ASPECTS OF CELLULAR PROPERTIES IN THE LENS OF THE CHICK

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Summary

Circadian rhythms in DNA synthesis have been shown to be present in the epithelium of whole chick lenses. These rhythms are a reflection of the mitosing epithelial cells and are strain specific. Three chick strains were studied: Hy-1, Hy-2 and N. The results suggest that the cell cycle in L.E. cells is under genetic control.

Synchronous cultures of L.E. have been induced *in vitro* by two different methods: mitotic selection and a cell cycle arrest method.

Protein synthesis studies of these synchronised cells showed that no qualitative differences were apparent at different times during the cell cycle. Several instances of quantitative changes were found including an increase in δ-crystallin synthesis during S-phase and an increase in actin synthesis during the transition into S-phase. An increase in newly synthesised actin associated with the membrane was also found at this time.

Few other changes in the L.E. cell membrane polypeptides during the cell cycle could be resolved by SDS-polyacrylamide gel electrophoresis. In apparent contrast, differences were found in the lectin binding capacity of L.E. cells during the cell cycle. Binding of all lectins under study was found to be greatest during mitosis. Strain differences were apparent: strains Hy-1 and Hy-2 showed greater lectin binding than strain N both during mitosis and interphase.

Insulin, foetal calf serum and a retinal extract were found to induce changes in growth rate, cell morphology and crystallin synthesis in L.E. cultures of the 3 strains of chick under study. In all cases strain differences were apparent. The role of external signals to the lens is discussed in the context of lens epithelial cell differentiation.

DECLARATION

I declare that this thesis has been composed by myself, and that the work contained within it is my own.

Fiona Randall.

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ABBREVIATIONS

LE	:	Lens epithelium
FCS	:	foetal calf serum
SDS	:	sodium dodecyl sulphate
Con A	:	concanavalin A
WGA	:	wheat germ agglutinin
PHA	:	phytohaemagglutinin
RCA	:	*Ricinis communis* agglutinin
RE	:	retinal extract
Ig	:	immunoglobulin
PFK	:	phosphofructokinase
PBS	:	phosphate buffered saline
CMF	:	calcium and magnesium free buffer
TCA	:	trichloroacetic acid

CONTENTS

CHAPTER 1. INTRODUCTION

1.1 Naturally Synchronous Cell Systems

In the past there has been an emphasis on the idea of constancy in physiological systems and that constancy is a characteristic of life. This attitude is rapidly changing in the light of the proliferation of data over the past 40 years pertaining to biological rhythms. It is now known that all levels of biological organisation, as well as all levels of animal life, exhibit some form of oscillatory phenomena ranging from the molecular level to the social level, from seconds to years. Oscillation rather than constancy seems to be a fundamental property of all life.

1.1.1 Biological Rhythms

These circannual, circadian and ultradian rhythms, so named because of their frequency, are endogenous self-sustained oscillations. They possess certain characteristics which are somewhat unorthodox in terms of biological properties. The rhythms are maintained despite variation in the temperature at which the organisms are kept. Drugs or chemicals which are known to alter the metabolic rate have little or no influence on the rhythm frequency. The oscillation itself also seems to be independent of protein synthesis as judged by chloramphenicol administration in some species at least. For example, the photosynthetic rhythm of acetabularia was unaffected as was the luminescent rhythm of the protozoan Gonyaulax (Vanden Dreissche, 1975). Such patterns of activity which recur daily in constant conditions can undergo a phase-shift by applying signals, or Zeitgebers as they are known, such as light or temperature at a changed time for a few experimental daily cycles. The resultant new phase relationships of the rhythm may then persist in continuing constant conditions. This, no doubt, would serve to

adjust the organism to periodic shifts in the environment.

In this discussion I am primarily concerned with biological rhythms at the cellular and biochemical level, although there is a considerable amount of literature concerned with rhythms at the behavioural level which I will not discuss here. However, it is worthwhile pointing out that social rhythmic behaviour may be discussed in terms of biochemical and cellular oscillations, relating them to the organism's biological clock. This will be mentioned in more detail in a later section.

1.1.2 The Cell Cycle

Progress through the cell cycle is usually assessed by observing two major cell cycle events which may be readily identified:

(a) DNA replication;

and (b) mitosis.

These events divide the cell cycle into four successive discrete phases: G_1, S, G_2 and M (mitosis) (Figure 1.1). The G_2 phase preceding mitosis, mitosis itself and DNA synthesis (S phase) have been recognised as being relatively invariable in their duration for any cell type when compared to the G_1 phase. This led to the belief that variation in the duration of the cell cycle was attributable to variation in the length of the G_1 phase. However data on the timing of cellular events in liver regeneration were found to be inconsistent with this view (Cater, Holmes and Mee, 1956). A period of cellular dormancy was proposed which involved the transition of cells into a phase outside the normal G_1-S-G_2-M

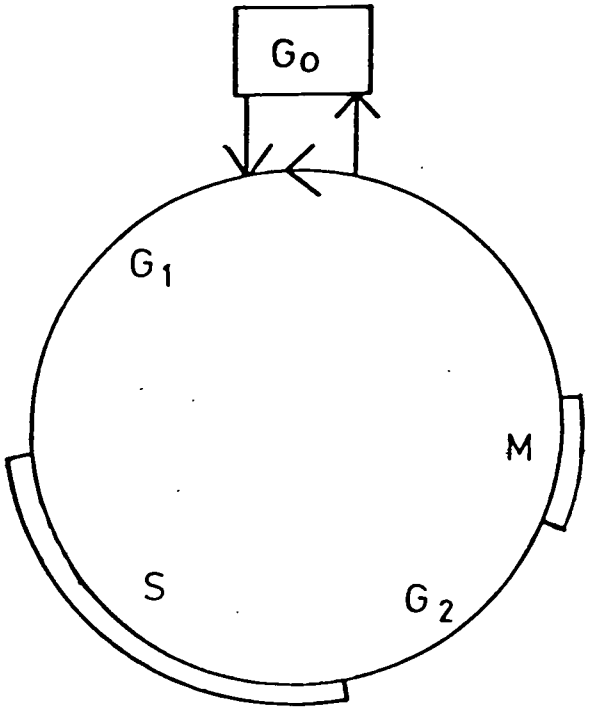


Figure 1.1

Diagrammatic representation of the cell cycle showing the various phases: G_1, S (DNA synthesis), G_2 and M (mitosis). G_0 represents cell cycle arrest.

proliferation cycle (See Figure 1.1). Lajtha (1963) coined the term 'Go phase' to describe it. Cells at this stage may or may not be triggered to re-enter the cell cycle and so undergo proliferation.

The cell cycle is another endogenous self-sustained oscillation. It is however, in principle, independent of biological rhythms and differs from their other characteristics outlined above. For example, the cell cycle is temperature dependent and single signals of light or temperature changes do not entrain the cell cycle although they do entrain circadian rhythms. The cell cycle is also dependent on RNA and protein synthesis. It may, however, be synchronised by biological rhythms, particularly the circadian rhythm, such that the length of the cell cycle or multiples of it is adjusted to the length of the circadian rhythm. The Zeitgeber signals produced by the circadian rhythm and received by the cell cycle in a multicellular organism could be amid a wide range of factors. These include biochemical oscillations of enzymes (Goldbeter and Caplan, 1976), periodic neuronal, hormonal or other blood borne signals (Bullough, 1962; Rensing, 1972) some of which have been discussed in this context by Rensing and Goedeke (1976) and will be mentioned in a later section.

1.1.3 Cellular Circadian Rhythms

This natural synchronisation of cells is evident from the observations of circadian fluctuations of DNA-replicating and dividing cells, each of which occurs in a discrete and easily monitored phase of the cell cycle. Every cell-renewing tissue studied so far have exhibited such circadian fluctuations.

Some of the first work on proliferative circadian rhythms dates back to the 1930's when Carleton (1934), among others, demonstrated the presence of a significant mitotic rhythm in the epidermis of various animals. Since then a wide range of epithelial tissues have been studied.

Von Sallman (1952), in an effort to evaluate the effect of X-ray radiation on mitosis in the rabbit lens, observed that there was a large variation in mitotic counts in the epithelium from untreated lenses. Using litter mates to minimise the amount of genetic variation, he found that the mitotic index at 21.00 hours was consistently greater than at 09.00 hours. He concluded that his findings were an expression of circadian variation but did not pursue it further. Studies of mitotic activity in the lens epithelium of rats, using larger numbers and 4 time points per day also revealed a circadian variation (Von Sallman and Grimes, 1966). Mitotic counts in epithelial flat mounts of animals killed at 24.00 hours and 06.00 hours were significantly higher than in the 12.00 and 18.00 hour groups. The highest average count was 1.6 times the lowest figure. No consistent pattern of circadian variation in ^{3}H-thymidine incorporation in the rat lens epithelium was evident, but it is possible that examinations at shorter intervals may have revealed that rhythmic fluctuations do exist.

Rhythmic uptake of ^{3}H-thymidine over a 24-hour period has been reported elsewhere for other tissues. For example, Pilgrim, Erb and Maurer (1963) employing autoradiography, demonstrated circadian fluctuations in the percentage of ^{3}H-thymidine labelled nuclei of cells in the tongue, oesophagus, fore stomach and abdominal epidermis of the mouse. No fluctuations however were determined in the jejunal crypt epithelium and the convoluted tubules of the kidney. From the data presented they determined that the peak in the ^{3}H-labelled cell index preceded the peak in the mitotic index by approximately 12 hours. Literature on the subject up to that time (reviewed by Bullough, 1962), tended to assume that fluctuations in the mitotic index was directly attributable to parallel fluctuations in the triggering of mitosis. This in turn may result in an accumulation of S-phase nuclei some time later. However, as Pilgrim *et al.* (1963) pointed out, it would be difficult to reconcile the observed sharp peak of thymidine incorporation with the known long and variable G_1 in the cell types which they studied. They concluded that the causes of fluctuations in the mitotic index were, no doubt complex, but suggested that they were partially attributable to synchronisation of S-phase cells.

Potten, Al-Barwari, Hume and Searle (1977) reported evidence for circadian rhythms of mitosis and ^{3}H-thymidine incorporation in the intestinal epithelium of adult mice which up till then was minimal. The conflicting evidence of the presence or absence of such rhythms and also variation of data in the literature concerning such things as peak time of mitosis or ^{3}H-thymidine labelling are due, at least in part, to differences of species, strain, age, handling conditions and the time of sampling. Potten *et al.* (1977) examined the

percentage of ^{3}H-thymidine labelled cells and mitoses by autoradiography of 3 epithelial tissues over 24 hours from the same mice - tongue, epidermis and intestine. Each of the 3 regions showed a clear circadian rhythm sharing a common single peak of tritium labelled cells at 24.00 - 03.00 hours determined using 3 hour interval time points. They suggested from this that the factors determining the circadian rhythm may be systemic affecting all the responsive tissues together, rather than local, although the latter was by no means ruled out.

A similar type of study was carried out on 7 day old rats, scoring the mitotic and ^{3}H-thymidine labelled cells at 3 hour intervals over a 24 hour period but on a different set of tissues (Bystrenina and Podderingina, 1976). Liver hepatocytes, epidermis and pancreatic acini from the same animals all exhibited significant circadian variation in the mitotic and labelling index. However, the dynamics of the changes differed in the different tissues. The peak times also varied from those of Potten et al. (1977), two peaks of tritium labelling occurring in the liver at 10.00 and 22.00 hours. These differences could be attributable to any varying factors between experiments as already discussed above, but it does suggest that either the circadian rhythm of these cells in different tissues are under separate local control or their sensitivity to some systemic factor is different in the various cell types. It is therefore quite likely that the relationship of the cell to the circadian oscillator is not a simple one.

Circadian fluctuations of mitosis have been shown to shift with age. Von Sallman et al. (1966) showed that in rat lens epithelium, a 6 hour shift of peak mitotic activity occurred in rats aged between 6 weeks and 12 months old.

Much work on these circadian proliferative rhythms have been carried out in various animals but a restricted amount, for experimental reasons, on humans. Fisher (1968), however, conducted a study of mitosis in the human epidermis and reported the presence of a significant circadian variation in the mitotic index reaching a peak about 03.00 hours. This conflicts with Scheving's report (1959) that two peaks were apparent, one in the early morning, and another smaller peak in the afternoon. Some of the discrepancy may be attributable to variation in sampling populations because Fisher demonstrated that exercise appeared to have a dampening effect on the frequency of mitosis. Prolonged exercise caused a reduction in mitosis to a value much lower than that normally recorded for that time of day. From this observation Fisher suggested that the circadian mitotic rhythm may be a result of reciprocal variation in the corticosteroids which can act as a mitotic inhibitor.

Circadian variation in mitosis and DNA synthesis have also been recorded in organisms other than mammals. For example, the epidermis of salamander larvae have been shown to exhibit rhythms in the mitotic rate and ^{3}H-thymidine uptake (Scheving and Chiakulas, 1964). Significant mitotic rhythms have also been reported in larval salamander corneal epithelium (Scheving and Chiakulas, 1962) and gastric mucosal epithelium (Chiakulas and Scheving, 1961). Scheving _et al_. (1964) reported two peaks of mitotic activity in epidermis and two of ^{3}H-thymidine incorporation. One peak of tritium uptake was correlated to a peak in mitosis occurring 9 - 10 hours later; another peak of uptake was correlated to a mitotic peak occurring 16 hours later.

From their data the suggestion was made that two sub-populations of cells existed which differed in their inter-mitotic times. This would suggest that cell differences are present in their response to the Zeitgeber.

Some years later Potten et al. (1977) proposed a model concerning cellular circadian rhythms involving two such sub-populations based on data from 3 epithelial cell types. They observed that circadian rhythmicity was most striking in cells at positions which correlated with presumptive stem cell activity. Their model suggested that epithelial tissues contain two types of proliferative cells - stem cells and their derived cells which are committed to further differentiation, and with a more limited division potential. The stem cells which had a longer cycling time, however, were the only cells responsive to the circadian oscillator controlling their cycling behaviour. The derived cells were not susceptible to these factors and exhibited less rhythm the further they were in time from their stem cell origin. Although this model fits in well with the data of Potten and colleagues, it would be of greater significance if it could be related to further data on the subject. The results could prove fruitful in drawing some possible relationships between endogenous circadian rhythms and differentiation.

Some tissues which exhibit a circadian rhythm in mitotic rate, such as the rodent liver (see Hardeland, Hohmann and Rensing, 1973 for a review) continued to express similar waves of mitosis when they regenerate. These rhythms in the regenerating tissue roughly coincide with the normal pattern. Barbason et al. (1974) noted that young rats did not exhibit a mitotic circadian rhythm until after the

20th day after birth. 15 day old rats showed no cyclical variation in mitotic rate in the regenerating tissue after partial hepatectomy. However when 25 day old rats were partially hepatectomised at 10.00 hours, a series of mitotic waves were evident occurring at approximately 24 hour intervals thereafter, during the day. When the same operation was performed at 20.00 hours, the first mitotic peak was delayed to coincide with the beginning of the 2nd day and at approximately 24 hours thereafter.

The rabbit lens, in response to injury by a fine needle inserted through the anterior face of the lens, stimulated thymidine incorporation in a large number of the cells surrounding, but outside of the injury zone itself (Harding and Srinivasan, 1961). A concentric wave of DNA-synthesising cells around the injury was propagated through the epithelium followed approximately 10 hours later by a second similar mitotic wave. A second wave of thymidine incorporation and mitosis was also evident. Von Sallman (1952), as previously noted, recorded a circadian rhythm of mitosis in the epithelium of rabbit lenses. Although no data are available to suggest that the response of the lens epithelium to injury is a reflection of the endogenous mitotic rhythm, it is a genuine possibility in the light of other related data.

1.1.4 Circadian Rhythms and Carcinogenesis

Rapidly proliferating cell populations tend to be particularly susceptible to carcinogens (Rajewsky, 1972) and the binding of a carcinogen to DNA in the nucleus is considered to be closely involved in the carcinogenic process, being a common feature of a large number

of known carcinogens (Brookes, 1977).

It has been shown, as outlined above, that many normal tissues undergo profound circadian variations in DNA replication and mitosis, and that most of these exhibit rapid proliferation. It is therefore not surprising that these tissues have become the focus of various experiments involving the effect of carcinogens (Laerum, 1976).

In an attempt to evaluate whether time-dependent variations of different biological processes were of importance in the process of carcinogenesis, Iversen, Iversen, Hennings and Bjerknes (1970) studied the effect of 20-methylcholanthrene (MCA), a strongly carcinogenic hydrocarbon, on the epidermis of the hairless mouse (hr/hr). One half of the mice under study were given a single application of MCA at 24.00 hours, when the rate of DNA synthesis was at a maximum. The other half were administered with the same dose at 08.00 hours when DNA synthesis was about 40% lower. Papillomas, squamous cell carcinomas and sarcomas were all observed up to a period of 20 months. In the mice treated when DNA synthesis rate was high, tumours appeared earlier and were 35% greater in frequency. The persistence of MCA and its metabolites in the cell were taken into account. However, Scheving et al. (1974) pointed out the value of studying carcinogenic effects over 24 hours with concommitant measurements in mitotic frequency, before final conclusions could be made.

Other studies such as that of Marquardt (1974) who applied various carcinogens to fibroblasts in cell cultures are consistent in yielding the highest frequency of malignant transformation when the carcinogen was administered during S-phase or early G_1.

Izquierdo (1977) carried out some interesting experiments in

which squamous cell neoplasms were induced by repeated administration of 7, 12-dimethyl-benzanthracene in the Syrian hamster cheek pouch. These tumours exhibited both circadian rhythms of DNA synthesis and mitotic activity. The presence of a circadian mitotic rhythm has already been demonstrated in normal hamster cheek pouch (Møller, Larsen and Faber, 1974). Fluctuations in the proportions of cells in DNA synthesis and mitosis in the tumours were approximately in phase with the circadian rhythms from normal precursor epithelium. This suggests that even after the process of carcinogenesis some remnant of the host cell's ability to respond to the circadian oscillator is maintained during neoplastic growth. These findings are consistent with other reports in which 24 hour variations in mitosis were found in mouse hepatomas (Nash and Echave Llano, 1971).

At any one time the proportion of cells in a particular cell cycle phase, e.g. S-phase, can be many fold greater than at other times in those tissues expressing cellular circadian rhythms. Biological rhythms therefore offer a unique *in vivo* opportunity of studying the process of carcinogenesis involving the relationship of a carcinogen with the target cell and the eventual outcome.

1.1.5 Circadian Rhythms and Medicine.

With a constantly changing biological system over the period of 24 hours, one could expect a differential response to identical stimuli at varying times of day, as discussed already with reference to carcinogens. Many therapeutic situations such as drug administration when given at a fixed dose and applied at various times of day may therefore elicit in Man responses of variable intensity. It may be possible by appropriate timing of the medication to reduce any

undesirable effects and so enhance the drug efficiency.

Such an achievement has been made by Haus *et al.* (1972) on leukaemic mice. They demonstrated that it is possible to design more effective treatment schedules by taking account of the circadian variation in DNA synthesis in the bone marrow and the circadian variation in the animals response to the carcinostatic drug arabinosyl cytosine (ara-C). By administering ara-C in sinusoidally increasing and decreasing 24 hour course, instead of courses of 8 equal doses at 3 hour intervals, the survival time of leukaemic mice was extended and the cure rate significantly enhanced. The technique, therefore, permits the same daily dose of the drug to be administered but with markedly reduced toxicity.

Scheving (1976) examined this possibility in Man and suggested that clinicians should become more aware of the presence of circadian rhythms and so exploit them in routine therapy. An area which promises to be useful in optimising treatment regimes is radiation therapy and chemotherapy for cancer treatment, such that their toxic effects may be minimised.

Chronobiological concepts therefore have a great future in clinical research and application.

I have discussed here the natural synchronisation of DNA synthesis and mitosis *in vivo* which are easily recognised phases of the cell cycle. However, certain factors make the analysis of a variety of cellular constituents during the cell cycle difficult.

(i) Few, if any, circadian rhythms in DNA synthesis and mitosis involve the complete population under study. A background level is often evident suggesting that complete synchrony is not present.

(ii) Contamination of other cellular types may be difficult to avoid when isolating the synchronous tissue.

(iii) The types of cells to be studied are limited to those exhibiting *in vivo* mitotic rhythms.

(iv) The timing of experiments may be rendered difficult by the pattern of the natural mitotic rhythm.

However there is the possibility of overcoming these problems by inducing synchrony in most cell types *in vitro*. In the next section I discuss the various methods which can be utilised for inducing synchronous cell populations, followed by a review of the literature concerning oscillations of cellular constituents during the cell cycle. These are then discussed in the context of their possible role in the functional whole organism.

1.2 Induced Synchronous Cell Systems

For any biochemical studies to be carried out on the cell cycle it is necessary to obtain cell populations which are synchronous with respect to the cell cycle phases. In exponentially growing cells in culture there is an asynchronous distribution of cells through the cell cycle in G_1, S, G_2 and M (mitosis) unless circadian rhythms, as already discussed are maintained in culture. Theoretically, cells can be synchronised in any one phase of the cell cycle. However the most common points of synchronisation are G_0, G_1 and M. G_2 arrest is difficult to achieve and S phase arrest can produce unpredictable results due to DNA damage. Synchronisation can be

achieved in a number of ways which can be divided into two basic categories:

(1) Cells can be selectively detached from the substratum or selected from a suspension culture in a particular phase of the cell cycle.

(2) Cells can be blocked at a specific point in the cell cycle by a drug or by some nutrient deficiency, followed by a restimulation into the growth phase.

A combination of both of these methods can also be used. Ideally, cell synchrony should be achieved by a method applicable to exponentially growing cultures without affecting the subsequent growth rate or biochemical balance of the cells. The resulting synchronous population should have a narrow age distribution with a sufficiently large yield of cells to permit biochemical analysis for which a minimum of approximately 10^6 cells is necessary. All of these criteria are difficult to fulfil in any one method, but I discuss below the various methods available, along with their advantages and disadvantages.

1.2.1 Selection Method

Mitotic Selection

The most attractive selective synchronisation method is that reported by Terasima and Tolmach (1961 & 1963). It is based on the observation that some types of cells, when growing in a monolayer culture, are tightly bound to the culture dish during interphase but abruptly loosen their attachment to the substratum and assume a spherical shape after entering mitosis. Cell division occurs while

the cells are in the loosely bound state, and later in early G_1, the daughter cells reattach themselves and spread out into the normal interphase morphology. During the loosely bound stage, the cells can be selectively removed by washing or shaking the monolayer with culture medium. The assets of this procedure are the minimal interference with the growth rate and the very narrow age band of the cell cycle which the selected cells occupy. Terasima *et al.* (1963) achieved a very high degree of synchrony with HeLa cell monolayers, more than 90% of the total yield being mitotic cells. A major disadvantage of this method, however, is that relatively small numbers of synchronous cells result from this procedure since in a random population only about 4-6% of cells are in mitosis and are therefore selectively removed, making biochemical analysis difficult unless vast initial cell numbers are used. By the time these synchronised cells and mitotic cells reach S-phase, the degree of synchrony can be markedly reduced. This is attributable to the variability of the G_1 phase. For example, Nias (1968) showed a spread of clone size over a period of 5 days growth in Chinese hamster cells (CHO) which was equivalent to $\pm$ 2.5 hours during one cell cycle. The reasons for this are not well understood. Different cell lines no doubt differ in their G_1 variability. Terasima *et al.* (1963) reported that 90% of mitotically selected HeLa cells reached S-phase in a short period of time. Irrespective of the initial percentage of cells in mitosis a decay in synchronisation is almost inevitable and may occur more rapidly in some cell lines, even after the first doubling period.

This disadvantage is also true of other synchronising methods and cannot be avoided unless repetitive synchronisation is carried out every few cell cycles. As a result much of the literature utilising induced synchronous cell systems for cell cycle analyses are only partially synchronised, i.e. less than 100% cells are at a precise point of the cell cycle at any one time. Mitotic selection is also limited to cell types which grow as monolayer cultures. Nias and Fox (1971) have reviewed mitotic synchronising methods in mammalian cells.

Inverted Mitotic Selection

The mitotic selection method can also be applied in the inverted sense, such that the experimental material can be the cells remaining as the monolayer rather than those removed. Pfeiffer and Tolmach (1967) treated HeLa monolayer cultures with vinblastine sulphate to accumulate mitotic cells over a defined period which were subsequently removed. The whole procedure was repeated resulting in residual monolayer cells within defined age boundaries. In principle one can accumulate larger populations in this manner but in practice the gain in population size is not impressive. Furthermore it is obtained only by a loss in temporal resolution.

Methods of Increasing the Mitotic Yield

The major disadvantage of the mitotic selection method is the low yield, a factor which various investigators have attempted to overcome. Peterson, Anderson and Tobey (1969) introduced the method of repeated mitotic selection, storing the cells at $4^{o}C$ to slow their passage through mitosis. Experiments have shown that a 4-hour period at $4^{o}C$ permitted mitosis to occur in the normal manner

when cells were returned to warm medium. A yield several times greater than that from a single selection can be obtained. However, as the number of accumulated cells increases the synchrony of the selected population decreases due to the difference in storage time between the first and last selection.

The use of colcemid to arrest cells in metaphase and so accumulate mitotic cells before mitotic selection was first devised by Stubblefield and Klevecz (1965) to increase the cell yield. They reversibly arrested Chinese hamster cells in metaphase by treatment with 0.06µg/ml colcemid for 2 hours. Nias et al. (1971) reported an increase in aberrant mitoses after prolonged exposure to colcemid, but no toxic effects were observed after periods of 3 hours or less. A considerably greater yield of cells is obtained and because colcemid blocks cells at metaphase, an even narrower age distribution of cells can be achieved by this method.

Other mitotic inhibitors used in this way are unsuitable because of their irreversible effects, e.g. vinblastine sulphate, or their greater toxicity, e.g. colchicine.

The elimination of calcium from the medium prior to mitotic selection diminishes cell-substratum attachment. Robbins and Marcus (1964) utilised this observation to obtain larger populations of synchronous cells. However, the effect on cellular binding capacity is non-specific resulting in the possibility of detachment of interphase cells also.

Gradient Separation

Other physical separation methods have not been very successful in obtaining a high degree of synchrony. The resolution of methods based on gradient techniques is limited and cells are selected on the basis of volume or density rather than on the basis of age. However they have the advantage of being applicable to suspension cultures. Everson, Buell and Rogentine (1973) used a Ficoll gradient to separate cells in order to obtain a modest degree of synchrony in the G_1 and S phases of cultured human lymphoblastoid cells. It is, however, an easy and fruitful method for obtaining large numbers. Linear sucrose gradients and foetal calf serum gradients have also been used and are reviewed by Nias et al. (1971). Although pure populations of G_1, S or G_2 can not be achieved, enriched populations of any of these phases can result.

Electronic Separation

Fulwyler (1965) developed an electronic cell separation method which involved volume measurement in a Coulter aperture. Cells were then isolated in droplets of medium which were given a charge according to the sensed volume. The charged droplets then entered an electrostatic field and were deflected in the appropriate direction into a collection vessel. With the recent development of complex microfluorometric apparatus, this could become a quick, easy method of selection, although the necessary apparatus would be very expensive. This method also suffers from the same drawbacks as other volume selection methods already discussed.

1.2.2 Cell Cycle Arrest Methods

Cell cycle blocking or arrest methods, as well as being used in conjunction with mitotic selection as already mentioned, can be used alone for obtaining a synchronised cell population. However, with these methods there is always the risk that cell cycle events may deviate from normal for a time following the block.

G_1/G_0 Synchronisation

Normal cells can follow either a proliferative or quiescent pathway when they reach G_1. The quiescent state has been termed G_0 (Lajtha , 1963), being kinetically and biochemically different from G_1 (see Pardee, Dubrow, Hamlin and Kletzien, 1978 for a recent review). G_0 cells take longer to reach S than do G_1 cells progressing to S from mitosis. Pardee (1974),using cultured mammalian cells provided evidence for a restriction point 'R' where various blocking agents act, e.g. high cell density, limitation of some amino acids or serum and the presence of certain drugs. The data suggests an arrest point positioned several hours before the beginning of DNA replication. This can be utilised in obtaining synchronised cultures by releasing cells from this arrest point simultaneously.

Ley and Toby (1970) described a synchronisation method applicable to suspension cultures and monolayers alike. They grew CHO cells in suspension culture in the absence of isoleucine and glutamine. The cells rapidly reached the stationary phase followed by prompt re-entry into the cell cycle again when the missing amino acids were added. Division in these cells was observed in a synchronous fashion 30 hours later. However this method is not applicable to all cell lines, some of which respond to isoleucine deprivation by

a poor level of cell synchrony (Ashihara and Baserga, 1979). The rate of entry of cells into S phase can vary from 1-2 hours after restimulation.

In a similar way, serum deprivation can be used to achieve synchrony by accumulating cells in G_0. The presence of 0.5%-1% serum during the arrest phase minimises any adverse effects on the cells. Chang and Baserga (1977) achieved good synchrony at the G_1/S boundary of mammalian BHK cells with no evident deleterious effect. They also used hydroxyurea 6 hours after restimulation from the serum arrest to accumulate cells at the beginning of S-phase. This had the effect of narrowing the age distribution of cells even further. However there is always the risk of additional toxic effects with the use of hydroxyurea.

