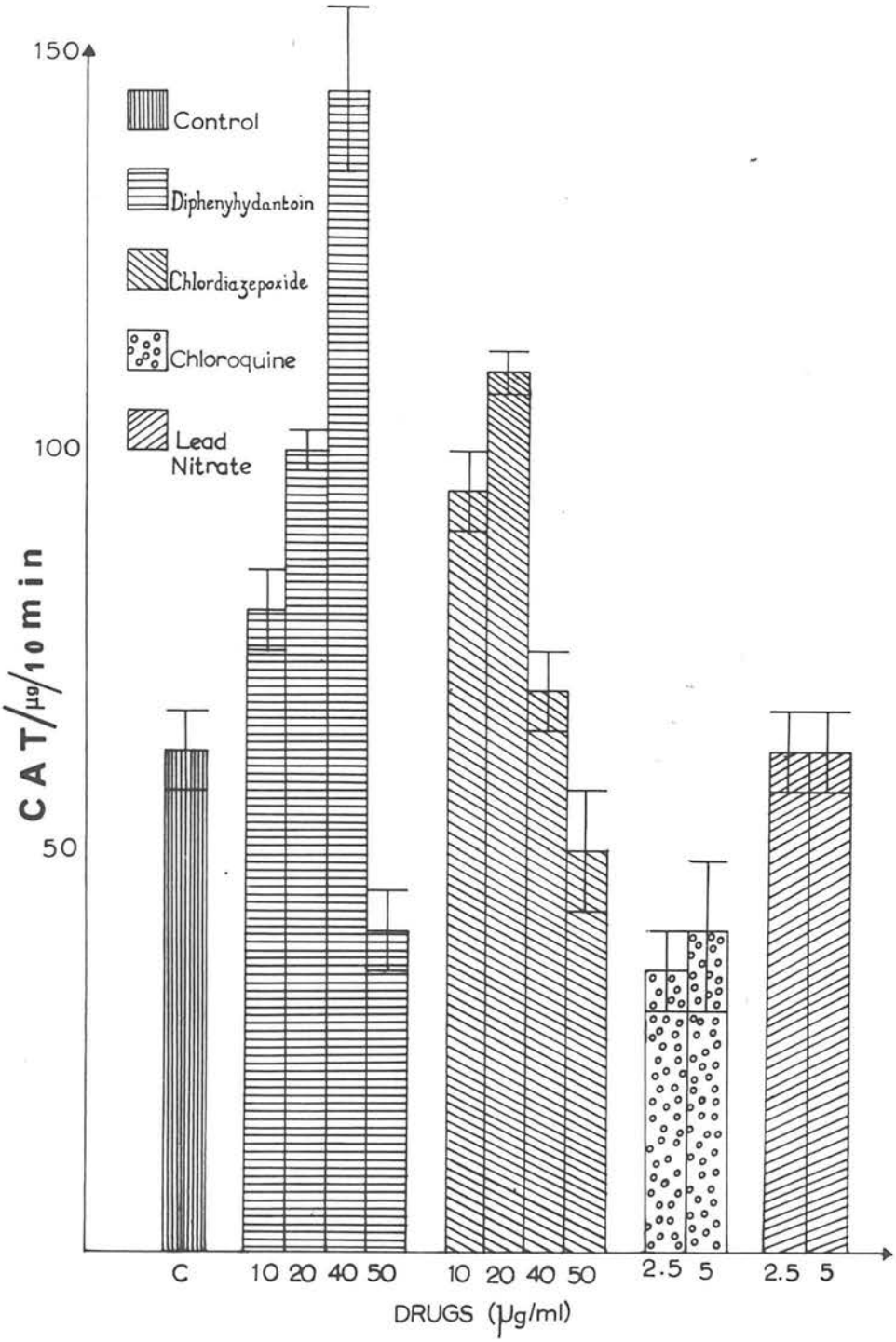


FIG. 14 CAT Activity in 10 day neural retina cultures treated with several agents on Day 9 of culture.

TABLE 11: Quantitative Densitometric Readings of Drug Treated and Control Cultures. The area of each peak Representing the numbered protein bands from plate 19Ba, Bc, Ca, Cb and Da, Db and Dc, expressed as a percentage of the total.

	Electrophoretic Separation : Accumulated Proteins					Translation in a Cell-free system.		
Peak No.	Control	C.D.P.	Control	C.D.P. 25 µg/ml	C.D.P. 50 µg/ml	Control	C.D.P.	**
1	4.3	3.9	5.2	5.0	5.0			(±0.9)
2	3.8	5.1	13.8	13.6	13.7			(±0.7)
3	5.7	5.7	11.8	12.1	16.4	6.9	3.2	(±0.2)
4	5.7	7.6	13.2	10.0	10.3	9.4	10.0	(±0.7)
5	7.0	7.8	16.0	16.4	15.0	15.7	19.1	(±0.8)
6	5.6	7.3	12.8	14.2	13.2	34.2	43.8	(±0.3)
7	10.4	10.1	3.5	3.7	4.1	22.2	13.0	(±0.3)
8	9.0	7.6	4.0	4.5	6.5	7.4	3.2	(±0.7)
9	5.2	5.8						(±0.9)
10	5.4	5.5						(±1.4)
11	3.4	4.0						(±0.9)

C.D.P.: Chlordiazepoxide hydrochloride.

**: Mean variation between duplicate samples.

TABLE 12. THE EFFECTS OF THE INJECTION OF MEPROBAMATE ON DEVELOPING CHICK EMBRYOS AFTER 48, 108 AND 192 HOURS OF INCUBATION.

Hours of incubation prior to injection	Dose mg/egg	No. of embryos injected	No. of survivors	Mortality at 6-17 days (%)
48	0.08	64	64	0
	C	32	32	0
108	0.25	80	70	10.0
	0.50	23	19	17.4
	0.80	30	21	30.0
	C	26	25	3.8
192	0.8	20	18	10
	C	16	16	0

C: Controls injected with the same volume of 0.9% NaCl

TABLE 13. THE EFFECTS OF THE INJECTION OF COBALTOUS CHLORIDE ON DEVELOPING CHICK EMBRYOS AFTER 48, 108 AND 192 HOURS OF INCUBATION.

Hours of incubation prior to injection	Dose mg/egg	No. of embryos injected	No. of survivors	Mortality at 6-17 days (%)	Abnormalities	
					Reduced size (%)	Opaque lens (%)
48	0.05	25	17	32.0	3(17.6)	2(11.7)
	0.10	28	7	75.0	0	0
	C	62	58	6.4	0	0
108	0.05	22	14	36.4	0	0
	0.10	40	21	47.5	0	0
	0.25	53	26	50.9	2(7.6)	1(3.8)
	0.50	43	18	58.1	0	2(11.1)
	C	104	99	4.8	0	0
192	0.25	65	43	33.8	5(11.6)	10(23.2)
	0.50	80	45	43.7	4(8.8)	7(15.5)
	C	97	91	6.2	0	0

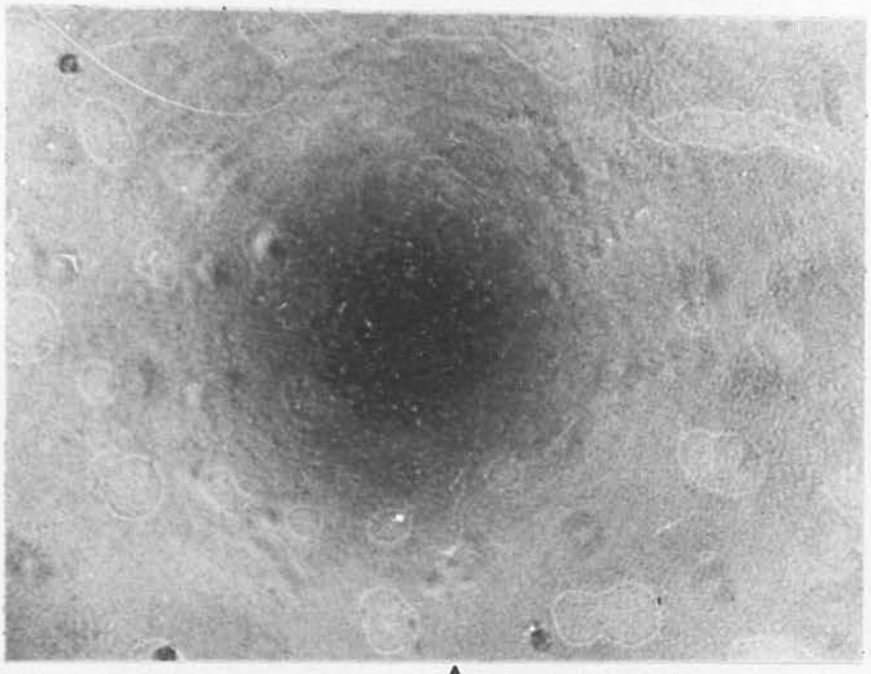
C: controls injected with the same volume of 0.9% NaCl

PLATE 20. The Effect of Cobaltous Chloride on the Chicken Lens *In Vivo*.

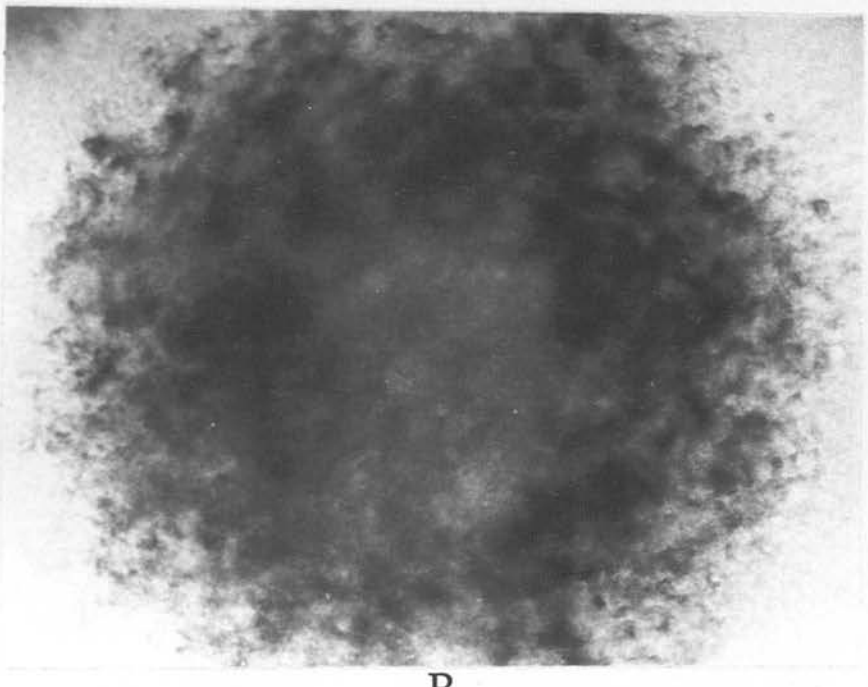
17-day embryo lenses seen from the anterior surface. The focus is just below the anterior epithelium (phase contrast 200 x magnification).

A. Lens of a normal control embryo, the fibre cells are seen as an orderly array.

B. Lens from the embryo injected at 4.5 days of incubation with 0.3 mg $CoCl_2$ per egg. The fibre cells are disorganised.



A



B

PLATE 20

PLATE 21. The Effect of Cobaltous Chloride and Lead Nitrate on Protein Synthesis by Cultured Cells.

The proteins were analysed as in Plate 2.

A. 15-day lens epithelial cell cultures. (a) Control grown in standard 6% FCS medium. (b) Treated with cobaltous chloride (4 μg/ml) trough days 4 - 14 inclusive, every 2 - 3 days when the medium was replaced.

B. 11-day kidney fibroblast cell cultures. (a) Control grown in standard 10% FCS medium. (b) Treated with cobaltous chloride (4 μg/ml) through days 4-10 inclusive, every 2-3 days when the medium was replaced.

C. 30-day neural retina cell cultures. (a) Control grown in Standard 10% FCS medium. (b) treated with lead nitrate (5μg/ml) through days 6 - 29 inclusive, every 2-3 days when the medium was replaced.

Arrows identify the components which show changes.

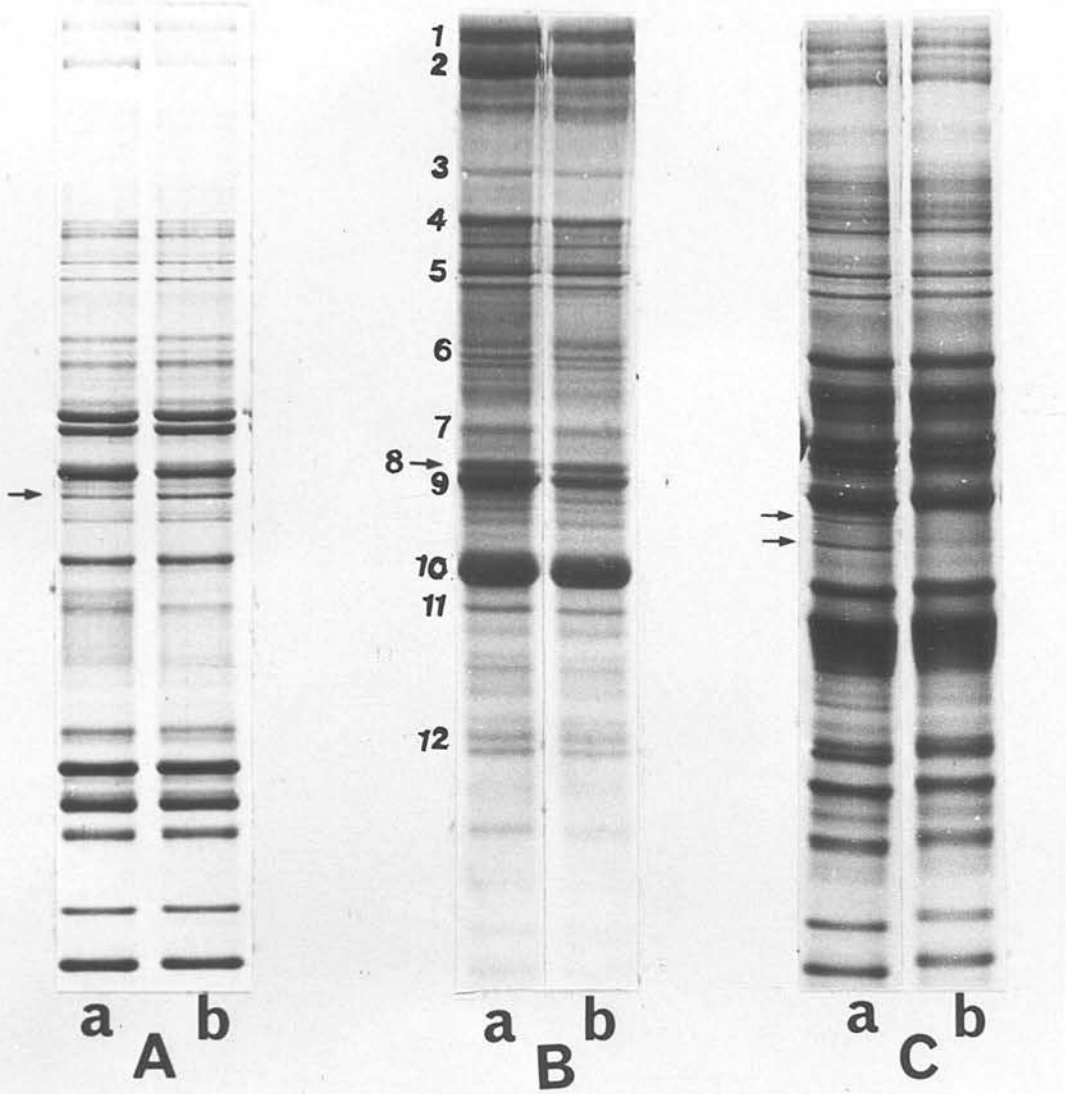


PLATE 21

FIGURE 15. Traces of Plate 21 Ba (a) and 21 Bb (b) respectively. Arrows identify the peak which shows components most affected.

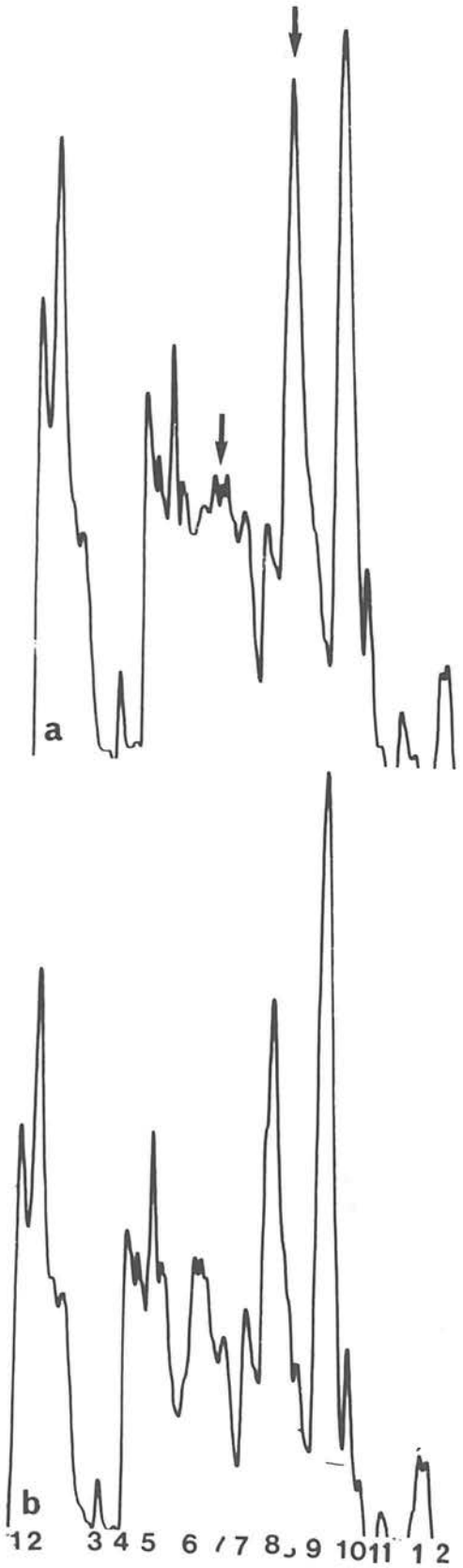


FIG 1 5

TABLE 14. THE EFFECTS OF THE INJECTION OF LEAD NITRATE ON DEVELOPING CHICK EMBRYOS AFTER 48, 108 AND 192 HOURS OF INCUBATION.