Picolinic acid has been used to reversibly arrest normal rat kidney cells in the G_0 phase of the cell cycle (Fernandez-Pol, Bono and Johnson, 1977). It may induce arrest by selectively withholding iron from the cells but little information is available about other possible deleterious effects. It could, however, be used as a possible alternative to the other methods discussed provided its effects were characterised on the cell type under study.

Another method of G_0 arrest which could be applied to obtain large amounts of synchronised cells, is contact inhibition. Certain cell types, when they reach monolayer density in culture inhibit their own growth by passing to the quiescent G_0 state of the cell cycle. Noonan and Burger (1973) succeeded in stimulating mouse embryo fibroblasts at the monolayer stage back into the cell cycle by a 5 minute pronase treatment. These cells entered S-phase in

a synchronous fashion several hours later. However, by this method a single cell cycle may only be obtained, the cells entering G_0 again during the next G_1.

S-phase Synchronisation

S-phase of the cell cycle may be blocked in order to obtain synchronised cultures, however with little success. For example the double-thymidine block method (Peterson and Anderson, 1964) has been thought to inhibit DNA synthesis and so accumulate cells at the G_1/S boundary. Although it has been reported frequently in the literature, more recent evidence suggests that it may be unsatisfactory as the sole synchronising agent. Studinski and Lambert (1969) studied thymidine effects on HeLa cells synchronised by mitotic selection. They reported that the concentration of thymidine normally used in randomly growing cultures and which inhibits the rate of cell division by more than 90%, still permits a considerable level of DNA synthesis to take place. In HeLa cells, therefore, the double thymidine blockade is ineffective, merely slowing down the passage of cells through S phase and this may apply to many other cell types. It could be used in conjunction with the mitotic selection method. However, as with other blocking methods care must be taken to minimise any toxic effect by careful manipulation of thymidine concentrations, because in this case widespread DNA damage can occur.

Other DNA synthesis inhibitors, such as amethopterin, hydroxyurea and cytosine arabinoside have also been used to synchronise cells in S-phase. Unfortunately, they all suffer from the same disadvantage of interfering with the DNA, possibly in a deleterious way.

G_2 Syncrhonisation

Numerous drugs utilised in cancer chemotherapy arrest cells in G_2, but are generally not reversible, for example neocarzinostatin, which is used in the treatment of human leukaemia (Tobey, 1975). These drugs are therefore unsuitable for achieving G_2 specific synchrony.

Optimisation of Methods

In both of the above sections I have outlined some of the most frequently used methods available for achieving synchronous cell cultures. However, although the basic methods may be applicable to most cell lines such factors as cell cycle and mitotic durations, drug sensitivity and a variety of other cellular parameters may vary. It is therefore necessary to optimise any method for the cell line under study.

Determination of Cell Synchrony

There are 3 methods which can be utilised in determining synchrony after induction by the above methods:

(i) autoradiography after ^{3}H-thymidine labelling;

(ii) liquid scintillation counting after ^{3}H-thymidine labelling;

(iii) flow microfluorimetry.

Autoradiography

The autoradiographic method involves continuous ^{3}H-thymidine labelling after the induction of synchrony followed by intermittent sampling of cells. The resulting autoradiographs must be analysed by microscopy and labelled nuclei scored at the various times. It is a very laborious and time-consuming task: However, a great deal

of information can result from such a procedure. The mitotic index at various times can also be calculated from the same samples.

Scintillation Counting

The necessary relevant information regarding the degree of synchrony of an induced cell culture can be quickly accumulated. The profile of thymidine incorporation can be quickly plotted by continuous or pulse labelling of a synchronous culture with ^{3}H-thymidine followed by liquid scintillation of the samples taken at intervals. Less information is obtained by this method but it has the major advantage of speed.

Flow MicroFluorimetry

Flow microfluorimetry is also a quick method based on the determination of DNA per cell. Fluorescent dyes are conjugated to the DNA of synchronous cells. The emitted fluorescence of single cells can be measured and can distinguish cells with a G_1 DNA content, G_2 or M DNA content or variable amounts corresponding to the passage through S-phase. By computer processing of the data, the percentage of cells in each of the 3 phases can be calculated with a high degree of accuracy, (reviewed by Gray and Coffino, 1979). This method suffers from the major drawback of being very expensive. It also does not yield the same amount of information as that gained by autoradiography.

Presentation of Data

In the literature there has been a tendency for cell synchrony profiles of DNA synthesis or mitosis to be plotted in a semilogarithmic manner (see Brooks, 1977 for example). This masks the scattering of data and gives a false impression of the degree of synchronisation. An arithmetic scale should therefore be used.

1.3 Cell Cycle Dependent Gene Expression

Synchronous cultures have permitted the examination of molecular events occuring throughout the cell cycle. As a direct result of development of these methods there has been a recent proliferation of data concerning cell cycle dependent synthesis of protein. Evidence in the literature suggests that some proteins, enzymes, RNA classes and membrane glycoproteins are synthesised at certain times in the cell cycle. However conflicting evidence is also documented particularly with respect to proteins, resulting in a very controversial field.

1.3.1 Proteins

(i) Histone Synthesis: The S-Phase Only Controversy

It has become fairly well established that the synthesis of at least the bulk of histones is coupled to DNA synthesis in the cell cycle. A variety of cell systems including synchronised cell cultures and also naturally synchronous systems as in the regenerating rat liver (Takai, Borun, Muchmore and Lieberman, 1968) have been used to investigate this relationship.

Prescott (1966) using elegant autoradiographic techniques demonstrated histochemically the concurrence of DNA and histone synthesis in the unicellular organism, Euplotes. DNA inhibitors such as high thymidine levels, cytosine arabinoside or hydroxyurea block histone production as judged by radioactive precursor uptake. The labelling of other acid-soluble, non-histone proteins is not affected. These conditions also provoke the disappearance of histone mRNA from the polyribosomes with a half-life of 15-30 minutes (see Elgin and Weintraub, 1975 for a review). Numerous

authors reporting studies of the synthesis of histone proteins have shown that these proteins are synthesised exclusively during the S-phase of the cell cycle. Robbins and Borun (1967) utilised the mitotic selection method for synchronising HeLa cells for a series of pulse-chase experiments of cells in G_1 and S phase. By examining cytoplasmic polysomes doubly labelled with radioactive tryptophan and lysine they showed that histones were synthesised in the cytoplasm on small polysomes. Initiation of DNA synthesis was accompanied by the activation of these cytoplasmic histone producing polysomes, while the arrest of DNA synthesis by cytosine arabinoside or fluorodeoxyuridine, caused not only a rapid decrease in histone synthesis but disruption of these specific polysomes. No effect was evident on polysomes of the same size when these inhibitors were added during G_1. A similar precise coupling of DNA and histone synthesis was reported by Gallwitz and Mueller (1969). Their experiments provided evidence for the synthesis and utilisation of some histone specific RNA species with the onset of DNA synthesis. Interruption of DNA synthesis led to the functional loss of these RNA species. They interpreted this as the activation of the histone genes under the direct control of the replicating DNA. A model of this nature has since been proposed (Butler and Mueller, 1973) inextricably linking DNA and histone synthesis. The model was based on results of their experiments, also on synchronised HeLa cells. It proposed that DNA replication opens template sites for transcription into histone mRNA's which are subsequently translated by cytoplasmic polysomes. When DNA replication ceases, the open template sites are again covered with incoming histones.

G_1 Histone Synthesis

In contrast to the conclusion made by these workers, Sadgopal and Bonner (1969) reported that all histones were synthesised in varying degrees in both G_1 and S phase. However, they did note differences in their degree of dependence on DNA synthesis. Spalding, Kajiwara and Mueller (1966) also reported histone synthesis through the cell cycle. A 16-hour labelling period with ^{14}C-leucine of G_1 HeLa cells resulted in the incorporation of 40-50% as much leucine into the basic nuclear proteins examined as did logarithmically growing control cultures. The histone proteins which they examined doubled during S phase. However the level of these proteins remained constant in G_1 cells which they suggested could be attributable to histone turnover. Gurley and Hardin (1969) investigated the possibility of histone turnover in CHO cells. In exponentially growing cultures prelabelled histone H1 was gradually lost from the chromatin while the other histones (H2a, H2b and H3) were conserved. When these cells were synchronised by the double thymidine block method all classes of histones showed turnover. They concluded from this that histone synthesis was not completely inhibited when net DNA synthesis was inhibited by high thymidine levels. This conclusion however may be doubtful in light of evidence that the double thymidine block method is really only successful in slowing down DNA synthesis. Studinski *et al.* (1969) reported that a considerable level of DNA synthesis was evident after a double thymidine block in HeLa cells.

Possible Factors Accounting for the Controversy

Several factors which vary between the experimental methods used by different investigators could account for the discrepancies found in the timing of histone synthesis.

(i) Synchronisation Methods.

Numerous synchronising methods have been used, which could be a direct reason for the differences reported. Excess thymidine or amethopterin (inhibits DNA synthesis by blocking purine synthesis) treatment are commonly used in these investigations. Both interfere with the synthesis of DNA and could affect it in unpredictable ways. Chromcsomal damage has been reported to occur with the excess thymidine methods.

(ii) Inadequate Synchronisation.

Perfect synchrony is difficult to achieve by whichever method is chosen. None of the investigators already cited in connection with histone synthesis in the G_1 phase discounted the possibility of a small cell population which was out of phase with the synchronised cells. This could therefore account for histone synthesis outside the S phase.

(iii) Contamination by Non-Histone Chromosomal Proteins.

Non-histone chromosomal proteins are synthesised throughout the cell cycle, perhaps with a few exceptions (Elgin and Weintraub, 1975). Cross-contamination of these proteins with the histone preparations could account for the variability in results.

Tarnowka, Baglioni and Basilico (1978) were aware of these variable factors between different laboratories and conducted a study of histone synthesis which excluded these possibilities. BHK cells were arrested in G_1 by isoleucine starvation, so overcoming

the use of DNA inhibitors. An extra purification step was added to eliminate any non-histone chromosomal protein contamination. They reported that H1 histones were synthesised in significant amounts during G_1. Furthermore, during S phase the molar ratio of histones synthesised was equivalent to that found in chromatin, but in G_1 arrested cells H1 histone was synthesised in a 2-4 fold higher molar ratio relative to the other histones. This latter observation discounted the possibility that a contaminating asynchronous population was responsible for G_1 histone synthesis.

(iv) Strain Differences.

Another possible source for the discrepancy which was not accounted for by Tarnowka et al. (1978) is genuine differences in histone synthesis between cell types and strains. Nadeau, Oliver and Chalkley (1978) investigated this possibility. They studied the degree of coupling of DNA synthesis and histone synthesis in different mammalian cell lines. HTC cells exhibited a limited degree of coupling of DNA and histone synthesis whereas it was stronger in HeLa cells.

This possibility is further supported by the findings of Pochron and Baserga (1979). They studied temperature sensitive mutants of the BHK cell line used by Tarnowka et al. (1978) and followed a similar methodology. However, they reported an insignificant level of H1 histone synthesis during G_1. Differences in histone synthesis appear to occur even although the cells have the same parentage but have undergone mutations.

Agreement has been reached at least on the timing of the bulk of histone synthesis. H1 histone, unlike the other histone

classes have been reported to be synthesised in excess amounts during G_1. It may be that those investigators reporting histone synthesis solely in S phase concentrated their efforts on S phase and did not examine G_1 sufficiently closely, or that genuine differences do occur in different cell lines. If this transpires to be the case, H1 histone may be different from the other histones with regard to the factors controlling its expression. The variability in the incidence of G_1 histone synthesis may lie in the nature of the coupling with DNA synthesis itself.

Transcriptional or Translational Control?

The molecular mechanisms involved in this coupling have been extensively explored (see Kedes, 1979 for a review). The general consensus of opinion in the literature suggests that the S phase regulation of histone mRNA activity is centred on a transcriptional control mechanism and the abrupt change in histone synthesis after S phase is attributable to changes in histone mRNA turnover or inactivation. Recent experiments by Melli, Spinelli and Arnold (1977) have suggested that this model may be wrong and that post-transcriptional processing of histone mRNA may be the key feature in histone-DNA coupling. By cross-hybridisation of HeLa cell poly(A) RNA with the heterologous DNA of a recombinant phage containing sea urchin histone genes, newly synthesised histone gene transcripts could be detected at different times in HeLa cells. These were synchronised by the double-thymidine block method. They showed by cross-hybridisation studies that histone mRNA of HeLa cells was synthesised throughout the entire cell cycle. By comparing the proportion of histone mRNA sequences in total and

cytoplasmic RNA of cells at different stages in the cell cycle they determined if the absence of coupling was attributable to cell asynchrony. If this was the case mRNA should enter the cytoplasm for translation at all phases of the cell cycle. Cytoplasmic histone mRNA in cells where DNA duplication was inhibited was 20-30 times lower than in controls. The presence of histone RNA in the nucleus throughout the cell cycle was therefore not attributable to incomplete synchronisation. Experiments from the same laboratory (Melli, Spinelli, Wyssling and Arnold, 1977) provided support for the presence of a high molecular weight nuclear histone RNA precursor to cytoplasmic histone mRNA. These data together suggest the continuous transcription of a nuclear histone precursor molecule followed by processing during S phase to provide translatable histone mRNA in the cytoplasm. This does not necessarily mean that these high molecular weight RNA's are polycistronic as no evidence is available firmly to suggest tandem repeating of histone genes in mammalian cells, although such evidence is available for sea urchins (Kedes, 1979).

This finding that histone mRNA synthesis is not coupled to DNA synthesis is in disagreement with Stein et al. (1975). They utilised a radiolabelled complementary DNA probe synthesised from histone cytoplasmic mRNA of HeLa cells to detect histone mRNA. Using mitotic selection to synchronise cells they confirmed that histone mRNA capable of hybridizing with the cDNA probe was detectable only from S phase polyribosomes. However they did not detect histone RNA sequences in nuclei of G_1 cells or cells incubated in the presence or absence of DNA replication inhibitors. Similarly in vitro transcription of isolated HeLa cell nuclei resulted in

cDNA hybridizable histone mRNA only from nuclei isolated from S phase cells (Stein et al., 1977a). This evidence, in contrast to Melli et al. (1977) suggests that histone gene expression is regulated, at least in part, at the transcriptional level. However as Kedes (1979) pointed out, Stein's approach would not have detected short-lived nuclear histone sequences. Resolution of this controversy must therefore await the development of a pure homologous probe for mammalian histone RNA which neither of these laboratories have yet achieved.

(ii) Immunoglobulin synthesis

Considerable effort has been devoted in recent years to elucidate whether immunoglobulin synthesis and secretion reflect a cell cycle dependent gene expression. Early histochemical studies resulted in the conclusion that IgG was expressed during limited periods of the cell cycle (Buell and Fahey, 1969). Immunoglobulins were detected in synchronised human lymphoid cells by fluorescein conjugated antisera. These cells were synchronised in mitosis by a thymidine block followed by colcemid treatment. The greatest degree of immunofluorescence detected was in late G_1 and S, reaching a maximum of 91% cells labelled. This fell sharply to 20% in G_2 and mitosis. This method did not discriminate between synthesis and storage of IgG. However the results were corroborated by ^{3}H-leucine labelling studies followed by specific immunoglobulin (Ig) co-precipitation. More recently, Garatun-Tjeldstø, Pryme, Weltman and Dowben (1976) came to a similar conclusion utilising a different cell line which was synchronised by a different method. Plasmacytoma mouse cells were synchronised by isoleucine deprivation.

They determined the synthesis and secretion of light chain Ig in particular, at various times by radiolabelling of proteins followed by immuno-precipitation of the Ig's and gel electrophoresis. Maximal synthesis and secretion of light chain Ig was observed in late G_1 and early S phase, of the 1st cycle after synchronisation. Byars and Kidson (1970) also made this observation in mouse myeloma cells using the double thymidine block method for synchronisation. However this was not confirmed by another group of investigators using the same cell line and identical synchronisation procedure (Liberti and Baglioni, 1973). Strain differences and synchronisation method in this case do not appear to account for the major discrepancy between the results. Some other technical variation in methodology could therefore account for it. Other reports in the literature do provide evidence for continuous Ig synthesis. Mouse myeloma cells synchronised by the double thymidine block method exhibited no pattern of Ig synthesis throughout the cell cycle.(Cowan and Milstein, 1972) using similar methods of analysis. They did report minor fluctuations occurring when concentrations of 4mM thymidine was used to synchronise the cells. However this is a greater concentration than that used by Byars et al. (1970) and Buell et al. (1969) who reported variation in Ig synthesis throughout the cell cycle. Various other synchronisation methods other than the double-thymidine block in lymphoid cells have been used such as thymidine-colcemid combination and isoleucine deprivation. Clearly, all these variables outlined create a complex situation and any combination of them could contribute towards the conflicting results obtained, in the same way as in the histone synthesis controversy. Damiani, Cosulich and Bargelles (1979) tried to overcome some of these problems in an

attempt to come closer to solving this controversy. They used the mitotic selection method to synchronise mouse myeloma cells, a method not previously used. Lymphoid cells are normally grown in suspension culture. However through mutagenesis and cloning they established a monolayer cell line. The synthesis and rate of secretion of IgG in particular was studied at 10 different points in the cell cycle. This was achieved by ^{14}Camino acid labelling of the cells followed by immunoprecipitation and quantitation of synthesised and secreted IgG. The data obtained were consistent with the hypothesis that synthesis and secretion of IgG are not cell cycle dependent in myeloma cells. They did not eliminate the possibility that differences in cell type and origin of the cell line may play some role in the differences. A study by any single group of investigators of various cell lines could resolve this point. There is also the possibility that Ig synthesis is different in myelomas from normal cells. Nevertheless it does provide a useful model system for analysis of possible time-dependent gene expression particularly because they produce a homogenous protein class which is readily identified and occurs in large amounts. Another possibility is that some immunoglobulins such as IgG may exhibit time dependent synthesis while other immunoglobulin gene products do not.

(iii) Cytoplasmic Protein Synthesis

Much of the recent data concerning cell cycle dependent protein synthesis has emerged as a result of ardent interest in biochemical events associated with the cell cycle. Protein markers specific for different phases would facilitate this analysis, for example the biochemical nature of the transition from quiescence (G_0) into the

cell proliferation cycle. Such problems have not yet been elucidated but interesting results have been reported regarding synthesis of the large numbers of cytoplasmic proteins present in the cell. Most of these studies have been concerned with mammalian cell lines although there are numerous reports on cell-cycle stage specific appearance of enzymes in micro-organisms. This will be discussed in the next section.

Most of the recent reports are in agreement that in mammalian cycling cells at least, there is no evidence of synthesis of detectable polypeptides which are confined to only one phase of the cell cycle (other than those already discussed). When differences appeared they were clearly variation in the rate of synthesis rather than in the appearance of new polypeptides. Bravo and Celis (1980) analysed 700 radioactively labelled cytoplasmic proteins at different cell cycle phases by high resolution 2-dimensional SDS electrophoresis. They used the mitotic selection method to obtain synchronous HeLa cell populations. Out of the polypeptides quantitated only a few were shown to vary consistently in S, M and in M and G_2, but no marker was found in G_1. They managed to identify α and β tubulin which they found to increase in M. Similar results with regard to the invariability of most cytoplasmic proteins, including actin, have been reported by Milcarek and Zahn (1978) in HeLa cells. Milcarek *et al.* reported 6 proteins which varied significantly particularly during mitosis. None were reported to vary in G_1.

There is however, one instance in the literature which contradicts these findings (Al-Bader, Orengo and Rao, 1978). Al-Bader *et al.* repcrted that at least 9 new polypeptides detected by gel-electrophoresis appeared after the transition of synchronised HeLa cells from the S phase across the G_2 phase. Irreversibly blocked

nitrosourea treated cells were deficient in these 9 polypeptides which they suggested may be necessary for G_2-mitotic transition. Synchrony may not have been sufficient in the G_2 phase of cells studied by other workers to detect any presence or absence of polypeptides. If cell synchronisation is achieved by the mitotic selection method, as in the case of Bravo et al. (1980), the level of synchrony is severely reduced by the time cells reach G_2.

Non-cycling cells have been shown to exhibit certain proteins which are not found in cycling cells, particularly associated with the G_0-S phase transition. Riddle, Dubrow and Pardee (1979) studied this part of the cell cycle in serum deficient, G_0 arrested Swiss mouse 3T3 cells. By isotope labelling followed by SDS gel electrophoresis methods they detected 2 major polypeptides synthesised preferentially by serum arrested cells. 4 other major polypeptides were synthesised preferentially by serum stimulated cells. One of these, a 42,000 dalton polypeptide, was identified as actin. It reached a maximum 4-6 hours after stimulation, returning to G_0 levels as S phase commenced. It showed both the earliest and greatest detectable change compared with the others. Riddle et al. (1979) have suggested that it may be a critical feature of transition from the quiescent to the proliferation status. Evidence that actin remains constant in cycling cells is provided by Milcarek et al. (1978). Two other groups of investigators, studying different mammalian cell lines, have also reported a very significant increase in a polypeptide at the same point in the transition from G_0-S phase (Ley, 1975; and Gates and Friedkin, 1978). Neither group have identified it as yet. However differences in the estimated molecular weight prevent any conclusions regarding similarities between the

findings. Riddle et al. have commented that the 50,000 dalton polypeptides found to increase at G_0-S by Gates and Friedkin, (1978). is, in their opinion, also actin. It may therefore prove to be a useful marker specific for G_0-S transition.

Any observations of protein synthesis may be dependent to some extent on the methods of synchronisation, which has been made clear in previous discussions in this chapter. Most of the above studies have tried to minimise the amount of biochemical upset by avoiding the use of drugs. Nevertheless, the results do suggest that many of the cellular 'housekeeping' functions continue irrespective of DNA synthesis and mitosis. This finding is quite remarkable when one considers the major changes occurring in the nucleus of cells during these phases.

Protein Synthesis in Mitosis.

A decrease in the rate of protein synthesis during mitosis is a well documented phenomenon in a large variety of cells from different organisms (Mitchison, 1971 for a review). The suspected step in controlling protein synthesis during mitosis is polypeptide chain initiation, since it appears that translational control in eukaryotic cells involves primarily regulation of chain initiation and not chain elongation or termination. Evidence from the reticulocyte system where α and β-globins are synthesised at different rates, it has been shown that initiation is the regulating factor (Lodish, 1976). A variety of components involved in the formation of the initiation complex could be responsible, including the availability of mRNA controlled by transcription or processing. Tarnowka and Baglioni (1979) have recently shown that in mitotic

HeLa cells met $tRNA_f$ binding to native 40S ribosomal subunits is not impaired *in vivo* during mitosis. This suggests that the subsequent step in initiation, mRNA binding to ribosomes, may be inhibited in mitotic cells. Therefore the decrease in protein synthesised during mitosis may not be due to selective inhibition of translation of particular mRNA species but to protein synthesis in general. This has already been confirmed in the previous section.

1.3.2 Enzymes

Over 100 cases of periodic enzyme regulation have been documented in organisms ranging from bacteria to mammals. Interest has grown in this field recently due to their putative link with biological clocks. Their possible role in mitosis and cell differentiation has been suggested and will be discussed later. Periodicity in enzyme reactions can be attributable to 2 causes:

(i) epigenetic control mechanisms at the transcriptional or translational level;

or (ii) metabolic regulation at the level of the enzyme itself. (Hess and Boiteux, 1971).

(i) Epigenetic Regulation.

A stepwise increase in some enzymes occurs once per cell cycle in some yeasts (Halvorson, Carter and Tauro, 1971) and in bacteria (Donachie and Masters, 1969). This catalogue of evidence of periodic enzyme synthesis led both of these groups to propose theories accounting for the phenomenon based on transcriptional or translational regulation. Halvorsen proposed the 'linear reading' or sequential transcription theory which postulated that genes were transcribed once during the cell cycle in the same order as their linear sequence on the

chromosome. Donachie's oscillatory repression theory, on the other hand, was primarily concerned with biosynthetic enzymes controlled by end-product repression. Data pertaining to periodic changes in enzymatic activity is available in other than these two categories of organism. However, speculations on the periodic nature of synthesis of individual enzyme proteins in other organisms is being viewed with some scepticism. Various researchers have reported assays of enzyme activity not synthesis, both of which are not necessarily synonymous. It is difficult to find clear examples of transcriptional regulation of oscillatory enzymes in the literature. This must await the detection of RNA transcripts from single genes using copy or cloned DNA hybridisation probes. This has been used to investigate a similar problem with regard to histones (Section 1.3.1). Unfortunately, Melli et al. (1977) and Stein et al. (1977a) conflict in their findings.

Mano (1970) has described cyclic protein synthesis in sea urchin embryos, correlated with cell division. 30-60 minute oscillations were found to occur only in fertilised eggs and in cell-free systems. The periodicity occurs in enucleated egg fragments and also in the presence of actinomycin so it can be attributed to translational control.

Elliot and McLaughlin (1978) investigated the possibility that oscillatory enzyme activities are attributable to metabolic oscillations rather than to periodic synthesis. They studied protein synthesis in Saccharomyces cerevisiae, an organism which has been well documented with regard to oscillatory enzyme activity (Halvorson et al., 1971). Elliot and McLaughlin's results suggested that periodic variations in the rate of synthesis of individual

proteins must occur in a minute fraction of proteins, if at all. They could therefore provide no support for the linear reading or oscillatory repression theories.

Metabolic Regulation.

Glycolysis

Glycolytic oscillations, which have been extensively studied, represent the most well understood example of metabolic oscillations. The periodicity is attributable to the oscillatory activity of a regulatory enzyme, phosphofructokinase (PFK). This phenomenon has been observed in yeast cells and cell-free extracts (reviewed by Hess et al., 1971), beef heart extracts (Frenkel, 1965), rat skeletal muscle extracts (Tornheim and Lowenstein, 1975) and in ascites tumour cells (Ibsen and Schiller, 1971). These oscillations are most conveniently monitored by recording the fluorescence of NADH. The requirement for generation of oscillation is a constant and relatively low input rate of glycolytic substrates. The frequency varies from 2-8 minutes in duration in yeast, depending on the input of substrates into intact cells. This frequency is less in yeast cell-free extracts where dilution of the glycolytic system occurs. In muscle extracts the periodicity has been observed to be about 20 minutes. This topic has been extensively reviewed (e.g. Hess et al. 1971; Goldbeter et al., 1976). The role of PFK in the oscillatory mechanism has been suggested by observations that fructose-6-phosphate, the substrate of PFK, is the last glycolytic substrate able to generate oscillatory behaviour. By-passing of the PFK step by the addition of fructose-1-6-diphosphate does not result in periodicity in glycolysis (Hess et al., 1971). The allosteric nature of PFK

and the autocatalytic control of the enzyme by adenine nucleotides are generally regarded as being responsible for the oscillations. Comparisons between yeast and beef heart extracts suggests a very close similarity in mechanism (Goldbeter et al., 1976).

Mitochondrial Systems.

Oscillations of light scattering due to swelling, and ion fluxes in suspensions of mitochondria have been recently reported and are being extensively studied. The first observations of this kind were induced by adding ionophoretic antibiotics such as valinomycin to pigeon heart mitochondria preparations (Chance and Yoshioka, 1966). They reported oscillations of K^+ and H^+ of approximately 50 seconds in duration. When K^+ entered the mitochondria during oscillation, H^+ is extruded and vice versa. The frequency changes in both ion concentrations were the same. Similar oscillatory phenomena have been reported without the use of such antibiotics. The addition of EDTA to rat liver mitochondria, which makes them permeable to Na^+ and Li^+, results in oscillations in swelling of mitochondria (Packer, Utsumi and Mustafa, 1966). This swelling depends on an energy source, recontraction occurring when the source is blocked. The period of oscillation was also shown to be dependent on pH and temperature, being in the order of 2-3 minutes. It is now known that all of these similar type oscillations require the presence of a monovalent cation, a permeable anion and are dependent on ATP. They are always associated with enhanced transport activity. The ratio of H^+ and K^+ movement has been shown to be about 4. Therefore anions and water may be expected to exchange across the membrane in order to balance electroneutrality and osmolarity (Hess et al., 1971). Concomitant with the oscillatory ion movements and volume changes of

isolated mitochondria, periodic variations in NADH fluorescence are also significant (Goldbeter et al., 1976). Particular interest in this has been shown because spectrophotometric analysis of intact slime mould and smooth rabbit muscle have shown similar fluctuations in the redox state of NAD and cytochrome C. Since both of these are mostly found in the mitochondria speculation that these may also oscillate in the mitochondria have been made (Berridge and Rapp, 1979). Gooch and Packer (1971) suggested that the oscillating phenomena observed in mitochondria could be due to synchronisation by an oscillatory flow of ADP and ATP, which they demonstrated by monitoring their effect on mitochondrion H^+ and K^+ transmittance, morphology and respiration rate. Membrane bound ATPase may therefore exhibit oscillatory activity and play a contributory role in the regulation of other mitochondrial oscillations. However it is conceivable that oscillations in mitochondrial metabolism may be generated by periodic input of pyruvate from the oscillating glycolytic pathway already discussed.

Adenylate Cyclase.

cAMP is the chemotactic agent of aggregating cells of Dictyostelium discoideum (slime mould). It was observed some years ago by time-lapse photography that this cell aggregation tended to occur in a periodic fashion (Arndt, 1937). It is now known that aggregating cells release cAMP periodically which is associated with changes in adenylate cyclase activity. These oscillations, about 5 minutes in duration, have been extensively studied by monitoring the cells' responsiveness to cAMP by utilising light-scattering changes and transient increases in the extracellular proton concentration as an indicator (Gerisch, Malchow,Roos and

Wick, 1979). cAMP binds to the cell surface receptors. This binding then triggers a train of responses within the cell including the activation of adenylate and guanylate cyclase. However this only occurs if the extracellular cAMP concentration changes with time.