Hours of incubation prior to injection	Dose mg/egg	No. of embryos injected	No. of survivors	Mortality at 6-17 days (%)	Abnormalities			
					Reduced size (%)	Brain damage (%)	Hydrocephalus (%)	Spinal Defects (%)
48	0.02	21	19	9.53	3(15.7)	0	0	2(10.5)
	0.05	25	19	24.0	5(26.3)	0	0	1(5.3)
	0.10	20	10	50.0	0	1(10.0)	0	0
	C	26	26	0	0	0	0	0
108	0.05	26	26	0	0	7(26.9)	13(50.0)	3(11.5)
	0.10	50	41	18.0	0	6(14.6)	24(58.5)	2(4.9)
	0.20	59	45	23.7	0	7(15.5)	20(44.4)	5(11.1)
	C	70	70	0	0	0	0	0
192	0.10	54	53	1.8	0	11(20.7)	0	0
	0.20	54	50	7.4	0	34(68.0)	0	0
	C	80	80	0	0	0	0	0

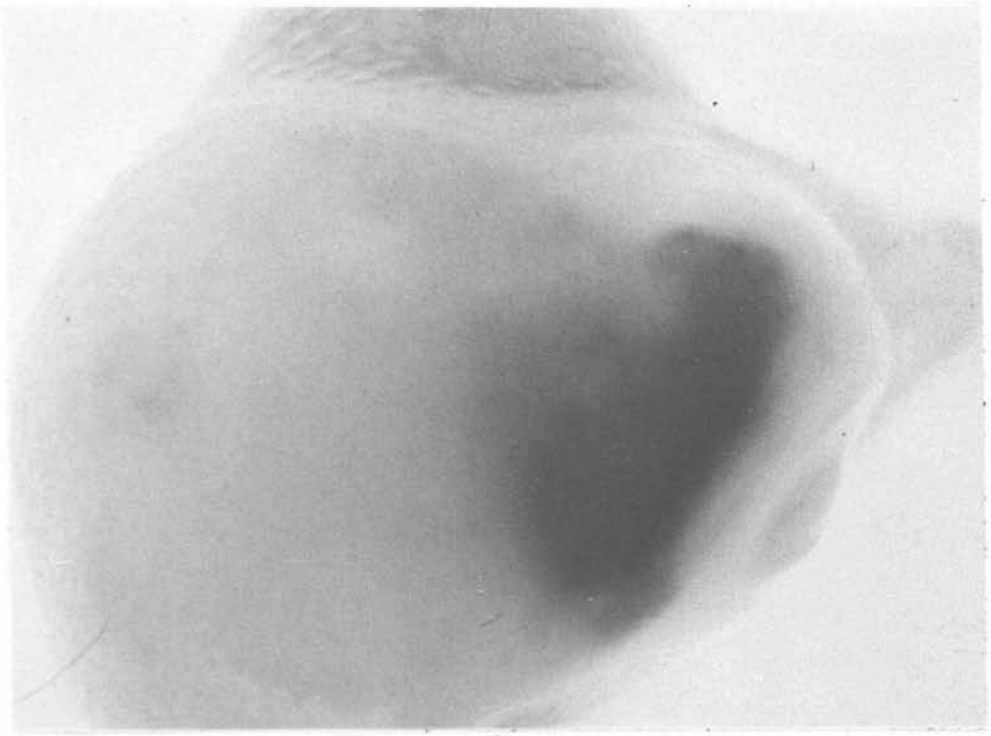
C: Controls injected with the same volume of 0.9% NaCl

PLATE 22. The Effect of Lead Nitrate on the Chick Brain *In Vivo*.

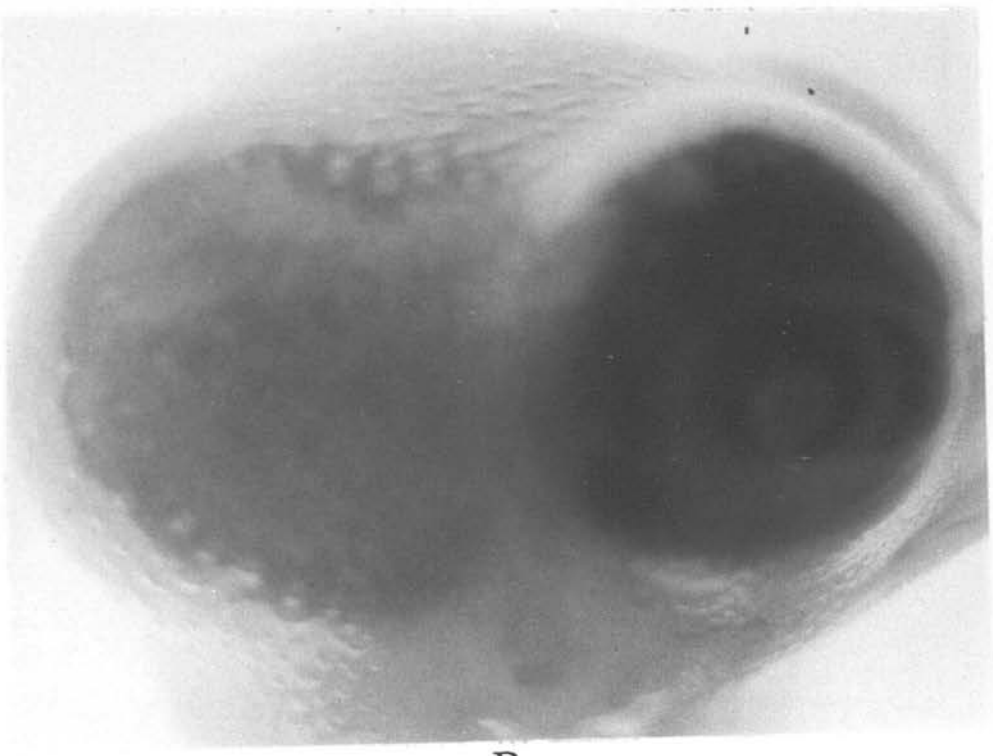
17-day embryo heads, showing hydrocephalus in lead injected embryos.

A. Head of a normal control embryo.

B. Head from the embryo injected with 0.1 mg per egg lead nitrate at 4.5 days of incubation.



A



B

PLATE 22

4. DISCUSSION

4.1 Teratologic Studies

Congenital malformations are diseases of differentiation, and teratogenesis results from alterations in the processes which control normal development. Many of these processes including cell and tissue-interactions can be made to occur in vitro, under experimentally controlled conditions, which allow the study of various biochemical and morphological aspects of normal development and teratogenesis. Teratogenic agents could affect an enzyme, act on a cell surface property, change mitosis, etc. We may consider, as an example, the effect of modification of cell surface receptors or cell surface properties. Studies both of the congenital abnormalities resulting from natural mutations and of those induced by teratogenic agents have suggested that these abnormalities are related to alterations in cellular and biochemical processes of cells.

Pathogenic agencies may be intrinsic, such as mutant genes or extrinsic, such as exposure to noxious agencies, or drugs. Disturbances in development must reflect the consequences of interference whether genetic or biochemical, with a target cell and could in principle affect an enzyme or a structural protein, or a cell membrane receptor or other membrane component, or disturb transcription or translation. Abnormalities in development may follow changes in cell surface properties leading to defects in cell-cell recognition, response to signals, or cell adhesion or changes in mitotic rate in protein synthesis in metabolism and so on. We may consider, as an example, some effects of modification of cell surface. For instance, in the chick limb skeletal mutant, talpid[3], the failure of the mesenchyme to separate properly from the epithelial tissue has been attributed to an increase

in cell adhesion, reduced cell movement and continued mitosis, all possible consequences of cell surface abnormalities (Ede and Agerbak, 1968; Ede and Flint, 1975a,b; Ede, Flint and Teague, 1975). These alterations in cellular processes have been well demonstrated by in vitro cell aggregation studies by the above authors. Other studies with limb mesenchyme cells from the brachypod mutant in mice showed that these cells are more adhesive than normal cells and fail to complete chondrogenesis in culture (Duke and Elmer, 1977). Studies in this laboratory with chick strains (Hy-1 and Hy-2) which have been selected for high growth rate, indicated that they have hyperplasia of the lens epithelium (Clayton, 1975). The lens cells of these hyperplastic strains are distinguishable from that of a normal control strain (N) by cell membrane composition (Clayton, 1979a; Odeigah, Truman and Clayton, 1979), and cell cycle intervals (Randall, Truman and Clayton, 1979). Still other studies from human disorders such as myasthenia gravis, which is characterised by muscular weakness and fatigability, indicated that the neuromuscular functions of patients with typical myasthenia gravis contain only 11-32% of the number of acetylcholine receptors found in normal control patients (Fambrough, Drachman and Satyamurti, 1973).

All of these studies suggest that abnormal developmental events are accompanied with changes in cell membrane composition which affects cell associations, and these changes can be investigated in cell culture studies.

Indeed cell aggregation studies by Hausman and Moscona (1976) showed that a glycoprotein (M.W. 50,000 daltons) is involved in the aggregation of chick neural retina cells in culture. They further reported that when these cells were treated with proflavin which inhibited the

accumulation of newly made RNA by 10% and reduced that of glycoprotein by 30%, then the histiotypic cell aggregation was totally prevented. When glycoprotein from untreated cultures were added to the drug treated cultures then the cells did aggregate suggesting that the reduced accumulation of glycoprotein is related to alterations in cellular aggregation. Other studies with drugs such as Vitamin A and its metabolite retinoic acid have suggested that the ability of these drugs to alter glycoprotein synthesis contributes to their teratogenicity in experimental animals (Kocchar, 1967, 1976). Alterations in the structure of a particular macromolecule may also lead to abnormal development. In this respect, β-aminoproprionitrite has been shown to inhibit cross-linking of newly synthesised collagen both *in vivo* and *in vitro* and, this action suggested it to be related to its teratogenicity in rats and mice (Martin *et al.*, 1961, 1966; Pinnel and Martin, 1968; Pratt and King, 1972).

Any *in vitro* method developed for teratogenicity tests must be assessed for its validity and reliability. It is essential to investigate the relationship between the observed abnormality of an organ system *in vivo* and the response of cells from that organ system to teratogenic agents *in vitro*. In order to assess the validity of cell culture tests it is important, first, to determine whether such teratologic problems as tissue-specific, dose and genotype related response and duration and stage dependent effects of teratogenic agents could be investigated.

In the present study seven agents were examined in an attempt to study the relationship between *in vivo* and *in vitro* tests. The data, together with the problems of in vitro teratogenicity tests using a cell culture method are discussed in the following section.

4.2 The Effects of Selected Agents on the Development of Chick Embryo In Ovo and on the Differentiation of Cultured Chick Tissues In Vitro.

4.2.1 Insulin

In the experiments reported here, insulin treatment of chick embryos caused various stage-dependent malformations of the tail, limb, beak and eye.

Short tail or tail-lessness was only observed in those embryos treated at 48 hours of incubation. This is in agreement with reports by other investigators that caudal abnormalities are observed only in those embryos treated with insulin before 72 hours of incubation, (Landauer, 1945; Moseley, 1947; Zwilling, 1951; Landauer and Rhodes, 1952). Limb, beak and eye abnormalities were found only among those embryos treated at later stages of incubation, and often all of them occurred in the same embryo. This finding is in agreement with those early observations by Landauer (1947, 1953).

Although there is this general agreement that insulin is teratogenic in the chick (Landauer, 1972) and that it can also be teratogenic in mammals, as judged by its effect on a number of laboratory animals, including rats, rabbits and mice (Smithberg and Runner, 1963; Miyamoto, Sakauchi and Midorikawa, 1977, 1979; Cole and Trasler, 1980), there is no conclusive evidence as yet to suggest that insulin is teratogenic to man.

Several investigators have suggested a possible relationship, between the hypoglycemic effect of insulin and some of the malformations induced by insulin treatment. For example studies by Zwilling (1949, 1951) showed that the degree of insulin induced malformations such as rumplessness and micromelia, was greater in those embryos which were

also hypoglycemic. Similarly Miyamoto,Sakauchi and Midorikawa (1979) showed that the occurrence of microphthalmia and anophthalmia was highest in the offspring of those rats with insulin induced hypoglycemia lasting more than 24 hours. However, it is not clear from these studies whether a direct relationship exists between the insulin-induced abnormalities and its hypoglycemic effect either on the embryo or the mother. The observed hypoglycemia could be a contributory factor to the insulin induced abnormalities but not the primary cause since some of these abnormalities were also observed in embryos with little or no hypoglycemia (Zwilling, 1949, 1951).

The present in vitro study on the action of insulin on cultured cells indicates a direct effect of insulin on cells of the target organ. For instance insulin affected lens epithelial cells in vitro and induced lens opacity in vivo. Further it affected limb fibroblasts in vitro and induced micromelia in treated embryos. These findings support the earlier suggestion that insulin induced abnormalities could be due to the direct effect of insulin on the cells of the target organ rather than its indirect hypoglycemic effect (Levin, 1965; Landauer, 1972; Hinchcliffe, 1974).

Moreover the present in vitro studies showed that the effect of insulin on cultured cells could be influenced by the sample of FCS used, the stage of the culture and the genotype of the chick from which the cells were originally taken.

Insulin treatment of limb fibroblast cell cultures stimulated the accumulation of a protein with a molecular weight of about 44K daltons but this specific effect was dependent on the level of FCS present in the medium. This finding is in agreement with earlier observations by

Hajek and Solursh (1975) who reported that the effect of insulin on mucopolysaccharide synthesis by cultured chick embryo chondrocytes increased significantly when the level of FCS was reduced from 5% to 2%. The present data together with that obtained by Hajek and Solursh (1975) suggest that higher levels of FCS in the medium could reduce the effect of insulin on cultured cells. However, the present in vitro studies also indicate that this apparently reductive effect of FCS on response to insulin could be tissue-specific since it did not seem to occur with neural retina and lens epithelial cell cultures, even when the medium was supplemented with 10% and 6% FCS respectively.

The fact that the effect of insulin on limb fibroblast cell cultures is relatively minor compared to that on neural retina and lens epithelium suggests that the latter tissues are more sensitive to insulin treatment. Therefore if FCS either inhibits or binds insulin, so that less insulin is available at the higher levels of FCS present in the medium, this effect on the more responsive tissue will be still discernible. However another possibility is that it is actually fibroblasts that are especially responsive, so that they rapidly reach a plateau of response to the low level of insulin present in all FCS, so that they will fail to respond to additional insulin if the serum levels are high, while if lens epithelial and neural retina cells are less responsive and less readily reach a plateau they may continue to respond over a greater range. The differential response to insulin could also be influenced by different numbers of insulin receptors in different cells types.

Studies with neural retina cell cultures showed that insulin treatment had a stage-specific effect on the synthesis and accumulation of δ crystallin subunits and also to a lesser extent some of the β crystallins.

It was found that treatments prior to lentoid formation induced a reduction in the relative proportion of the 50K dalton δ crystallin but an increase in that of the 48K dalton δ crystallin. However total δ crystallin rose by about 8% as compared to total proteins in treated cultures and to the δ crystallin in control cultures. The relative amount of some β crystallins was decreased while others increased and still others did not alter significantly. The latter finding was also observed in insulin treated lens epithelial cell cultures. These results suggest that the synthesis of each crystallin subunit may be regulated noncoordinately. Longer duration treatments of neural retina cell cultures continuing until after lentoids had fully developed, did not seem to show this greater effect observed at earlier stages of the culture. This was also the case in studies by de Pomerai and Clayton (1980). In their study they showed that insulin treatment of neural retina cell cultures up to the 28th day of inoculation caused a considerable increase in total δ crystallin accumulation but this effect was largely obviated when insulin was added only from 18 days onwards. One of the main differences between the earlier results and the present observations was that the total increase in δ crystallin accumulation was much greater in the former than in the present study. Second, from the earlier study it is not evident whether the ratio of the two δ crystallin subunits was altered by insulin treatment, since the technique cannot distinguish the total effect from that on the individual crystallin subunits.

The discrepancy between the percentages of total increase in δ crystallin could be, first, due to the fact that the assay they used, haemagglutination inhibition, is more accurate than the SDS polyacrylamide

gel electrophoresis. Therefore it is possible that an even smaller increase is quantifiable by haemagglutination inhibition assay. Alternatively, any alteration in the relative proportion of non δ crystallin protein, which has the same molecular weight as δ crystallin, will in turn affect the relative proportion of δ crystallin assayed by SDS polyacrylamide gel electrophoresis. Secondly, it is evident from both the earlier studies (de Pomerai and Clayton, 1980) and the present findings that the developmental stage of the culture at which the treatment was first initiated influences the degree of alteration when the cultures are terminated. In the earlier study the effect was greater when the cultures were kept in insulin containing medium from day 1 of inoculation, while in the present study the treatment was initiated at a later stage of inoculation. Finally it is possible that the difference is due to the genetic differences between the chicks used in the earlier studies and in the present investigation, since in this study with lens epithelial cell cultures, it was found that the response of the cells to insulin treatment varies considerably between the two genetically different chicks, hyperplastic (Hy-2) and a normal control (Db) strains.

The apparent lack of response in neural retina cell cultures at later stages of in vitro differentiation may be due in part to massive increase in the total proportion of crystallins in the total protein as the number of lentoids increases. These lentoids come to contribute the bulk of the total crystallins (de Pomerai and Clayton, 1978). In addition the lentoids are comprised of terminally differentiated fibre cells and these appear to be less responsive to insulin.

In studies with lens epithelial cells it was noted that insulin treatment stimulated both mitosis and the synthesis and the accumulation

of several proteins. This effect of insulin was stage dependent as was the case in neural retina cultures, and also significantly influenced by the genotype of the cells.

Treatment of lens epithelial cell cultures, established from the Db strain, with insulin between days 4 to 16 stimulated mitosis and increased both the synthesis and accumulation of the 50K dalton δ crystallin subunit and of actin. The products synthesized in the cell-free translation system, using mRNA extracted from such cultures indicated that the action of insulin was to affect the available translatable mRNA, either by regulating transcription or processing. However, there was a difference between the effect of insulin on the intact cells and the effect on the cell-free system, which showed differential effects on the β and α crystallin subunits as well as on δ crystallin and actin.

These data indicate firtly that the synthesis of crystallins is affected non-coordinately; secondly that there is an effect at the level of transcription or translation, and finally, that there is some translational or post-translational regulatory feature in the cell which does not entirely reflect the changes in the translatable mRNA population.

The studies with lens epithelial cells also indicated that just as in the neural retina cultures, the terminally differentiated fibre cells are less susceptible to insulin treatment. There are several possible explanations for the diminishing effect of insulin on differentiated cultures. The terminal differentiation of lens fibre cells is accompanied by a cessation of nuclear function. If the effect of insulin is on transcription or processing as suggested above, no effect would be expected in cells without a functional nucleus. However, other changes may also occur which could be significant at

slightly earlier stages. For example there may be changes in the number of or availibility of insulin receptors during terminal differentiation since the effect of insulin is mediated via an interaction with cell membrane receptors (Levin, 1965; Butcher, et al., 1973; Czech 1977).

The present in vitro studies with lens epithelial cells further indicate that the effect of insulin on mitosis and protein synthesis and accumulation varies with the genotype. The lens epithelial cells derived from the normal (Db) strain were more responsive to insulin treatment as compared to those cells derived from the hyperplastic (Hy-2) strain.

There is some evidence, from the earlier studies, that genetic differences exist in the response of lens cells to several agents affecting growth conditions in culture (Clayton et al., 1976b). The data obtained in the present study confirms and establishes that the cells of the fast growing Hy-2 strain were less responsive to the effects of insulin than the slower growing Db strain. The data obtained from the cell-free translation shows that this growth difference in response is reflected in the differences in the translatable mRNA from the treated cells of these two genotypes.

The fast growing Hy-2 strain might be less responsive because lens fibre differentiation appears to occur several days sooner in these cultures than in those from Db strain. The number of insulin binding sites on the cell surface may be diminished in Hy-2 cells since there is evidence that diminished response to insulin is associated with a reduced number of its receptors (Kahn et al. 1978). Certainly the cell surface of the fast growing (Hy-2) strain has been shown to differ in

several respects, e.g. the membrane polypeptides and their lectin binding ability, from a normal control (N) strain (Odeigah, Truman and Clayton, 1977).

It would appear from the present data that genetic differences may be investigated in a cell culture system.

In conclusion, the present study with insulin indicates that a correlation may exist between at least some of the *in vivo* and *in vitro* effects. One of these is the response of lens epithelial cells *in vitro* and the formation of cataract *in ovo*. Although the response of limb fibroblasts is obtained only at low serum levels in the medium, this still suggests a possible relationship to the mesodermal effects obtained *in ovo*. Finally, it is evident that there is considerable difference in the responses of the tissues tested. Some of these differences in response are due to the state of differentiation of the culture at which the cells were treated and some could be due to genetic differences. Thus while there is a coicidence in the sensitivity of lens and limbs *in ovo* and the response of lens and limb cells *in vitro*, the findings suggest that a practical *in vitro* teratogen test would require considerable experimentation into culture conditions used in order to avoid experimental errors.

4.2.2 Diphenylhydantoin (Epanutin)

In the present in vivo studies no gross malformation was observed following diphenylhydantoin treatment of chick embryos at various stages of incubation. In contrast to the present investigation diphenylhydantoin has been reported to be teratogenic in man and laboratory animals. Evidence suggests that treatment of pregnant women with anticonvulsant drugs including diphenylhydantoin increases the incidence of cleft lip and palate and mental retardation in the offspring (Ajodhia and Hope, 1973; Hanson, 1976; Yang et al., 1978; Mallow, Herrick and Gathman, 1980). It is often difficult to assess the effect of a particular drug on the human embryo because the mother may be receiving several medications during a single pregnancy (Mirkin, 1970). However, studies with laboratory animals such as mice, rats and rabbits have shown that diphenylhydantoin treatment at various stages of gestation induced a wide array of malformations in the offspring including cleft palate, foetal haemorrhages, limb and orofacial defects (Paulson, Paulson and Treissaty, 1979; Petter, 1979; McClain and Langhoff, 1980).