These oscillations are under developmental control as they only appear at a specific time in the life cycle of D. discoideum. Evidence suggests that these oscillations are necessary for differentiation to occur in D. discoideum. For example, in one strain of D. discoideum inhibition of aggregation was achieved by a continuous influx of cAMP, whereas small pulses of cAMP stimulated differentiation (Gerisch, Fromm, Heugsen and Wick, 1975c). The absence of cell differentiation in a phosphodiesterase mutant which exhibited no oscillations underlines their importance. Gerish et al. (1975c) also demonstrated the enhancement in expression of a specific glycoprotein on the cell surface after exposure to cAMP pulses. It could be involved in cell adhesion at this aggregation stage.

A model has been proposed for these intracellular periodicities which centres upon two membrane-bound enzymes which are involved in the synthesis of cAMP-adenyl cyclase and ATP pyrophosphohydrolase. Goldbeter (1975) showed that the allosteric properties of adenyl cyclase could give rise to sustained oscillations in the synthesis of cAMP by positive feedback.

If Goldbeter's model predictions are correct concerning the autoregulation of oscillations of adenyl cyclase activity, speculations on the role of cAMP in differentiation processes in other organisms could be far reaching.

cAMP and the Cell Cycle.

In fact various other investigators have recorded the periodic variation of cAMP levels in other cell types, but over a longer time course. Abell and Monohan (1973) conducted a study of cAMP throughout the cell cycle of 13 different mouse fibroblast cell lines. They found in all cases that cAMP levels were inversely proportional to the rate of DNA synthesis. When 3T3 cells were transformed with SV40 an increase in growth was evident. This was correlated directly with a corresponding decrease in cAMP levels.

Marks and Grimm (1972) provide other evidence of a connection between cAMP levels and the cell cycle. They determined intracellular cAMP levels of mouse epidermis. A significant circadian rhythm in cAMP levels was evident reaching a maximum when mitotic activity was low. The evidence available concerning cAMP in mammalian cells implies some role in the regulation of growth which in turn could affect differentiation. No data is available concerning adenylate cyclase activity in these examples cited.

1.3.3 Other Metabolic Oscillatory Systems.

Various other types of oscillatory processes have been observed recently in biochemical systems. The oscillatory phenomena have yet to be identified but probably originate from metabolic control.

Enzyme activity oscillations have also been reported in mammalian cells. Klevecz and Ruddle (1968) assayed the activity of lactate dehydrogenase and glucose-6-phosphate dehydrogenase in synchronised CHO cells. They found both enzymes to reach peaks every 3-4 hours. Although no control mechanisms have been proposed to account for these oscillations it is likely that they are metabolic

in origin and may even be linked to the glycolytic oscillations.

The plasmodia of Physarum show different oscillatory phenomena in their contraction behaviour and their protoplasmic flow. The oscillatory period was found to be only about 1.3 minutes (Wohlfarth-Botterman, 1979) and stems from the periodic contractions of the cytoplasmic actomyosin within the ectoplasm.

Sustained oscillations of acetylcholine (ACh) and ATP have been found to be triggered by nerve stimulation in the electric organ of Torpedo (Israel et al., 1979). Both metabolites oscillate in phase with each other, the periodicity depending on the level of stimulation administered. Two types of oscillations were observed. The 'slow wave' changes of ACh and ATP were of approximately 120 seconds duration with a 5Hz stimulation. Superimposed on this were rapid oscillations of ACh and ATP of 5 second periods. Israel and colleagues (1979) suggested that these oscillations may result from regulation of the enzymes involved in the synthesis of the cholinergic transmitter.

1.3.4 Cell Membranes.

Properties of the cell surface which are available to the extracellular environment are amenable to analysis by lectin and antibody binding. Lectin analysis in particular has recently become widely used for this purpose (reviewed by Brown and Hunt, 1976). Lectins are proteins which can bind noncovalently to specific carbohydrate groups, in this case on the cell surface. They have more than one specific carbohydrate-binding site which enables them to cross-link the sites available. This results in cell agglutination. Numerous lectins have been isolated both from plant and animal

sources each with different specificities for certain sugars. A wide range of cell surface glycoprotein sites can therefore be studied.

Utilising these lectins as probes for the cell surface of cells in vitro it was observed that transformed cells exhibited a cell surface architecture to which lectins readily bind. Untransformed cells appeared more resistant to lectin binding (Noonan and Burger, 1973). Such observations led to a closer analysis because of the link with tumorigenicity. Shoham and Sachs (1974) found that normal hamster fibroblasts became agglutinable with low concentrations of Concanavalin A (Con A) and wheat germ agglutinin (WGA) specifically during mitosis, but that the cells reverted to the non-agglutinable state during interphase. This pattern of lectin binding was found to be present in a variety of normal cell lines such as BHK cells (Glick and Buck, 1973) and mouse 3T3 cells (Collard and Temmink, 1975). This mitotic membrane configuration was shown to be associated with increased binding of fluorescein-tagged WGA lectin (Fox, Shepherd and Burger, 1971) and of ^{3}H-Con A (Noonan, Levine and Burger, 1973). This suggests that the number of specific glycoprotein sites on the membrane exposed to the extracellular environment change during the cell cycle. Alterations in the configuration of these sites could also contribute to the increased lectin agglutinability. Edidin (1974) has demonstrated differences in the membrane antigen mobility.

Transformed cells in vitro permanently display the surface architecture observed in normal cells only during mitosis, cell agglutination by lectins and lectin binding being significantly increased. These cells also vary in their sensitivity to lectin agglutination during mitosis, but some become more and others less

agglutinable. For example, hamster fibroblasts transformed by dimethylnitrosamine are agglutinated by Con A or WGA during interphase but not during M phase (Shoham et al., 1974), while SV-40 transformed 3T3 cells (Collard et al., 1975) and Epstein-Barr virus transformed human lymphocytes (Smets, 1973) are most agglutinable during mitosis.

Other cell surface changes have also been reported during the cell cycle. Cikes and Friberg (1971) demonstrated that a cell-surface localized H2 antigen is expressed more in S phase than at any other time in the cell cycle of mouse bone-marrow derived cells in vitro.

Utilising lymphoma suspension cells, Bosmann (1974) has shown that cell surface glycosyl transferases showed peak activity during the S phase of the cell cycle. Sialyl-, galactosyl- and N-acetylglucosaminyltransferase activity all behaved similarly, and with virtually no activity during mitosis.

Oscillations in ion transport are not restricted to mitochondrial membranes. Nelson and Henkart (1979) reported periodic changes in the cell membrane potential of a variety of cells of mesenchymal origin. Large hyperpolarizations of the membrane were recorded, lasting several seconds. They have been attributed to an increase of the surface membrane permeability to potassium ions.

Various secretory cells such as the pancreatic islet β-cells which produce insulin, exhibit oscillations in membrane potential (Mathews and O'Connor, 1979). These oscillations are related to the control mechanisms responsible for release of materials by exocytosis.

Regular oscillations in membrane potential are also responsible for rhythmical contractions of heart and smooth muscle.

Changes in the membrane permeability of cellular constituents other than ions have also been recorded. The uptake of both glucose and deoxyglucose by chicken fibroblast cells in culture were found to

reach a maximum as the cells progressed into S-phase (Smith and Temin, 1974). Similar timing was obtained for a several fold increase in the transport of leucine across the membrane of human fibroblast cells (Costlow and Baserga, 1973) and of certain nucleosides into rat hepatoma cells in culture (Plagemann, Richey, Zylka and Erbe, 1975).

1.3.5 Conclusions

I have discussed in this section several examples of the wide range of cellular constituents which exhibit some form of cell cycle dependent or oscillatory gene expression. In some cases, such as protein synthesis, this is accompanied by an equal volume of data against such oscillatory gene expression. In an attempt to resolve such controversies, I have tried to emphasise the important role that experimental techniques and other variable factors such as strain differences may play in such conflicting data.

The question of whether there is any physiological significance in this phenomenon now arises. Oscillations could be nothing more than a manifestation of the allosteric properties of enzymes, as illustrated by phosphofructo kinase. On the other hand, they could provide timing mechanisms for the cell cycle. Data concerning cell-cycle dependent protein synthesis, enzyme activity and membrane configuration may lead the way to a biochemical understanding of the cell-cycle which for many years has remained elusive. They may also provide timing mechanisms for differentiation and morphogenesis. Much speculation has been made about their possible role as biological clocks responsible for behavioural circadian rhythms, amongst others, in multicellular organisms. These possibilities are now briefly discussed.

1.4 The Physiological Significance of Biochemical Oscillations.

1.4.1 Biochemical Oscillations and the Biological Clock.

Despite extensive suggestions concerning the presence of an internal oscillator, there is still no proof that such an oscillator exists which is responsible for any form of rhythmical activity from the cell to the whole organism. Nonetheless various attempts to pinpoint a cellular mechanism for circadian rhythms have been made by means of theoretical modelling. Two conceptually different approaches have emerged in the literature. The first implicates the occurrence of discrete biochemical events in a strict sequence. However very little evidence of sequential gene transcription can be found to substantiate this. The other approach, pursued by various researchers, envisages the biological clock as a biochemical network with self-sustained oscillations stemming from feedback of the whole biochemical system. Biochemical oscillations can be shown theoretically to be the predictable consequence of gain and feedback.

Enzymes have become popular as the basis of biological clocks because certain features are common to both. Enzymes have been shown to have a stable period of oscillation, the ability to be phase-shifted by external stimuli and to be entrained by a periodic driving force in a similar way to circadian rhythms.

The glycolytic pathway is a good example because it has been extensively studied both theoretically and experimentally. The frequency of the oscillatory enzyme in the sequence, phosphofructokinase has been shown to be independent of substrate input. This could therefore provide a reliable timing mechanism. Analysis of concentrations of glycolytic intermediates has shown them to vary between 10^{-5} to 10^{-3}M in yeast cells (Hess, 1979). Such oscillations

could be capable of driving a range of observed oscillatory phenomena. Glycolytic oscillations have been shown to have generated cyclic changes in the pool of adenine nucleotides which also affects the enzymes phosphoglycerate kinase and pyruvate kinase. Both of these enzymes are involved in ATP production. Many other metabolic enzymes therefore would respond to changes in energy availability to the cell, which could lead to far reaching propagation of these oscillations stemming from PFK.

A major discrepancy in the relationship between enzyme oscillations and circadian rhythms in many cases is the relative time span. However, theoretical analysis shows that the period of an oscillatory enzyme can be extended to circadian values by sufficiently lowering the enzyme concentration and the rates of substrate input and end-product removal (Boiteux, Goldbeter and Hess, 1975). Unfortunately, these levels could easily be outside of the physiological limits.

A circadian period has also been shown theoretically to arise from the interaction of many smaller period oscillations. Winfree (1975) termed this as the 'clock-shop' phenomenon.

None of these models can explain the observed temperature independence exhibited by circadian rhythms. This is a drawback of implicating enzymes as the biological clock. Njus, Sulzmann and Hastings(1974) proposed another model incorporating the network idea but identified ion changes and the membrane-bound ion transport system as the timing mechanism. They proposed that the distribution of glycoproteins in the lipid bilayer could be regulated by oscillating ion concentrations. Periodic changes in the membrane permeability would then be generated which could be temperature-independent.

The 'Clock shop' model of Winfree (1975) could be taken a step further such that the cell itself, as a summation of all its oscillating constituents, could be the biological clock from which circadian rhythms are timed. One of the difficulties with such a theory is the variety of cell types and the presence of both cycling and non-cycling cells in multicellular organisms. A specific cell type at a specific locus with a constant cell cycle period would then need to be implicated as the oscillator in multicellular organisms. The period of the cell cycle is much nearer circadian values than many other biochemical oscillations. However, as pointed out in section 1.1, the cell cycle does differ in certain characteristics from the properties of circadian rhythms.

1.4.2 Biochemical Oscillations and the Cell Cycle.

Cells, in the majority of instances *in vivo* are asynchronous. However they can be entrained by the circadian rhythm such that the phases of the cell cycle are synchronous, as discussed in section 1.1. This could suggest that the cell cycle is not the circadian oscillator but is susceptible to it. The question of how these cells are synchronised is a difficult one. We do not understand the mechanism of the transition between one phase of the cell cycle to another, so it is difficult to postulate sites of control. However, it is likely that the circadian signals will affect certain important steps in the cell cycle such as the G_1 - S transition or the earlier G_0 arrest point (see Figure 2.2).

Any oscillating cellular constituent could be responsible for entraining certain events of the cell cycle such as DNA initiation or mitosis. Rensing and Goedeke (1976) collated some of the available

data concerning cellular circadian rhythms in rat liver. They compared the timing of the rhythms and found a definite temporal sequence. Increases in the active transport of α-amino isobutyric acid and the level of arginine were paralleled by an increase in RNA polymerase II activity. Maxima of nuclear non-histone protein synthesis and of nuclear protein kinase activity followed. DNA synthesis then commenced followed by mitosis 8 hours later. Whether this sequence of events is related to the cell cycle in a causal manner, or is only fortuitous is speculative.

If cells are synchronous, it is only to be expected that the metabolic pathways are co-ordinated also. Therefore, changes at specific points in the cell cycle such as the protein levels enhanced during the G_0 - S phase transition (Riddle et al., 1979) or the levels of enzymes, may not be causally related to the cell cycle. The question of which is the cart and which is the horse is an open one.

There is also the possibility that in multicellular organisms cells may be entrained by external blood borne or neural signals. Numercus hormones have been shown to exhibit fluctuations on a circadian basis (Scheving, 1976). Tutton (1973) implicated variations in the levels of glucocorticoid hormone levels in the circadian rhythm of crypt cells of the rat small intestine. Bullough (1962) has discussed circadian mitotic rhythms in terms of waking and sleeping, implicating the associated changes in hormone levels such as a high secretion of adrenalin while the animal is awake and active, and a low rate of secretion while asleep.

These oscillations in hormone secretion could stem from the endogenous oscillations in secretory cell membranes such as that found in insulin secreting pancreatic cells (Mathews and O'Connor, 1979).

A specific hormone concentration could act as Zeitgeber to the cells at a single critical point of the cell cycle. However, since not all cycling cells exhibit circadian rhythms in DNA synthesis and mitosis only particular cell types may be competent to respond to the Zeitgeber. This competence to respond could in itself be an intercellular or group of oscillations specific to these cells. If this is the case then the data available suggests that epithelial cells have this capability, being virtually the only cells investigated which exhibit cellular circadian rhythms. In any one organism, however, these cell types which exhibit such rhythms are not necessarily in phase with one another, mitosis occurring at various times during the day. The various tissues of any one organism which exhibit circadian rhythms of DNA synthesis do not respond in a similar way to the same hormone levels in the blood at any one time. This could be explained by different times of cellular competence or different hormone threshold levels in the various tissues.

1.4.3 Oscillations, Differentiation and Morphogenesis.

Differentiation and morphogenesis are highly ordered processes occurring at specified times in development and in relationship to the whole organism. This implies that time is an important element of these processes. How time is intertwined with such events is a major outstanding problem of develcpmental biology. Clearly, such oscillations as I have discussed in this chapter, ranging from high frequency oscillations of ion permeability to circadian rhythms in DNA synthesis, offer a very strong reliable timing mechanism to which differentiation and morphogenesis could be tuned.

I have already discussed evidence which suggests enhancement

in expression of certain proteins, glycoproteins and enzymes at specific times during the cell cycle. If cells are synchronised this would result in a significant rise in the concentration of such cellular constituents across the whole population. In this way time-limited signals could be generated which are necessarily above a certain threshold for them to elicit the required response, for example the next step in the differentiation process. If cells are not sufficiently synchronised then such threshold levels would not be gained. Cellular circadian rhythms could therefore be closely coupled to differentiation in epithelial cells.

An additional problem in morphogenesis is the specification of positional information in morphogenetic fields. Goodwin (1969) theoretically analysed the potential that oscillatory metabolic processes may have in fulfilling such a role. He concluded from his analysis that oscillatory reactions, in the presence of diffusion, could provide a means for the fast transmission of a chemical signal over macroscopic dimensions with little attenuation. Metabolic oscillations could, therefore, be involved such that spatio-temporal positional information is specified in the process of morphogenesis. If these oscillations are also cell cycle dependent or related, then the intermitotic time would be expected to vary in cells across the morphogenetic field.

CHAPTER 2. THE VERTEBRATE LENS

2.1 Advantages as a Model System for Cellular Differentiation

The vertebrate eye lens is a transparent organ consisting of a self renewed epithelial stem population which terminally differentiates to form lens fibre cells containing several specific protein classes collectively termed crystallins. These proteins form the bulk of the lens. This system therefore offers an ideal opportunity for the study of cellular differentiation and its genetic regulation (Reviewed by Clayton, 1979). Other attributes which make the lens a valuable model system for such studies are discussed below.

2.1.1 Whole Lens Culture

The lens is avascular, not innervated, and is completely enclosed within a membrane synthesised by the epithelial cells, the lens capsule. These factors facilitate its isolation and through special culture techniques permit the examination and manipulation of epithelial cells in the whole, intact, isolated lens in culture (Gierthy, Bobrow and Rothstein, 1968). *In vivo* the lens is bathed in the nutritive fluids of the vitreous and aqueous humors, which are the sole sources of nutrition due to the absence of vascularisation. In a similar way the explanted lens *in vitro* is bathed in a specified nutrient medium. It is therefore possible to study the processes of cellular differentiation in an entire intact cell population of an organised tissue as near to the *in vivo* situation, in the structural whole organ, as is possible.

2.1.2 Lens Epithelial Cell Culture

Isolated lens epithelial (LE) cells have been established as monolayer cultures with various animal materials such as chicken (Kirby, Eastey and Tabor, 1929), rabbit (Tamura, 1965) and calf lenses (Van der Veen and Heyen, 1959). Okada, Eguchi and Takeichi (1971) demonstrated that chick LE could be dissociated, grown in *in vitro* culture and exhibit morphological and biochemical changes corresponding to differentiation of lens fibres *in vivo*. Structures termed 'lentoids' were observed in epithelial cultures, which, on examination by electron microscopy, consisted of a multilayered lens-like arrangement of elongated lens fibres, depleted in mitochondria and endoplasmic reticulum but with large numbers of polysomes. Immunofluorescence studies indicated a high accumulation of crystallins in these 'lentoid' bodies (Okada, Eguchi and Takeichi, 1973). Cell culture, therefore, offers a powerful tool for the study of undifferentiated cells, their differentiation potential and cellular behaviour during this process. These studies are facilitated by the fact that no extraneous cell types are present to complicate experimental results.

2.1.3 Transdifferentiation.

The vertebrate lens is of further interest with respect to cellular differentiation, because other than the normal developmental pathway described later, lenses can be derived from other developmentally unrelated eye tissues. These tissues lose their definitive characteristics and acquire those features which normally characterize completely different differentiation pathways. This

phenomenon, termed 'metaplasia', or more recently, 'transdifferentiation', was first reported in the classical example of Wolffian lens regeneration in which a normal lens is regenerated from the dorsal iris epithelium after lentectomy in newts (Wolff, 1895; and reviewed more recently by Yamada and McDevitt, 1974). Recently transdifferentiated lens cells have been derived from a variety of eye tissues in vitro. 9 day old chick embryo pigmented epithelium from the retina lacks the ability of regenerating the lens in situ, but Okada and Eguchi (1973) showed convincingly, by establishing single cell clones, that it could 'transdifferentiate' into crystallin synthesising cells in vitro. Similarly, iris from the newt (Eguchi, 1967) and neural retina from chick embryos (Okada, Itoh, Watanabe and Eguchi, 1975) have been shown to develop into lens-like cells in tissue culture.

2.1.4 Lens Development

The basic features of lens development are common to all vertebrates resulting in a remarkably uniform structure. The vertebrate lens is derived from the competent head ectoderm in response to an inducing stimulus from the developing optic cup. A vesicle becomes formed from a single layer of epithelial cells which begins to elongate on the posterior face adjacent to the neural retina. These cells terminally differentiate to form the primary lens fibres which fill up the lumen. The germinal zone of epithelial cells around the equator of the lens undergoes division to form cells which are displaced posteriorly and which subsequently differentiate to form meridionally orientated secondary lens fibres which are added to the body

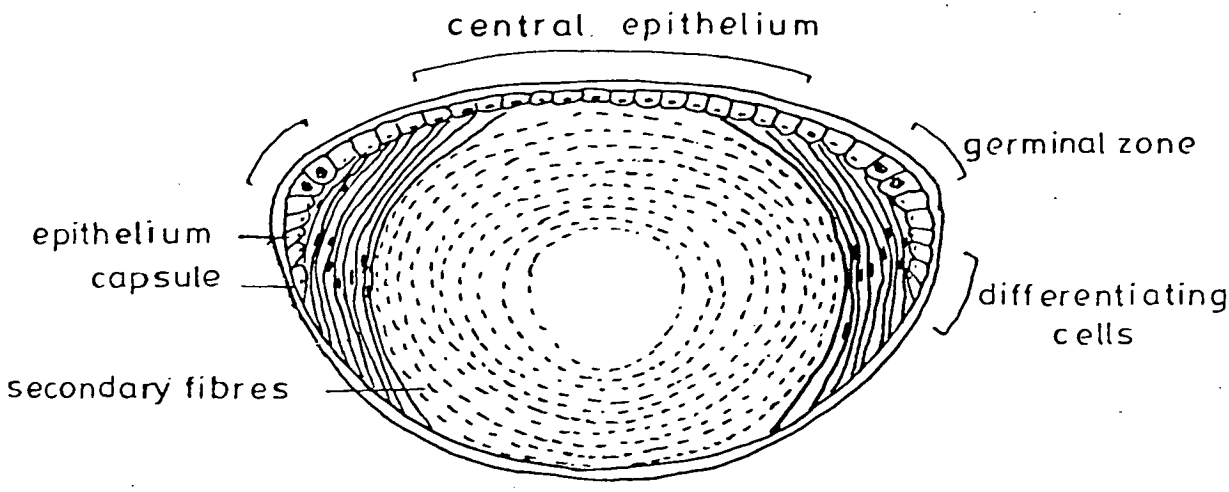


Figure 2.1

Diagrammatic cross-section of the vertebrate lens.

of the lens in concentric layers (Fig. 2.1). The developmental history in terms of differentiation products is, therefore, contained within the lens capsule.

2.2 The Lens Epithelium

The entire population of epithelial cells exists as a monolayer over the anterior face of the lens and exhibits a relatively uniform thickness in the normal lens. The lens epithelium whole mount procedure has permitted the examination of DNA synthesis and mitotic patterns in the lens (reviewed by Harding, Reddan, Unakar and Bagchi, 1971). By autoradiography and counting of mitotic figures it has been established that cell division and DNA synthesis can take place over the whole anterior epithelium in embryonic lenses. In the adult they become essentially confined to a region around the periphery of the epithelial layer, the germinal zone, and in the pre-equatorial region of the epithelium (Von Sallman, 1952; Reddan and Rothstein, 1966). Von Sallman (1952) also found that the total number of mitotic cells in the rabbit lens epithelium decreased as a function of age. Mitosis in the rabbit (Von Sallman, 1952) and in the rat lens (Von Sallman and Grimes, 1966), exhibited patterns of Circadian variation, as already discussed.

This equatorial zone constitutes a renewal system whereby cells are provided which subsequently further differentiate into fibres throughout life. The epithelial cell populations with no mitotic activity have become arrested in the G_1/G_0 phase of the cell cycle (Riley and Devi, 1967). Gelfant (1977) has proposed a model for cell and tissue proliferation based on the idea that cycling cells

can arrest at 3 stages in the cell cycle: early G_1/G_0, in late G_1 and in late G_2. This was presented in the light of a large amount of data from a variety of cell types, for example mouse ear epidermis. However, there is no evidence for mitotic blocks in a phase of the cell cycle other than G_1 in the lens epithelium.

The quiescent cells of the central anterior epithelium can be restimulated to divide under certain conditions. In vitro culture conditions (Harding et al., 1971), probably attributable to the presence of foetal calf serum stimulated proliferation in these cells. Lens injury (Harding et al., 1961), and some cataractous conditions, for example, as a result of diabetes (Kuwabara, Kinoshita and Cogan, 1969) also result in mitotic stimulation of this region.

2.3 The Lens Fibres

The differentiation pathway from epithelial cells to the fibres leads to biochemical, morphological and ultrastructural changes. Cells elongate, the nuclei of which become pycnotic. Endoplasmic reticulum and mitochondria are lost while polysomes increase dramatically due to the high levels of crystallin synthesis.

Mörner (1894) showed that biochemical fractionation of the lens yielded the water soluble crystallins and a water insoluble fraction which is now known to consist of a membrane-intercellular matrix complex (Lasser and Balazs, 1972). Lasser and Balazs (1972) further fractionated this complex, isolated from the calf lens, into the urea soluble intercellular matrix and the urea insoluble cell membrane fractions. Alcala, Lieska and Maisel (1975) have since isolated the proteins and glycoproteins from the isolated membranes of the bovine lens by Triton solubilisation.

2.4 The Lens Crystallins

Certain evolutionary constraints have been exerted on the developing lens:

(a) it must remain transparent so that light can pass through it,

and

(b) it must be of the correct refractive index so that it can focus light onto the retina.

These constraints have resulted in highly conserved crystallin proteins. The lens fibres are characterized by a high concentration of these crystallins which constitute 80-90% of the total soluble protein in the lens. These range in molecular weight from 20,000 to 50,000 daltons (reviewed by Clayton, 1974). Three gene families of crystallins termed α, β and γ are to be found in the mammalian lens. However γ crystallins are substituted by δ crystallins in birds and some reptiles. These factors offer an ideal system for the investigation of gene expression during differentiation. In addition, these crystallins are readily identified and their synthesis can be regulated *in vitro*.

Crystallins form very close and specific packing arrangements resulting in successive changes in protein concentration. This is necessary as a lens increases in size during development, in order to conserve its optical efficiency by changes in the refractive index. (Philipson, 1969). These changes arise as a result of the changing pattern of crystallin classes and their quantitative regulation in the differentiating lens fibres. In the chick embryonic lens δ synthesis is high in the early fibres forming the centre of the lens where the refractive index is necessarily high. δ crystallin is

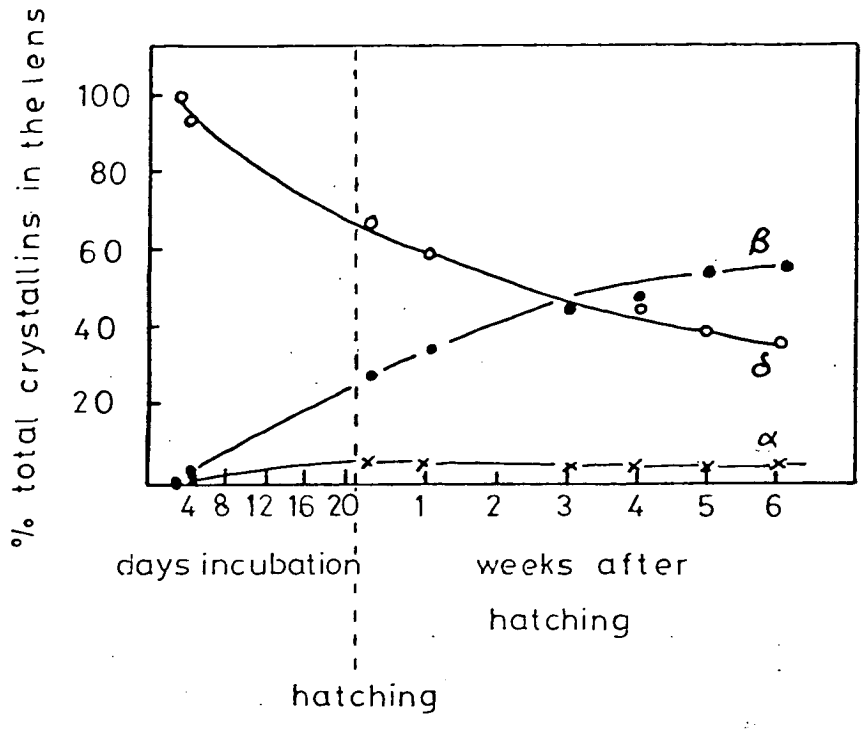


Figure 2.2

Changes in the protein composition of the chick lens during development. The composition of the lens as shown by quantitative immunoelectrophoresis. From Truman, Brown and Campbell (1973) (By courtesy of Dr D.E.S. Truman).

therefore thought to permit a closer molecular packing than the α and β crystallins in the periphery of the lens. α and β crystallins are synthesised in greater amounts as δ crystallin decreases at the time of hatching, as determined by quantitative immunoelectrophoresis (Truman, Brown and Campbell, 1972). Figure 2.2 shows the relationship of these 3 crystallins during development of the lens of the chick. This changing ontogenic sequence of the crystallins leads to questions concerning the regulatory mechanisms of the qualitative and quantitative gene expression during lens development.