The failure to observe gross malformations in the chick embryo following diphenylhydantoin treatment may be due to technical problems. It is possible that some of the abnormalities reported in the offspring of experimental animals treated with this drug may also occur in chick, but these cannot be detected, if they are not severe, without histological examination. There may also be the problems of dosage and dispersion of the drug in ovo. It is evident from this study that diphenylhydantoin crystallises within 1-2 hours of exposure to air. This problem may affect its activity, dosage and dispersion in chick embryos. It is

also possible that the teratogenic action of diphenylhydantoin both in man and laboratory animals may be secondary to changes induced by diphenylhydantoin treatment of the mother. It is known that diphenylhydantoin has large numbers of side effects such as reduced calcium metabolism (Bowden, 1974), insulin and thyroxin blood levels (Fariss and Lutcher, 1971) and increased catabolism of Vitamin D, (Kutzman, 1969) and the level of cytochrome P450 (McColl et al., 1980). It also causes folic acid deficiency (Reynolds, 1972) in patients receiving long-term treatment with diphenylhydantoin. Of the range of effects of diphenylhydantoin observed in man, the mental deficiency may be due to a permanent deficit in neurones, neuronal connections or a permanent functional deficit. Any such effect would not be observed in any of the animal experiments reported to date nor would it be possible to detect such alterations in the present study.

In vitro studies showed that diphenylhydantoin treatment affected the synthesis and accumulation of several protein components in lens epithelial and neural retina cell cultures. Treatment of lens epithelial cells with diphenylhydantoin through days 4 to 15 reduced the synthesis and accumulation of the 50K δ crystallin and actin and that of a 38K dalton molecular weight component. Shinohara and Piatigorsky (1977) studying with intact embryonic chick lenses showed that Ouabain treatment in vitro changes the ratio of the two δ crystallin subunits in favour of the higher molecular weight subunit and this was associated with an altered Na^+/K^+ ratio in the lens oells. Thus the effect of diphenyl-hydantoin on the ratio of the two δ crystallins in lens epithelial cell cultures may be related to its action on Na^+/K^+ ratio in the cells since it is known to alter the intracellular concentrations of these

ions (Sohn and Ferrendeli, 1976; Hamstrom and Jones, 1979).

Studies with neural retina cells showed that the effect of diphenylhydantoin on protein synthesis was stage dependent. In 10-day old neural retina cell cultures which possess both neuronal and neuroepithelial cells, lower levels of diphenylhydantoin stimulated and higher levels reduced choline acetyltransferase activity. Only with the higher levels did diphenylhydantoin affect protein synthesis causing a reduced synthesis and accumulation of a protein with molecular weight of about 40K daltons. However, the same dose-level did not affect either the synthesis or the accumulation of proteins in later stages of neural retina cultures when neuronal cells are normally lost spontaneously. This suggests that diphenylhydantoin affects neuronal cells rather than neuro-epithelium. This has been suggested to be the case in chick brain cell cultures treated with diphenylhydantoin (Culver and Vernadakis, 1979).

It is interesting to note that the effective level of diphenylhydantoin which altered CAT activity in vitro is within the therapeutic range normally detected in human blood (McLain, Martin and Allen, 1980). It is also important to note that diphenylhydantoin treatment of neural retina cell cultures could, at low levels, affect CAT activity without affecting protein synthesis. This emphasizes the need for testing several levels of a particular drug, and for assessing several cellular and biochemical processes when investigating the effect of an agent on cultured cells.

Other in vitro studies have also shown that diphenylhydantoin affects protein synthesis in several cell types such as human lymphocytes (MacKinney, Vyas and Walker, 1978) and chick embryo brain cell cultures (Culver and Vernadakis, 1979). It causes reduced synthesis and

degradation of collagen in both rat platal mucosa and calvaria (Bergenholtz and Hamstrom, 1976; Dietrich and Duffield, 1980), and in chick embryo tibias (Villareale et al., 1978). Diphenylhydantoin has been reported to inhibit the phosphorylation of acetylcholine receptors in receptor-enriched membranes (Gordon, Davis and Diamond, 1977; Gordon, Milfay and Diamond, 1979). At present it is not clear whether the stimulatory effect of diphenylhydantoin on CAT activity in neural retina cells is related directly to its action on acetylcholine receptors but the inhibitory effect at higher levels could be due to a possible selective loss of neurones resulting from an irreversible cytotoxic effect. This suggestion seems to agree with those findings by Culver and Vernadakis,(1979), who showed that at especially higher levels, diphenylhydantoin reduces the size of neuronal aggregates and their processes in brain cell cultures.

Although the mechanism by which diphenylhydantoin affects CAT activity and protein synthesis is not clear, earlier studies suggest that diphenylhydantoin may act by altering cell membrane permeability and affecting membrane components and intracellular concentration of Na^+ and K^+ ions (Sohn and Ferrendeli, 1976; Hamstrom and Jones, 1979), but the effect on CAT activity could be due to its cytotoxic action on neuronal cells (Culver and Vernadakis, 1979).

4.2.3 Chloroquine Sulphate (Nivaquine)

Treatment of chick embryos with chloroquine sulphate at various stages of incubation induced no gross morphological changes. However, subtle histological changes, which were not investigated in the present investigation cannot be discounted which in the long term, may lead to gross abnormalities. This would not be detected in the present study

in view of the short 20-day embryonic life of the chick, since the reports suggest that chloroquine-induced pathological changes in the eye tissues of treated animals and humans often appear long after the treatment has been terminated (Linquist and Ulberg, 1972; Heckenlively, Martin and Levy, 1980).

Present in vitro studies showed that chloroquine sulphate has a direct effect on cultured cells, especially those of neuronal tissues (neural retina and brain). Of particular interest was the fact that in lens epithelial, neural retina and brain cell cultures, chloroquine sulphate treatment inhibited the synthesis and accumulation of two proteins which appeared to be the same in all cases as judged by their molecular weights. Other in vitro studies have shown that chloroquine also inhibits protein synthesis in rat adipocyte cultures (Seglen, Grinde and Solheim, 1979), and enhances the secretion of lysosomal hydrolases in human skin fibroblasts (Gonzales-Noeriga, et al., 1980). However, chloroquine has also been shown to inhibit the lysosomal pathway of protein degradation both in rat (Wibo and Poole, 1974) and chick embryo (Libby, Bursztajn and Goldberg, 1980), fibroblast cell cultures. These studies together with the present investigation suggest that the effect of chloroquine on protein biosynthesis may be at several levels. In addition chloroquine treatment reduced CAT activity in 10-day neural retina cells cultures. This effect could be due to a selective loss of neurones resulting from the toxic effect of the drug on neuronal cells, since some of them were observed to lose their axons and dendrites and to detatch from the underlying neuro-epithelial sheet. The observed sensitivity of neural retina as well as that of brain cell cultures to chloroquine treatment is of particular

interest since evidence does suggest that Chloroquine is a neuro-toxic drug which specifically accumulates in the eye tissues, brain and spinal cord and causes retino-pathological changes (Linquist and Ulberg, 1972; Majumdar, 1977; Rosenthal *et al.*, 1978; Heckenlively, Martin and Levy, 1980), while the effect on intelligence and the 8th nerve have been referred to above.

The present *in vitro* studies suggest, first, a possible relationship between chloroquine-induced abnormalities *in vivo* and its effect on cultured cells and indicate that the effect on neuronal tissue *in vivo* may be the direct action of the drug on the target organ systems.

4.2.4 The Minor Tranquillizers

In vivo studies with the two minor tranquillizer drugs, chlordiazepoxide HCl and meprobamate, showed that the formaer but not the latter caused a significant growth retardation in treated chick embryos. The reduction of the hind limbs was especially noticeable.

Although there is no conclusive evidence to suggest that chlordiazepoxide HCl is teratogenic in man, it has been reported to be teratogenic in rats causing severe growth retardation and impaired learning ability (Harris and Case, 1979). Possible behavioural changes were however outside the scope of the present investigation.

In the present study with cultured cells it was observed that chlordiazepoxide HCl treatment reduced the synthesis and accumulation of several proteins both in limb and kidney fibroblast cell cultures. One of the proteins affected appeared to be actin as judged by its molecular weight. These findings may suggest a possible relationship between the action of chlordiazepoxide HCl on overall embryonic development and its effect on cultured cells.

Studies with 10-day neural retina cell cultures showed that chlordiazepoxide HCl had a dose- and stage-dependent effect on these cells. At low levels although it had no discernible effect on protein synthesis it significantly increased CAT activity, while at the higher level, of 50 μg/ml, it reduced CAT activity. With this higher level chlordiazepoxide HCl also reduced the synthesis and accumulation of several proteins. The effect was related to the stage of differentiation of the culture, since this effect was no longer evident if the treatment continued for a further 15 days, by which time the cultures had trans-differentiated into lens fibre-like cells, while most of the neuronal cells had been lost. Similarly there was no effect in those cultures in which the treatment was first initiated after most of the neuronal cells were lost.

This stage-dependent effect suggests that neurones are particularly sensitive to chlordiazepoxide HCl treatment, a result found also for chloroquine sulphate and diphenylhydantoin. It also seems likely that the terminally differentiated lens fibre-like cells in the neural retina culture are insensitive to diphenylhydantoin and librium at the subtoxic levels used, for the same reasons as suggested earlier for the response to insulin. Since the lentoids form the bulk of these cultures, the possible effect on the neuroepithelial cells remaining cannot be ascertained from this data.

The finding in the present study that chlordiazepoxide HCl caused severe growth retardation in the chick embryo is in agreement with the reported growth retardation in rats after the drug treatment (Harris and Case, 1979). The possibility of behavioural change in treated chick embryos was not investigated but it remains as a possible effect since

chlordiazepoxide is known to alter learning ability in rats (Harris and Case, 1979; Morris, Tremmel and Gebhart, 1979) and in the present study specifically affects neuronal cells in vitro.

The second of the two minor tranquillizer drugs tested, meprobamate induced no gross malformations in the chick embryos at the dose-levels used. However, studies with cultured cells showed that meprobamate treatment increased the synthesis and accumulation of a particular protein in limb fibroblast cell cultures. This effect was, however, not consistently observed but was clearly stage dependent. Meprobamate is known to cause behavioural changes in rats (Hoffeld and Webster, 1965; Harris and Case, 1979) but such effects were not investigated in the present study. Further study is required on the action of these two minor tranquillizer drugs on brain cells which may provide information about the mechanism by which the drugs induce behavioural changes.

It is important to note that all of the three drugs tested, diphenylhydantoin, chloroquine sulphate and chlordiazepoxide HCl, have a greater effect on neuronal cells than on glial cells in neural retina cell cultures. This finding may suggest a possible relationship between the effects of these drugs on neural function in vivo (Voorhees and Butcher, 1982) and on neuronal cells or on their processes in vitro.

4.2.5 Cobaltous Chloride

Cobaltous chloride treatment of chick embryos induced growth retardation often associated with a pallor of colour and lens opacity in those embryos which survived up to the 17th day of incubation. Grwoth retardation was profound amongst the embryos treated at the earlier stages while the incidence of lens opacity was especially high in those embryos treated at 192 hours of incubation. DeMatties and

Gibbs (1976) and Igarachi, Hayashi and Kikuchi (1978) have reported that colbaltous chloride reduces haem and cytochrone P450 levels in the treated rats. Induction of anaemia by cobaltous chloride has earlier been reported in chick embryos (Kury and Crossby, 1968). In the present study the marked pallor of the affected embryos was characteristic of anaemia but no blood counts were made.

The development of lens opacity was often associated with microphthalmia. The nuclear region of the lens in each case was dark-brown in colour and the nuclear fibre cells were disarranged. Studies with cultured cells showed that cobaltous chloride treatment increased the synthesis and accumulation of a minor protein, with a molecular weight of about 41K daltons, in lens epithelial cells. The sensitivity of lens cells to cobaltous chloride treatment in culture indicates a relationship between *in vitro* and *in vivo* tests, since in the latter it caused lens opacity.

In kidney cell cultures it reduced the synthesis and accumulation of a protein which has a molecular weight similar to that of actin. Although the particular sensitivity of the lens cells is shown both *in vivo* and *in vitro*, the possibility of changes in the kidney in ovo was not examined histologically, however, no gross abnormality was visible. The particular effect of cobaltous chloride on protein synthesis by kidney cells shown here and on guanylate cyclase activity in kidney homogenates (Tsai, Manganiello and Vaughan, 1978) may suggest that if sufficient cobalt accumulates in the kidney *in vivo*, then the abnormal development could be of concern.

4.2.6 Lead Nitrate

In the present study stage-specific gross abnormalities were observed in the chick embryos treated with lead nitrate. At the early stages of incubation lead nitrate treatment induced spinal injury while treatment at later stages caused abnormalities of the central nervous system and included the appearance of haemorrhagic fluid in the brain of treated embryos. The latter finding is in agreement with earlier studies which have also shown that lead treatment of chick embryos induces abnormalities of the central nervous system (Ridgway and Karnofsky, 1952; Catisone and Gray, 1974). Further it was noted that the cerebellum was the first and most severely affected region of the brain. This finding supports the observations in rats, by Goldstein Asbury and Diamond (1974), that the cerebellar region is more sensitive to lead treatment than the other brain regions.

The finding in the present study that lead induces stage dependent abnormalities in the nervous system of chick embryos is in agreement with the stage-dependent abnormalities observed in the offspring of lead treated rats (McClain and Becker, 1975). The finding that lead treatment induced growth retardation especially in embryos treated at earlier stages of incubation is in agreement with observations in the offspring of mice (Jacquet and Geber, 1979) and rats (McClain and Becker, 1975; Dilts and Ahokas, 1980), treated with lead at various stages of gestation. In addition, it was found that the rates of mortality and malformations were greatest when the higher levels of lead nitrate were administered. Similar findings have been reported in hamsters (Ferm and Ferm, 1971).

From the present and earlier studies it is evident that the teratogenic

effects of lead in chicks are similar in many respects to those observed in the offspring of lead treated mice, rats and hamsters. These findings are of special importance since a substantial volume of evidence points to its toxicity to man (Chisolm, 1973) and high blood lead levels increasing the incidence both of foetal death and of mental retardation in the babies of mothers exposed to lead (Baker, 1960; Engle and McIntyre, 1964; Moore and Medith, 1977, Moore, 1980).

Studies with cultured neural retina cells showed that at subtoxic levels used, lead nitrate inhibited both the synthesis and accumulation of two as yet unidentified proteins. These were also the same proteins as judged by their molecular weights affected by chloroquine sulphate treatment of neural retina cell cultures. In the case of lead, continuous treatment was required for about 20 days before the effect became apparent. This suggests that the effect is probably due to a selective cell loss over the long period of treatment.

However treatment of neural retina cell cultures with subtoxic levels of lead did not seem to affect CAT activity. This observation was made only after 24 hours of the treatment, thus longer treatments may affect CAT activity. It is also possible that the effect of lead on specific neuronal cells may not be detected in culture, since in neural retina cell cultures there are several types of neuronal cells as judged by their morphology. The sensitive type of neuronal cells may not grow well under the culture conditions used in the present study. This is likely the case since Culver and Vernadakis (1979) showed that neuronal cells from the cerebellum of chick embryos do not grow in culture as do the neuronal cells from other regions of the brain.

4.3 Correlations Between In Vivo and In Vitro Screening Tests

Four of seven agents tested proved to be teratogenic in chicks and to induce detectable morphological and biochemical alterations in cultured cells, and there were correlations in the sensitivity of cells and the effects obtained in vivo. The remaining three agents, diphenylhydantoin, chloroquine sulphate and meprobamate, did not induce any detectable gross malformations in chicks but induced alterations in cultured cells. However all of these agents have been shown to be teratogenic in experimental animals such as rats, rabbits or mice, and are known to be teratogenic in man. The effects recorded, such as cleft palate, would not have been detected in the chick embryos without detailed histological studies, which were not undertaken. Those effects such as lowered intelligence or deafness, are also not testable on chick embryos. At present therefore, it is not possible to point to definite correlations between embryonic and cellular effects, but they are certainly not ruled out, since the response of the chick tissues is compatible with some of the effects reported for embryos of other species. Future studies would therefore require detailed investigation of *in vivo* teratogenicity both histologically and biochemically and the data compared with the results obtained from such analysis of *in vitro* studies.

The observed discrepancies between the *in vivo* and *in vitro* findings in the present investigation could be due to several factors. It is possible that the proteins which were affected *in vitro* may not be affected *in vivo*. This may be due to the fact that either cultured cells are more sensitive to the treatment or it may be that the proteins affected are artifacts of culture due to *in vitro* adaptation of the cells

to the culture conditions and are not present in the embryonic tissues at the time of treatment. It is evident from the present *in vitro* studies that, in general, continuous treatment is essential to induce alterations in cultured cells and even then the effect is reversible within 24-48 hours of the last treatment, except treatments with chloroquine sulphate, as discussed earlier (Section 4.2). It is also evident that not all proteins are affected by a particular drug, and that the effect is only apparent if such a protein is present in detectable quantities at the time of treatment. If the protein is not present at the time of treatment then the effect is not apparent.

Therefore these alterations observed *in vitro* could be insignificant when compared with *in vivo* effects, in that either the similar changes *in vivo* are repaired by the embryo or do not lead to any gross malformations or they may not occur at all for the reasons described above. However, a transient change at a critical stage of development, as during a critical induction, could still have lasting consequences. Thus further analysis of more cellular and biochemical changes must be made, presumable *in vivo* after multiple treatments and soon after the last treatment in order to establish whether a direct correlation exists between *in vivo* and *in vitro* experiments. This is essential because subtle changes which do not lead to gross abnormalities nevertheless could induce behavioural changes (Vorhees and Butcher, 1982) and also because pregnant women are often under long-term drug therapy.

Teratologic studies both in humans and experimental animals such as rats, rabbits and mice have shown that the type and the degree of defect is dependent on the amount of the drug administered and the stage of development at the time of treatment (Saxen and Rapola, 1969; Wilson,

1973; Shepard, 1975, 1979) and this was shown to be the case in the present study both with chick embryos and cultured cells.

In vivo studies it was noted that chick embryos treated with high levels of insulin or lead nitrate showed a significantly higher incidence of defects than those receiving only half the amount of teratogens. In earlier studies by Zwilling (1948) a direct relationship was established between dose and the incidence of insulin-induced limb defects in chick embryos. It was noted at different stages of culture development that the level and probably the type of proteins vary and the effect of a particular agent is only apparent on some of the specific proteins. There was no case in which all the proteins were affected by the agent tested.

It is therefore evident from this study that if a given malformation is due to alterations in any one of the cullular or biochemical processes then the correct amount of the drug must be present at the correct stage of differentiation.

Furthermore with the present in vitro studies a tissue-specific response was evident as judged by the analysis of water-soluble proteins. As discussed earlier it was found that some agents affected either one or several tissues but not all of the tissues investigated. There was no case in which a particular drug affected all the tissues. However, it is possible that a given agent may have several reactive sites and therefore multiple effects, alternatively a vulnerable structure or a metabolic pathway may be distributed between several cell types. This appears to be true since it was found in this study that the levels of diphenylhydantoin and chlordiazepoxide HCl which did not have a discernible effect on proteins resolved by SDS electrophoresis but significantly

altered CAT activity in neural retina cell cultures. Similarly other cellular of biochemical processes could have been affected by the levels of the agents tested, but these were not investigated in the present study. Thus the tissue-specific effect here is based largely on the action of the agents on the synthesis and accumulation of water-soluble proteins.

The tissue-specific effects observed may also be due to the different number of cells susceptible to a particular treatment in a relatively heterogeneous cell population, such as exists in retina, brain and kidney cultures and to changes in the cells at different stages of culture differentiation. The state of differentiation of each tissue is not the same even if they are taken from the same developmental stage of the embryo. In addition most of the proteins affected in different susceptible cell types are not universally distributed and were not detected in all tissues at the time of treatment. All of these variations between the cultures of different tissues must reflect tissue specificity, and they appear to contribute significantly to the response of cells derived from various organ systems.

Finally, when different responses were seen between cells from different organ systems yet these were not in full agreement with the effects seen <u>in vivo</u>, this phenomenon could be related to the amount of drug available for a particular tissue at the time of treatment since it has been established in teratologic studies that tissue specificity is in many cases directly related to the level of the agent in that specific tissue. Studies with mice have shown that arsenic, diphenylhydantoin, hexachlophene, nicotin and salicylic acid accumulate to a greater extent in the neuroepithelium than in other embryonic

tissues and this is associated with defects of the central nervous system (see review by Ulberg, Dencker and Danielson, 1982). Similarly, a correlation has been established between the ability of chloroquine to bind and accumulate in the eye tissue and inner ear and induction of foetal eye and eighth-nerve damage (Hart and Naunton, 1964; Matz and Naunton, 1968; Linquist and Ulberg, 1972). Quintart et al., (1979) reported that chloroquine crosses the blood-brain barrier, and the brain and spinal cord contain 10-30 times the amount present in the plasma. If such levels were used for the cultured cells no doubt all the treated cells would die since it was observed in the present study that even 5 μg/ml was cytotoxic to neuronal cells. Furthermore it is known that lead which accumulates and is retained at especially high levels in the brain causes damage to central nervous system (see review by Ulberg, Dencker and Danielson, 1982). Some at least of this damage being secondary to the formation of massive haemorrhages.

Thus it seems probable that if the actual level of each drug in a particular tissue in vivo is known and such levels tested in cell cultures then a positive relationship could be established between the observed abnormalities in vivo and the effect of teratogenic agents on cultured cells. The present study nevertheless suggests that in vitro cell culture test shares certain common aspects with in vivo tests, in that it allows one to investigate such phenomena as dose, stage and tissue specific effects of a particular agent both at the cellular and molecular levels. Finally, the present study of the in vitro effects of seven agents indicate, when compared with their teratogenic actions in vivo (Page 101), the possibility that with additional work, in future, positive correlations may be established between in vivo and in vitro screening tests.

TABLE 15: Teratogenic actions of the seven agents in vivo are compared with their effects on cultured cells.

Agents Tested	Pharmacological class	Major Effects In Vivo	Tissues Effected In Vitro				
			LE	NR	BR	LF	KF
Insulin	Antidiabetic	skeletal (in chicks and other animals) not in man	++	++	nt	+	-
Diphenylhydantoin	Anticonvulsant	Cleft palate and lip. Behavioural teratogen.	+	++	nt	-	-
Chloroquine sulphate	Antimalarial	Posterior column, 8th nerve, mental retardation (man) Retinopathy (man and animal)	+	++	++	±	±
Chlordiazepoxide HCl	Anti anxiety	Behavioural teratogen (rats and possibly man). Growth retardation (chicks).	±	++	nt	++	+
Meprobamate	Anti anxiety	Similar effects as Chlordiazepoxide HCl.	-	±	nt	+	±
Cobaltous chloride	Used in the treatment of anaemia	Lens opacity, anaemia and growth retardation (chicks), may also cause brain damage.	+	±	nt	±	+
Lead nitrate	No pharmacological use	Damage to CNS, behavioural teratogen (man). Spinal and brain injury. (chicks).	-	+	±	-	-

+: There is an effect

-: No observed effect

±: There may be an effect at the higher dose

nt: Not tested.

5. CONCLUSIONS AND SUGGESTIONS FOR FUTURE WORK

In the study of mechanisms of teratogenicity, *in vitro* techniques have a clear practical advantage over whole-animal studies. Whether they offer a predictive advantage over standard testing *in vivo* is uncertain.

In vitro methods permit one to investigate and compare the direct effects of a given agent on target and non-target tissues, under controlled culture conditions, and the mechanism of action of teratogenic agents can be studied both at the cellular and molecular levels. Using the cell culture screening tests it is possible to investigate the immediate as well as the long-term effect of an agent in long-term cell cultures by several passages of the cells and in principle to determine the affect on cell differentiation. Further, the technique permits one to study whether the cells which share certain common metabolic pathways or surface receptors respond to a particular agent in a similar manner. The technique could also be used to investigate the effect of those agents which are not in themselves teratogenic but require maternal activation. This can be achieved by using the amniotic fluid of drug treated animals to investigate the activity and the effect of the actual drug or its metabolite on cultured cells, since this would permit maternal metabolism and maternal foetal partition to be taken into account. Finally it would be possible to investigate the effects of teratogenic agents on tissues from human sources and compare these with the results obtained from the studies using animal tissues. This is essential for the validity of *in vitro* screening tests.

However, up-to-date cell culture tests appear to have certain limitations as an assay system screening for potential teratogenic

agents. One of the major differences between these *in vitro* tests and the whole-animal or the human situation is the absence of the complex maternal-foetal placental relationship. Thus it is not possible to screen such teratogenic agents which either affect placental function or affect primarily the mother, the embryo being affected only indirectly.

The complexity of growing various types of cells in culture must also be appreciated. Such parameters as enzyme activity, protein synthesis and the activity and metabolism of a teratogenic agent may be altered by (1) the type of culture medium and the batch of serum used (Milunsky *et al.*, 1970; Gielen and Nebert, 1972; Hajek and Solursh, 1975), (2) the pH of the medium which is a determinant of cellular growth and contact inhibition (Cessarini and Eagle, 1971), (3) the cell cycle and generation time (Nebert and Geiboin, 1968), (4) cellular senescence (Robbins, Levine and Eagle, 1970), (5) degree of cell confluency (Deluca and Nitowsky, 1964; Nebert and Geiboin, 1968) and (6) variation for instance in the level and activity of enzymes among different tissues of the same individual (Nebert and Geiboin, 1968; Uhlendorf and Mudd, 1968; Milunsky *et al.*, 1970; Shih and Littlefield, 1970; Shih and Schulman, 1970). Thus all these variations should be taken into consideration when extrapolating the observations made *in vitro* to the human situation *in vivo*. However *in vitro* examination of these secondary consequences which arise *in vivo* from the interaction of tissues, such as that between inducer and competent tissue, would require additional technical procedures.

The present study was limited in the sense that with the *in vivo* work only gross morphological abnormalities were recorded, and in general with the cultured cells only the water-soluble proteins were investigated in significant detail. Clearly in the future work, other

histological, cellular and biochemical parameters should be investigated both *in vivo* and *in vitro* in order to assess more completely the sequence of events which link cell susceptibility to morphological consequence. The *in vivo* studies whould be extended to investigate such parameters as (1) whether the agents affect the structure or functional activity of a particular organ system, (2) the influence of species and strains, (3) whether the level of a particular agent accumulated in a given tissue is related to the teratogenic effect, and (4) the role of maternal activation or inactivation by investigating amniotic fluid from the treated animals. Such studies would be relevant to problems of the mechanism of action of various teratogens and also give an indication of the importance of pharmacogenetic variation in a population. The *in vitro* studies should be extended to include the effect of agents on such parameters as (1) changes in the synthesis, accumulation and structure of cell surface and extra cellular components, (2) the activity of various enzymes, and (3) alterations in cell contact, adhesion and cell movement all of which play important roles in normal differentiation. Such studies being also relevant both to mechanisms of drug action and to investigations of the relationship between cell, tissue, organ and organism.

In an effort to establish further the validity of *in vitro* tests and to see what correlations exist between results from *in vivo* and *in vitro* assays, future studies should also include a greater number of teratogenic and non-teratogenic (controls) agents and should investigate their effects both *in vivo* on animals from different genetic backgrounds and *in vitro* on tissues from these and other animals. Such studies would be necessary to determine the degree of reliability as a predictive

method for teratogen screening. Conceivably, in the future, the *in vitro* cell culture assay technique will be capable of predicting an acceptable level of risk to the human population of selected new environmental agents, by studying their metabolism and by quantifying the effects of these agents or of their metabolites in foetal cell cultures.

Although the *in vitro* tests will probably never completely replace that of whole-animal testing, it nevertheless could be a reasonable adjunct to these tests.

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DEVELOPMENTAL BIOLOGY AND LENS CELLS CULTURE

FORMATION AND BEHAVIOUR OF LENS METAZYMES DEPENDENT ON CULTURE CONDITIONS

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Many enzymes are sustaining postsynthetic changes during aging. Smallest changes within the enzyme molecule effect a decreased stability of its conformation, which can be expressed by increased heat lability of the specific enzyme activity. Various enzyme forms are thereby appearing, the so-called metazymes. Postsynthetic changes may also be induced in vitro by external influences.

Besides chemicals and irradiation, culture conditions affect the formation and the lifespan of metazymes of phospho-fructokinase, PFK (EC 1.7.1.11), glucose-6-phosphate-dehydrogenase, G-6-PDH (EC 1.1.1.49). A 24-hr incubation in Ringer-solution caused a shift from the native PFK to the more labile metazyme, whereas the incubation in TC 199 induced the transition from the labile meta - zyme to a catalytically inactive form. Such changes could not be observed with G-6-PDH.

MODIFICATIONS OF CRYSTALLIN SYNTHESIS BY CELL CULTURE CONDITIONS AND CELL GENOTYPE.

R.M. Clayton, J. Bower, C. Patek, F. Randall, N. Wainwright and A. Zehir.

Chick lens epithelial cells have been grown in media with various additives, including insulin, different levels of F.C.S., retina extract (Courtois, Arruti, Barritault et al.,) and several drugs.' The relative balance of crystallins and other proteins (including actin) is affected differently by these media. Effects are apparently non-coordinate and three genotypes, characterised by intrinsically different rates of mitosis and biochemically modified cell membranes each produce a characteristically different response. Some implications in the data are discussed.

22A5 Electrical Coupling Between Neuroectoderm and Chorda-mesoderm Cells in Amphibian Embryo.
S.ITO*and Y.IKEMATSU. Dept of Biology, Kumamoto University, Kumamoto.

It has been previously reported neuroectoderm cells of Cynops embryo are electrically coupled to the adjacent chorda-mesoderm cells during the initial stage of gastrulation(St. 12C). Subsequently, coupling between these different tissues diminishes(St. 15-16) and finally disappears(St. 22-23, DGD, 23, 407).

To clarify the spatial patterns of electrical coupling along the embryonic axis, measurement of the coupling ratio between close membrane contact in these tissues has been examined at the anterior, middle and posterior parts of embryos at four stages(12C, 13C, 15-16 and 18). Measurement of electrical coupling in the intact whole embryo usually encounters difficulty in intracellular recording of chorda-mesoderm cells which are overlaid by the neuroectoderm. The embryos were cut into halves along the median line by microdissection and the chorda-mesoderm layer was exposed to the medium. A single electrode was visually inserted into each of the tissue layers. One electrode was used for current injection and voltage recording.

Local difference of electrical coupling could not be observed anywhere along the embryonic axis during development. Coupling ratios obtained commonly ranged from 0.05 to 0.20.

22A6 Developmental Changes of Avian Pepsinogens.
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The developmental changes of pepsinogen (Pg) isozymes in the chicken and quail proventriculus were studied in vivo and in culture. The peptic activity increased rapidly at the time of hatching and reached a plateau 2-3 days after the hatching in both species. In concomitant with this rapid increase, electrophoretic patterns of Pg isozymes shifted from the embryonic to the adult type. This shift occurred even in non-fed fowls, indicating that the changes of isozyme patterns were not regulated by feeding stimuli. Electrophoretic patterns and immunological studies with the purified Pgs revealed that the embryonic and adult proventriculi of both species have embryo- and adult-specific Pgs, respectively, and suggested that the Pgs of chicken and quail are very similar molecules, though their electrophoretic mobilities are slightly different.

When the proventricular endoderm of 5-9 day chicken and quail embryos was associated with homo- or heterospecific proventricular mesenchyme and cultivated on the chicken chorio-allantoic membrane for 6 or 10 days, the endoderm produced Pgs according to its own genetic informations. The stage-specificity of Pg isozymes in such grafts was affected by the age of the hosts.

22A7 Cleavage Pattern and Graph Theory.
H.DOI Dept. of Biophysics, Kyoto Univ., Kyoto.

A graph represents an embryo at a cleavage stage, whose nodes correspond to cells and edges do to cell-cell adjacency. Then we obtain a series of graphs according to the development. We derive this series from the graph generating system by means of matrix baced on the incidence matrix of graph theory, which the author proposes. We can keep the development in this system mathematically, treat it by computer and extract the characteristics from the cleavage pattern. The system consists of node substitution rules and edge renewal rules. The cleavage pattern of the ascidian, Halocynthia roretzi was put into this system. The result was node substitution rules are closely related to cell lineages and future cell fate. This findings implies that each isolated blastomere shows the individual division patterns according to the fate of itself.

22A8 Intrinsic programming of neural retina degeneration in a mutant chick.
H.KONDOH*, T.S.OKADA. C.RANDALL. J.BRODY. A.ZAHIR and R.CLAYTON. Dept. of Biophysics, Kyoto University & Dept. of Genetics, University of Edinburgh.

A recessive mutation(p) in chick has been discovered which shows degeneration in the retina in homozygotes (Vet. Rec. 105). The degeneration is confined to outer layers of the neural retina(NR), from bipolar cells to photoreceptors. The lesion appears at the 15th day in embryonic stage and progressively develops in post-hatching period.

We obtained embryos from eggs layed between a homozygote(p/p) and a heterozygote(p/+), and cultured NR cells from each embryo.

The embryos were divided into two distinct classes according to the fate of NR cells in culture. One class, presumably representing heterozygotes, appeared normal. Neuronal cells as well as epithelial cells are maintained up to 4 weeks. The other class, representing putative homozygotes, initially produced both neuronal and epithelial cells, and later initiated the neuronal degeneration. The degeneration began synchronously in cultures of this class; on the day 10 from 6-day-old embryos, and on the day 5 to 6 from 8-day-old embryos. Thus, the neuronal degeneration proceeded in vitro in the same time course as in vivo.

These observations indicate that the retinal degeneration in homozygous mutant is programmed in NR cells themselves, and that the neuronal cells in NR cultures may represent cells in outer layers of NR.

CHAPTER THREE

THE USE OF CELL CULTURE METHODS FOR EXPLORING TERATOGENIC SUSCEPTIBILITY

Ruth M. Clayton and Ahmet Zehir

CONTENTS

Contents

I. Introduction

According to data summarised by Hemminki *et al.*, in 1979, there were then some 63 000 chemicals in common use, and around 1 000 (or 2 000 according to Shepard, 1979), new compounds are marketed each year. About 7 000 had been tested for carcinogenicity when they wrote, and Maugh (1978) estimated the rate of such necessary tests at around 500 a year. The only source of direct information on the teratogenic effects of various substances on humans is epidemiological, so that it rests on an accumulation of family tragedies (Taussig, 1962; Saxén and Rapola, 1969; Weatherall and Haskey, 1976).

Although both micro-organisms and eukaryote cells are now used in tests of mutagenicity or carcinogenicity (McCann *et al.*, 1975; Purchase *et al.*, 1976), such systems have not been considered suitable for teratogenicity testing. Mutagens and carcinogens may have properties which also make them potential teratogens (Harbison, 1978; Brusick, 1978), but many teratogens are not carcinogens or mutagens (Miller, 1977; Brusick, 1978; Shepard, 1979). This is to be expected: the production of a teratogenic effect is the result of the exposure of a pregnant female to an agency which may damage any part of the interacting and changing systems of the embryo or fetus. A temporary, perhaps even a minor, interference with a crucial embryonic process such as inductive contacts or the migration and sorting of cells may have consequences which may lead to multiple effects, and to gross changes in the morphology and function of various structures and organs, which have been affected during the course of their development. The response to teratogenic agencies is characterised by

different periods of fetal sensitivity, physiological variables affected by the routes, times and duration of drug administration, and the range of exposure levels. Tests for teratogenicity therefore pose special problems. The currently approved tests of new drugs and other agencies for possible teratogenic effects on the human fetus generally include the examination for morphopathology and for histopathology of very large numbers of rodent fetuses of various ages, obtained from pregnant females injected or fed with suspected teratogenic substances, at different stages of pregnancy and at different dose levels. There are, however, different levels of susceptibility between species, and indeed some agencies do not affect all species. For this reason, some investigations now use more than one rodent species, and occasionally a small number of some non-rodent, such as the rabbit or the dog. Large numbers of animals must be exposed to obtain a statistically significant result.

Apart from their slowness and expense, experimental assays on intact animals lead to a number of quite serious problems. Some variability in results springs directly from the processes of embryonic development, but other problems are the product of evolutionary divergence between species. These have been discussed in detail elsewhere (Clayton, 1980), but some of the problems are outlined here.

This article does not attempt to be a complete review. Some major problems in testing for embryotoxicity, and problems in *in vitro* testing will be outlined, and a few examples given of results obtainable from cell-based assays, both from the literature and our own experiments.

II. Genetic Variables

Species differences include such features as placental structure as well as pharmacogenetic characteristics. Such differences may not affect the response to some drugs, but may be of major importance for others. (A well-known example of species difference in response is that of thalidomide; Schumacher *et al.*, 1968; Scott *et al.*, 1977).

There are also numerous reports of strain differences in susceptibility to particular teratogens occuring within a species. Examples include responses to galactoflavin (Kalter and Warkany, 1959), to 6-aminonicotinamide (Goldstein *et al.*, 1963), and to corticosteroids (Kalter, 1965; Biddle and Fraser, 1977, 1979), to diphenylhydantoin (Gibson and Becker, 1968), to alcohol (Randall and Taylor, 1979; Chernoff, 1980), and to insulin (Cole and Trasler, 1980).

The possibility that the genotype of an animal may confer either relative unresponsiveness or especial hazard with respect to a particular teratogen has implications both for the necessary conduct of tests and for problems confronting the human population. Genetic variants in man which predispose to adverse effects of drugs (which are acceptable to the population at large) are found in increasing numbers (see for example, Vesell, 1976; Propping, 1978). Such genetic variation is known for several substances which are known teratogens, such as alcohol, phenothiazines, anticonvulsants, anti-malarials, halothane, barbiturates, dicoumarol, etc. In principle (Clayton, 1980) one would anticipate that a fetus homozygous for a susceptible function in a physiologically normal heterozygous mother may be at risk from an agent pharmacologically acceptable to the mother. In addition, the genotype of the mother may affect the circulating levels of a given drug and the duration of exposure of the fetus. Rapid metabolisers will presumably expose their fetus, of whatever genotype, to less hazard than slow metabolisers of a potentially hazardous substance. Several lines of evidence point to this as a real possibility, and a few examples may be outlined.

Phenytoin (diphenylhydantoin) causes a dose-dependent incidence of cleft palate in mice (Elshove, 1969), and can lead to a cluster of defects in man, which include mental defect, cleft lip, cleft palate, dysmorphic facies, congenital heart defects and other changes (Shapiro *et al.*, 1976; Hanson, *et al.*, 1976). Hanson *et al.* (1976) found an incidence of severe effects in 11% and less severe effects in 33%, including impaired intellectual performance. (Discussion of the teratogenicity of phenytoin and other anticonvulsants can be found in Ajodhia and Hope, 1973; and Smith, 1977.) Agencies increasing the rate of metabolism of phenytoin decrease its teratogenicity in mice, those that decrease the rate of metabolism increase teratogenicity (Harbison and Becker, 1970). Strain differences in susceptibility have also been observed in mice (Gibson and Becker, 1968; Johnston *et al.*, 1979), and genetic variants for the rate of metabolism of phenytoin in man are known (reviewed by Propping, 1978). The drug accumulates in genetically slow metabolisers, increasing the risk of toxic side effects. Since maternal plasma phenytoin is rapidly equilibrated with the fetal plasma (Mirkin, 1971), it is likely that the hazard to the fetus will be increased in slow metabolisers.

Alcohol teratogenesis is dose-dependent in the mouse (Randall and Taylor, 1979), and Chernoff (1980) found that the risk of alcohol teratogenesis differed between inbred strains of mice exposed to the same dose. This risk was related to the maternal alcohol concentration, which is

regulated by genetically different levels of activity of alcohol dehydrogenase. Thus fetal damage is greatest in the offspring of low metabolisers. Alcohol causes direct damage to the rodent fetus (Brown *et al.*, 1979), but other mechanisms are also possible (Chernoff, 1980), one via a possible change in cellular NAD/NADH ratios after induction of alcohol dehydrogenase, another via the formation of acetaldehyde which is teratogenic to mice (O'Shea and Kaufman, 1979).