2.5 Regulation of Crystallin Synthesis

Coulombre and Coulombre (1963) demonstrated unequivocally that differentiation of lens epithelium was not only a function of age but depended on the location within the eye. By surgical lens reversal in 5 day old chick embryo eyes, new fibres elongated and developed from the previous epithelium. The original fibres, now on the anterior face of the lens, ceased to elongate. A new epithelium then began to form over the anterior face of the lens derived from cells in the equatorial region. Eguchi (1969) also showed that the proportion of cells differentiating appears to be related to the distance between the lens and retina. The conclusion has, therefore, been made that the neural retina is a source of some factor which promotes lens differentiation. This regulatory control, however, only suggests an 'on/off' regulation of crystallin synthesis, insufficient to account for the differential synthesis of crystallin classes which have been observed. Clayton, Odeigah, de Pomerai, Pritchard, Thomson and Truman (1976b), studied changes in crystallin

polypeptide synthesis in the LE and whole lenses of 3 chicken strains as a result of variation of *in vitro* culture conditions. They concluded from this study that regulation of a crystallin polypeptide in response to particular conditions was independent of other polypeptides. This non-coordinate regulation of crystallin synthesis suggests the possibility of several regulatory mechanisms involved in the synthesis of each crystallin.

2.5.1 Possible Regulatory Mechanisms

(i) Clayton *et al.* (1976b) demonstrated that insulin treatment and the presence of foetal calf serum, factors enhancing cell division, altered the crystallin synthesis profile in chick lens epithelial cultures. Differences in crystallins were also found when 3 genetic strains of chick with different intrinsic mitotic rates in the epithelium were compared. The distribution of mitosis in the epithelium of chick embryos is more extensive, when δ crystallin synthesis is high, than in the adult when α and β crystallins take its place (Modak, Morris and Yamada, 1968). This correlative evidence points to the cell cycle and mitotic distribution as a possible regulatory factor.

(ii) Hanna and Keatts (1966), in a study of chick epithelial cell production and migration by autoradiography, noted that cells of the germinative zone of lenses of 2 and 10 day old chickens migrated towards the equator and formed new fibres at a faster rate than those of the 6 week old chicken. The rate of differentiation of lens fibres clearly

differed according to age, and could be another source of crystallin synthesis regulation.

(iii) In one of the first reports of differentiation of lens epithelium into lens-like structures *in vitro*, (Okada *et al.*, 1971) it was noted that these lentoids only developed in regions of extensive cell contact. Similarly, *in vivo* the epithelial cells on the anterior face of the lens which show signs of differentiation are those which have contact with other epithelial cells on all surfaces, the outermost layer always remaining undifferentiated (Clayton, Eguchi, Truman, Perry, Jacob and Flint, 1976a). This suggests a third possible regulatory level possibly mediated by way of the cell surface.

(iv) Hanna and Keatts (1966) observed that, at hatching, aqueous humor formation was accelerated resulting in a greater supply of oxygen and nutrients to the anterior surface of the lens. Enforced metabolic changes in lens epithelial cultures by the addition of sodium salicylate (inhibition of cation movement across cell membrane) and 6-amino nicotinamide (inhibits NAD-linked dehydrogenases) resulted in alterations of crystallin synthesis (Clayton *et al.*, 1976b). The metabolic status of the epithelial cells and adjacent tissue may therefore play some role in regulating differentiation of these cells.

Regulatory factors involved in lens fibre formation *in vitro* have been widely discussed recently by Clayton (1979a and b).

2.5.2 The Cell Cycle and Crystallin Synthesis Regulation

One of the above possible regulatory mechanisms of crystallin synthesis in the chick lens which has been investigated in this research project is the cell cycle. Data has been accumulating concerning a relationship between cell cycle duration and δ crystallin synthesis. de Pomerai, Clayton and Pritchard (1978), using haemagglutination inhibition and gel electrophoresis to quantify the levels of crystallins in cultures of lens epithelium, demonstrated that the age of the tissue from which the culture was derived determined the crystallin composition of fibres differentiating *in vitro*. For example, 12 day and 15 day embryos and 1 day post-hatch chicks resulted in decreasing production of δ crystallin respectively. Similarities between the *in vitro* and *in vivo* situation are therefore apparent, δ crystallin synthesis in the lens decreasing markedly around the time of hatching (Truman *et al.*, 1972). It should be noted, however, that although the trend is similar, δ crystallin levels in cultures do not approach those found in lens fibres from the same stage in the intact lens, possibly due to incomplete fibre differentiation in *in vitro* culture conditions (de Pomerai, Pritchard and Clayton, 1977). Embryonic LE cells also have a greater intrinsic mitotic rate in culture than post-hatch LE cells. This suggests that the cell cycle may be a factor in the promotion of δ crystallin synthesis. This idea is further supported by the finding that LE from different genetic strains (described in detail in Section 2.6) with different growth rates in the log phase in culture (Eguchi *et al.*, 1975; de Pomerai *et al.*, 1978) synthesise different levels of δ crystallin - the faster growth corresponding to high δ crystallin synthesis. However it remains to be elucidated whether indeed any causal relationship exists.

2.6 Genetic Differences in the Lenses of 3 Chick Strains

Comparative investigations of genetic differences between strains have proved to be a useful tool in the dissection of biological processes. Genetic modifications may therefore help to illuminate the regulatory mechanisms involved in the expression of the crystallin classes in the lens. In this project the cell cycle is of particular interest. The lens of the chick provides an ideal system for such studies as a wealth of information concerning it has already been established (reviewed by Harding, Reddan, Unakar and Bagchi, 1971).

2.6.1 Cellular Properties

Two genetically unrelated strains of chick, Hy-1 and Hy-2, have been rigorously selected for high early growth rate over a period of several years. Hy-1 is an inbred strain and Hy-2 is the F_1 between two inbred strains. Both show hyperplasia of the LE and a variety of differences in cellular properties when compared with a strain (N), not selected for rapid growth (Clayton, 1975). In Hy-1 and Hy-2 the lens epithelium contains an excessive number of cells as compared to normal and it forms a multi-layered structure across the anterior face of the lens between the capsule and fibre body. Normally, recruitment of the epithelial cells for differentiation into fibre cells must be related to the number of new cells derived by mitosis such that the epithelium remains as a monolayer. This apparent failure to regulate the mitotic rate appears, from autoradiographic studies after ^{3}H-thymidine incorporation, to be attributable to the retention of the capacity for mitosis in the whole epithelium and not just in the germinal zone as in normal day old lenses

(Clayton et al., 1976a). Therefore the abnormality of high growth rate in Hy-1 and Hy-2 becomes manifest at the time when the incidence of mitosis normally drops in the central epithelium in later embryonic development but in these hyperplastic strains is maintained. The morphological arrangement of these epithelial cells suggests that they are deficient in contact inhibition on their upper and lower surfaces compared with normal lenses and a tendency for short fibre-like cells to form from excessive epithelium between the layers (Clayton, 1975).

Investigations concerning the properties of the epithelial cells from the Hy-1 strain have shown it to differ in numerous aspects from a normal control strain (N). Eguchi et al. (1975) demonstrated that the growth rate in the log phase in culture was approximately double that of N strain LE cells in culture over the same period and under identical conditions. By growing single cell clones and scoring the increase in cell number in each colony, they were able to show that Hy-1 LE cells consisted of two populations. One of these showed similar growth kinetics to that of N, while the other showed a much greater growth capacity. Hy-1 LE cells also displayed a high affinity for one another, forming aggregates readily after dissociation. This resulted in a lower percentage of cells attaching to the culture plate than N cells. These differences between the 2 strains appear to be fundamental properties of the cells themselves, since the differences were persistent through many cell divisions and 3 culture passages.

2.6.2 DNA, RNA and Protein Metabolism

Further studies on these strains and Hy-2, concerning DNA, RNA and protein metabolism, have lead to the discovery of other differences. Pulse-chase experiments on whole lenses indicated that the rate of synthesis of rRNA, tRNA and mRNA was specific for each strain, large differences occurring between them. This suggested that the rate of synthesis of RNA classes was under genetic control (Truman, Clayton, Gillies and MacKenzie, 1976). Thymidine incorporation into day-old lenses of all 3 strains was greater in both hyperplastic strains than normal (N). The autoradiographs showed that more nuclei were labelled, visible in any of the layers of the multi-layered epithelium (Clayton et al., 1976a).

Similarly, differences were found in these strains in the rate of crystallin synthesis and also qualitative differences such that δ crystallin synthesis in particular was enhanced in the hyperplastic strains, as previously discussed.

2.6.3 Membrane Composition

Numerous cases are reported in the literature of growth modifications of particular cell types being closely correlated with a change in a cell surface marker (e.g. lymphocytes in AKR-CBA mouse chimaeras, (Tuffrey, Barnes, Evans and Ford, 1974). Odeigah, Clayton and Truman (1979) showed qualitative differences in the protein and glycoprotein composition of the membranes. The Triton X100 solubilised membranes yielded different electrophoretic polypeptide and glycoprotein profiles in all 2 strains. A marked deficiency of gap junctions and a raised sialic acid content in membranes from strains Hy-1 and Hy-2 was also evident. Cell agglutination of dissociated

day old LE cells by Con A, Ricinis communis agglutinin 120, Phytohaemagglutinin P and anti-H lectins was found to be greater for each lectin in both hyperplastic strains when compared to normal. The cell surface changes between the strains appeared to be extensive and more complex for straightforward correlation of growth modifications and genetic cell membrane alterations. However these modifications may be related to the abnormal behaviour and hyperproliferation of the LE in vitro exhibited by these hyperplastic strains.

All of these biochemical differences in the LE between the 3 genetic strains which are described above, have been elucidated without knowledge of possible circadian variation in metabolic events, which this thesis reports. It is possible that those differences may be less prominent, or indeed even more marked in the light of this evidence.

CHAPTER 3. MATERIALS AND METHODS

All chemicals and reagents used were of Analar grade and obtained either from B.D.H. Chemicals Ltd. (Poole, England), or Fisons Ltd. (Loughborough, England) except where otherwise stated.

3.1 Animals

Day old chicks of the Hy-1 and Hy-2 strains and of the control strain (N) were supplied by Ross Poultry Ltd., Newbridge, Midlothian and the Poultry Research Centre, Edinburgh.

3.2 Culture Methods

Numclon tissue culture dishes and flasks were obtained through Gibco Biocult Ltd., Glasgow, Scotland.

Eagle's minimal essential medium (MEM) x 10, Medium 199, Earle's salts, foetal calf serum, penicillin-streptomycin mixture, L-glutamine (2mM) and sodium bicarbonate (22mg/ml were obtained from Gibco Biocult Ltd.

Whole lenses and lens epithelial cells were incubated at 37°C in an automatic CO_2 incubator (Forma Scientic, Ohio) with a humidified atmosphereof 95% air and 5% CO_2.

3.2.1 Whole Lens Culture

Intact lenses were dissected from day old chick eyes in a horizontal laminar flow hood (South London Electrical Equipment Company, Surrey, U.K.) to minimise bacterial and fungal contamination. Lenses were cleaned of adhering iris by gently rolling on sterile filter paper (Whatman No. 1) and washed in pre-warmed medium 199 containing 200 i.u./ml penicillin-streptomycin mixture. On completion of the dissection, the lenses were transferred to 2ml pre-warmed medium 199

supplemented with 10% (v/v) foetal calf serum (FCS) and 200 i.u. penicillin-streptomycin mixture. The medium was double labelled with radioactive precursors for DNA and RNA synthesis as indicated in Section 3.

3.2.2 Lens Epithelial Cell Culture

Whole eyes were removed from day old chicks, sterilised in 70% ethanol for 60 seconds then washed three times in warmed 0.9% (w/v) saline. The front of the eye was cut off in phosphate buffered saline (PBS), the lens capsule punctured with sharp forceps and the lens fibers extruded by gently squeezing around the lens equator. The lens epithelium (LE) including most of the annular pad was then pipetted into calcium and magnesium-free Hank's saline (CMF) until the dissection of eyes was completed.

The lens epithelia were dissociated by 0.125% (w/v) trypsin (Sigma Chemical Co.Ltd., London) in CMF for one hour with occasional agitation with a pipette. The cells were then washed three times in Eagles minimal essential medium supplemented with 6% FCS. Small clumps of cells were removed by centrifugation for 30 seconds.

The final cell suspension, consisting mainly of single cells, were counted using a haemocytometer. They were inoculated at a density of either (a) 3×10^5 cells per 6cm diameter plastic tissue culture grade plates each containing 4ml of Eagles minimal essential medium plus 6% FCS (Okada, Eguchi & Takeichi, 1971), or (b) 2×10^6 cells in 150 x 110mm plastic tissue culture flasks containing 30ml of nutrient medium as above.

The medium was changed every 2-3 days.

3.2.3. Cell Counting

Monolayer cultures were trypsinised with 0.2% trypsin for 1 hour at 37°C, agitated by gentle pipetting, then treated for 10 mins with 0.01% EDTA to ensure that all the cells were completely separated. The cells were then harvested by centrifugation at 1000g, resuspended in a known volume of CMF and counted using a Fuchs-Rosenthal haemocytometer.

3.2.4 Cell Synchronisation Methods

See Chapter 4 for the methods, results and discussion of the synchronisation procedures investigated and subsequently used.

3.2.5 Additional Supplements to LE Cell Cultures

Bovine crystallin insulin was obtained from B.D.H. Chemicals Ltd. (Poole, England) and was used in a concentration of 10 µg/ml in 6% FCS supplemented minimal essential medium.

Retinal extract (RE) was donated by courtesy of C. Arruti, D. Barritault and Y. Courtois (Unité de Résearche de Gérontologie, I.N.S.E.R.M., Paris) and was used in a concentration of 50µg/ml in 6% FCS supplemented minimal essential medium.

3.2.6 DNA Estimation of LE Cell Cultures

Cells were harvested and the DNA content/cell estimated by the method of Giles and Myers (1965).

3.3 Radioactive Labelling

^{3}H amino acid mixture, ^{14}C amino acid mixture, (methyl-^{3}H) thymidine, (5-^{3}H) uridine and iodine-125 were purchased from the Radiochemical Centre, Amersham, Bucks.

3.3.1 Whole lenses

Whole lenses were explanted at varying times over a 24-hour period and transferred to 2ml M199 supplemented with 10% FCS and 200 i.u. penicillin. The medium was labelled with radioactive precursors for DNA and/or RNA synthesis according to the experiment. The pulse label was one hour in duration at 37°C in 5% CO_2 in air.

The maximum time-lapse between decapitation of the chick and the commencement of the radioactive pulse was 25 minutes.

Whole lenses were processed according to either (i) or (ii):

(i) Lenses were double-labelled with 100µCi/ml ^{3}H thymidine and 5µCi/ml ^{14}C uridine, washed in cold saline and transferred singly to a 2mm square of chromatography paper. The lens capsule was punctured squashed firmly onto the paper and then transferred to 5% TCA. The non-aborbent surface of polythene backed paper (Benchkote, Whatman) was used directly beneath the chromatography paper to prevent the loss of any material. Each lens was washed twice separately in 5% TCA, in absolute ethanol and then in ether. The radioactivity incorporated was determined by scintillation counting (Section 3.4.1).

It has been verified that incorporated precursor was adequately washed from the squashed lenses, by labelling lenses for varying periods from zero-time to 90 mins. The plotted results of disintegrations / minute/lens against time showed a smooth curve which passed through the origin.

(ii) Lenses were labelled with 100µCi/ml ^{3}H-thymidine, prepared for histological sectioning followed by autoradiography of the sections (see Section 3.4.2).

3.3.2 Cell Cultures

Protein labelling

Random monolayer cultures of lens epithelium were labelled with 50μCi/ml ^{3}H mixed amino acid mixture in medium supplemented with 6% FCS for 3 hours at 37°C in 5% CO_2 in air. Lens epithelial cultures which were synchronised before labelling for protein analysis, and their asynchronous control cultures, were labelled for 30 minutes with 10μCi/ml ^{14}C mixed amino acids in medium supplemented with 6% FCS at timed intervals. In all cases the cells were washed thoroughly with cold saline to remove FCS proteins and unincorporated radioactivity. Water-soluble and membrane fractions were prepared as below (Section 3.5) and the samples run on polyacrylamide gels. A fluorograph of the gel was then prepared (Section 3.4.3).

DNA and RNA Labelling

Monolayer cultures were labelled for 1 hour with 100μCi/ml ^{3}H-thymidine, or 100μCi/ml ^{3}H-thymidine and 5μCi/ml ^{14}C-uridine at timed intervals. The cells were washed thoroughly with cold saline, precipitated with 5% TCA then filtered onto discs of Whatman glass microfibre (GFA-1) and washed with 5% TCA, absolute ethanol and ether. After drying the radioactive incorporation was determined by scintillation counting (Section 4A).

Aliquots of synchronous cell suspensions (see Chapter 4) were removed and labelled with 100μCi/ml ^{3}H-thymidine and/or 5μCi/ml ^{14}C-uridine for 1 hour. Samples were precipitated with 5% TCA after the addition of bovine serum albumin to a total concentration of 50μg/ml. This acts as a carrier to precipitate small concentrations of the labelled RNA and DNA. The samples were filtered as above and radioactivity determined by scintillation counting.

Assay for Binding of Iodinated Lectins

Preparation of Labelled Lectins

Wheatgerm agglutinin (WGA), Ricinis communis agglutinin-120 (RCA-120), Phytohaemagglutin (PHA) were obtained from Miles Laboratories Ltd. (Stoke Poges, Slough, England). Concanavalin A (Con A)(Grade IV from Jack Beans) was obtained from Sigma Chemical Co. Ltd. (London).

The specific sugar inhibitors for the various lectins are α methyl-D-mannoside (Con A), D-galactose (RCA-120) N-acetyl-D-galactosamine (PHA) and N-acetyl-D-glucosamine (WGA) and were obtained from Sigma Chemical Co. Ltd. (London).

The lectins were labelled with ^{125}I (carrier free) by the Chloramine-T method of Hunter and Greenwood (1962) 100µg of lectin was dissolved in 10µl of phosphate buffered saline. The lectin was iodinated by adding 1mCi of carrier free ^{125}I and 10µl of 1% chloramine-T in PBS solution. The solution was allowed to stand for 10 minutes at 20°C before adding 10µl of 1% sodium metabisulphite in PBS. The mixture was left for 10 minutes with occasional gentle shaking and diluted to 1ml with PBS. The solution was dialysed against PBS for 24 hours at 4°C with frequent changing of the PBS in order to remove any unreacted isotope.

Reaction of Iodinated Lectins to Cells

Aliquots of synchronous lens epithelial cells were mixed with either an equal aliquot of iodinated lectin solution or iodinated lectin solution plus the appropriate inhibitor in a 2ml sterile serum tube. The cells were then incubated at 37°C for 30 mins, washed three times in PBS and the pellet suspended in 70% ethanol. The samples were precipitated with 5% TCA and filtered, washed and

dried as above ready for scintillation counting. To calculate the amount of iodinated lectin bound specifically, the amount bound in the presence of the inhibitor was subtracted from the amount bound in the absence of the inhibitor.

3.4 Assay of Radioactivity

3.4.1 Scintillation Counting

The triton/toluene scintillator used contained 25g PPO and 1.5g dimethyl POPOP in 3.5 litres toluene and 1.5 litres triton X-100. All of these chemicals were obtained from Koch-light Laboratories (Colnbrook, Bucks.).

Samples were precipitated with TCA, washed and dried as above (Section 3.3). The filters were placed in vials containing 5ml of the triton/toluene scintillator and counted in a refrigerated inter-technique scintillation spectrometer.

3.4.2 Autoradiography

Whole lenses were fixed in Carnoy's fixative for 4 hours after radioactive labelling, washed in 70% alcohol several times followed by several washes in 95% alcohol over a period of 48 hours. They were then transferred to terpineol for 48 hours. The terpineol was allowed to reach 60^{o}C for 15 mins and paraffin wax added to make a 1:1 ratio for 15 mins. Several changes of wax were made over a period of 10 hours. The lenses were then put into block and cut into 10μm sections. The sections were stained with haemotoxylin and eosin. Coating of the slides with Ilford L 4 gel type nuclear emulsion was carried out using the dipping method of Messier and Leblond (1957) and exposed in the dark at 4^{o}C for 4-6 days.

The slides were developed using Kodak D19B for 4 mins, rinsed in distilled water then fixed with Ilford Hypam for 8 mins. They were washed in running water, dried and examined. Photographs were taken with a Carl Zeiss Ultraphot.

3.4.3 Fluorography

In order to detect labelled proteins after separation on polyacrylamide slab gels, they were analyzed by fluorography (Bonner and Laskey, 1974) using pre-flashed X-ray film (Kodak X-OMAT R film) to give a quantitative response to radioactive disintegrations (Laskey and Mills, 1975). A Kipp and Zonen integrating densitometer was used to scan the fluorographs.

3.5 Preparation of Lens Epithelial Cell Fractions

3.5.1 Water-Soluble Fraction

Cells were harvested using a rubber policeman, washed thoroughly to remove FCS proteins and either homogenised at $4^{o}C$ in 10mM phosphate buffer pH 7.2 containing 10mM β-2-mercaptoethanol or repeatedly frozen in liquid N_2 and allowed to thaw each time (5 times). This freeze/thaw method was particularly suitable to obtain maximum yield of protein from small cell culture samples. The proteins obtained were comparable with those obtained by homogenisation. The samples were centrifuged at $4^{o}C$ for 20 mins at 10,000 r.p.m. in an Ependorf bench centrifuge (Model 5412). The supernatant contained the water soluble proteins. The pellet was washed 6-10 times in the above buffer, after which no soluble crystallins were detectable in the supernatant using immunoelectrophoresis and an antiserum to total chick lens soluble proteins.

3.5.2. Urea-Soluble Fraction

The pellet was homogenised in the phosphate buffer as above with 8M urea and left overnight for 18 hours at 4°C. The homogenate was centrifuged at 16,000 r.p.m. for 30 mins. The supernatant contained all the urea-soluble proteins. The pellet (urea-insoluble membrane rich fraction) was washed,repeatedly in 8M urea until crystallins were no longer detectable in the supernatant.

3.5.3 Cell Membrane Fraction

Method

The urea-soluble pellet was homogenised in the "solubilising solution" of Miner and Heston (1972). This contained 50mM K_2CO_3, 8M urea, 10% β-2-mercaptoethanol and 5% Triton x -100, which permits differences in charge to be analysed. Triton X-100 was substituted by 0.2% SDS for separation of the subunits by molecular weight. The homogenate was centrifuged at 16,000 r.p.m. for 30 minutes at 7°C. The supernatant was used for analysis on polyacrylamide gels. Clayton et al., 1976b,c, and Odeigah et al., 1978 reported that a 15-20 minute sonication period prior to centrifugation increased the yield of membrane proteins. Results using this method however were unsatisfactory when sonication of that duration was used. An investigation was therefore carried out on the effect of sonication on the membrane proteins which were analysed by SDS and IEF rod gels (see Section 3.6). Whole lenses from mixed chick strains were used for this analysis. A Soniprobe Type 1103A (Dave Instruments Ltd., London) was used for sonicating the samples.

The supernatants were prepared as above with the following modifications:

(a) unsonicated membrane sample as above.

(b) the pellet of sample (a) resolubilised for 18 hours

(c) 30 second sonication

(d) intermittent sonication for 5 minutes.

Results

The profiles of membrane protein polypeptides fractionated by IEF and SDS gels are shown in Figures 3.1 and 3.2 respectively. Additional membrane polypeptides and an increase in yield was evident after the supernatant of the second solubilisation period was compared to that of the initial solubilisation of the same pellet (Gels B and C in Fig. 3.1, and A and B in Fig. 3.2). The bands of the IEF gels show a decline in resolution as the sonication time increased. It was noticeable that the presence of polypeptides in the pI range of 5.0 - 6.5 increased, particularly in the sample sonicated for 5 minutes. The number of bands in the 7.0 - 7.5 pI range diminished, however. In the SDS gels the polypeptides in the lower molecular weight region are reduced in amount while those in the higher molecular weight region show a greater density, particularly a major band at 103,000 M.W. A greater deposit of material which did not enter the SDS gels is evident in the sonicated samples.

Discussion

Prolonged solubilisation of the urea insoluble pellet with Miner and Heston's (1972) "solubilising solution" resulted in both a qualitative and quantitative increase in the yield of membrane

FIGURE 3.1

IEF polyacrylamide gels of membrane polypeptides from whole lenses treated in different ways. (A) The water soluble proteins from day old chick with the crystallins labelled as markers. (B) Unsonicated membrane sample. (C) Sample resulting from 18 hour resolubilisation of the pellet from (B). (D) 30 second sonication of membrane sample. (E) Intermittent sonication of membrane sample for 5 minutes.

FIGURE 3.2

SDS polyacrylamide gels of membrane polypeptides from whole lenses. (A) Unsonicated membrane sample. (B) Sample resulting from an 18 hour resolubilisation of the pellet from (A). (C) 30 second sonication of membrane sample. (D) Intermittent sonication of membrane sample for 5 minutes. The molecular weight markers represent α-phosphorylase, δ-crystallin and α_2 crystallin in order of decreasing size.

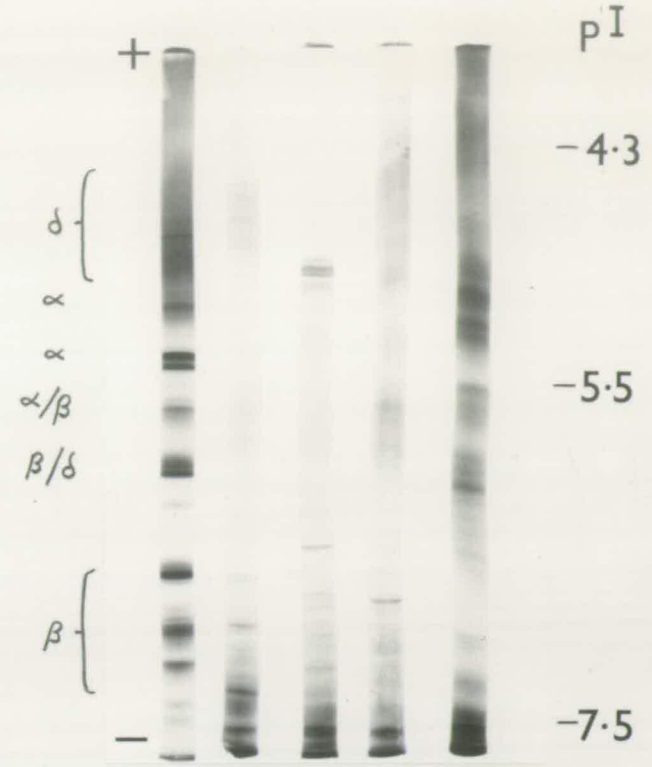


FIG 3.1

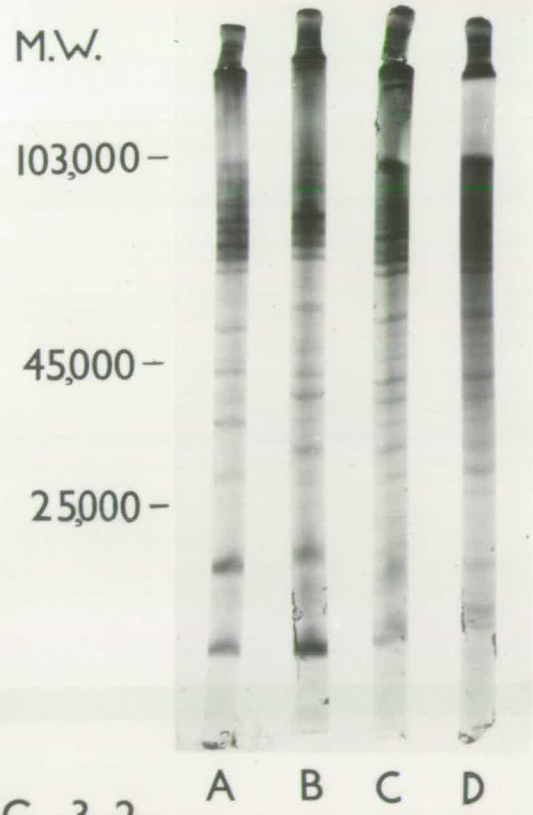


FIG 3.2

polypeptides. Sonication resulted in qualitative differences in the membrane polypeptides, when compared to the unsonicated samples. In the IEF gels certain bands were present while others were absent. The SDS gels show a reduction in the amount of low molecular weight material with an increase in the higher molecular weight material in the sonicated samples. Certain factors could contribute to these differences such as oxidation of membrane glycoproteins leading to cross-linking or fragmentation of the membrane such that parts of the lipid components are attached to the membrane glycoproteins, so changing their charge and size. It was found that 20 - 30 seconds was the maximum time of sonication which could be carried out without overheating of the sample. 15 to 20 minutes therefore seems an excessive duration for sonication to be continued. Due to the large proportion of detergent in the sample excessive frothing occurs during sonication. Care must be taken to ensure that the Soniprobe tip remains submerged in the sample. The profiles of membrane polypeptides in Clayton *et al.* (1976b,c) and Odeigah *et al.* (1978) suggest that this may have occurred, as bands in the IEF gels were well resolved unlike gel E of Figure 3.1, sonicated for 5 minutes. This suggests that effective sonication was not carried out for the duration stated. Variation in the amount of sonication administered to each sample could, however, account for some of the differences reported in the membrane composition of the different chick strains compared. No strain comparisons were made in this study.

It is therefore concluded that the above mentioned method of membrane solubilisation of the urea insoluble pellet with the addition

of 18 hours in the "solubilising solution" prior to centrifugation gives a higher yield of membrane polypeptides with a consistent profile.