Alcohol is teratogenic in man (Jones *et al.*, 1973; Mulvihill and Yeager, 1976; Ouellette *et al.*, 1977), and genetic variants in the efficiency of alcohol metabolism are also known (reviewed by Vesell, 1976; Propping, 1978). The relative importance of maternal genotype and of the level of alcohol intake probably depend on the values of both these variables in any individual case.

A condition in which the relative contributions to fetal hazard of maternal and fetal genotypes depend, in any pregnancy, on their respective genotypes, was investigated in the mouse by Shum *et al.* (1979). In the mouse there is a locus (Ah) which controls the inducibility of arylhydrocarbon hydroxylase and cytochrome P450. This locus affects the response to polycylic aromatic hydrocarbons (see Nebert and Felton, 1976), and it has also been shown to be correlated with teratogenesis by this class of compounds (Lambert and Nebert, 1977). The alleles Ah^d and Ah^b are distinguished by the inducibility of this enzyme and the several enzymes associated with cytochrome P450. This regulates the production of carcinogenic intermediates from polycyclic hydrocarbon compounds. Mice homozygous for Ah^b (responders) produce toxic metabolites, and Shum *et al.* (1979) showed that a mother of this genotype exposes her fetuses, whether heterozygotes or homozygous responders, to equal risk. However, in a homozygous Ah^d (non-responding) mother, the heterozygous (Ah^d/Ah^b) fetus is more affected than the homozygous Ah^d fetus.

This enzyme system is found in a wide variety of fetal tissues although it is highest in the liver (see Shum *et al.*, 1979, for further references), and sensitive and resistant genotypes may be assayed in cultures of such cell types as express this enzyme (Nebert, 1973). A similar locus exists in man (Atlas *et al.*, 1976; Kellerman *et al.*, 1975; Nebert and Felton, 1976). Shum *et al.* (1979) also discuss the possibility that the inducibility of P450 may be included in the metabolic pathways of a wide range of substances and may be significant in determining the teratogenic potential of such teratogens as anticonvulsants, anticoagulants, oral contraceptives, etc. Genetic variants for the metabolism of these substances are already known, and if this view is substantiated it would point to a complex regulation of risk

for any teratogen, governed by maternal dose and maternal genotype, both for the immediately relevant locus but also for other loci (such as Ah) and fetal genotype.

Other single-gene differences which affect teratogenic risk include muscular dystrophy, in the chick, which is associated with a genetic defect in acetylcholinesterase which renders the embryo more sensitive than normal to the teratogenic effect of cholinomimetic drugs (Landauer *et al.*, 1976), and the production of exencephaly among the range of responses to a teratogenic dose of insulin in mice. The frequency of this condition, affected by two different genes (Cd and Rf), are both recognised by skeletal effects (Cole and Trasler, 1980). Genetic differences in response to insulin have also been reported by Smithberg and Runner (1963), and we have found striking differences in response to insulin of cells of different genotypes in cell culture conditions (Clayton *et al.*, 1976; Clayton *et al.*, 1980; and see Section VIII C.).

Other examples may be expected to come to light in the future, and one might speculate as to the possible risk to individuals with such genetic idiosyncracies as are exemplified by some variants of the X-linked locus for glucose 6-phosphate dehydrogenase (G6PD) in man, which confer sensitivity of affected males to sulphonamides, analgesics, antimalarials and certain other drugs, many of which are teratogenic to animals and some of which are teratogenic to man. A male fetus may be at some risk if the physiologically normal heterozygous mother ingests any of these substances at a period critical for the fetus.

III. Developmental Variables

It has been amply demonstrated that the incidence and nature of teratogenic defects depend on the stage of development of the fetus at the time of insult. The earliest effects will include failures of implantation or early fetal loss which will be recorded as reduced fecundity or reduced litter size. Eye and brain defects will occur earlier than forelimb defects and these, in turn, will occur earlier than hind-limb defects (Kochhar, 1973; Nelson, 1960; Nowack, 1965; Russel, 1950; Wilson *et al.*, 1953; Palmer, 1974; Saxén and Rapola, 1969; Wilson, 1973; Shepard, 1979; Goldman, 1979). The sequence of processes required for the determination, morphogenesis and differentiation of any structure is normally spread out over a period of embryonic life, so that an overlapping series of curves may be drawn, each representing a relatively protracted period of susceptibility

for an embryonic structure, with a peak period of maximum sensitivity, when the teratogenic dose required to produce a given defect is lowest (for examples, see Chapters 1 and 4).

IV. The Cell as Target

A teratogenic substance must exert its immediate effect on the cells of the embryo or fetus by affecting cellular properties, unless the effects are wholly secondary to a change in maternal physiology or placental function, or to an extrinsic change in fetal physiology. Modifications of cell division leading to changes in relative growth rate, or cell orientation or adhesion, could affect necessary inductive contacts, or lead to disproportionate growth. An increase or decrease in the rate of cell death will have morphological consequences: either by a loss or diminution in cell number which cannot be regulated, or by a failure to eliminate cells which are 'programmed' to die in specific regions as part of a morphogenetic process. Changes in properties of the cell surface or of extracellular components will affect cell contact, cell recognition, adhesiveness or cell movement. A teratogen may act by blocking a receptor site, or affecting the synthesis of extracellular matrix, or the integrity of a component of the cytoarchitecture. Modified cell-surface properties have been shown to exist in several mutants with severe developmental defects (see Bennett, 1975; Clayton, 1980). Finally, a teratogen may affect cell inducibility or interfere with cell metabolism, (for example as an enzyme poison, inhibitor, or as a metabolic analogue) or affect the synthesis of nucleic acids or proteins.

The developmental anomalies after any of these types of change may include both direct and indirect consequences. Cellular sensitivity to the action of a teratogen, and the possible developmental consequences, may vary according to the time of insult and the developmental processes affected. This subject is discussed by Saxén (1976a, b), Moscona (1976), and Clayton, (1980).

Restricted periods of sensitivity for a particular defect imply either that a specific vulnerable process is occuring temporally or that a biochemical characteristic does not appear before a certain stage of differentiation.

In whole-animal tests, each potential teratogen must be assayed over a wide range of doses and the tests must span the whole period of fetal development. Culture-based assays, on the other hand, can assess the effect of a substance on differentiation in a relatively short time, and

sample representative tissues; for example, a pre-cartilage rudiment can stand in for all differentiating pre-cartilage rudiments over the whole embryonic period.

An example would be the effect of vitamin A on cells in the early stage of chondrogenesis (Lewis *et al.*, 1978). Since the initiation of chondrogenesis in the limb follows an orderly sequence along a proximodistal gradient it is not surprising that the precise effect on the limb depends on the period of drug administration. All the evidence from cell culture studies points to an important regulatory effect of vitamin A on the differentiating chondrocyte cell surface, probably by regulating the synthesis of specific glycoproteins. (Vitamin A is discussed further in Section VIII A.) The proposition that the action of a substance on the cell surface is one of the possible mechanisms of teratogenesis may be illustrated by a few brief examples.

At 5 days of incubation, the chick embryo retina is a simple neuroepithelium. If it is explanted into culture it develops normal histiotypic organisation and cell differentiation; furthermore, dissociated cells, if permitted to reassemble, show histiotypic reassembly and cell differentiation. In both cases, glutamine synthetase is inducible by hydrocortisone. (Monroy and Moscona, 1980, review the considerable body of experimental work on this system.) 5-Bromodeoxyuridine(BUdR) is teratogenic, producing a range of defects according to the time of administration: the CNS is particularly vulnerable (Chaube and Murphy, 1968; Ruffolo and Ferm, 1965). Brief exposure of 5-day embryo retina to BUdR in culture leads to very disorganised histogenesis, indicating a failure of cell recognition and assembly. The cells adhere, but do so non-selectively, and glutamine synthetase induction is impaired or prevented (Mayerson and Moscona, 1979; Moscona and Moscona, 1979).

Cleft palate has been obtained in mice after high doses of diazepam (Valium) (Miller and Becker, 1975), but rats were not affected (Beall, 1972). There is some evidence that an increased incidence of cleft palate may occur in children exposed to diazepam during the first trimester (Saxén and Saxén, 1975; Aarskog, 1975). Clark and Ryan (1980) tested a number of benzodiazepines on a line of Friend cells in culture and found diazepam one of the two most active in affecting induction. They consider that this effect is mediated via changes in the cell surface, in which the lipophilic benzodiazepines are soluble, and suggest that the teratogenic effects of tranquillisers in large doses may be related to this effect.

The possibility that many teratogens may act by affecting cell-surface properties is also supported by the inhibition by teratogens of the

attachment of tumour cells to lectin-coated surfaces (Braun *et al.*, 1979), and by the teratogenic effect of concanavalin A (Con A) on the rabbit fetus (De Sesso *et al.*, 1977). The range of morphological defects are presumably related to the known quantitative differences in the distribution of Con A or other lectin-type binding in different tissues (Robertson *et al.*, 1975), to the changes in such distribution during differentiation (Kleinshuster and Moscona, 1972), and to the effect of blocking these sites on such cellular activities as cell migration (Moran, 1974).

V. *In Vitro* Assays

A cell-based assay would require that there be a necessary relationship between fetal morphology, cell function, cell interactions and detectable aspects of cell biochemistry. Evidence which indicates that this is the case has been reviewed and discussed by Clayton (1980). Cell tests are possible in principle because different types of embryonic or fetal cells grown in appropriate conditions may continue to demonstrate specific characteristics, including the capacity for reassembly of histiotypic organisation, inducibility, and differentiation as judged by the development of characteristic ultrastructure and cell products (for reviews see Kaighn, 1976; Morris, 1976; Clayton, 1980). Several investigators have tested *in vitro* assay systems (see Nebert, 1973; Rajan, 1974; Kochhar, 1975; Beck, 1976; Clayton, 1976, 1980; Kaighn, 1976; Karkinnen-Jääskeläinen and Saxén, 1976; Morris, 1976; Saxén, 1976a, b; Shepard and Pious, 1978; Wilk *et al.*, 1980).

The major advantage of *in vitro* assays for teratogens are their greater rapidity than whole-animal tests. They could also be applied to scarce primate material. Organ cultures require growth measurements and histological investigation. Cell cultures, being more homogeneous (and being also exposed to the possible teratogenic agent more homogeneously) are more suited to biochemical assay, but cell behaviour, cell-cell recognition properties, histiotypic assembly and cell replication can also be measured accurately. However, the drug must be applied to the cells in the correct biochemical form and at concentrations equivalent to those experienced by fetal cells *in vivo*. A concentration in the culture dish derived directly from the dose per body weight of the mother does not allow for the effects of maternal-fetal partition, or of rapid elimination by the mother, which would rapidly reduce the fetal dose. The *in vivo* doses of several drugs producing limb teratology in the mouse, calculated

as mg/g body weight, appear to bear no relationship to the dose in μg/ml found to produce definite effects in limb organ culture systems (Kochhar, 1975). For example the required *in vitro* concentration of 6-aminonicotinamide was 100μg/ml and the hydroxyurea 10μg/ml, but the *in vivo* concentrations were 25μg/g and 3mg/g respectively.

A serious problem for organ and cell culture systems (see Clayton, 1976, 1980) is the form in which the substance should be presented, since some substances administered to the mother *in vivo* do not exert their teratogenic effects directly on the fetus, but through a toxic or persistent metabolite, while other substances which are toxic *in vitro* may be metabolised to a harmless agent *in vivo*. This problem of administration of drugs in the correct form to cultured cells has been approached in two ways: by using the amniotic fluid of drug-injected mothers as culture medium, which allows for metabolism and partitioning (Clayton, 1980), or by adding the drug together with a maternal liver postmitochondrial fraction and a NADPH-generating system in a dialysis sac and suspending this in the culture medium which permits metabolism to occur (Wilk *et al.*, 1980). Cell behaviour was recorded in both sets of experiments; the differentiation of neural crest cells into pigment cells or neurones and the amount of stainable matrix synthesised by chondrocytes was used as a further index by Wilk *et al.* (1980) and the effect on the profiles of accumulated protein and of protein synthesis by Clayton (1976, 1980). In both sets of experiments a correlation was found between the teratogenicity of a compound and its effect in cell culture.

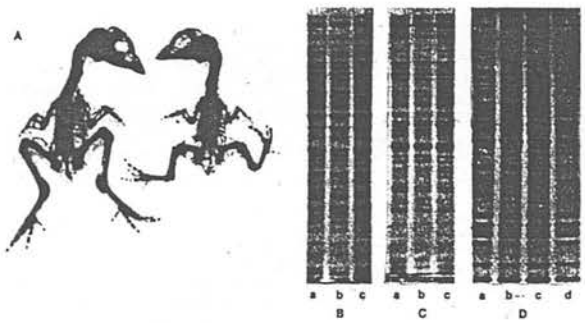
VI. Substances with Little or No Effect

A. *Chlordiazepoxide*

At sufficiently high doses almost any normally innocuous substance would be expected to affect cultured embryonic cells. The necessity for caution in setting up cell-based assay systems may be illustrated by the effect of a minor tranquilliser, the benzodiazepine chlordiazepoxide HCl (Librium), which was not found to be a teratogen in one study (Hartz *et al.*, 1975) and only slightly teratogenic in another (Milkovich and van den Berg, 1974).

We have not observed any morphological effects in chick embryos injected at 4 days' incubation with 0.5mg or 1.25mg per egg. Calculating the relative dose on the basis of the average weights of a human adult and a chick egg is most unsatisfactory, since the 4-day embryo is only a fraction

Figure 3.1: The Effect of Chlordiazepoxide (Librium) on Embryos and Cultured Cells. A: 17-day chick embryos, cleared skeletons. Left, untreated; right, injected with 2mg Librium per egg at 8 days' incubation. No effects were observed at doses from 0.5mg to 1mg per egg. At 1.5mg per egg a very slight reduction in size was found in some embryos. 2.5mg/egg was embryolethal. B: Limb fibroblasts from 8-day embryos, transferred to medium containing Librium on day 4, with medium changes every 2-3 days, and harvested on day 12. Accumulated proteins were separated by SDS polyacrylamide gel electrophoresis (as in Laemmli, 1970). (a) 25μg/ml Librium; (b) 50μg/ml Librium; (c) control. Two proteins are affected, actin and another protein as yet not unambiguously identified. C: Kidney fibroblasts from 8-day chick embryos, transferred to medium containing Librium on day 4, with medium changes every 2-3 days, and harvested on day 12. (a) 25μg/ml Librium; (b) 50μg/ml Librium; (c) control. Actin is affected. D: 16-day lens epithelium cultures from 1-day-old chicks. Transferred to medium containing Librium on day 6, with medium changes every 2-3 days and harvested on day 16. (a) Control; (b) 25μg/ml Librium; (c) 50μg/ml Librium; (d) 75μg/ml Librium. At the highest dose the lower molecular weight δ-actin and some β-crystallins are diminished. Close inspection shows components affected.



of the weight of the egg, and no allowance is made in either case for drug distribution in the various compartments of man or egg, nor of turnover. In the absence of pharmacokinetic data on Librium in the egg, it may still be noted that on this per weight basis these doses are 10 and 20 times higher than a particularly high daily intake of 60mg for an adult (15 and 30mg daily is a more likely dose). The true excess is probably considerably greater. However, at 2mg and 2.5mg per egg an overall reduction in embryonic body size was obtained (Fig. 3.1), and there was 70% lethality at 2.5mg/egg. In cell culture, a range of unphysiological high-dose concentrations were tested, but effects on the cells were not seen below 50μg/ml for limb fibroblasts and 75μg/ml for lens epithelium. At these values, which are not less than 40 and 60 times a high human dose, an apparently specific effect was observed: a marked diminution of actin in limb and kidney fibroblasts and in lens epithelium (Fig. 3.1). Additional changes in lens epithelium were also observed. The available translatable actin-mRNA was found to be diminished by exposure of limb fibroblasts to very high concentrations of Librium (Fig. 3.2).

An effect on postnatal learning ability has been reported for anticonvulsants and tranquillisers (see Voorhees and Butcher, Chapter 9). Hartz *et al.* (1975) found no evidence for a diminution of IQ levels in children after Librium treatment of their mothers. Although we have not yet assessed the effect of Librium on brain cultures, we observed an effect, at 50μg/ml, on 10-day cultures of 8-day chick embryo neural retina, when there are abundant neurones with axonal connections (Fig. 3.3A). However, no effect was observed on such cultures at a later stage of growth, when the neuronal cells are no longer present and the culture comprises only neuroepithelial cells (Okada, 1976; Clayton *et al.*, 1977).

B. *Meprobamate*

Meprobamate, a substituted propanediol, may only rarely have teratogenic effects, but the evidence is not unambiguous (Milkovich and van den Berg, 1974). We have not found it to produce morphological effects on chick embryos *in ovo*, but 0.8mg/egg was embryotoxic to 30% of 4.5-day embryos and 10% of 8-day embryos. At 20 or 40μg/ml no effects were observed in cell culture, and at 60μg/ml it was cytotoxic. At 50μg/ml it did not affect the protein profiles of the cells tested, with the exception of a slight effect on one component only (of molecular weight between 23 and 30K daltons) in limb fibroblasts.

Figure 3.2: Fluorograph of Translation Products of mRNA from 12-Day Cultures of Limb Fibroblasts. Culture medium contained 50μg/ml Librium, as in Fig. 3.1. Procedures as in Thomson *et al.* (1979). Arrows indicate proteins affected in Librium treated cultures (B) compared with control cultures (A).

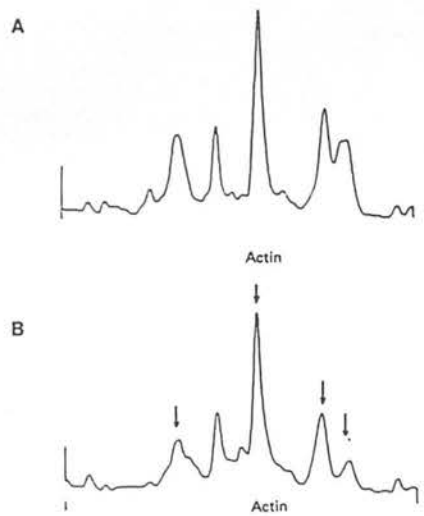
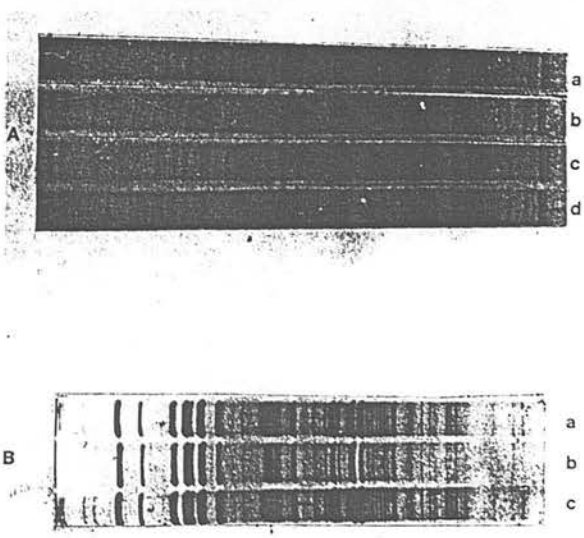


Figure 3.3: A. The Effect of Several Substances on 10-Day Cultures of Neural Retina Established from 8-Day Chick Embryos. Both neuroepithelium and neurones are normally present in 8-day embryo neural retina cultures at this stage. (a) Control; (b) phenytoin (Epanutin), 50μg/ml; (c) chloroquine sulphate (Nivaquine), 4μg/ml; (d) chlordiazepoxide HCl (Librium), 50μg/ml. Close inspection reveals the components affected. Actin is severely affected by Nivaquine.

B. The Effect of Several Substances on 16-Day Cultures of Lens Epithelium from 1-Day-Old Chicks. (a) Control; (b) phenytoin (Epanutin), 50μg/ml; (c) meprobamate (Equanil), 50μg/ml. Actin is diminished, and the higher molecular weight δ-crystallin is depressed by Epanutin. Meprobamate does not affect the protein profile. Close inspection reveals the components affected.



VII. Toxic Effects

A. *Diphenylhydantoin*

Diphenylhydantoin or phenytoin (Epanutin) is an anticonvulsant which prevents both cardiac arhythmia and epileptic discharges. It acts by stabilising cell membranes, thus affecting Na^+/K^+ flux (Kizer *et al.*, 1970). Anticonvulsants are teratogenic (Lefebvre *et al.*, 1972; Ajodhia and Hope, 1973), and this has been suggested to be due to folic acid antagonism (Netzloff *et al.*, 1979).

We did not observe gross abnormalities in 17-day chick embryos *in ovo* injected at 4 days' incubation with 0.25mg per egg, when 5% lethality was obtained, but some embryotoxicity appeared at 0.5mg/egg, with a 41% incidence of death. This dose was 100% embryotoxic at 2 days' incubation and 30% at 8 days' incubation. No effects were discernible in cultured cells at concentrations of 20 or 40μg/ml, but 60μg/ml was cytotoxic. At 50μg/ml, effects were observed on cultured cells. Again, actin was affected in all cells, but other effects were found in lens epithelium and neural retina. In lens epithelium the ratio of the 50K dalton and 45K dalton δ-crystallin polypeptides is strongly affected by the drug (Fig. 3.3B). This ratio is known to be modified by changing the Na^+/K^+ balance (Piatigorsky, 1980), so that this effect would seem to be reasonably related to the action of phenytoin.

We have observed a diminution in axon formation and outgrowth in cell cultures of 8-day chick embryo brain and neural retina (A. Zehir and R.M. Clayton, unpublished work). Brain-cell proteins are still to be tested, but (as with high levels of Librium) we observed that 10-day cultures of neural retina, containing both neuroepithelium and neurones with axonal connections are affected (Fig. 3.3A) but later cultures, when all neurones have been lost, are not affected.

B. *Daunomycin*

Daunomycin has been used as an antibiotic and in cancer chemotherapy; it is teratogenic in the rabbit and rat (Thompson *et al.*, 1978). We found that at sub-cytotoxic concentrations it produced distortions of the profile of protein synthesis in cultured cells, of each of three different genotypes, but the patterns of response were quantitatively different for each genotype (Clayton *et al.*, 1976).

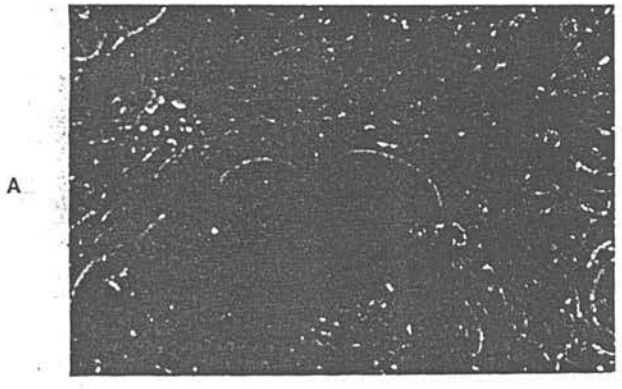
C. *6-Aminonicotinamide*

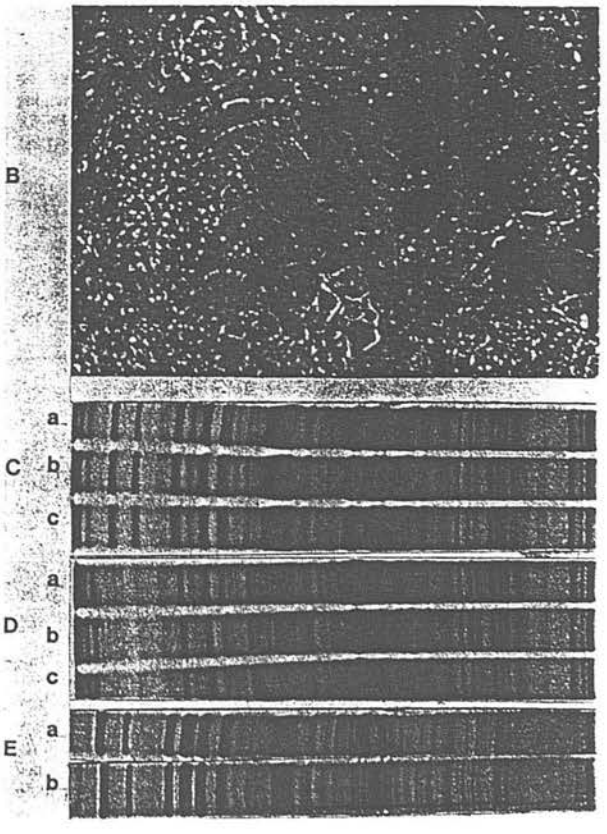
Nicotinamide analogues are teratogenic, causing a variety of defects in mice and chicks, including cleft palate and skeletal defects. Genetic differences in sensitivity to 6-aminonicotinamide have been found (Goldstein *et al.*, 1963; Biddle and Fraser, 1979). Caplan (1970, 1972) was able to relate the effects observed on chick development *in ovo* to those obtained on limb cells in culture conditions, and showed that the differentiation of mesoderm into muscle and chondrogenic cells is inhibited. Seegmiller *et al.* (1980) have shown that cartilage epiphyses *in vitro* in medium supplemented with 6-aminonicotinamide synthesise chondrotin sulphate with a reduced molecular weight and the sulphate moeity in an abnormal position. Similar changes were found in myogenic cultures (Caplan, 1972a). 6-Aminonicotinamide was also found to affect the protein profile of lens cells in culture (Clayton, 1976, 1980) and to affect the protein profiles of neural retina, limb and kidney fibroblasts in culture (C. Smart and R.M. Clayton, unpublished work).

D. *Chloroquine*

Chloroquine sulphate (Nivaquine) is used as an antimalarial drug and also for rheumatoid arthritis, when the daily dose may be from 200 to 400mg. Retinopathy is among the more common toxic side effects in adults, and the teratogenic effects reported include damage to the 8th nerve and cochlea, mental retardation, neonatal convulsions and posterior column defects (Maltz and Naunton, 1968). At 200-300ng per egg, we observed no effects in the 4.5- and 8-day embryo, but 400ng/egg was embryotoxic to 100%, 67% and 28% of 2-, 4.5- and 8-day embryos, respectively. In cell culture, we found that 2μg/ml was without discernible effect, and above 4μg/ml Nivaquine was cytotoxic. At 4μg/ml, cultures survived if exposed only once; cells survived and a number of specific effects were observed, the same protein being affected in lens epithelium, (Fig. 3.4E), neural retina (Fig. 3.4C), and brain cultures (Fig. 3.4D). Several components are affected in neural retina (Fig. 3.4C). In early cultures a new pair of bands normally appears in SDS gels (molecular weights 50-55K daltons); these are abolished by Nivaquine. In later cultures, two components of about 40K daltons appear, which are lost on Nivaquine treatment (Fig. 3.4C-E). Cell morphology is also affected (Fig. 3.4A and B).

Figure 3.4: The Effect of Chloroquine Sulphate (Nivaquine) on Cultured Embryonic Cells. The effects of Nivaquine on 16-day neural retina cell cultures, established from 8-day chick embryos. A. Control culture: neuronal cells have disappeared, as is normal at this stage, leaving neuroepithelium. B. Treated once with Nivaquine at 4μg/ml on day 14, cells are abnormal and nuclei swollen. C. 30-day cultures set up as above, harvested and analysed as described in Fig. 3.1. (a) Controls; (b) treated once with Nivaquine as in B above, on day 29; (c) treated once with Nivaquine as in B above, on day 15. D. 30-day cultures set up from 8-day chick embryo brain, harvested and analysed as above. (a) Controls: neuronal cells survive for about 15 days, but at this stage the cultures are largely neuroepithelium; (b) treated with Nivaquine as above on day 29; (c) treated with Nivaquine as above on day 15. E. 16-day lens epithelial culture set up from 1-day-old chick lens (as in Eguchi *et al.*, 1975), harvested on day 15 and analysed as above. (a) Controls; (b) treated with Nivaquine as above on day 15.





E. *Cobalt*

Cobaltous chloride caused 'brain softening' and defective eye development including anophthalamia and microphthalamia, and anaemia (Ridgway and Karnofsky, 1952; Kury and Crosby, 1968). It reduces concentrations of haem and cytochrome P450 in intact rats (Guzelian and Bissell, 1976), and in monolayer primary cultures of rat hepatocytes (Guzelian and Bissell, 1976).

We observed dose- and stage-dependent lethality to cobaltous chloride: mortality in the 2-day chick embryo rose from 32% at 0.05mg/egg to 75% at 0.1mg/egg and 100% at 0.3mg/egg. However the 4.5-day embryo was more resistant, the lethality being 47% at 0.1mg/egg and 51% at 0.3mg/egg. Furthermore, the 8-day embryo was not killed by 0.1mg/egg: lethality at 0.3mg/egg was 34% and at 0.5mg/egg 44%. Although no red cell counts were made, the very marked pallor of these embryos would be compatible with the previously reported anaemia.

At 0.3mg/egg, chick embryos injected at 4.5 days developed lens opacities by 7-8 days; those injected on the eighth day developed lens opacities by 9-10 days (Fig. 3.5A). The survivors also had thin limbs.

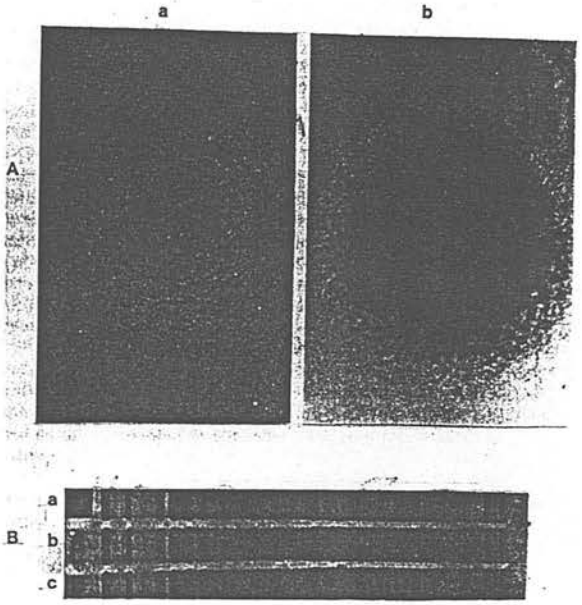
In cell culture it is cytotoxic above 4μg/ml, but limb and kidney fibroblasts are more affected than neural retina or lens cells. Actin is affected in kidney cells and one band is absent in polyacrylamide gel analyses of treated lens cells (A. Zehir and R.M. Clayton, unpublished work; Fig. 3.5B).

F. *Lead*

Lead salts are teratogenic in chicks, hamsters, rats and mice (Ridgway and Karnofsky, 1952; Butt *et al.*, 1952; Ferm and Carpenter, 1967; Murakami *et al.*, 1954; McClain and Becker, 1975). Reduction of body size, brain damage, and posterior axial defects including the urorectocaudal syndrome have all been reported. In man there is some evidence of an increase in fetal deaths (Angle and McIntyre, 1964), and of lead-associated mental retardation (Moore *et al.*, 1977).

We observed stage- and dose-related embryotoxicity for chick embryos injected with lead nitrate. Embryos injected at 2 days' incubation developed spinal and caudal defects, those injected at 4 or 8 days developed brain defects including hydrocephaly and exencephaly. At 2 days' incubation, 0.025mg/egg produced 10% lethality, rising to 24% at 0.05mg/egg, and 50% at 0.1mg/egg. Older embryos were less susceptible; 4.5-day embryos

Figure 3.5: The Effect of Cobaltous Chloride. A. 8-day embryo lenses, seen from the anterior face (phase contrast x 200) and focused below the epithelium. (a) Control lens; (b) lens from embryo treated with cobaltous chloride at 0.3mg/egg at 4.5 days. B. 12-day cultures of lens epithelium set up from 1-day-old chicks. Harvested and analysed as in Fig. 3.1. (a) Control cultures; (b) treated with 2μg/ml cobaltous chloride at 11 days; (c) treated with 4μg/ml cobaltous chloride at 11 days.



were not killed at 0.05mg/egg, but 18% lethality was obtained at 0.1mg/egg and 24% at 0.2mg/egg. By 8 days of incubation, 0.1mg/egg led to 2% lethality, and 0.2mg/egg to only 7.5%. In cell culture, levels of 6μg/ml were cytotoxic to all tissues, at 5μg/ml brain and neural retina cells were affected. The effects seen on polyacrylamide gel electrophoresis were similar to those observed with chloroquine sulphate for these two tissues.

VIII. Effects Including Changes in Differentiation

A. *Vitamin A*

The importance of vitamin A and related substances in regulating developmental processes is evident from the teratogenic effects obtained in animals both by deprivation and by excess. The commonest effects of hypovitaminosis A in the rat include ocular and urinogenital defects, but cardiac and other anomalies are also common (Wilson *et al.*, 1953; Kalter, 1968). Hypervitaminosis A and retinoic acid produce a wide range of effects depending on the state of development of the fetus (Cohlan, 1954; Wilson *et al.*, 1953; Kalter, 1968; Shenefelt, 1972; Geelen, 1979). The effects include skeletal anomalies, cleft palate, eye defects and neural-tube defects. In the early embryo there is interference with closure of the neural tube (Giroud and Martinet, 1956), but later treatment affects learning and motor ability (see Vorhees and Butcher, Chapter 9).

Sporn *et al.* (1976) review the evidence that retinoids including vitamin A regulate the normal differentiation of many epithelial cells. The effect on epithelial growth, differentiation and metaplasia has been studied in organ culture for several systems besides the chondrogenic cells already mentioned, for example prostate (Lasnitzki, 1962), epidermis (Sporn *et al.*, 1975), somite, notochordal cells, and mesoderm in general (Marin-Padilla and Ferm, 1965; Marin-Padilla, 1966).

Teratogenic effects include limb defects, and vitamin A can be shown to interfere with chondrogenesis *in vivo* (Dingle *et al.*, 1966; Kochhar, 1973). Limb rudiments cultured in vitamin A excess develop abnormally (Nakamura, 1977; Rajan, 1974) and cartilage matrix is lost (Fell and Dingle, 1963; Kochhar and Aydelotte, 1974). The effects of vitamin A on limb differentiation is produced only during the period of early chondrogenesis; direct effects on chondrocytes in cell culture have been shown with matrix synthesis being inhibited (Shapiro and Poon, 1976; Vasan and

Lash, 1975). Lewis *et al.* (1978), using organ culture, found that growth in the presence of vitamin A preserves the type of cell-cell contacts, and the presence of a glycoprotein characteristic of prechondrocyte mesenchyme. The appearance of a high molecular weight glycoprotein and changes in cell-to-cell contacts and in the intercellular matrix characterising chondrocyte differentiation were prevented.

B. *N-Methyl-N-nitro-N-nitrosoguanidine*

N-Methyl-N-nitro-N-nitrosoguanidine (MNNG), an alkylating agent, is both mutagenic and carcinogenic. It has been shown to have teratogenic effects in mice (Inouye and Murakami, 1978), with multiple defects of brain, skeleton and palate. It has been shown to cause transdifferentiation in the amphibian eye (Eguchi and Watanabe, 1973), and we have found that it disturbs the pattern of protein synthesis in both lens and neural retina cells in culture. The quantitative changes in protein profiles differed for the two genotypes tested. Exposure to 7μg/ml MNNG for 1 hour also produces distortions in the pathway of differentiation and transdifferentiation of these cells in culture (Clayton *et al.*, 1980). 8-day chick embryo neural retina in cell culture can give rise to neuronal cells, pigment cells or lens under appropriate culture conditions (Eguchi, 1976; Okada, 1976; Clayton, 1978). We have found that the effect of exposure to a subcytotoxic dose of MNNG depends on the state of differentiation that the cells have acquired during their period of cell culture. Early exposure (at 9 and 11 days after plating) totally prevents the differentiation of pigment cells which normally appear from about day 30 onwards, delays the appearance of lens cell differentiation by 7-10 days, and also leads to a late reappearance of neuronal cells, which are not normally seen after about 16-18 days in normal culture conditions. Exposure to MNNG at a later stage (19 and 21 days) leads to a delay of 10 days or more in pigment-cell appearance, which are abnormal and die after a few days. The appearance of lens cells is only slightly delayed, and again neurone-like cells reappear at a late stage. Thus the effect of MNNG is to affect the future pathways of differentiation of embryonic cells (determination) (Clayton *et al.*, 1980; C. Patek and R.M. Clayton unpublished work).

C. *Insulin*

Insulin has not been found to be teratogenic in rats, but defects of brain, skeleton or both are produced in rabbits, mice and chicks (reviewed by Kalter, 1968; Landauer, 1972). Genetic differences in responses of mice to insulin have been described above (Section II). We have confirmed all the teratogenic effects for the chick *in ovo* reported by Landauer (1972). At 2 days' incubation 37% and 57% of embryos were killed by doses of one and two IU of insulin, respectively. The lower dose was not lethal to 4.5- and 8-day embryos, but lethality of the higher dose remained the same.

In addition to the age- and dose-dependent lethality and the skeletal and other effects, we also observed that insulin *in ovo* causes cataract, (Fig. 3.6). We have explored the reasons for this effect by examining the response of lens epithelium to insulin *in vitro*.

Insulin stimulates mitosis in a wide range of tissues such as liver, (Gerschenson *et al.*, 1972), chondrocytes, (Hajek and Solursh, 1974), fibroblasts, (Paul and Pearson, 1960), myoblasts (Kumegawa *et al.*, 1980) and lens epithelium (Reddan *et al.*, 1972), although in lens cell sheets from the early embryo it promotes cell elongation: a process which characterises lens fibre cell differentiation (Piatigorsky *et al.*, 1973). We find that the genotype of the cell may affect the growth rate response to insulin (Fig. 3.7a and b). Insulin affects the survival of neurones in culture (reviewed by Waymouth, 1977). It also affects microtubule assembly (Soifer *et al.*, 1971; Piatigorsky, *et al.*, 1973) and nucleic acid and protein metabolism (Hickey and Klein, 1970). Insulin binds to membranes, including the plasmalemma and Golgi membranes (Carpenter *et al.*, 1979), nuclear membranes and both rough and smooth endoplasmic reticulum (Goldfine *et al.*, 1978).

In the lens cell, the effect of insulin is to change the relative balance of crystallins synthesised, and in lens epithelium from embryos and 1-day-old chicks the proportion of δ-crystallin is increased (Milstone and Piatigorsky, 1977; de Pomerai and Clayton, 1978; de Pomerai *et al.*, 1978). This effect is also obtained in lens cells transdifferentiating from neural retina cells in culture (de Pomerai and Clayton, 1980). However, many other crystallins are also affected by insulin treatment (Clayton *et al.*, 1976, 1980; and Fig. 3.8). This response is modified by the genotype of the cell (Clayton *et al.*, 1976, 1980). These changes are effected by changes in the available mRNA population, for example the amount of δ-crystallin-mRNA increases preferentially in the 6-day-old embryo lens (Milstone *et al.*, 1976). We find

that other crystallin-mRNAs are also affected in 1-day old lens non-coordinately, and this effect is also modulated by the genotype (Clayton *et al.*, 1980; and Figs. 3.9, and 3.10).

Figure 3.6: The Effect of Insulin on the Chick Lens *In Vivo*. The centre area of whole lenses of 17-day chick embryos are seen from the anterior surface, with focus just below the anterior epithelium (phase contrast x 200). A. Control: the types of the fibre cells are seen in orderly array. B. Lens from embryo injected at 4.5 days' incubation with 2 IU of insulin. Swollen, disorganised cells are seen in the centre of the lens, replacing the fibres.

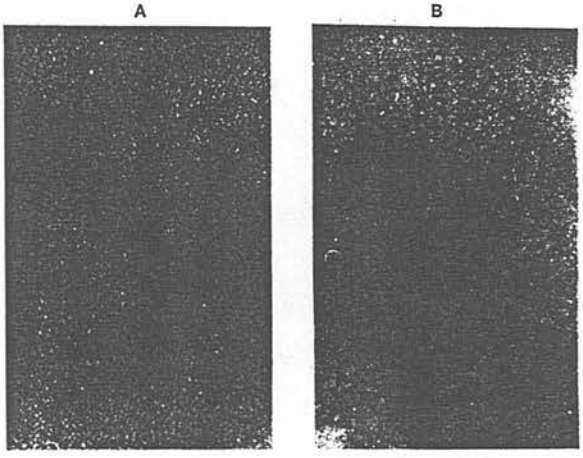


Figure 3.7: The Effect of Insulin on Cell Growth *In Vitro*. Growth curves of 1-day-old chick lens epithelium cells in culture, conditions as in Eguchi *et al.* (1975). Dotted lines, control cultures; solid lines, grown in medium containing insulin.