3.5.4 Protein Determination

Protein concentration was determined using the commercially available Biorad protein assay (Biorad Laboratories, Munich) and by reading the absorbancy at 595nm. Bovine serum albumin was used as a standard.

3.6 Polyacrylamide Gels.

3.6.1 Isoelectric Focussing on Slab Polyacrylamide Gels

The isoelectric focussing technique was based on the method of Wrigley (1968) modified so that the gel contained 50ml 9M urea/ 1% ammonium persulphate solution, 4.8ml 1% temed and 0.9ml of each ampholine in the pH range 3.5-10, 4-6 and 6-8 (Burns, 1975), and 18ml of a 30% acrylamide/bis-acrylamide solution. 1.25% Triton x 100 was added to the gel mixture to prevent precipitation or re-aggregation of the membrane components. The commercially available "multiphor apparatus" was used for running these gels (L.K.B. Instruments Ltd., South Croyden). Ampholines were also purchased from L.K.B. Instruments Ltd.

Samples were loaded at a concentration of 50µg on 5 x 10mm Whatman 3mm paper. The electrode solutions consisted of 1M NaOH (cathode) and 1M H_3PO_4 (anode).

The gel was run at a starting current of 45-50mA, increasing the voltage every 5-10mins until 1,100V was reached. The gel run was completed after 2 hours.

The gels were fixed in 12.5% TCA at 65°C for 30 mins and the ampholytes removed.

3.6.2 Isoelectric Focusing on Rod gels

The above solutions were used to make 9.6 x 0.5cm rod gels by filling 0.5cm internal diameter glass tubes sealed at one end with plasticine. The gels were immediately overlayed with iso-butanol to obtain a flat top.

The anodal electrode solution consisted of 0.2% sulphuric acid containing 10mM 2-mercaptoethanol. The cathodal electrode solution consisted of 0.4% ethanolamine also containing 10mM 2-mercaptoethanol. The gels were aligned vertically in a disc electrophoresis tank with the anodal solution in the upper compartment and the cathodal solution in the lower compartment. The gels were pre-run at a constant current of 0.5mA per tube for 30mins at 4°C. Samples were layered onto the top of the tubes in a concentration of 250-300µg/gel. The gels were run for 16 hours at 4°C with a constant current of 0.5mA/tube.

The gels were removed from the tubes by injecting a jet of water with a hypodermic syringe between the gel and tube.

3.6.3. SDS Slab Polyacrylamide Gels

A 12% acrylamide lower separation gel was used with a 3% acrylamide upper stacking gel. The lower gel consisted of a mixture of 20ml of a 30% acrylamide/0.8% bisacrylamide solution, 25ml of 0.75M Tris pH 8.8; 0.5ml of 10% sodium dodecyl sulphate, 9ml distilled water; 0.2ml of 10% ammonium persulphate and 20µl of TEMED. This was poured, overlayed with water and allowed to set. The stacking

gel consisted of 2.5ml of 30% acrylamide/0.8% bisacrylamide solution; 5ml 0.25M Tris pH 6.8; 0.25ml of 10% SDS; 17ml of distilled water; 0.1ml of 10% ammonium persulphate and 10µl of TEMED.

50µg of sample protein was mixed with an equal volume of sample buffer, consisting of 0.025M Tris pH 6.8; 2% SDS; 10% glycerol 5% 2-mercaptoethanol and 0.002-0.004% Bromophenol blue, and then loaded onto the gel. The gel was run with a constant current of 10mA until the bromophenol blue reached the lower gel. The current was then increased to 30mA. The gel run was complete after 4-6 hours, when the bromophenol blue reached the bottom.

The electrode buffer consisted of 0.025M Tris, 0.192M glycine and 0.1% SDS.

3.6.4 SDS Rod Polyacrylamide Gels

The above solutions in Section C were used to make 10 x 0.5cm rod gels. The lower gel was allowed to set prior to overlayering with the larger pore gel solution. Samples of about 150µg protein were mixed with the sample buffer described in the previous section, then layered on the top of the gels. Electrophoresis was carried out at room temperature for 4 hours at a constant current of 3mA per gel tube in the tris-glycine buffer previously described. The run was complete when the marker dye reached the bottom.

3.6.5 Staining of Protein

The protein staining method was based on that of Crambach, Reisfeld, Wychoff and Zaccar (1967). 0.2% Coomassie Brilliant Blue in an ethanol-water-glacial acetic acid mixture (45:45:10) was used

for staining at 65°C for 30 mins followed by destaining in an ethanol-water-glacial acetic acid solution (25:65:10). The gels were stored in 7% acetic acid at room temperature prior to being photographed.

3.6.6 Photography of Gels

Ilford Pan F film was used for the photography of stained gels using a yellow filter.

3.6.7 Scanning of Gels

Positive transparencies of the gel photographs were scanned with a Kipp and Zonen densitometer (Model KS3 Microdensiscan, F.T. Scientific Instruments Ltd., Yateley, Surrey).

3.7 Time Lapse Photography

Lens epithelial cultures from strains Hy-1, Hy-2 and N were set up as previously noted. Cells were seeded at a density of 1 x 10^5 cells/6cm diameter plastic tissue culture grade plate. Cells were allowed to plate and grow for 4 days under the previous noted conditions. On day 5 of culture the medium was changed and the conventional tissue culture lid replaced with a Cooper dish lid (Nunclon) ready for filming. This type of lid avoided an air interspace between the cell medium and the lid which would interfere with the photography. Cell growth studies were carried out to see if any difference was apparent in cultures with the two types of tissue culture lids. It was possible that the lack of an air/CO_2 interspace between the culture medium and the Cooper dish lid could limit equilibration of nutrients and waste products. No significant

difference was found in the growth rate between the two types of culture.

A Gillet and Siebert inverted phase microscope was housed in a polystyrene temperature controlled hot box. The temperature was maintained at $37^{o}C \pm 0.5^{o}C$ throughout filming. Phase contrast was used with a total magnification of x36. A Kodak ciné camera specially modified for motor driven single frame records was used for filming in conjunction with a Wild intervelometer. The frame interval was 10 minutes. Ilford Pan F film was used for the analysis.

The culture plate was housed in a platform shown in diagrammatic form in Figure 3.3. Gas inlets for humidified 95% air/5% CO_2 maintained the culture medium at the correct pH. A moistened foam ring around the periphery of the culture dish maintained the humidity. Filming was carried out for periods of up to 4 days. Medium changing within this period was carried out. The arrangement of the microscope platform permitted exactly the same field to be returned for filming after each medium change.

Single frame analysis was carried out using a Lytax single frame analyser (now obsolete).

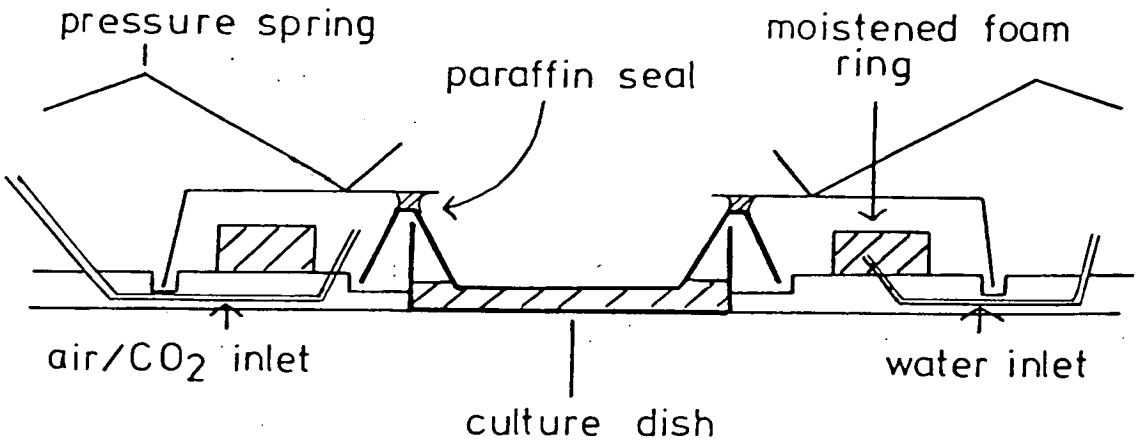


Figure 3.3

Diagrammatic representation of the culture-dish housing during time-lapse photography.

CHAPTER 4. INDUCTION OF SYNCHRONOUS LENS EPITHELIAL CULTURES: TWO METHODS

4.1 Mitotic Selection

4.1.1 Method

Only strain Hy-2 was used for examining the possibility of inducing synchronous cultures of lens epithelium.

Freshly dissociated lens epithelial (LE) cells were seeded in flasks as above. A method of cell synchronisation based on the mitotic selection method of Terasima and Tolmach (1961 and 1963) was used. When the cells were in exponential growth (Day 6 after seeding) the monolayers were briskly agitated for 15 seconds and the supernatant was carefully decanted into sterile tubes and either discarded (in the case of the first few selections), or concentrated by low speed centrifugation. The time interval, ΔT, between agitations varied according to the experiment.

4.1.2 Results

(a) The Effect of Agitation of Exponentially Growing LE Cultures

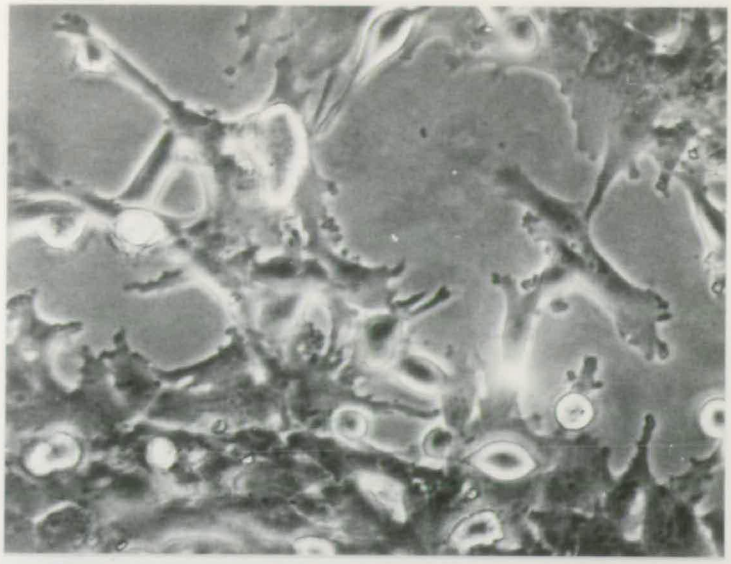
Lens epithelia were dissociated, seeded and grown in flasks as previously described. Photographs of cultures were taken before and after agitation of the flasks (Plate 4.1). The agitation applied to the flask was sufficient to release all loosely attached cells. The cells in the supernatant were counted and scored as either mitotic or interphase. The average yield obtained was 1.63×10^5 cells for every 10^6 cells in the flask. The average mitotic fraction obtained was 0.57. This percentage increased with successive agitations of the same monolayer cultures with a 15 minute interval between them (Fig. 4.1). The mitotic fraction increased from 0.57 in the first

PLATE 4.1

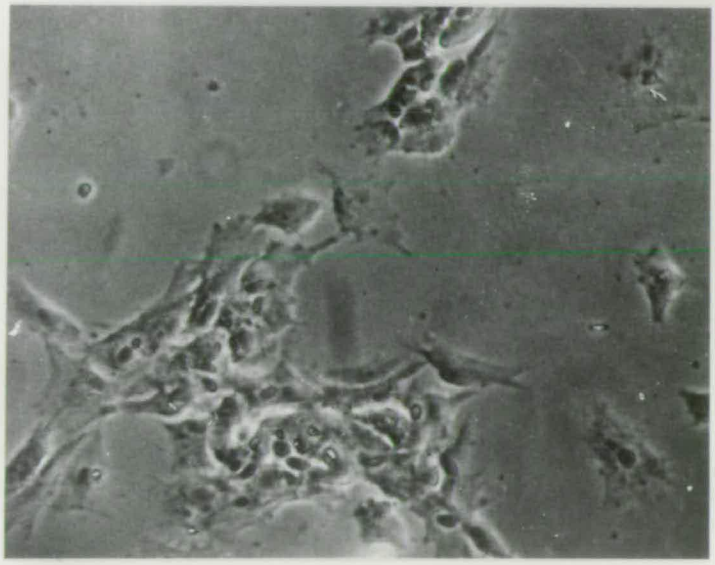
The effect of agitation of exponentially growing lens epithelial cultures (strain Hy-2).

(A) shows a culture immediately prior to agitation. Mitotic cells can be seen loosely attached to the monolayer.

(B) shows the same culture immediately after agitation of the flask, removal of the medium, and followed by replacement by fresh medium.



A



B

PLATE 4.1

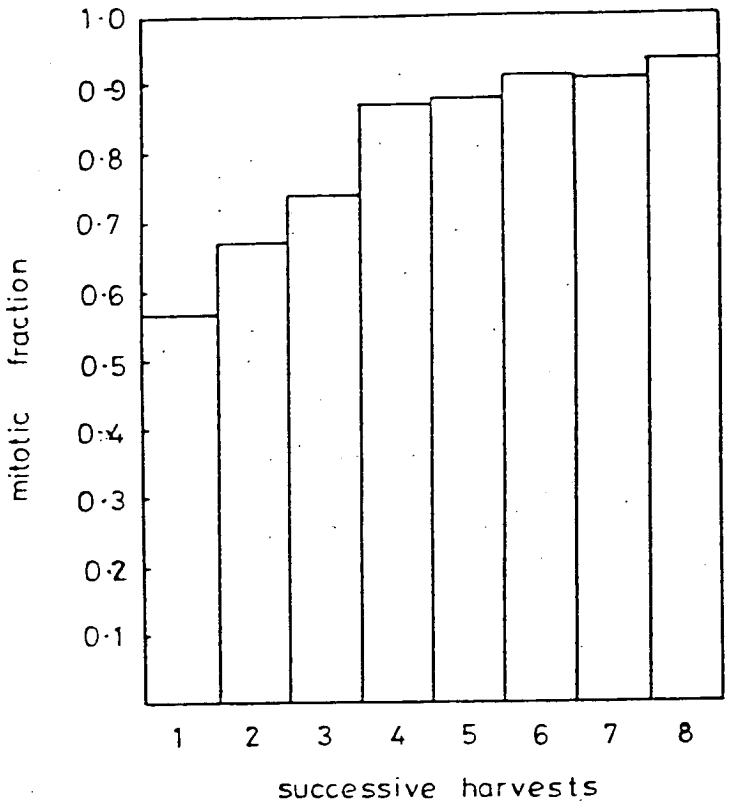


Figure 4.1

Mitotic cells were scored after each harvest from the same monolayer culture at intervals of 15 minutes. 8 successive harvests were scored. The mitotic cells were expressed as a ratio of the total cells present:- the mitotic fraction. The mitotic fraction is plotted against each successive harvest.

selection to 0.92 in the sixth selection. The yield, however, decreased to 2.9×10^4 cells for every 10^6 cells in the flask.

(b) Determination of the ΔT Interval Between Harvests to Optimise the Mitotic Fraction

Monolayers of LE cells, 6 days after seeding, were successively agitated four times and the supernatant with the detached cells discarded each time. The time interval (ΔT) between agitations ranged from 5-40 minutes. The fifth harvest was scored for mitotic cells. The results are plotted in Figure 4.2. The highest mitotic fractions were obtained after harvesting at 10 minute and 15 minute intervals, and were 0.91 and 0.94 respectively.

(c) Determination of the Age Distribution of Selected Cells

Populations of LE cells were selected as in (b) using different values for the ΔT interval between successive agitations. Cells were isolated only after 4 cycles had been discarded. These populations were concentrated and seeded as suspension cultures and the mitotic fraction determined as a function of time (Fig. 4.3). In all cases the mitotic fraction fell rapidly irrespective of the initial mitotic fraction, and approximated to zero at 30 minutes after selection.

(d) Maximisation of Yield of Synchronised Cells

(i) Pooling of Successive Harvests

Successive harvests of selected LE cells were pooled. Four successive harvests with the optimum ΔT interval of 15 minutes determined in Section (b), resulted in a population size of 1.2×10^5 cells but with an age distribution of 90 minutes.

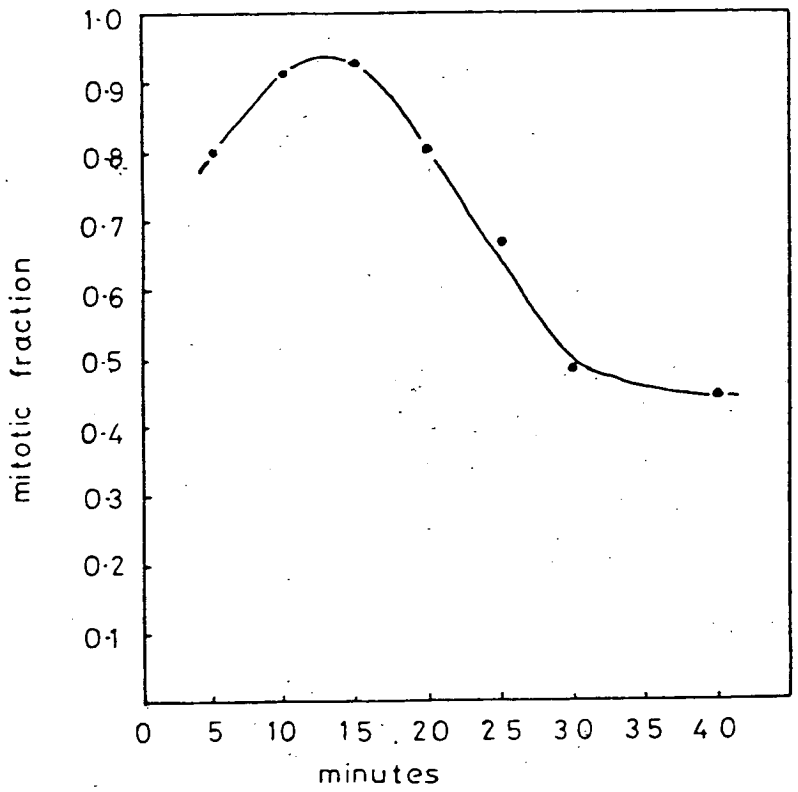


Figure 4.2

Determination of ΔT interval between harvests to optimise the mitotic fraction. The 5th successive harvest of each culture, with intervals between them ranging from 5-40 minutes were scored for mitotic cells. The mitotic fraction is plotted against the time interval between successive harvests.

FIGURE 4.3

LE cells were seeded as suspension cultures after mitotic selection. Populations were harvested with different ΔT intervals ranging from 5-40 minutes. The cells in each culture were monitored by photographs at six minute intervals. The mitotic fraction was determined by scoring from the photographs, and plotted against time.

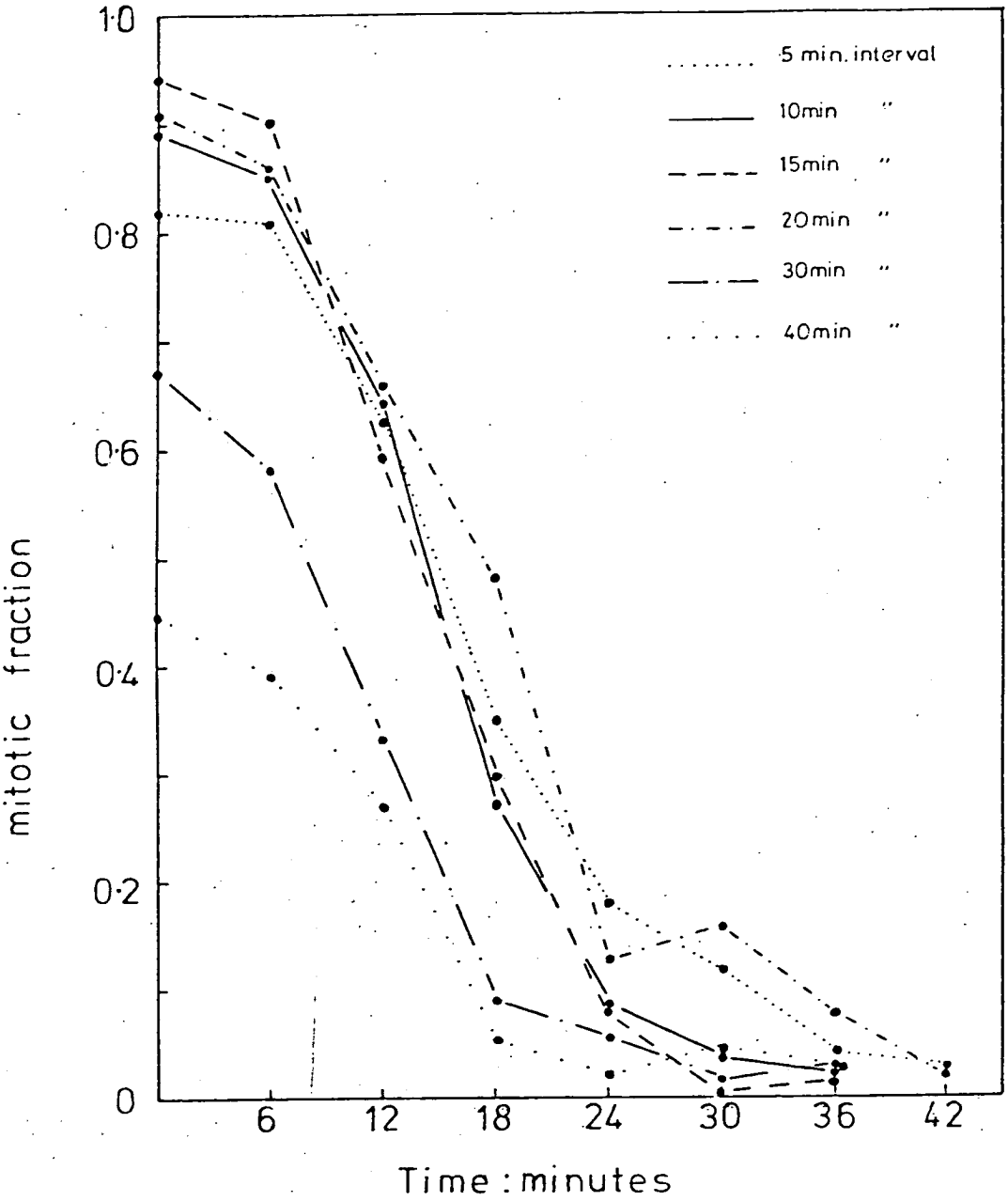

1·0
0·8
0·6
0·4
0·2
0
mitotic fraction
6
12
18
24
30
36
42
Time : minutes
5 min. interval
10min "
15min "
20min "
30min "
40min "

Figure 4.3

(ii) Effect of Cold on Advance of Mitosis.

Selected populations of mitotic LE cells were seeded as a suspension culture as previously described. They were cooled to 4°C for 2 hours and the mitotic fraction was scored at intervals. The results are plotted in Figure 4.4. The low temperature reduced the number of cells reaching cell division. After 2 hours the mitotic fraction was 0.71 after an initial fraction of 0.93.

(iii) Colcemid Treatment as a Means to Increase Yield

Colcemid (demecolcine) was used in an attempt to increase the yield of mitotic cells from monolayer LE cells (Stubblefield et al., 1967).

Different concentrations of colcemid, ranging from 0.5µg/ml - 0.001µg/ml were used to determine the minimal amount which would accumulate metaphase cells in exponentially growing cultures. 6 day old LE cultures were dosed for 2 hours and scored at the end of the period as having either an increase or no change in the frequency of mitotic cells as compared to an untreated control. 0.01µg/ml was the minimal concentration which would increase the number of metaphase cells over a 2 hour treatment period. Plate 4.2 shows (A) an untreated control compared with (B) a culture treated with 0.01µg colcemid/ml. (C) is a photograph of the same culture, one hour later showing the reversibility of the colcemid treatment. The large accumulation of mitotic cells visible in (B) have completed mitosis and have reattached to the culture dish.

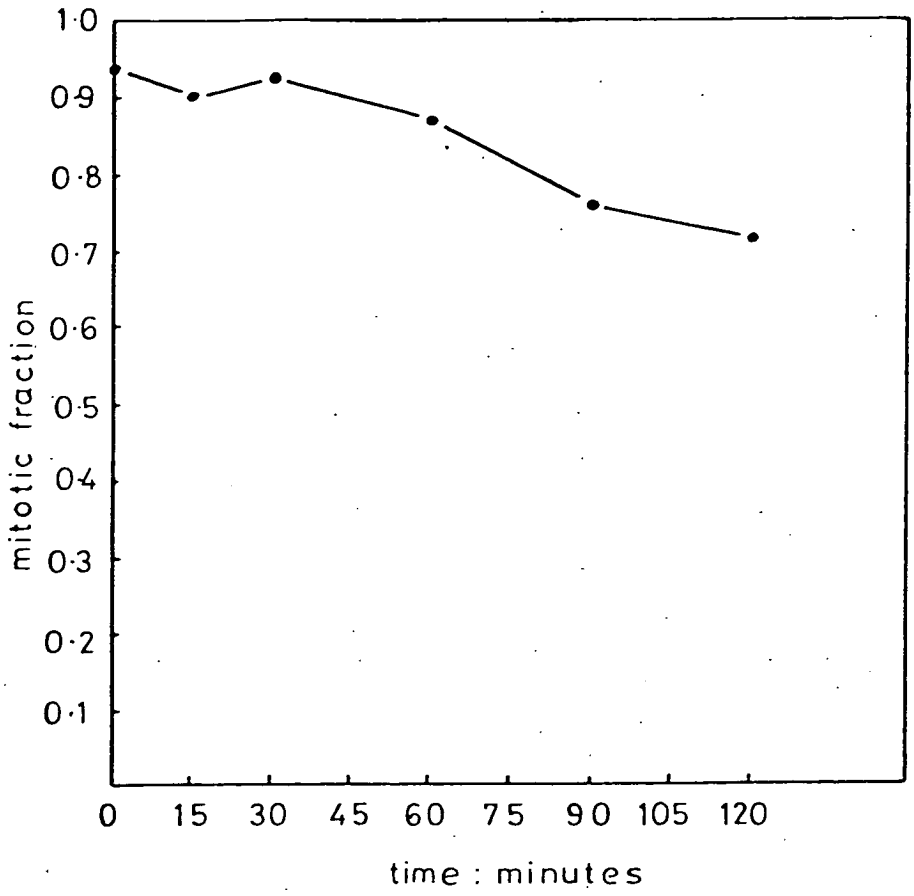


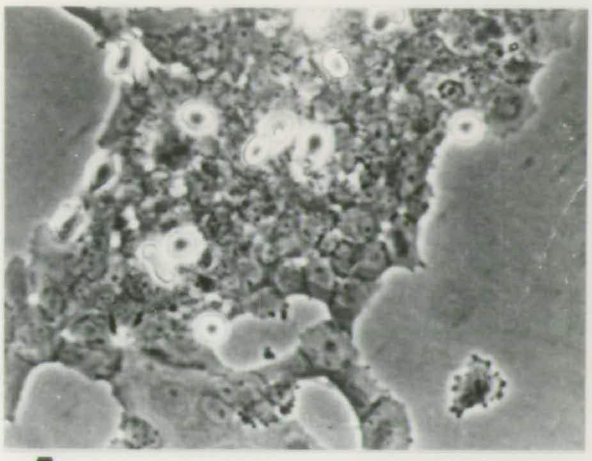
Figure 4.4

The effect of cold on the advance of mitosis. Selected mitotic cells were cooled to 4°C immediately after harvesting, and the mitotic fraction was scored at intervals. The mitotic fraction is plotted against time.

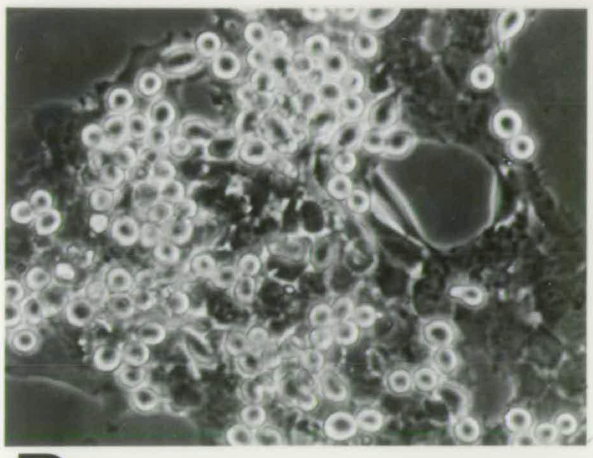
PLATE 4.2

Photographs of LE cultures.

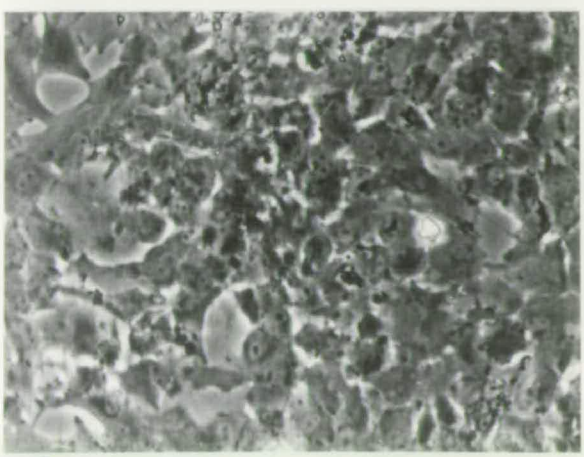
(A) An untreated control compared with (B), a culture treated with 0.01μg colcemid/ml. (C) is a photograph of the same culture as (B), one hour later after the removal of colcemid.



A



B



C

PLATE 4.2

Protein Analyses of Colcemid Treated Cultures

6 day old LE cultures of strains Hy-2 and N were treated for 2 hours with 0.01µg colcemid determined in the previous section as the minimum concentration which increased the number of mitotic cells. The cultures were washed and subsequently grown in the presence of normal medium. Control and treated cultures were labelled with ^{3}H-mixed amino acids for three hours at various intervals after treatment: immediately; 8 hours; 24 hours; 3 days and 6 days later. The water soluble protein fractions from the cultures were run on IEF gels and fluorographed as described elsewhere. Figure 4.5 shows densitometer traces of the radioactively labelled polypeptides. No significant differences were detectable in profiles of the treated samples (A) when compared to the untreated controls (B).

Effect of Colcemid on Yield of Mitotic Cells

A 2 hour treatment of 0.01µg/ml colcemid resulted in a mitotic fraction of 0.15, composed predominantly of metaphase cells. This compared with a mitotic fraction of 0.06 from untreated cultures, which also contained interphase cells (see Section 4A.a)

4.1.3 Discussion

The method of mitotic selection as a means of obtaining synchronous cells, described by Terasima and Tolmach (1961 and 1963) can be applied successfully to chick lens epithelium. However, two criteria must be fulfilled in order to obtain a population of synchronous cells suitable for further experimentation.

(i) cells with as narrow an age limit as possible, and

(ii) as large a yield as possible.

The optimisation of conditions for the selection procedure in order to fulfil these criteria was the object of the experiments outlined.

FIGURE 4.5

The effect of 0.01μg/ml colcemid treatment on protein synthesis. LE cultures were treated with 0.01μg/ml colcemid for 2 hours, followed by a 3 hour pulse label of ^{3}H-mixed amino acids. A) immediately, B) 8 hours, C) 24 hours, D) 3 days and E) 6 days later. Untreated controls were labelled simultaneously in each group. Water soluble proteins were run on IEF gels and the fluorographs exposed for 26 days. Densitometer traces of the radioactively labelled proteins are shown above for each class. The upper trace in each, represents the colcemid treated. The lower trace represents the control.

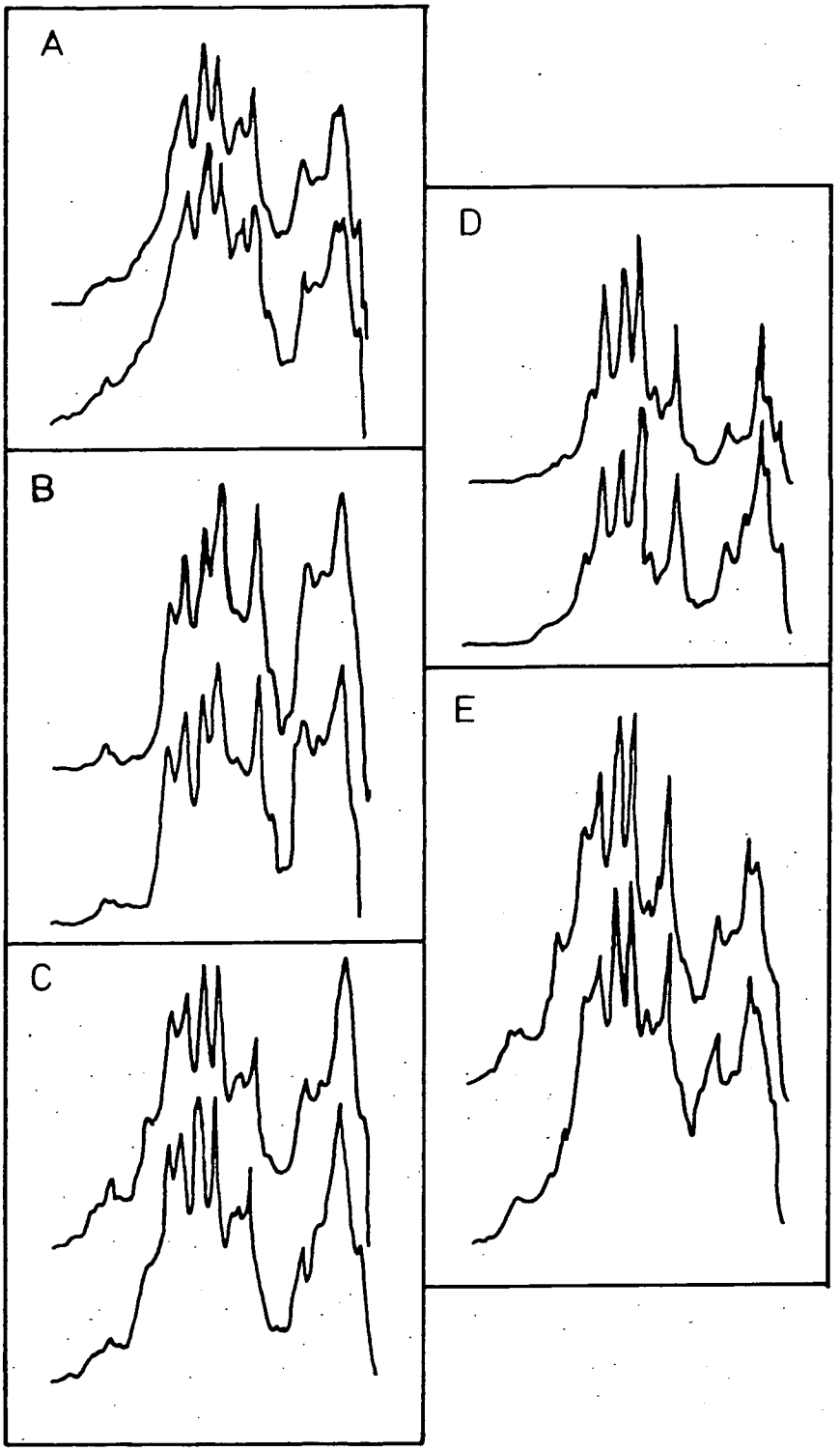


Figure 4.5

Monolayers are initially unsuitable for mitotic selection because of loosely attached early G_1 cells, as well as other floating cells and debris. The contaminants may be removed by a series of preliminary agitations. Figure 4.1 shows the effect on the mitotic fraction with each successive harvest. The mitotic fraction increases up to the fifth harvest resulting in a narrower age-spread of the selected cell population. However the yield is decreased when compared to the first harvest.

The time interval between harvesting is an important parameter. If T_R represents the age of release of a cell from the culture dish as it enters mitosis, and T_A represents the age of reattachment of the daughter cells in early G_1, then the ΔT between T_R and T_A is of considerable importance. If the time interval between harvesting (ΔT) is greater than $\Delta T_A - T_R$, each selection will repeatedly yield mitotic plus early G_1 cells. If ΔT is sufficiently small, i.e. smaller than $\Delta T_A - T_R$, then selection yields mitotic cells within definable boundaries. If, however, ΔT is too short, the yield will be minimal since not many more cells will have reached T_R. Figure 4.2 shows the optimum interval between harvesting as 15 minutes, with a mitotic fraction of 0.94. The first five harvests were subsequently discarded and only the sixth used for experimental purposes. Figure 4.3 shows the decline in the mitotic fraction with time, irrespective of the initial mitotic fraction. It approximated to zero at about 30 minutes after selection. This suggests that the time required for cells in mitosis from the point of detachment to reach division is approximately 30 minutes. This represents the age distribution of the newly selected population when the mitotic fraction is 1.0.

In order to increase the population size, harvesting can be repeated at 15 minute intervals but at the expense of an equal broadening of the initial population width (Peterson et al., 1969) Figure 4.5 shows that a low temperature slows the progress of cells through mitosis to cell division, such that after two hours the mitotic fraction was 74% of the original. After one hour the mitotic fraction was 93% of the original and therefore would not seriously affect the population width. This could allow four harvests to be successively pooled and used together for an experiment. The total yield, however, would still be only about 1-2 x 10^5 cells, which is insufficient for biochemical analysis.

Colcemid has been used to increase the yield of mitotic selection by accumulating mitotic cells (e.g. Stubblefield, Klevecz and Deaven, 1967). However, in using biochemical interference of the cells there is always a danger of upsetting the biochemical balance of the culture.

The minimal concentration which increased the accumulation of metaphase cells by disruption of the microtubule assembly in these lens epithelial cells was determined as 0.01μg/ml of colcemid for 2 hours. Analysis of the crystallins synthesised both immediately after colcemid treatment and at intervals up to 6 days after treatment showed no detectable differences in the polypeptide profiles when compared to untreated controls of the same age. Morphological differences were not detected with no observed change in the onset of lentoid development. Plate 4.2 shows the recovery of the microtubule assembly in the form of mitotic division and passage into G_1 as soon as colcemid is removed.

This however does not eliminate possibilities of other side effects on the cells as a result of colcemid administration. Beebe, Feagans, Blanchette-Mackie and Nau (1979), have recently shown that colchicine, at concentrations lower than those that dissociate microtubules, blocks cell elongation and the associated increase in cell volume in embryonic chick lens epithelial cell cultures. It did not however interfere with δ crystallin synthesis, or the accumulation of δ crystallin messenger RNA, events associated with lens fibre differentiation.(Beebe and Piatogorsky, 1977). It may also be a side effect specific to colchicine and not to other microtubule depolymerisation drugs such as colcemid (demecolcine). The use of colcemid succeeded in significantly increasing the yield of synchronous cells from 5.7% to 14.9% while still maintaining a narrow age limit throughout the population. Colcemid blocks cells in metaphase, a stage which therefore accumulates. Cells at a later stage in mitosis when colcemid treatment commences continue through the cell cycle as normal. No late mitotic or early G_1 cells are present after 2 hours. Preliminary washing of the monolayer cultures to eliminate early G_1 cells is therefore no longer necessary.

4.1.4 Conclusion

In light of the data presented, mitotic selection with the aid of colcemid to increase the yield is the simplest and most effective way of obtaining a synchronous culture. A narrow age band of cells is obtained in this way. Although synchronous cell numbers are increased by the use of colcemid, the number obtained is still severely limiting for studies involving aliquots at different

cell cycle stages. This problem is enhanced by the restriction of initial cell numbers for primary cultures, imposed by the nature of the tissue under study.

4.2 Cell Cycle Arrest

4.2.1 Method

LE cultures from strain Hy-2 were grown in the presence of normal 6% FCS medium for 5 days as previously described. The cultures were washed and treated with medium plus 0.5% FCS for a period of up to 60 hours. Normal 6% FCS medium was then replaced. Control cultures were treated with 6% FCS medium throughout the experiment.

4.2.2 Results

(a) The Effect of FCS Depletion on Exponentially Growing LE Cells

Cell counts were made at intervals throughout the culture period, the method of which is described in Chapter 3. The growth curves of the FCS depleted cultures and of the controls are plotted in Figure 4.6. The depletion of FCS from the cultures for sixty hours resulted in a lag in the growth of cells. The reintroduction of 6% FCS into the culture medium caused cell growth to resume at the same rate as in the control, but with a smaller total number of cells per culture plate.

An FCS deficient period of 36 hours was found to be sufficient to result in the complete absence of mitotic cells in the culture.

(b) Determination of Phase of the Cell Cycle in which Arrest Occurs

(i) 5 day old LE cultures were treated for 36 hours with 0.5% FCS medium followed by the addition of normal 6% FCS medium (Chang et al., 1977). The cultures were then continuously labelled with 0.5μCi/ml ^{3}H-thymidine and harvested at intervals thereafter, up to 24 hours. The

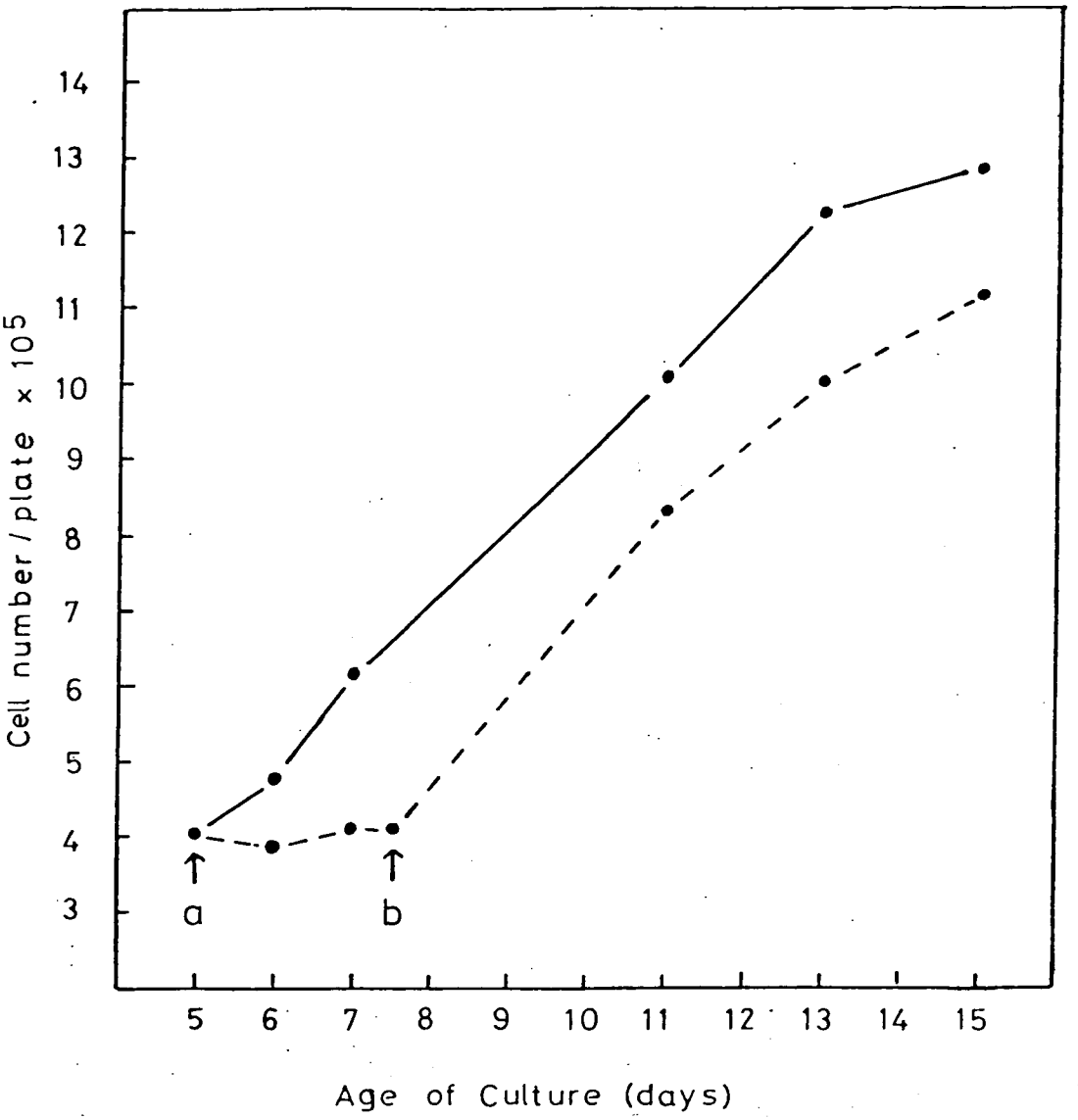


Figure 4.6

Growth curves of FCS depleted LE cultures (---) and of the controls (——). Each count was carried out on triplicate culture plates. The above represents the average of three separate experiments. Arrow (a) denotes the time of removal of the FCS. Arrow (b) denotes the readdition of FCS to the culture. The control was maintained with a constant 6% FCS throughout.

TCA precipitable radioactivity was expressed as a ratio of the protein concentration of each sample. The results are plotted against time in Figure 4.7. After a short lag period the c.p.m./protein concentration increased rapidly until 15 hours after restimulation by addition of FCS.

(ii) 7 day LE cultures were harvested after 36 hours in FCS deficient medium. Control cultures were harvested at the same time after being grown continuously in 6% FCS medium. The amount of DNA was estimated in both cases. The relative amount of DNA/cell in the arrested, FCS reduced cultures was 62% that of normal asynchronous cultures.

4.2.3 Discussion

Deficiency in certain medium constituents normally present for cell growth to be maintained in culture results in the arrest of growth (Pardee et al., 1978). This permits the synchronisation of cell cultures by a cell cycle block, followed by a restimulation of growth due to the reintroduction of the essential growth mediating compound (Section 1.1).

Figure 4.6 shows the effect of 0.5% FCS on the growth rate of LE cells for 60 hours instead of 6% FCS medium in the case of the normal control. A period of no growth is evident when the FCS concentration is reduced but which is completely reversible on the reintroduction of 6% FCS to the arrested cells in the culture. The rate of growth after arrest is similar to that of the control as judged by the gradient of the growth curve, although there are fewer cells in the culture plate. A small concentration of FCS provides any necessary

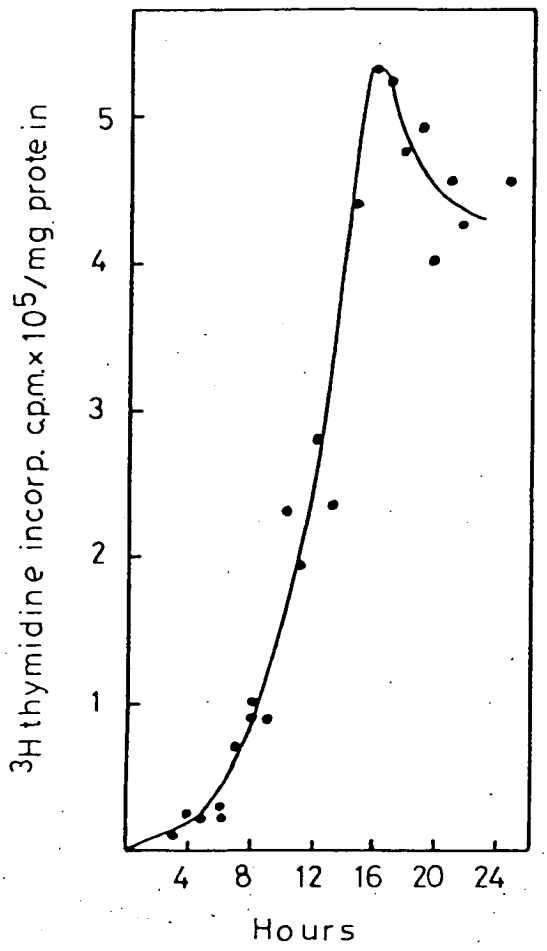


Figure 4.7

^{3}H-thymidine incorporation into LE cells after FCS deprivation followed by its readdition 36 hours later. ^{3}H-thymidine incorporation/mg protein is plotted against the hours after the readdition of FCS.

factors for the normal maintenance of the cells, but there is insufficient for promotion into the cell cycle. This serves to minimise any detrimental effect on the cells during this period. No toxic effects from this method have been reported (e.g. Chang et al., 1977).

The question then arises concerning the phase of the cell cycle in which arrest occurs. This is critical when intending the cells for synchronised cell culture analyses, as it is necessary to know where the cells are in the cell cycle at a particular time. Cells generally become arrested at the G_1/G_0 boundary by, for example, isoleucine, glutamine and FCS deprivation (Pardee et al., 1978). This point of arrest has been termed the restriction point 'R' located in mid-G_1. It is regarded as the critical switching point at which cells can shift back and forth from the cycling to the non-cycling state. However cells can also arrest in G_2. Gelfant (1977) quoted numerous examples of various cell types which undergo G_2 arrest when quiescent, being restimulated by a variety of factors. He suggests that a G_2 block may be more common than originally thought as judged by his reinvestigation into numerous cell types. G_1 and G_2 blocked cells can be distinguished by their patterns of uptake of ^{3}H-thymidine, the timing of their mitotic activity and by the amount of DNA per cell. In autoradiographs prepared at intervals after ^{3}H-thymidine labelling of arrested-restimulated cells no mitotic cells were visible during the first ten hours. A peak of mitosis would be expected almost immediately after restimulation if a G_2 block had occurred. Figure 4.7 shows a pattern of thymidine incorporation which suggests a G_0 arrest. A short lag period is evident before a rapid increase in uptake of ^{3}H-thymidine after about 8 hours. ^{3}H-thymidine incorporation would be expected to increase after a longer lag period if a G_2 block

was present, as the cells would need to traverse M and G_1. This suggests a G_0 blocked population of major size, there being some range in the time of completion of G_1 after arrest till S-phase commences. A lag period between the stimulation from G_0 to the commencement of S-phase fits into the model suggested by Pardee (1974) already mentioned, of an 'R' point mid-way in G_1. Cells therefore still have to traverse part of G_1 after leaving G_0 before reaching S phase. The variability in the time taken for cells to reach S phase and the resultant sigmoid curve in Figure 4.7 can be accounted for by the transition probability model of Smith and Martin (1973). Their model portrays cell replication as a random event resulting from the probability of cells leaving G_0 (or the 'A' state to use their own terminology). Environmental factors such as serum influence this transition probability by lowering the threshold and permitting more cells to pass back into the cycle (or 'B' state). Because it depends on a probabilistic event all cells cannot be expected to enter the 'B' state simultaneously.

A low background level of DNA synthesis was present in arrested cultures. This was evident in autoradiographs of arrested cultures where a few cells were labelled with ^{3}H-thymidine. This accounted for only 1-2% of the total cell population. It was pointed out in Chapter 2.1 that absolute synchrony is difficult to achieve. Section 5.2 demonstrates that considerable synchrony is achieved by this method.

DNA estimations of arrested cultures showed that the DNA content per cell was 38% less than the asynchronous control. This shows that more cells are in the prereplicative G_1 phase than in the asynchronous control. More DNA per cell would be expected if a G_2 block was

present when compared to the controls. This verifies that the depletion of serum from the medium of strain Hy-2 LE cells results in a G_1/G_0 block in at least the majority of cells.

4.2.4 Conclusion.

The above data suggest that cell cycle arrest by FCS deprivation followed by restimulation into the cell cycle is an effective method for obtaining a synchronous culture of LE cells. It has the advantage of resulting in large numbers of cells in synchrony, with the minimum of upset to the cells themselves.

CHAPTER 5. RESULTS

5.1 DNA and RNA Synthesis in the Epithelium of the Lens of the Chick

5.1.1 ^{3}H-Thymidine Incorporation

When freshly excised chick lenses were incubated in medium containing ^{3}H-thymidine for one hour, the level of incorporation of radioactivity into DNA varied according to the time of day. The patterns of incorporation were reproducible in different batches of chicks and were consistent in three independent experiments under similar conditions (Fig. 5.1). The fluctuations of incorporation observed over a 24 hour period were greater than the standard deviations of the observations on the individual lenses made at one time. Comparisons of lenses from different genetic strains of the chick showed that the pattern of diurnal variation was strain specific. The number of cells engaged in DNA synthesis (as judged by the amplitude of the peaks) and the synchrony (as confirmed by autoradiography, Plate 5.1), are both greater in the strains with hyperplasia of the lens epithelium (Hy-1 and Hy-2) than in lenses of more normal morphology (N). The statistical significance of the differences between the maxima and minima were evaluated by Student's t-test. All three peaks of incorporation of strain Hy-2 are statistically highly significant within a 24 hour period. In strain Hy-1 4 peaks are statistically significant. Strain N also shows 4 peaks. Because of the limited number of observations within a 24 hour period, and because each observation is of one hour duration, the precise times of maxima and minima of incorporation can only be estimated by interpolation. In some instances, additional observations have been interpolated even although they were from a single experiment. These have no standard deviations but represent the mean of at least 8 lenses.

FIGURE 5.1

Plot of the mean values and standard deviation of ^{3}H-thymidine incorporation of groups of freshly explanted lenses of the three strains of day-old chick (N, Hy-1 and Hy-2) at intervals over a 24-hour period. Three independent experiments are represented. Mean values of disintegrations/minute/lens are plotted at the time of commencement of pulse labelling and therefore correspond to the following hour. The length of the bar represents twice the standard deviation. Additional observations have been interpolated even though they were from a single experiment. These have no standard deviations but represent the mean of at least 8 lenses.

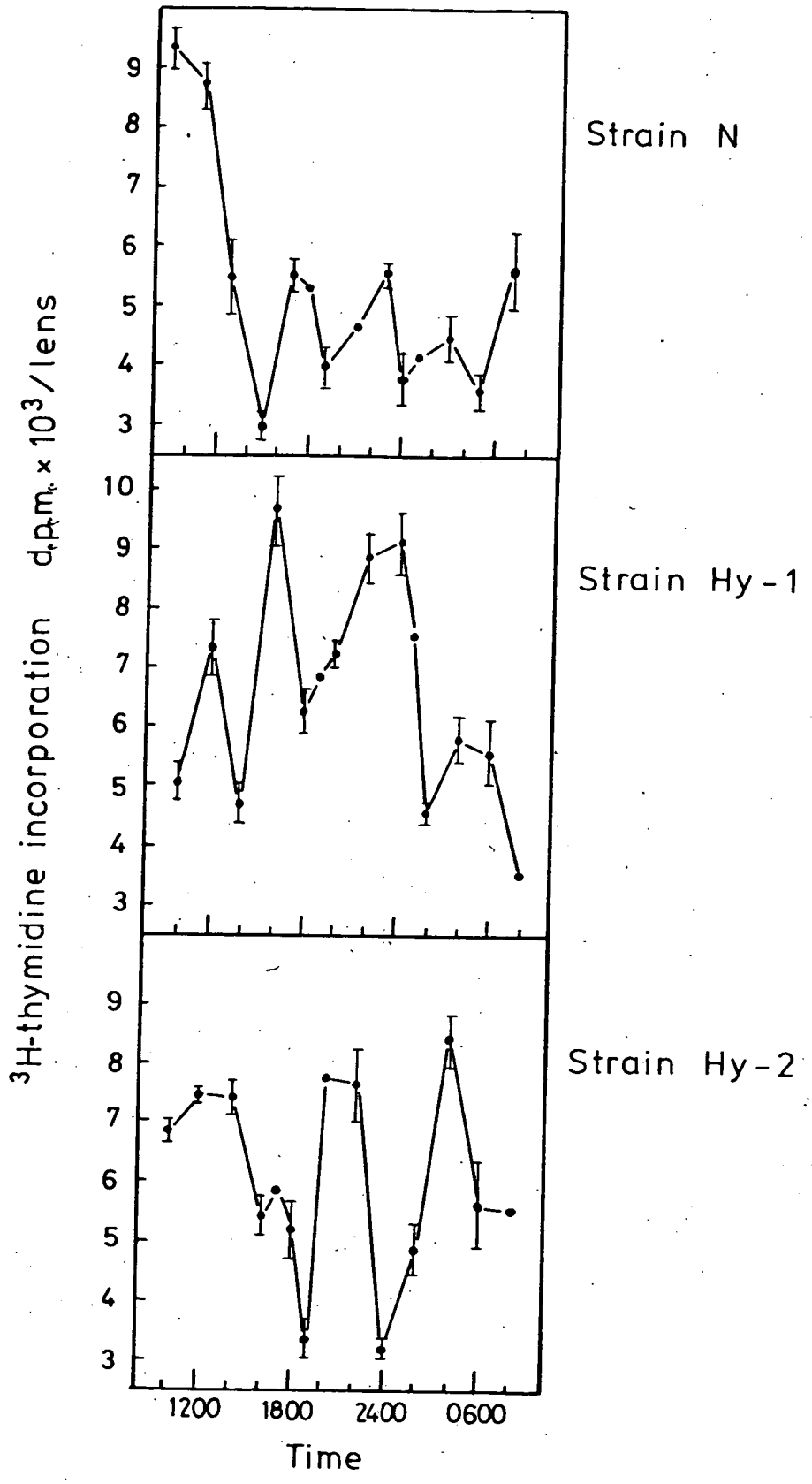

Strain N
Strain Hy-1
Strain Hy-2
^{3}H-thymidine incorporation d.p.m. x 10^3/lens
9
8
7
6
5
4
3
10
9
8
7
6
5
4
3
9
8
7
6
5
4
3
1200
1800
2400
0600
Time

Figure 5.1

PLATE 5.1

Autoradiographs showing incorporation of ^{3}H-thymidine in day old strains of Hy-1, Hy-2 and N chick lenses. Freshly explanted lenses were labelled for one hour at varying times of day, embedded sectioned, stained and then dipped in photographic emulsion. They were exposed for 4 days.

A Strain Hy-1 lens labelled between 23.30 - 00.30, a period of high level incorporation (see Fig. 5.1).

B Strain Hy-1 lens labelled between 14.10 and 15.10, a low level of thymidine incorporation (see Fig. 5.1).

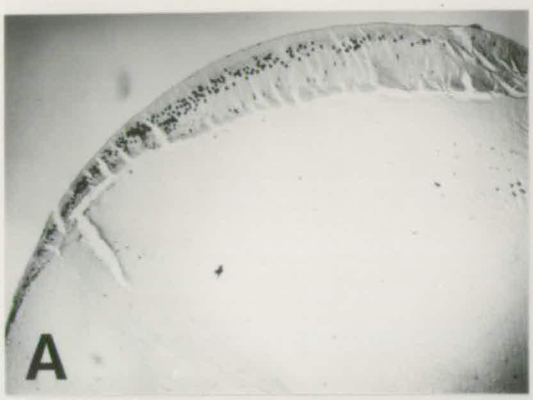
C Strain Hy-2 lens labelled between 12.30 - 13.30, a maximum incorporation period (see Fig. 5.1).