Cells were transferred to medium containing 10μg/ml insulin, which was replaced every 3 days. Insulin stimulates growth but the Hy-2 genotype response is slight, that of Db more marked.

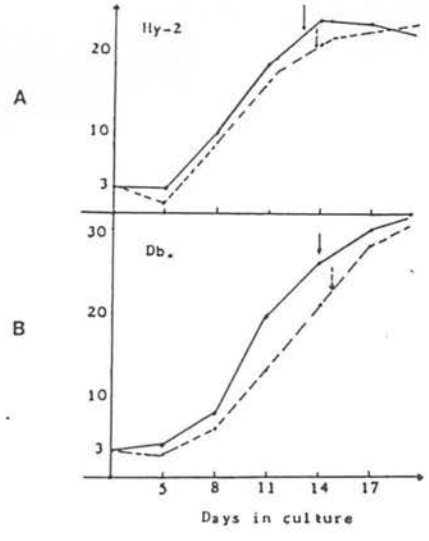


Figure 3.8: The Effect of Insulin on Protein Synthesis in Cell Culture. Traces obtained by integrating densitometer from fluorographs of SDS polyacrylamide gel electrophoretic separation of proteins from cultured lens cells from 1-day-old chick Db genotype cells, grown as in Fig. 3.7, labelled for 3 hours with ^{3}H-amino acid mixture and harvested on day 13, as described in Thomson *et al.* (1979). A. Control. B. Cells transferred to medium containing 10μg/ml insulin and transferred every 3 days as for Fig. 3.7. Arrows indicate proteins most affected.

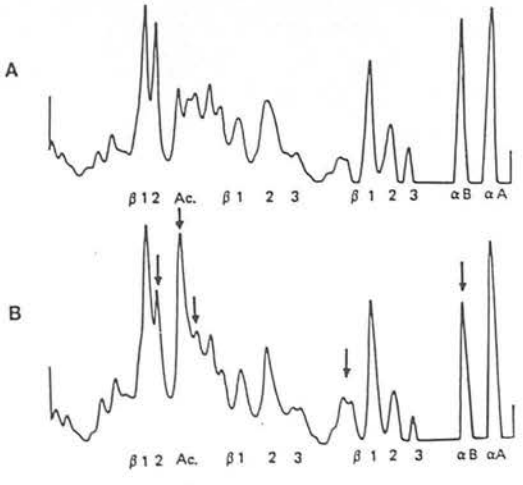


Figure 3.9: Fluorographic Analysis of the Effect of Insulin on mRNA Translation in Cell Culture. Fluorographs of SDS polyacrylamide gel electrophoresis of products translated in a cell-free system from mRNA obtained from cultures of 1-day-old lens epithelium, grown and analysed as described for Figs. 3.7 and 3.8. (a) Control cells; (b) insulin-treated cells. A. Db genotype. B. Hy-2 genotype.

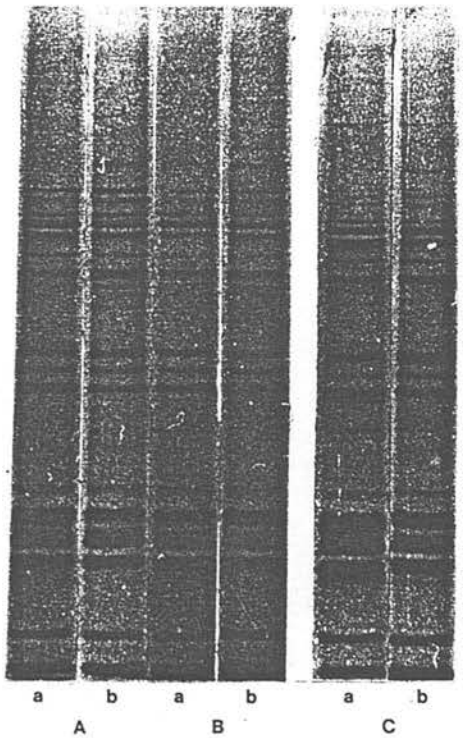
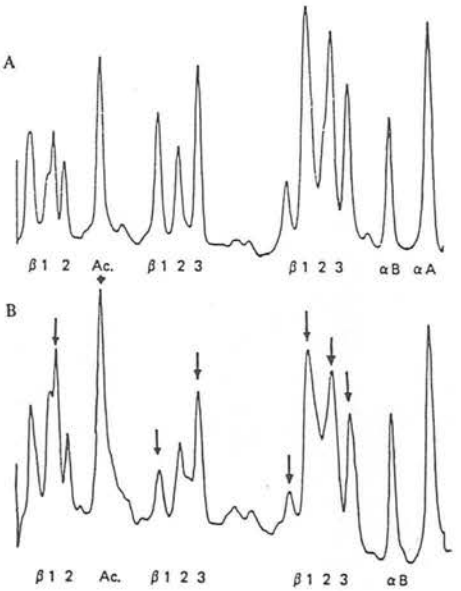


Figure 3.10: Densitometric Analysis of the Effect of Insulin on mRNA Translation in Cell Culture. Showing the reduced effect of insulin treatment on more differentiated cultures. Db lens epithelial cells from 1-day-old chick embryos, harvested on 15 days when mature lentoids are developed in the cultures. All procedures as in Thomson *et al.* (1979). A. Translation products of mRNA from control cells. B. Translation products of mRNA from insulin-treated cells. Arrows indicate proteins most affected.



IX. Conclusions

There seems to be reason to be optimistic regarding the possibility both of using *in vitro* assays as preliminary tests of compounds (and probably to apply these to human material), and of using such methods to elucidate some of the primary actions of a teratogen. However, both problems require that the question of the form of the drug and the relevant dose be investigated fully, for the reasons given above (Section V). It is very likely that teratogens which exert their effect indirectly, for example by affecting maternal physiology, may not be testable by *in vitro* methods. On the other hand, some problems not considered suitable for *in vitro* investigation may nevertheless be accessible. One such problem may be that of low-level neurotoxic effects.

Gross disturbances in neuroanatomy, intelligence or neurological function do not cause difficulties in assessment, but assessment of lesser changes in postnatal behaviour or in learning capacity, which could be expected as part of the spectrum of impairment, may lead to difficulties or conflicting reports, (see Vorhees and Butcher, Chapter 9, for discussion). Learning or behavioural deficits have been reported in rats after halothane administration in the second trimester (Smith *et al.*, 1978), subjecting the pregnant female to continued stress which would be expected to affect maternal endocrinology (Barlow *et al.*, 1979), and after exposure to alcohol (Bond and Digusto, 1976), or to some psychotropic drugs (see Buelke-Sam and Kimmel, 1979; Mello, 1975; Kolata, 1978; and Vorhees and Butcher, Chapter 9, for discussions). Buelke-Sam and Kimmel discuss the problems of different systems of measurement of performance. A different approach might be to assess the effect of a suspected substance on neuronal outgrowth, and synaptic connections, in cell or tissue culture systems. This possibility is suggested by the effect of some teratogens on fetal brain cells in mice (Manning *et al.*, 1971; Langman and Cordell, 1977). Barbiturates are teratogenic in animals (McColl *et al.*, 1963) and may lead to a dose-related diminution in learning capacity (Martin and Mackler, quoted in Smith, 1977), and a reduction in embryo brain-cell number (Manning *et al.*, 1971). We have made preliminary observations of the effects of barbiturates on protein synthesis in neural retina (Clayton, 1980), and of phenytoin, chloroquine and lead on brain and neural retina, (Figs. 3.3 and 3.4; and A. Zehir and R.M. Clayton, unpublished work), and changes in growth, differentiation in culture and protein profiles of neuronal cells may be of relevance in this context.

The development of cellular techniques for assessment of teratogens

may prove to be relevant not only to fundamental problems of development and cell biology, but also to help in providing a rapid assessment of hazards with a sociobiological effect which produce both physical and intellectual handicap in the human population.

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CELL CULTURE IN THE INVESTIGATION OF NORMAL AND ABNORMAL DIFFERENTIATION OF EYE TISSUES

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KEYWORDS

Cell culture, crystallins, lens, neural retina, transdifferentiation, carcinogen, insulin, mRNA, genetic.

ABSTRACT

We have studied chick lens epithelium and embryo neural retina of three different genotypes in several cell culture conditions. The genotypes are distinguished by cell behaviour, cell cycle and cell membrane composition. The culture medium additives chosen are known to affect cell growth patterns. We report here on the effects of brief exposure to N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), a carcinogen, and the effects of growth in the presence of insulin, retina extract or different levels of foetal calf serum. In all cases the relative rates of synthesis of different crystallins is affected both qualitatively and quantitatively as compared to controls. In two cases it has also been found that the mRNA population is differentially affected, as judged by translation in a cell free system. The specific effects are related to the different medium conditions, and modulated by the cell genotype. In addition to these effects, there are delayed effects of MNNG treatment. The appearance of unexpected morphological cell types may involve phenomena similar to transdifferentiation. A relationship between the effects observed and the stage of the cell cycle is suggested.

INTRODUCTION

Cell differentiation is the product of differential gene expression and the pattern of this expression may itself affect the capacity for response to such external signals as lead to further cellular changes. Cell culture methods permit cells of different genotypes, from different tissues and stages of development to be exposed to selected and controlled conditions. For example, the effects of media containing cell growth promoters or inhibitors, or culture conditions favouring cell dispersion or cell contact, can be assessed not only in terms of the consequences for cell viability or cell morphology, and differentiation in culture, but also in molecular terms, i.e. qualitative and quantitative changes in mRNA and protein synthesis.

The cells of day old chick lens and embryo chick neural retina have especial advantages for studies of this kind. The vertebrate lens is composed only of epithelial cells and the terminally differentiated fibre cells derived from them, and this terminal differentiation can be obtained in cell culture. The major gene products of the bird lens are the α, β, and δ crystallins. Throughout development new fibres form from epithelial cells. The display of crystallins in a cell changes as it differentiates into a fibre, in both successively developing epithelial and in fibre cells. Thus the crystallin composition is also modified with increasing developmental age (Clayton, 1974). Regulatory factors affecting a lens cell in vivo could include cell mass, cell position, cell contact, rate of mitosis, and metabolic factors (Clayton and colleagues, 1976), and cytoarchitectural components. The effects on crystallin synthesis of culture conditions affecting some of these parameters shows that crystallin synthesis is affected non-coordinately, (for example, Clayton and colleagues, 1976; Vermorken and Bloemendal, 1978; Mousa and Trevithick, 1977; de Pomerai, Clayton and Pritchard, 1978).

Embryo neural retina (NR) cells in culture can differentiate into neuronal cells and neuroepithelium, but can also transdifferentiate into pigment (PE) and lens cells (Okada, 1976). Differentiation of lens epithelial cells and transdifferentiation of embryo retina cells into lens cells both involve changes in the crystallin mRNA population. For example, a selective increase of hybridisable δ-crystallin mRNA characterises the early stages of embryo chick lens cell differentiation, (Piatigorsky and colleagues, 1976), and changes were found in translatable crystallin mRNA at later stages (Thomson and colleagues, 1978b). During transdifferentiation from retinal cells, crystallin mRNAs move from the intermediate into the high abundance class (Thomson and colleagues, 1979; Yasuda and colleagues, 1979; Clayton, 1979 b,c). These massive increases in crystallin mRNA must involve transcriptional controls: although differential increases in crystallin mRNA stability do occur (e.g. Clayton, Truman and Hannah, 1974; Delcour Odaert and Bouchet, 1976), these changes in stability could not be sufficient to account for the increase observed. The initial events may however be non transcriptional. The relationship between the frequency and rate of transdifferentiation and crystallin mRNA levels, (Clayton, Thomson and de Pomerai, 1979) and also the effects on these two parameters of certain culture conditions, (Clayton, de Pomerai and Pritchard, 1977; Araki and Okada, 1978) imply that there may be selection within a cell for expression of particular mRNAs from amongst those already present (Clayton, 1979 b,c), and indeed, in both normal and transdifferentiating systems there is evidence that various post transcriptional regulatory mechanisms may occur, (Beebe and Piatigorsky, 1977; Thomson and colleagues, 1978, Clayton, Thomson and de Pomerai, 1979).

There is a class of cellular changes which may turn out to have molecular parallels with transdifferentiation. These include those tumours which produce products, such as hormones, which are normal to the organism but not to the tissue from which the tumour was derived and those tumours which may give rise to cell lines which revert to normal, suggesting that genetic loss is not involved, but rather a reversible shift in gene expression (Coggin and Anderson, 1973; Mintz, 1976; Uriel, 1979). Eguchi and Watanabe (1973), found that N-methyl-N'-nitro-N-nitro soguanidine, a potent carcinogen, caused transformation of both dorsal and ventral iris cells into lens tissue in situ in the intact amphibian eye. Although dorsal iris can give rise to a lens following lentectomy in vivo, ventral iris does not, but once the potential for lens formation had been elicited following MNNG treatment, it was stable for at least a year: no tumours were observed. The changes in the cellular commitment of cell lines which lead to reversible malignancy, or to expression of heterologous cell products, clearly differ in one important respect from transdifferentiation into lens: the lens fibre is a non dividing terminal cell: indeed, any change in differentiation characteristics which, (although not appropriate to the site within the body), does not also give

rise to cells with a higher rate of replication than the original one is likely to pass undetected.

We report here on investigations into changes, in four chick genotypes, of the balance of crystallin synthesis in lens cells grown in the presence of three agents affecting mitosis; insulin, foetal calf serum (FCS) (Reddan and Wilson, 1978) and retina growth factor (RE) (Arruti and Courtois, 1978), and the effect on both lens and neural retina cells of brief exposure to MNNG. The lens cells of three of the genotypes, N, Hy-1 and Hy-2, are known to be distinguished by cell membrane composition (Odeigah, Truman and Clayton, 1979; Clayton, 1979a) and cell cycle interval (Randall, Truman and Clayton, 1979).

MATERIALS AND METHODS

Cell culture Lens epithelium (LE) cells from day old chicks and neural retina (NR) from 8 day embryos were seeded at 3×10^5 and 5×10^6 cells per dish respectively and cultured as in de Pomerai, Pritchard and Clayton (1977). Cells were labelled as in Thomson and colleagues (1978a) and Randall, Truman and Clayton (1979).

Medium modifications 1. Insulin, (B.D.H.) was added at 10µg/ml to medium with 6% F.C.S. from the 4th to the 14th day of culture. 2. Retina extract (R.E.) (Arruti and Courtois, 1978) was added at 20µg/ml to medium with 6% F.C.S. from the 4th day of culture onwards. 3. L.E. cultures were exposed to MNNG at 7.5 µg/ml for 1 hour on the 7th day of culture. N.R. cultures were exposed to MNNG at 10.0 µg/ml for 1 hour on the 19th and again for 1 hour on the 21st day of culture. These doses are subtoxic but growth rate is slowed thereafter. 4. L.E. cells were also grown in medium with 10% and 15% F.C.S.

Cell synchronisation Cells were arrested in G_1 by 24 hours in culture in the absence of F.C.S., induced to re-enter the cell cycle by transfer to medium containing 6% F.C.S., and pulse labelled for 30 minutes at intervals, with 10µCi/ml 14C aminoacids. The time points were those in which a high proportion of cells were in G_0, G_1, S, or G_2/M, as determined from ^{3}H thymidine incorporation as in Randall, Truman and Clayton (1979).

Choline Acetyl Transferase C.A.T. was assayed according to Fonnum (1975) as modified by Crisanti-Coombes and colleagues (1978).

Translation Polysomal and post polysomal mRNA was prepared and translated in a cell free system as in Thomson and colleagues (1978b), mRNA was quantified and its poly A content determined according to Bishop, Rosbash and Evans (1974).

Protein analyses Cell proteins were extracted as in de Pomerai, Pritchard and Clayton (1977). The haemagglutination inhibition assay (HIA) and antisera used were as described in this paper. Electrophoresis in 12% S.D.S. polyacrylamide gel was according to Araki and Okada, (1978) and fluorography as in Thomson and colleagues (1979).

RESULTS AND DISCUSSION

Insulin Insulin affects embryo lens cell morphology and ultrastructure (Piatigorsky, Rothschild and Wollberg, 1973) increases the rate of cell division in older lens epithelium (Reddan and Wilson, 1978) and increases both cell division and the levels of δ crystallin in lentoids transdifferentiated from neural retina cultures (de Pomerai and Clayton, in press). It is teratogenic in the chick, producing limb, beak and tail defects (Landauer and Clark, 1962) but if injected on the 4th day of incubation we find it also produces cataract (Fig. 1a, b). Such lenses, at 17 days of incubation, show 13% increase in α crystallin, 11% increase in β crystallin and a 13% fall in δ crystallin compared with controls. Table 1 and Fig. 2a,b, show the effect of culture with insulin on accumulated protein (HIA and electrophoresis) and Fig. 2c,d,e,f, Fig. 3 and Table 1 show the results of translation in a cell free system with mRNA taken from control and treated cells. Crystallin synthesis and accumulation is markedly more affected

in Db cells than in the rapidly dividing Hy-2 cells. This could be due to differences in receptors or in effectors, but Thomopoulos and colleagues (1976) have found that the number of insulin binding sites on fibroblasts falls when they divide rapidly; it may therefore be useful to compare the number of such sites on these genotypes.

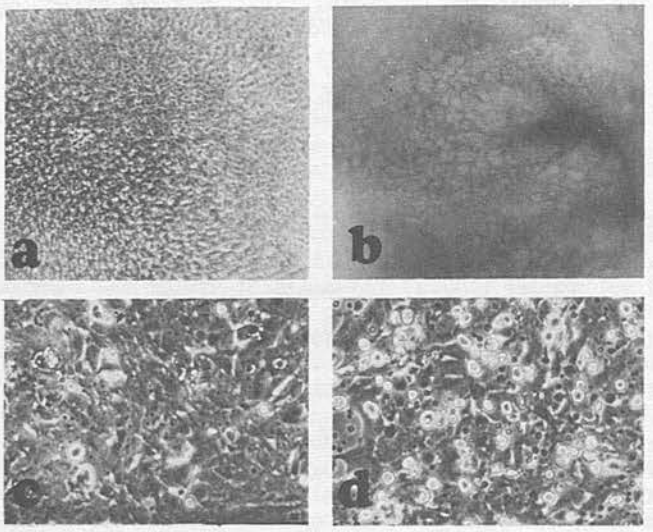


Fig. 1.

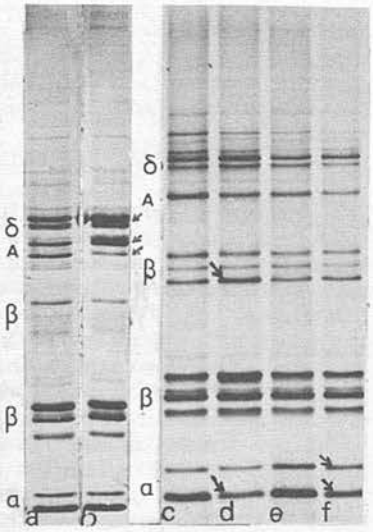


Fig. 2.

Fig. 1. 17 day embryo chick lenses. a) control. b) injected with 2 i.u. insulin at 4 days in vivo. c)d) Day old chick LE cultures. c) control, few mitoses, d) 10μg/ml insulin, numerous mitoses. Fig. 2. a)b) Accumulated protein from Db cells in culture. a) control. b) insulin at 10μg/ml. Crystallins translated in a cell free system by mRNA from cell cultures: c)d) Db cells, e)f) Hy-2 cells. c)e) controls. d)f) insulin.