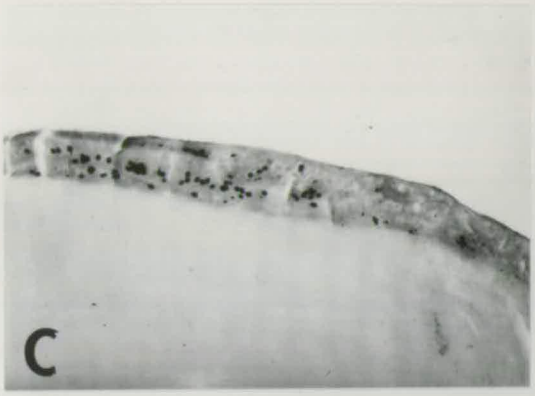
D Strain Hy-2 lens labelled between 23.55-00.55, a minimum incorporation period.

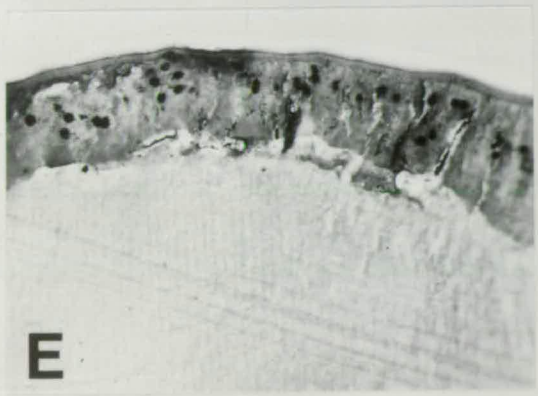
E Strain N lens labelled between 14.00-15.00, a high level of incorporation.

F Strain N lens labelled between 11.00-12.00, a minimal incorporation period.

G High power magnification showing labelled nuclei in an autoradiograph x 1000. Hy-1 lens.







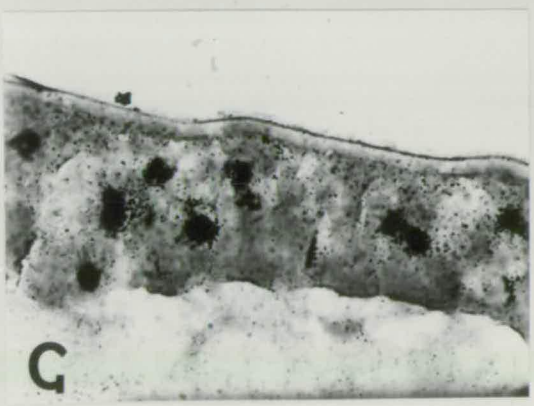


PLATE 5.1

5.1.2 ^{14}C-Uridine Incorporation

Freshly explanted lenses incubated in medium containing ^{14}C-uridine for one hour also exhibited partial synchrony and a diurnal rhythm in incorporation of the RNA precursor which was characteristic for each strain (Fig. 5.2). As in the thymidine incorporation, synchrony is greater in the strains with hyperplasia of the lens epithelium than in the control strain (N).

A definite relationship exists between thymidine and uridine uptake of the same lenses. The frequency of the peaks of incorporation are similar in both profiles, particularly Hy-1 and Hy-2. In these profiles, however, the uridine maxima appear to occur slightly later than the thymidine maxima.

5.1.3 Autoradiography of ^{3}H-Thymidine labelled lenses.

Freshly excised lenses of the 3 strains were incubated in medium containing ^{3}H-thymidine. The times of labelling were chosen from the thymidine incorporation profiles (Fig. 5.1) to correspond to a maximum and a minimum for each strain. Autoradiography of these lenses has confirmed that only the lens epithelium incorporates thymidine during a one hour pulse in all 3 strains. The results are consistent with the incorporation data in that the level of precursor uptake varies with time. Lenses labelled at a time coincident with maximal thymidine incorporation, as judged by a previous experiment showed a greater number of labelled nuclei in autoradiographed serial sections than lenses labelled at a time corresponding to a minimal level (Plate 5.1) in all 3 strains. At least 10 lenses of each strain labelled at each time point were examined in this way.

Plate 5.1 (A-G) shows photographs of transverse sections taken

FIGURE 5.2

Plot of the mean and standard deviation of ^{14}C-uridine incorporation of groups of freshly explanted lenses of day old chicks over a 24-hour period. The graphs represent the averaged data of 3 independent experiments. Each lens was pulse labelled for one hour. Mean values of d.p.m./lens are plotted at the time of commencement of pulse-labelling and correspond to the following hour. The length of the bar represents twice the standard deviation.

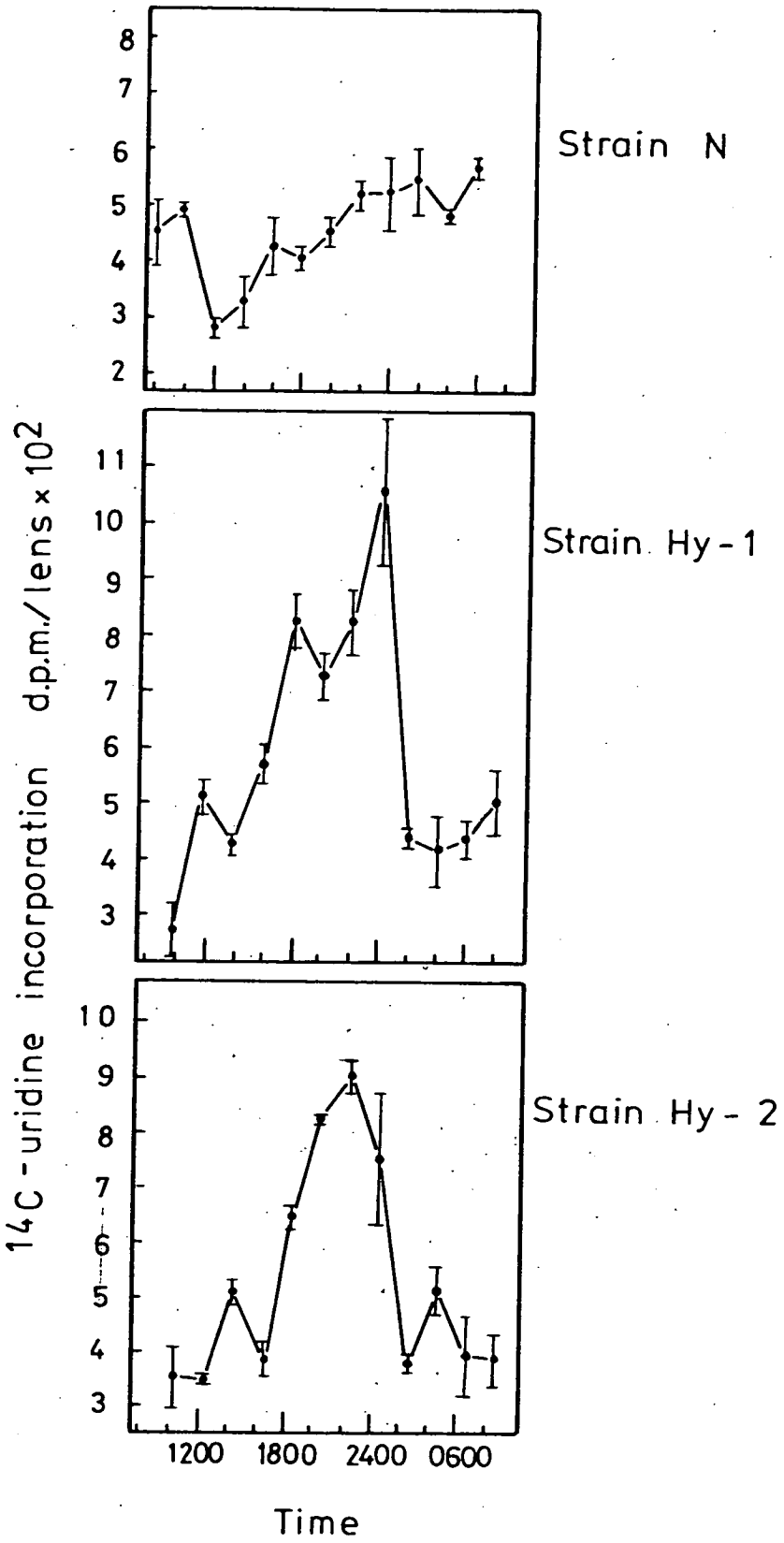

Strain N
Strain Hy-1
Strain Hy-2
14C-uridine incorporation d.p.m./lens x 10²
1200
1800
2400
0600
Time

Figure 5.2

from similar positions in lenses from the different chick strains. A-D show the multi-layered nature of the epithelium in strain Hy-1 and Hy-2 in contrast to the monolayered epithelium in strain N (E and F). Judging from examination of serial sections of lenses from the Hy-1 and Hy-2 strains, the variability observed between the lenses at any one time point in uptake of thymidine, appeared to be at least partly attributable to the amount of multi-layering of the epithelium. Of the lenses examined within each strain, some showed multi-layering over the whole anterior face of the lens while others were only multi-layered in the peripheral regions. At the times of maximum incorporation of ^{3}H-thymidine in strains Hy-1 and Hy-2, the central multi-layered epithelium exhibited the highest level of nuclei labelling. However, in those lenses exhibiting a single layered epithelium across this central region, the highest level of ^{3}H-thymidine incorporation was in the multi-layered epithelium immediately adjacent to this. Labelled nuclei were not observed in single layered central epithelia in these strains. At the times of minimal incorporation in these 2 strains (judged as previously noted) the number of labelled nuclei observed was considerably less. Furthermore the distribution pattern of labelled nuclei was different at this time in comparison to the time of maximal incorporation of ^{3}H-thymidine. This reduced number of labelled nuclei was found in the peripheral regions of the epithelium, around the whole circumference of the lens.

At the time of maximal ^{3}H-thymidine incorporation in strain N, labelled nuclei were evident across the whole of the epithelium although fewer were observed in the central region. Labelled nuclei were found only in the peripheral epithelium at the time coincident with the minimum level of ^{3}H-thymidine incorporation.

Plate 5.1 (G) shows a high power photograph of a section through labelled, autoradiographed Hy-1 lens epithelium. The labelled nuclei can be seen to consist of a high concentration of grains. A background level of grains is evident.

In regions of the epithelium where labelled nuclei were not evident, mitotic indices were visible. Their distribution pattern showed similarities to the distribution of labelled nuclei but at a different time. In strains Hy-1 and Hy-2 mitotic cells were evident in the multi-layered central epithelium but were not found in single layered epithelium in this region. However, mitotic cells were evident in the multi-layers bordering this single layered central epithelium. This synchrony in mitosis has established that ^{3}H-thymidine incorporation profiles are a reflection of the mitosing epithelial cells.

5.1.4 ^{3}H-Thymidine Incorporation in Dissociated Lens Epithelial Cells

Lens epithelia were dissected from day old chicks of the Hy-1, Hy-2 and N strains. The epithelia from each strain were dissociated into single cells and seeded onto plastic petri dishes and incubated at 37°C in an atmosphere of 5% CO_2 in air. After 5 days of growth the plates were labelled with ^{3}H-thymidine for one hour at intervals, and the level of incorporation of radioactivity was measured by scintillation counting. Replica plates were dissociated with trypsin and the single cells counted with an haemocytometer. At least three separate plates were averaged. The level of incorporation for each strain was expressed as disintegrations per minute per 10^{4} cells (Fig. 5.3). The graphs represent an average of 3 independent experiments.

FIGURE 5.3

Plot of the mean values and standard deviation of ^{3}H-thymidine incorporation of lens epithelial cultures from 3 strains of chick over a period of 18 hours. Three independent experiments are averaged. The incorporation of radioactivity is expressed as d.p.m./10^{4} cells and is plotted at the time of commencement of a one hour pulse. The length of the bar represents twice the standard deviation.

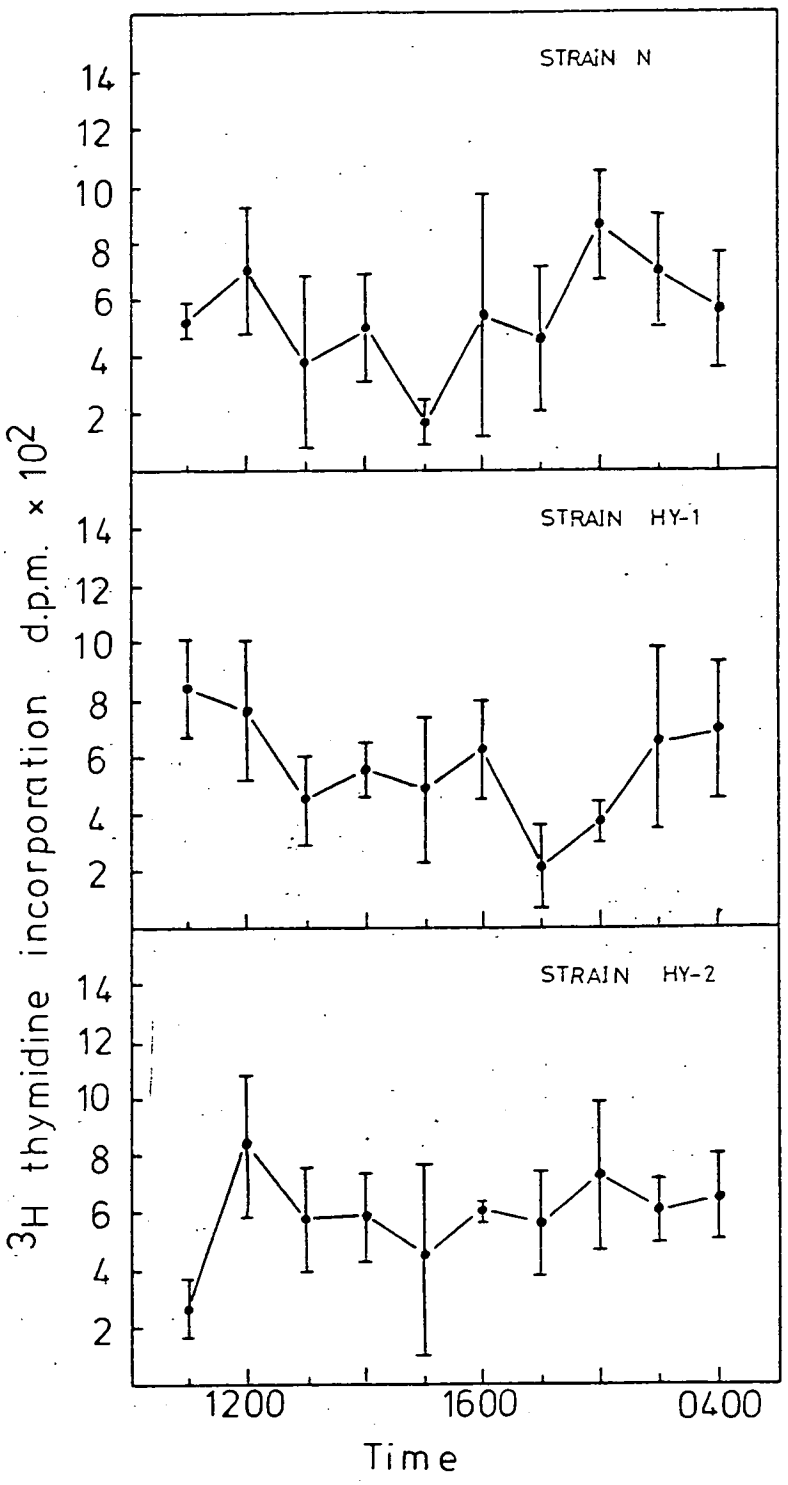

STRAIN N
STRAIN HY-1
STRAIN HY-2
14
12
10
8
6
4
2
^3H thymidine incorporation d.p.m. × 10^2
1200
1600
0400
Time

Figure 5.3

It was found that there was no significant pattern of thymidine incorporation in any of the strains over a period of 18 hours, in contrast tc the previous data using whole freshly explanted lenses.

These results suggest that other methods must be applied to lens epithelial cultures in vitro in order that synchronous cell cultures may be obtained.

5.2 Macromolecular Synthesis in Synchronised Cultures.

5.2.1 ^{3}H-Thymidine Incorporation After Synchronisation of L.E. Cells - Verification of Synchrony.

(i) Mitotic Selection Method.

Mitotic cells were harvested from colcemid treated Hy-2 cultures, L.E. counted, reseeded and aliquots labelled with ^{3}H-thymidine at intervals thereafter. The radioactivity incorporated was plotted against time (Fig. 5.4). The level of incorporation of radioactivity into DNA varied according to the time lapsed after mitotic selection. The data plotted represent two independent experiments. A steady rise in ^{3}H-thymidine incorporation was evident immediately after synchronisation. A maximal peak was reached at 6 hours. A steep decline in incorporation followed, reaching a minimum about 10-11 hours after mitotic selection. A second rise was evident in both experiments but did not coincide completely.

(ii) Cell Cycle Arrest Method

Hy-2 L.E. cultures were treated for 24 hours with FCS deficient medium to arrest growth, followed by the readdition of 6% FCS to the cultures. They were pulse labelled with ^{3}H-thymidine at varying intervals thereafter for one hour. Duplicate culture plates were

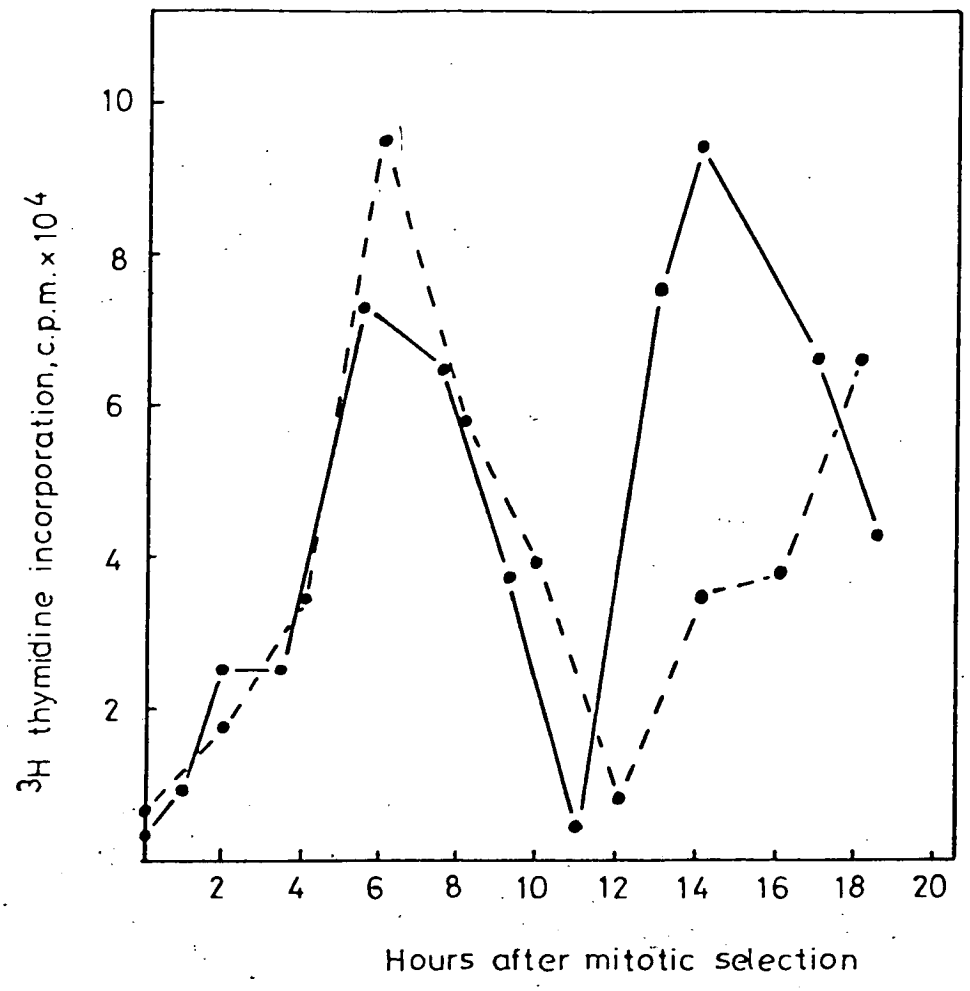


Figure 5.4

^{3}H-thymidine incorporation, after a 30 minute pulse, into LE cells plotted against the time after mitotic selection. Two separate experiments are represented. c.p.m. is expressed per 10^5 cells.

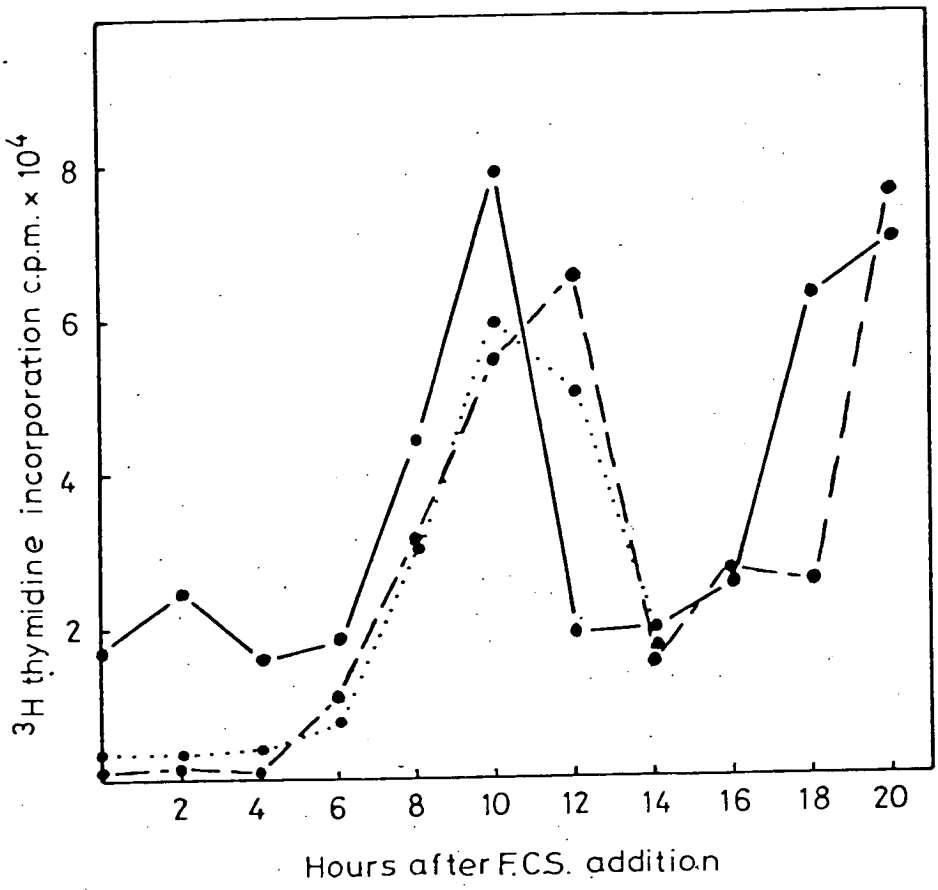


Figure 5.5

^{3}H-thymidine incorporation, after a 30 minute pulse, into LE cells from strain Hy-2 plotted against the time after FCS addition following cell cycle arrest. Three separate experiments are represented. c.p.m. is expressed per 10^5 cells.

trypsinised and the dissociated cells counted. Radioactivity incorporated per aliquot of cells was plotted against time. Figure 5.5 shows 3 independent experiments. After a lag period of 6 hours there was a steep rise in radioactive incorporation followed by a decline 4 hours later. In two experiments which were continued up to 20 hours, a second rise in radioactivity at 16 hours was evident.

5.2.2 ^{14}C-Uridine Incorporation

(i) Mitotic Selection Method

Aliquots of cells from synchronised cultures of strain Hy-2 were labelled with both ^{3}H-thymidine and ^{14}C-uridine at varying intervals. The ^{3}H-thymidine incorporation profile is part of Figure 5.4 and is plotted concurrently with the ^{14}C-uridine incorporation profile in Figure 5.6. There was a similar pattern of ^{14}C-uridine incorporation when compared to the ^{3}H-thymidine profile, but with less profound peaks. A single experiment is represented.

(ii) Cell Cycle Arrest Method

Hy-2 L.E. cultures, arrested for 24 hours with FCS deficient medium, were labelled with ^{3}H-thymidine and ^{14}C-uridine for one hour at intervals thereafter. Duplicate culture plates were trypsinised and the dissociated cells counted. Radioactivity incorporated per aliquot of cells was plotted against time for both ^{3}H and ^{14}C. The ^{3}H-thymidine incorporation profiles are part of Figure 5.5 and are plotted concurrently with the ^{14}C-uridine incorporation data for the same cultures (Fig. 5.7). The ^{14}C-uridine incorporation profiles were identical in two independent experiments. They follow a similar trend to that of the ^{3}H-thymidine incorporation data reaching a maximum after 10 hours followed by a decrease, a trend which was also observed

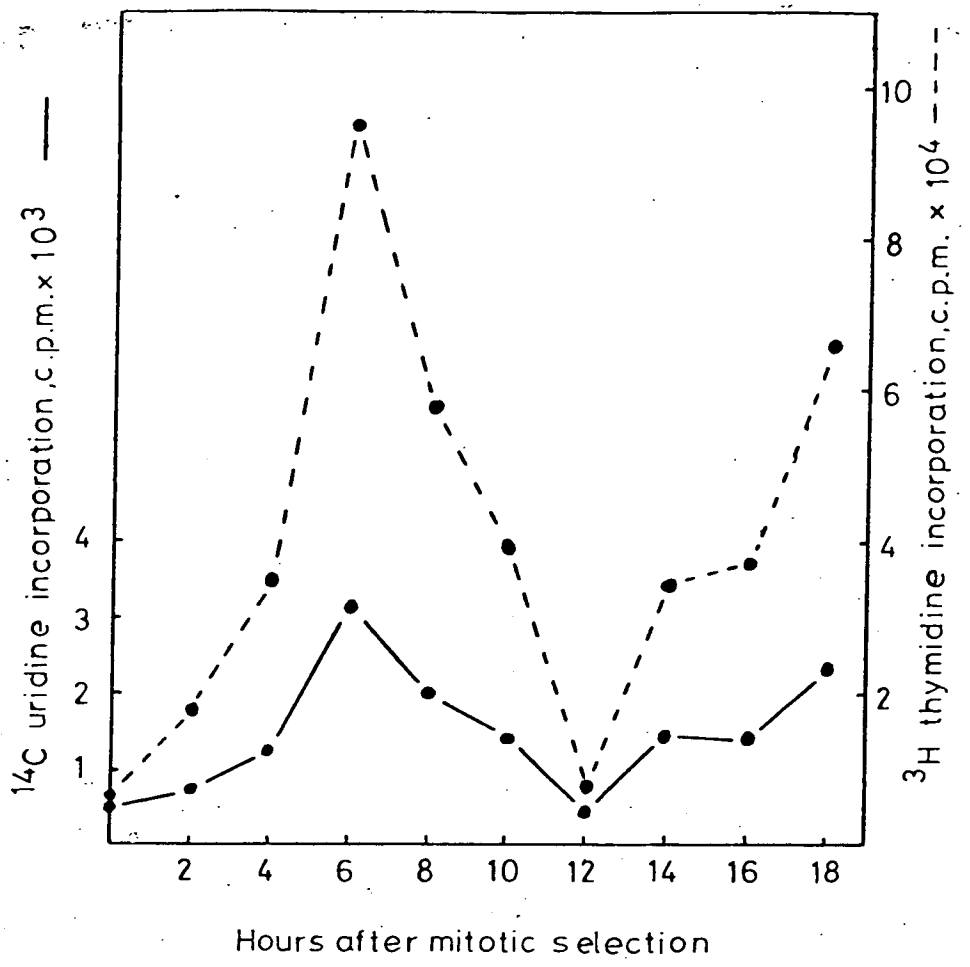


Figure 5.6

^{14}C-uridine incorporation, after a 30 minute pulse, into LE cells synchronised by mitotic selection. These cells were simultaneously labelled with ^{3}H-thymidine. The incorporation of both isotopes are plotted against the time after mitotic selection and expressed as c.p.m./10^5 cells.