Accumulated proteins Haemagglutination Inhibition					Electrophoretic Separation		Trans. in a cell free system			
	Db strain		Hy-2 strain		Db strain		Db strain		Hy-2 strain	
Crystallin	Cont.	Ins.	Cont.	Ins.	Cont.	Ins.	Cont.	Ins.	Cont.	Ins.
α1	7.5	5.11	2.13	2.51	13.7	13.5	5.0	5.0	13.0*	7.7
α2							3.9	14.4*	12.8	15.7*
β1	25.0	34.09	18.95	22.32	40.5	36.5	6.6	8.5*	5.3	4.6
					8.6	4.2				
β2							1.7	3.8*	2.7	2.9
β3							6.7*	5.7	5.4	6.1
β4							21.7*	12.8	11.7	11.0
β5					8.5	10.1	14.8	14.4	13.8	14.2
β6							13.7*	10.6	9.4	9.1
δ1	15.0	27.27	11.35	13.39	31.8	38.5	7.5	8.4*	5.5	5.8
δ2							4.9*	3.6	2.0	3.2*
SpA					8.1	2.3	3.5	4.8*	3.3	4.5*

TABLE I Cells grown in presence of insulin at 10 μg/ml from 4th day of culture onwards, and harvested on the 14th day. Percentages of total.

F.C.S. The effects include promotion of cell plating and cell division. F.C.S. also affects the ratio of δ to β in transdifferentiating neural retina (de Pomerai and Clayton, in press). The effects on the relative proportions of β4, 5 and 6, especially in Hy-2 cells, is shown in Fig. 3a,b,d,e, Cells in arrest in the absence of F.C.S. have a different pattern of protein synthesis from cells in G_1, S and G_2 (Fig. 10). R.E. Cell division is stimulated by a retina extract (R.E.)

prepared by Arruti and Courtois (1979). Hy-1 lens cells have an intrinsically higher rate of mitosis than normal (N) cells, and Hy-2 are intermediate (Randall, Truman & Clayton, 1979). Cells from N, Hy-1 and Hy-2 were adjusted to 1.5 x 10^5 cells per dish on the 4th day of culture. On the 8th day, Hy-1 was at 9 x 10^5, Hy-2 at 7 x 10^5 and N at 6 x 10^5. The strains respond differently to RE. N and Hy-2 cells both rise to 10 x 10^5 but Hy-1 cells are unresponsive, and this clearly cannot be due to an upper limit to mitotic rate for day old chick lens cells. The crystallin profile of the cells is affected (Fig. 3c, f.), but shows genetic differences in the changes obtained in the proportions of the three high molecular weight β crystallins: a reduction in β4 in both genotypes, but β3 is reduced in Hy-2 only. δ1 is reduced and δ2 increased, especially in Hy-1. Changes in cell content in response to RE were also found by Barritault and colleagues (1979)

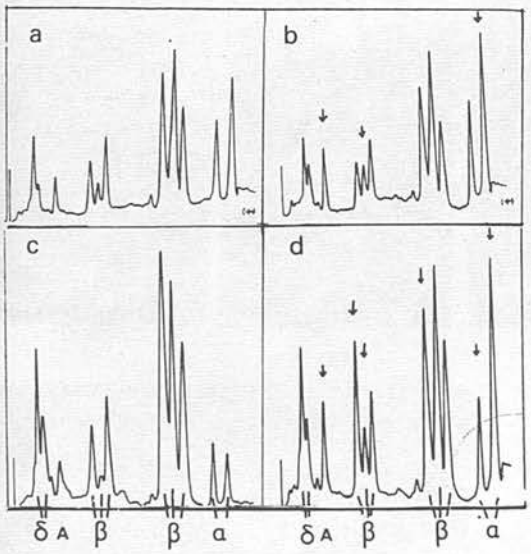
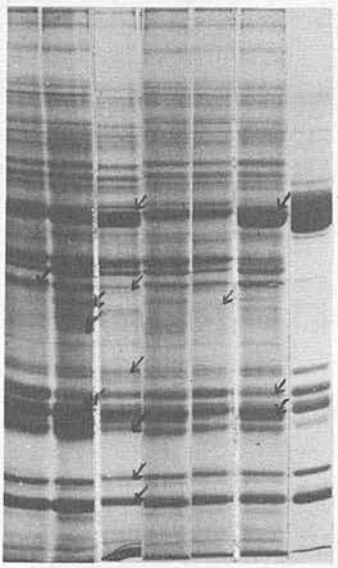


Fig. 3.



Aa b c Ba b c C

Fig. 4.

Fig. 3. Densitometer traces of gels in 2; a) Hy-2 control. b) Hy-2 insulin. c) Db control. d) Db insulin. Arrows indicate the components which have been affected.

Fig. 4. Effect of F.C.S. and R.E. on proteins from LE cells. A) Hy-2 cells. B) Hy-1 cells. C) Crystallin standard. a) 6% F.C.S. Ab) 10% F.C.S. Bb) 15% F.C.S. c) RE: 20μg/ml. Arrows indicate the components most affected by medium.

MNNG: Cellular response: (1) Neural retina. 8 day embryo neural retina differentiates within a week after plating, into neuroepithelial cells and neural cells with connecting axonal out-growths (Okada, 1979). The latter begin to disappear after two weeks and by the 16th - 18th day no axon-bearing cells, remain, but clumped cell aggregates are seen. Some lentoids may be derived from such aggregates (Okada and colleagues, 1979). In 8 day NR, lentoids are seen from 25 days onwards and at the cell densities employed here, pigment cells transdifferentiate from 30 days onwards (Clayton, de Pomerai and Pritchard, 1977) (Fig. 6a). In cultures exposed to MNNG on the 19th and 21st days lentoids appear with about 2-3 days delay compared to controls. There are also three major changes seen:- 1. Cells with fusiform morphology (Fig. 6b). 2. Pre-pigment cells appear 7 days later than normal and only about 20% of these pigment over the next 7 days. Thereafter they gradually change shape, swell, and detatch (Fig. 6d). 3. Cells resembling neurones with axonal out-growths appear, mainly at about the 46th day, 25 days after MNNG treatment (Fig. 6c,e). Choline acetyl transferase (C.A.T.) a

marker for neuronal cells (for example Crisanti-Coombes and colleagues, 1978), is 2-6 times higher in 35 day MNNG treated Hy-1 cultures than in controls. Selective elimination of cells by MNNG is not a possible explanation for the appearance of these new, late appearing neurone-like cells. A hypothesis which we hope to test is that the differentiation of some neuroepithelial cells may become diverted, after MNNG treatment, giving rise to these cells of neuronal appearance.

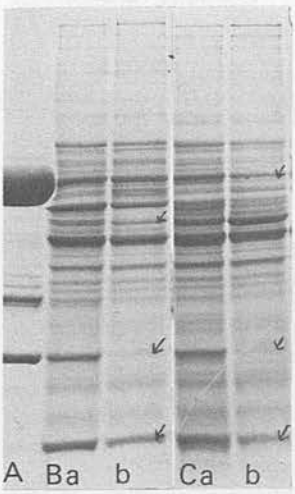


Fig. 5.

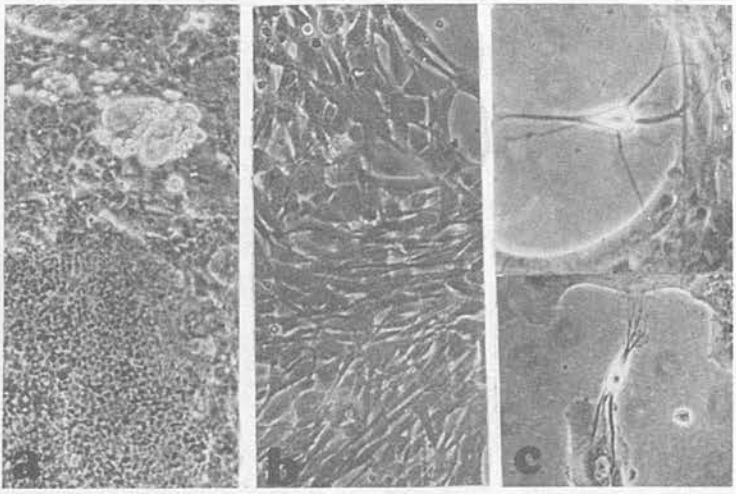


Fig. 6.

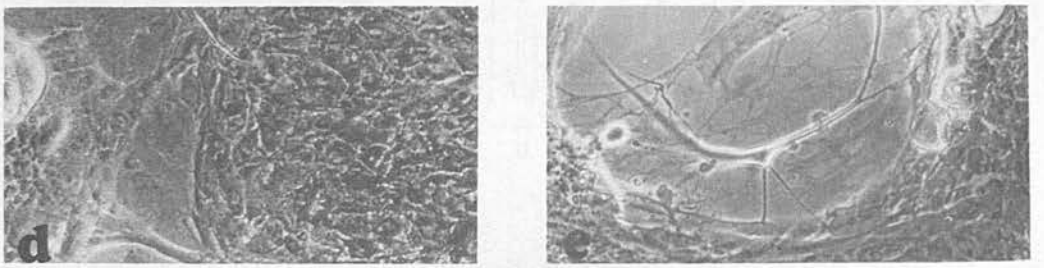


Fig. 6.

Fig. 5. Effect of MNNG on proteins of neural retina cultures 8 days after treatment. A) crystallin standard. B) N cells. C) Hy-1 cells. a) control. b) MNNG.
Fig. 6. Neural retina cells in culture. a) control terminal culture with neuroepithelial cells, lentoids and pigmented cells. b) 30 days after MNNG treatment, fusiform cells. c)e) neurone-like cells after MNNG treatment, 50 day cultures. d) degenerating pigment cells, 26 days, MNNG.

2. Lens Epithelium Lentoids appear in primary cultures, which grow slowly but still appear morphologically normal at 30 and 40 days. Secondary cultures, established from 9 day old MNNG primary cultures developed lentoids, but show abnormalities which are not found in control secondary cultures. These include fusiform cells (Fig. 7b) bipolar cells with some arborisation (Fig. 7d) at the two ends, and a small number of cells also appear after 25-30 days which are neurone-like in appearance (Fig. 7c). No laboratory has so far ever reported such cells from lens epithelium cultures. Cytochalasin D can produce arborisation around the periphery of lens epithelial cells in culture, (Mousa and Trevithick, 1977), but this effect is rapidly reversible, and is neither delayed nor persistent as here. Neural retina mRNA sequences are found in the intermediate abundance class of lens mRNA (Jackson and colleagues, 1978) and it is possible that some of these mRNAs may become expressed in a proportion of lens cells after MNNG treatment.

We hope to examine this problem further by specific identification of the molecular properties of these axon-like cells.

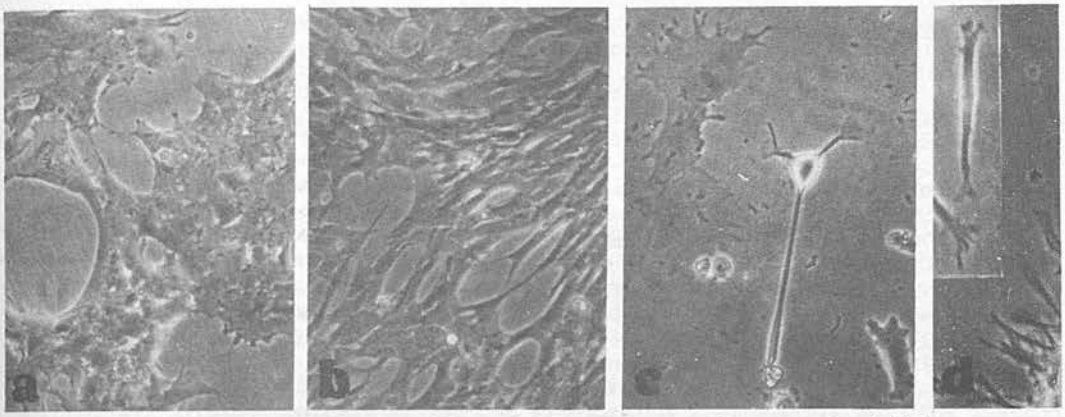


Fig. 7. Lens epithelium cells in secondary culture. a) control. b)c)d) cells in cultures derived from MNNG treated primary culture. b) fusiform cells. c) neurone like cell and epithelial cells. d) bipolar cells from edge of culture, inset, two bipolar cells.

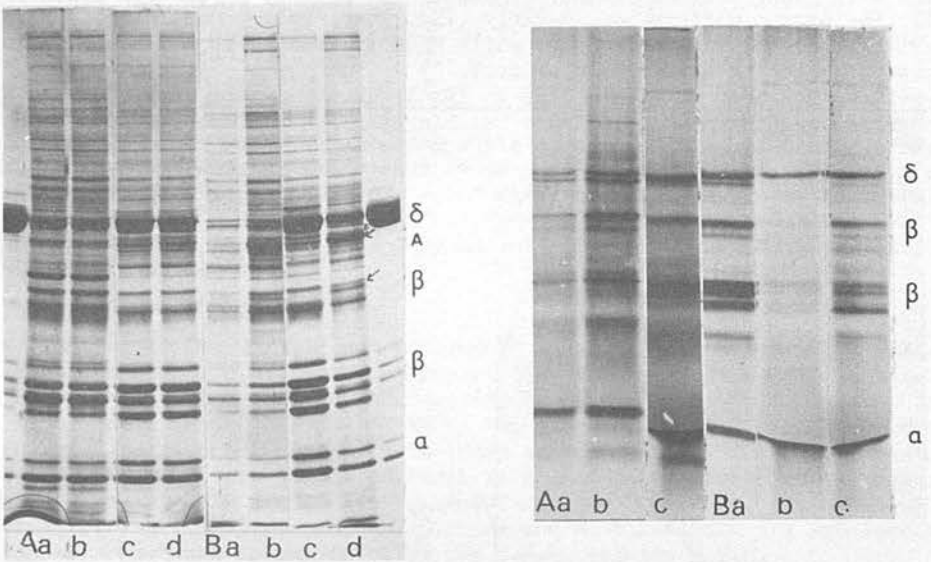


Fig. 8. Proteins from LE control cells and cells after MNNG treatment at 7 days. A) Db. B) Hy-1. Aa) Ba) 9 days, control. Ab) Bb) 9 days, MNNG treated. Ac) Bc) control, 24 and 21 days respectively. Ad) Bd) MNNG treated, 24 and 21 days.
Fig. 9. mRNA translation products from MNNG and control cultures: A) polysomal, RNA. B) post polysomal RNA. a) 3 hours, control. b) 3 hours after MNNG. c) 48 hours after MNNG.. PAG-SDS electrophoresis and densitometer traces.

MNNG: Biosynthesis (1) Neural Retina. The protein profile of NR cell cultures was examined 8 days after MNNG treatment in N and Hy-1 strains. These cultures; at 28 days after plating, are transdifferentiated and contain crystallins.
In both strains, there is a fall in α crystallin and a considerable fall in a β

crystallin in experimental compared to control cultures, (Fig. 5). There is also a slight diminution of δ crystallin in Hy-1, and of a high MW component ('A') in N strain.

Lens Epithelium Little cell death is seen at the dose levels used and is unlikely therefore to contribute significantly to the results. The protein profile of LE cultures shows both an ontogenic change and strain specific differences (Fig. 8). 2 days and 17 days after MNNG treatment, the profiles of Db control and treated cells remain similar, but Hy-1 cells show several changes by 14 days after treatment, mainly in 'A' and high MW β crystallins. The effect on mRNA has so far been examined mainly on Db cells. 1 hour after MNNG, Db cells have less total polysomal RNA but the translation of equal amounts of total mRNA is more efficient, suggesting a high mRNA:rRNA ratio. Much of the poly A-containing RNA has moved to the post polysomal fraction. This suggests an immediate effect on polysome stability. At 3 hours the translation profiles of total RNA are similar in control and treated cells (Fig. 9 Aab). 8 hours after MNNG, the poly A content of total RNA was 3 times higher in Hy-1 and 11 times higher in Hy-2 control cells compared to MNNG treated cells. Equal amounts of total RNA from control cultures translated 2.3 times as efficiently as from treated cultures in Hy-1, and 3.6 times as efficiently in Hy-2. If the efficiency of translation is a measure of mRNA, these data suggest that the average poly A length is shorter in treated cells. The poly A tail on mRNA is progressively shortened as a function of time, so these results may indicate a short-fall in new mRNA. 48 hours later (at 9 days of culture, when lentoids are beginning to appear), MNNG treated Db cells resemble control cells in the total amount of RNA present, the association of mRNA with polysomes, and the efficiency of translation. However the pattern of protein synthesis in a cell free system differs considerably (Fig. 9 Ac, Bc). Control cells synthesise δ crystallin as the main component, while MNNG treated cells still synthesise other crystallins in appreciable amounts.

Differential Response of Crystallins to Insulin, R.E., Levels of F.C.S., and MNNG. Although our results are preliminary, strain specific differences in response have emerged. The basis of the genetic differences in response requires investigation. Pulse labelling of synchronous cells shows that there is some cycle-dependent regulation of specific syntheses (Fig. 10). Since a higher proportion of cells

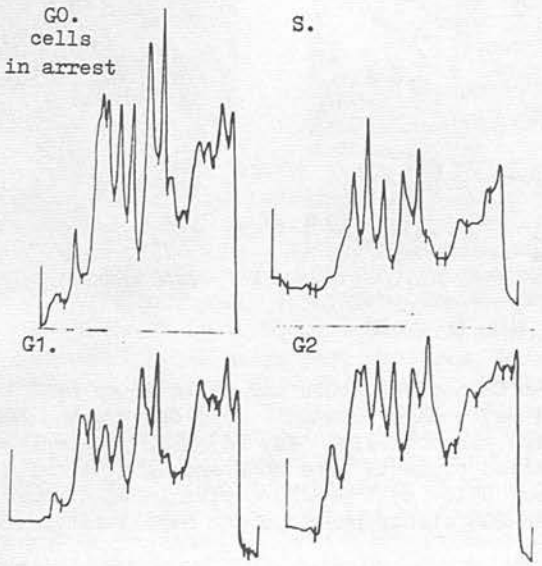


Fig. 10. Densitometer trace from fluorograph of PAG i.e.f. analysis of pulse labelled crystallins from synchronised LE dells. The majority of cycling cells were determined to be in G_1, S, G_2/M, by pulse labelling with ^3H thymidine as described in Randall, Truman and Clayton (1979).

in a population may be of a given cycle stage in rapidly growing cells, any cell cycle dependent susceiptibility to an agent (e.g. to carcinogen, Peterson and colleagues, 1974), may be related to the known strain differences in cycle time. However Hy-1, Hy-2 and N strains also differ in cell membrane ultrastructure and composition (Odeigah, Truman and Clayton, 1979), and in RNA metabolism and mRNA turnover (Truman and colleagues, 1976). R.E., insulin and F.C.S. all stimulate cell division, but there are some differences in the effects on the profile of crystallin synthesis for a given genotype: this suggests that there may be different receptors and effector systems involved , and that there are probably several levels at which synthesis of a particular polypeptide may be regulated. Differential rates of synthesis of specific crystallin subunits in response to various conditions have been reported or reviewed elsewhere (Vermorken and Bloemendal, 1978; Clayton, 1979a; Piatigorsky, 1980). All the conditions reported here also affect protein synthesis differentially; crystallins, therefore, appear to be regulated non-coordinately.

In the case of insulin and MNNG, differential effects in the mRNA have also been found, as judged by translation in a cell free system. The possibility that the effect on cell differentiation is related to the type or degree of disturbance of the profile of available mRNAs requires further study. The range of crystallin compositions which obtain during normal ontogeny and during lens cell differentiation show that some change in representation in a cell is compatible with normality and is actually required during development. The changes brought about by insulin range from shifting of the balance of crystallins synthesised in cell cultures of day-old chick lens (this report) or in cultures of 8 day embryo neural retina (de Pomerai and Clayton, 1980) to pathological changes in lens cells, as in the embryonic cataracts reported here. However the short term effects of MNNG on mRNA are more extreme, and the long term effects in culture include pathological changes and the appearance of unexpected cell types. The process of transdifferentiation from neural retina into lens cells is accompanied by a steady process of radical change in the abundance of crystallin mRNAs (Thomson and colleagues, 1979). We have not yet examined the long term molecular effects of MNNG, but the possibility of major shifts in the mRNA population of some of the exposed cells or their descendants, leading in some cases to a process resembling transdifferentiation, now requires investigation.

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