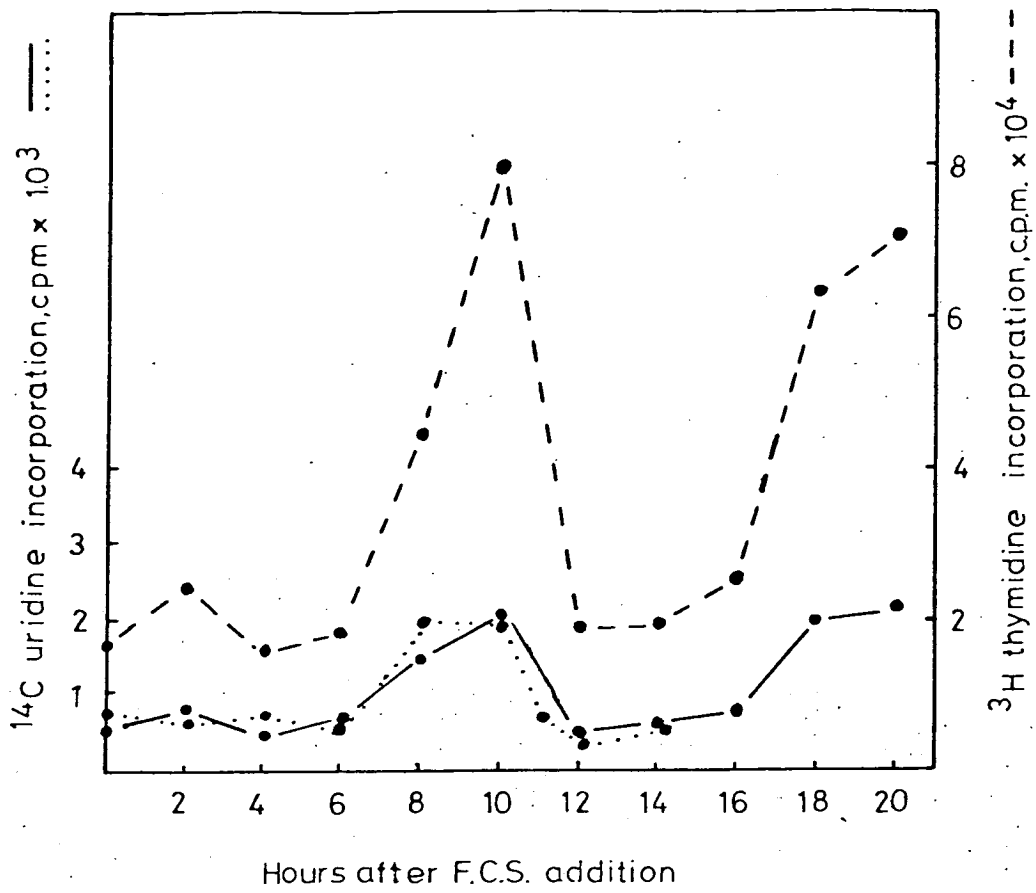


Figure 5.7

^{14}C-uridine incorporation after a 30 minute pulse, into LE cells at different times after synchronisation by the cell cycle arrest method. A ^{3}H-thymidine incorporation profile from Figure 5.6 is included as a marker. The incorporation of both isotopes are plotted against the time after FCS readdition following cell cycle arrest. c.p.m. is expressed per 10^5 cells.

in the previous section.

5.2.3 Analysis of Water Soluble Proteins Synthesised at Different Times after Cell Cycle Arrest Synchronisation.

L.E. cultures of strain Hy-2 were synchronised by removal of FCS from the medium and its subsequent reintroduction 24 hours later as previously described. Cultures were then given a 30 minute pulse of ^{14}C mixed amino acids at intervals after the synchronisation procedure, determined to be representative of different phases in the cell cycle (Fig. 5.5). The intervals taken were 0, 2, 6, 10 and 14 hours after the synchronisation procedure and were correlated with G_0, G_0/G_1, G_1/S, S and G_2/mitosis respectively. Cell cultures which had not undergone cell cycle arrest were used as controls. The water soluble proteins were separated by SDS polyacrylamide gel electrophoresis. The densitometer traces of a typical experiment after fluorography are shown in Figure 5.8. Qualitative differences were not evident in the polypeptides at the different times of labelling when compared with each other and the controls (Figs. 5.8 and 5.9). Such changes, if they were present, may not be detectable at this level of resolution of the polypeptides synthesised. Some quantitative differences in the labelled polypeptides at different times of labelling of the synchronised cultures were present and were consistent in three separate experiments. These changes were not found in asynchronous control cell cultures, also labelled at the same time intervals (Fig. 5.9). An increase was evident in a polypeptide in the 52000-55000 molecular weight range (marked 'u'). The synthesis of this polypeptide is enhanced by 82% 6 hours after synchronisation when compared to 0 hours (Table 5.1). This enhancement in synthesis coincides with the

FIGURES 5.8 and 5.9

Densitometer traces of fluorographs of the water soluble fraction from ^{14}C-amino-acid labelled LE cells. The fractions were run on SDS polyacrylamide gels. Figure 5.8 shows profiles of polypeptides at intervals after cell synchronisation. Profiles A-E represent polypeptides labelled at 0, 2, 6, 10 and 14 hours after synchronisation. Figure 5.9 shows profiles labelled at the same time intervals as the corresponding profiles in 5.8, but the cells were not synchronised. The arrows in 5.8 mark those peaks which show changes in synthesis between time points.

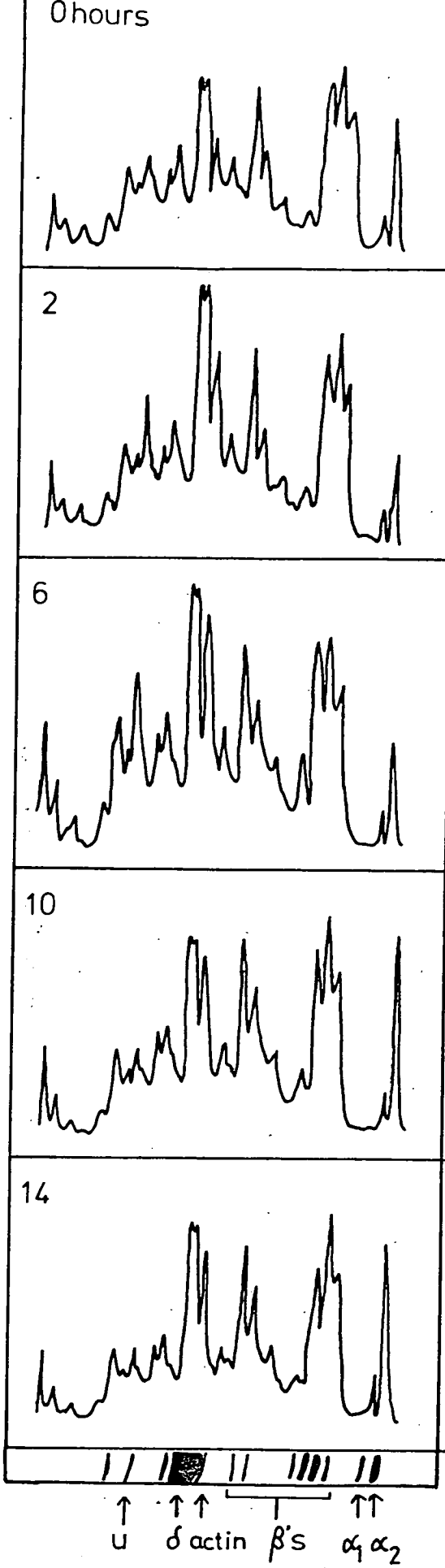


Figure 5.8

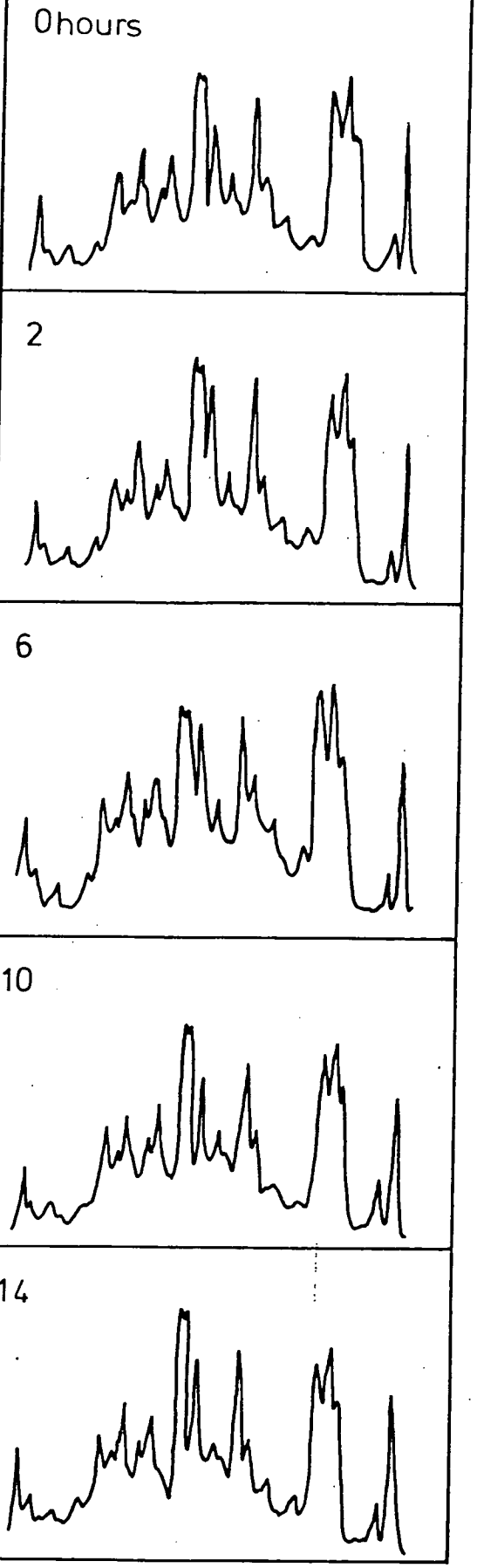


Figure 5.9

TABLE 5.1

Tabulated below are the changes in synthesis of 4 polypeptides through the cell cycle relative to the same polypeptides from asynchronous control cultures. The data was obtained by running ^{14}C-labelled cellular proteins from LE cultures on SDS gels. The radioactive bands were quantitated by integration of the peaks from densitometer tracings. The values are expressed as the percentage of the asynchronous control for each time point. (see Fig. 5.9). The average of two separate experiments are represented.

Polypeptide	Time after synchronisation	Percentage of controls
u 52-55,000	0 hours	73%
	2	115%
	6	155%
	10	99%
	14	70%
δ crystallin 45,000	0	96%
	2	113%
	6	120%
	10	92%
	14	93%
actin 42,000	0	71%
	2	157%
	6	234%
	10	154%
	14	114%
α_2 20,000	0	99%
	2	69%
	6	69%
	10	153%
	14	144%

transition of cells from G_1 into S-phase. 10 hours after synchronisation when most L.E. cells are in S phase, this polypeptide decrease in synthesis and by 14 hours the level of synthesis was similar to that found at 0 hours.

Two detectable changes were evident in the quantitative synthesis of crystallins at different times after the cell synchronisation procedure. δ_{1+2} crystallins exhibited a maximum increase of 28% in synthesis between 6 and 10 hours after synchronisation. α_2 also exhibited a change in synthesis at the different times after synchronisation. The level of synthesis was increased by 80% between 6 and 10 hours after synchronisation. The percentage levels of synthesis relative to the averaged controls are given in Table 5.1. α_1 and the β crystallins exhibited little or no change in synthesis at the different times after synchronisation, or when compared to the asynchronous control cell cultures.

Another non-crystallin polypeptide with a molecular weight of about 42,000 also showed a quantitative variation in synthesis at the different times after synchronisation when compared to the controls. This has been identified as actin (personal communication with A. Zehir), and is labelled in Figure 5.8 as 'act'. The maximum level of synthesis, 150% of the averaged controls, occurred 6 hours after synchronisation. This coincides with the traverse into S-phase and with an increase in synthesis of the unidentified polypeptide labelled 'u'. Table 5.1 shows the percentage levels of synthesis relative to the averaged controls.

5.2.4 Analysis of Membrane Proteins at Different Times after Cell Cycle Arrest Synchronisation.

The pellets remaining after extraction of the water-soluble proteins, the analysis of which is outlined in the previous section, were further solubilised to obtain the membrane fraction. These detergent soluble polypeptides were separated on SDS polyacrylamide gels. A typical experiment is shown in Figure 5.10. Some quantitative differences can be identified although this is restricted to the major polypeptides. None was found to change by more than 15% of the asynchronous controls. A large number of very faint bands were visible, but quantitation was very difficult as seen by the densitometer traces. A major band which showed a quantitative variation at different times after synchronisation co-migrated with that already identified as actin. It increased 6 hours after synchronisation, a similar time of increase to the corresponding polypeptide in the water-soluble fraction. No polypeptide was identified in the membrane fraction which co-migrated with 'u' from the water-soluble fraction and which showed a variation in the level of synthesis.

5.3 Lectin Binding to L.E. Cells after Mitotic Selection.

Lens epithelial cells from strains N, Hy-1 and Hy-2 were cultured in flasks and the mitotic cells harvested as outlined in section 4.1.4. The mitotic selection method was chosen for these experiments because single dissociated cells resulted from the procedure. This exposed the maximum cell surface area for binding of the lectins and eliminated the need of trypsinisation of otherwise plated cells. Trypsin may transiently affect the cell membrane

FIGURE 5.10

Densitometer traces of fluorographs of the membrane fraction from synchronised LE cells run on SDS-triton polyacrylamide gels. Each profile, A-E is of membrane polypeptides from cells 0, 2, 6, 10 and 14 hours after synchronisation respectively. The peak indicated by an arrow has been correlated with that evident in the cytoplasmic water soluble fraction shown in Figure 5.8 and identified as actin.

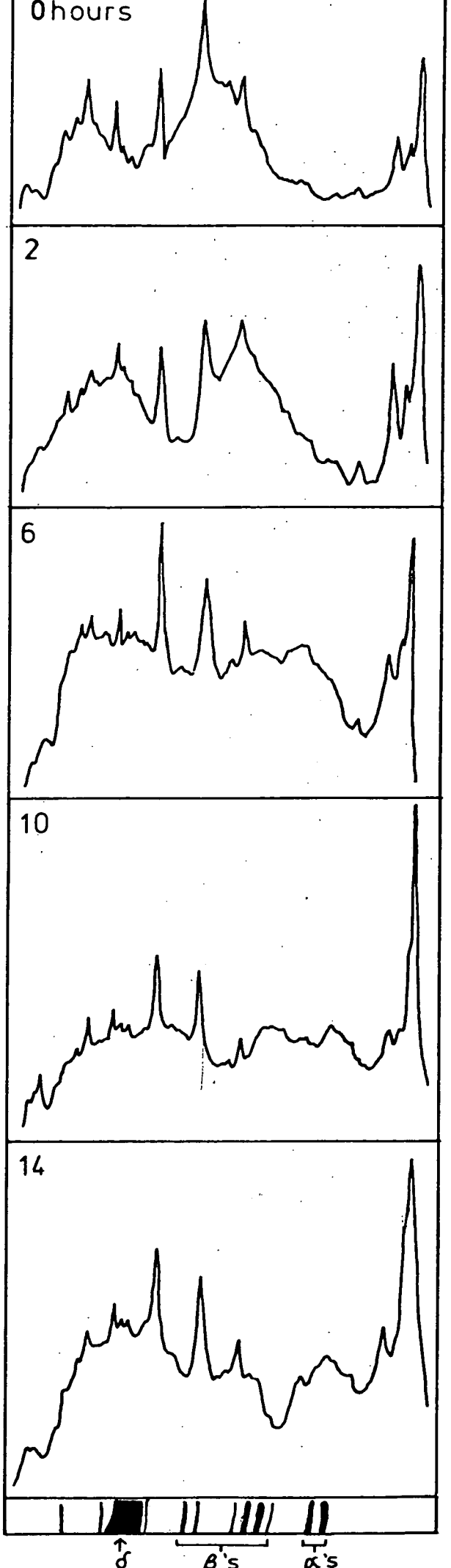


Figure 5.10

glycoproteins. Large numbers of cells were not necessary as in previous experiments where the cell cycle arrest method was used.

Concanavalin A (Con A) wheat germ agglutinin (WGA), Ricinis communis agglutinin (RCA) and Phylohaemaglutinin (PHA) were iodinated as outlined in 3.3.2. Aliquots of the synchronous lens epithelial cells were labelled with ^{125}I-lectin with and without the appropriate lectin binding inhibitor (see 3.3.2) at different times after mitotic selection. The amount of lectin bound specifically to the cell membrane was determined by subtraction of the amount bound in the presence of the inhibitor from the amount bound in the absence of the inhibitor. Counts per minute per 10^3 cells are plotted against time in Figures 5.11-13 for each strain. The data represents an average of 2 experiments.

In all 3 strains and in the presence of each of the lectins investigated, a higher level of lectin binding was found immediately after mitotic selection, i.e. when more than 90% of the cells were in mitosis (see Chapter 4). All strains showed the greatest decrease in binding of all 4 lectins at 2 hours after mitotic selection. The percentage decrease at this time was greater in strain N than in either strain Hy-1 or Hy-2. Strains Hy-1 and Hy-2 therefore exhibited greater binding of these lectins during interphase when compared to strain N. This difference was particularly obvious for Con A, WGA and RCA. The amount of each lectin bound to L.E. cells during mitosis was greatest for strain Hy-1. Strain Hy-2 was intermediate between Hy-1 and N.

The averaged amount of each lectin bound at different times for each strain shows that strain N has the least amount of each lectin

FIGURES 5.11 - 5.13

Graphs of ^{125}I-lectin binding to synchronised cells. Each graph represents a different chick strain. Aliquots of cells synchronised by mitotic selection were incubated at intervals with one of the following:

a. ^{125}I-Con A.

b. ^{125}I-Con A plus inhibitor.

c. ^{125}I-WGA.

d. ^{125}I-WGA plus inhibitor.

e. ^{125}I-RCA.

f. ^{125}I-RCA plus inhibitor.

g. ^{125}I-PHA.

h. ^{125}I-PHA plus inhibitor.

Radioactivity binding to the cells specifically was determined by subtracting any bound in the presence of the lectin and its specific inhibitor. Counts per minute per aliquot of cells were plotted against the appropriate time interval after the selection procedure.

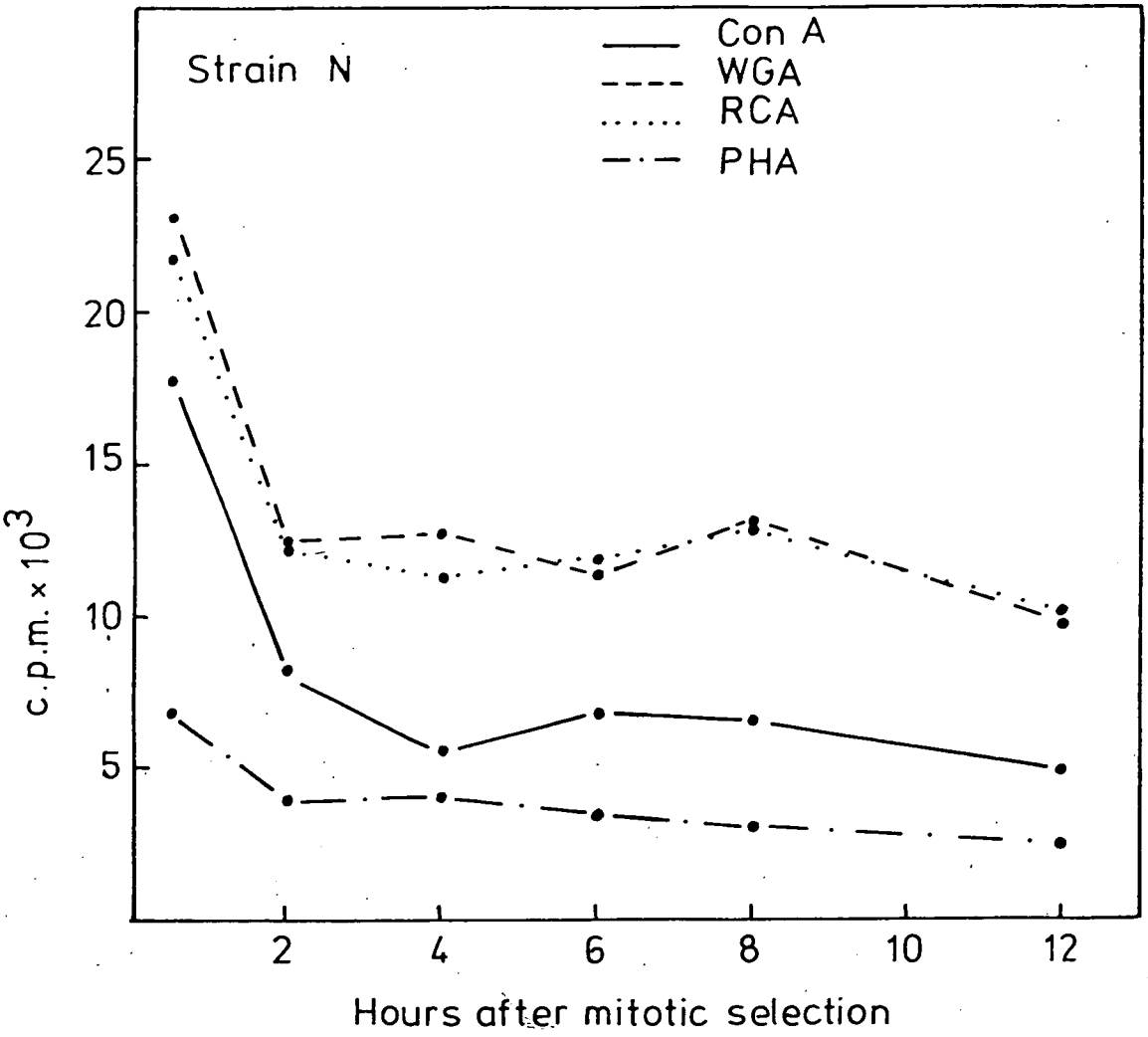

Strain N
Con A
WGA
RCA
PHA
25
20
15
10
5
c.p.m. × 10³
2
4
6
8
10
12
Hours after mitotic selection

Figure 5.11

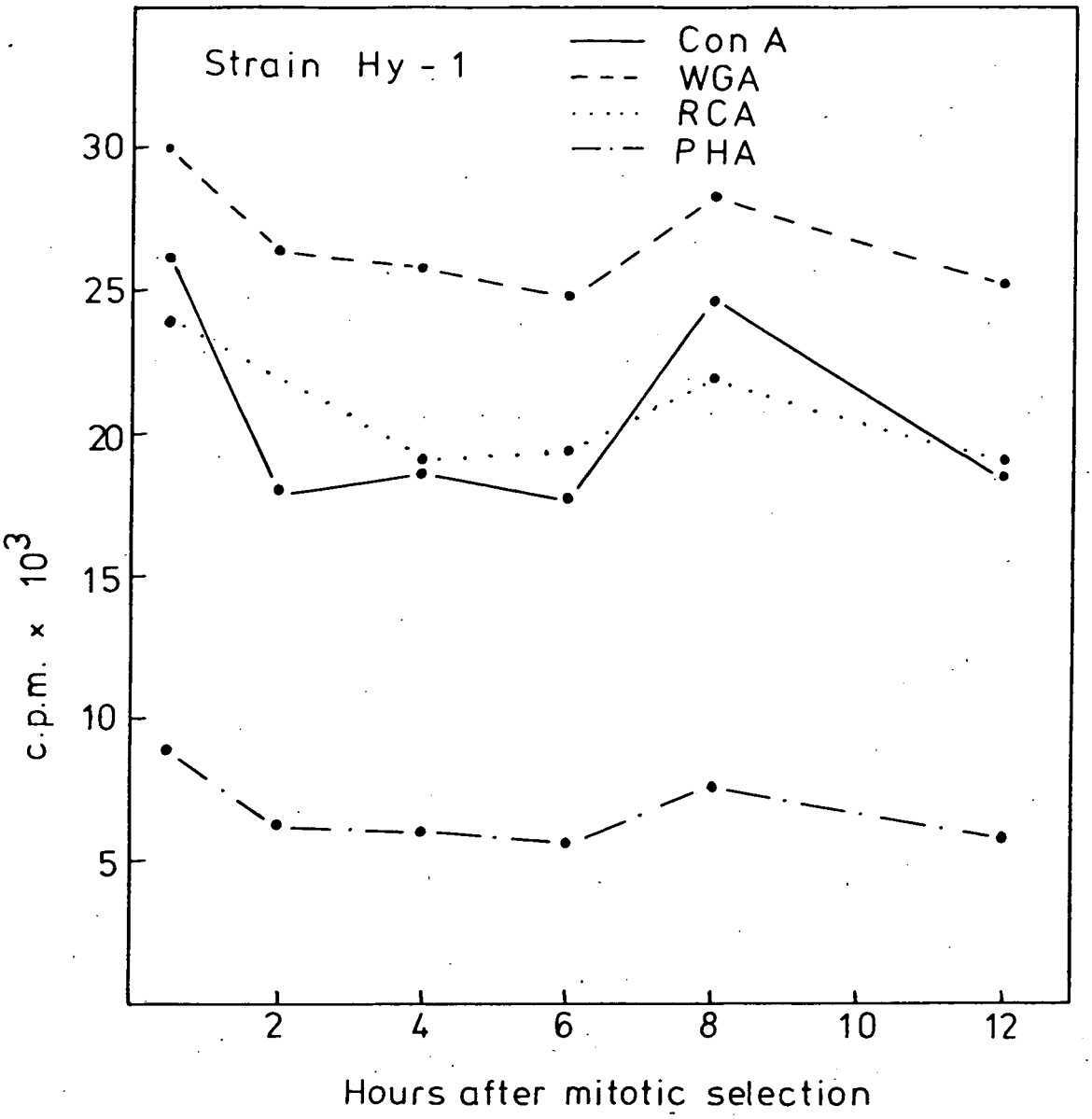

Strain Hy - 1
Con A
WGA
RCA
PHA
30
25
20
15
10
5
c.p.m. × 10³
2
4
6
8
10
12
Hours after mitotic selection

Figure 5.12

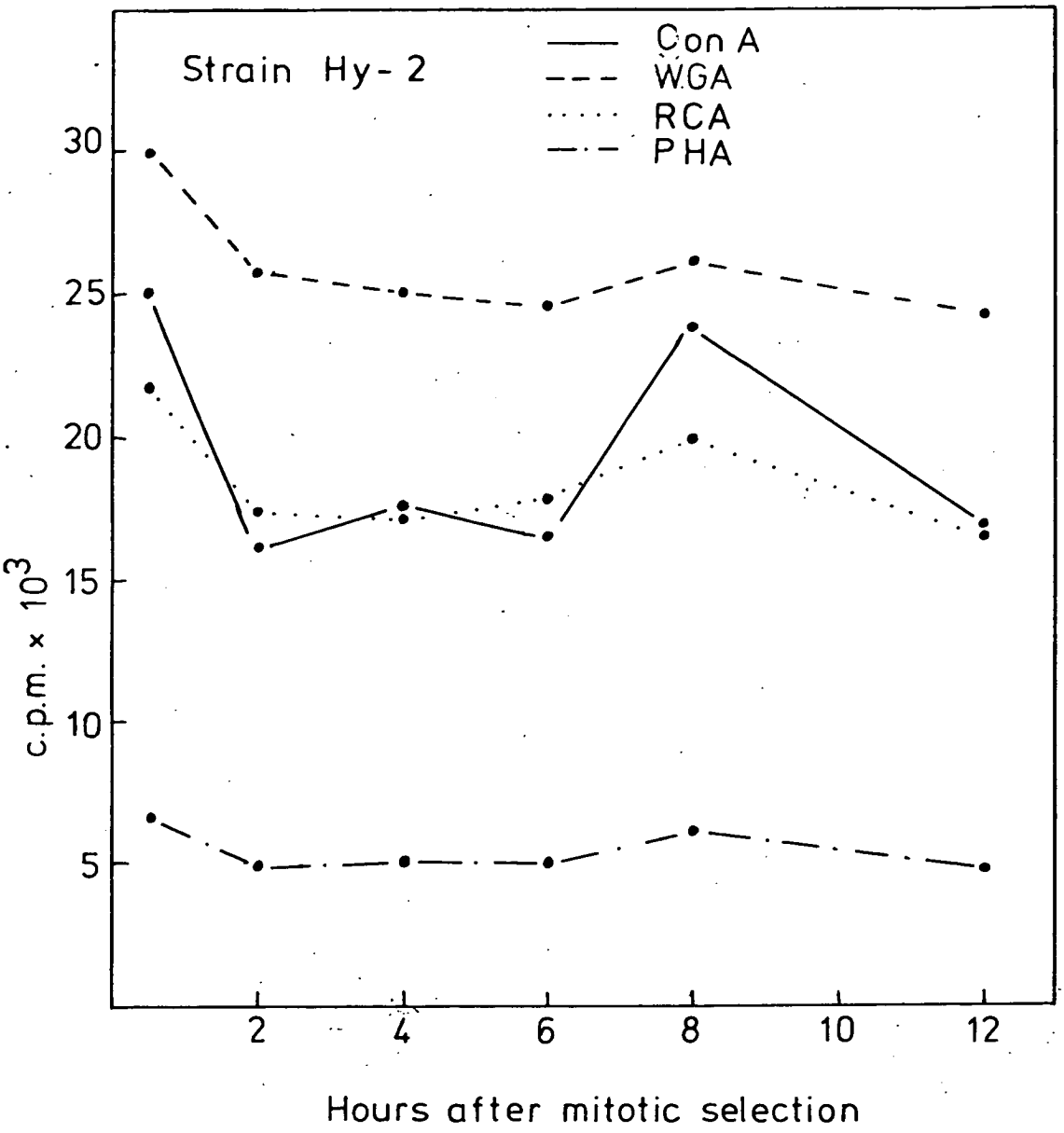

Strain Hy-2
Con A
WGA
RCA
PHA
30
25
20
15
10
5
c.p.m. x 10^3
2
4
6
8
10
12
Hours after mitotic selection

Figure 5.13

bound followed by strain Hy-2 and then by strain Hy-1. This data therefore corroborates the lectin binding data of Odeigah (1977) for Con A, RCA and PHA for the 3 chick strains of lens epithelium.

A rise in the binding of each lectin particularly to L.E. of strains Hy-1 and Hy-2 was evident 8 hours after mitotic selection. This could suggest another smaller peak in mitosis.

The relative binding of the different lectins to L.E. cells is not necessarily a measure of the relative number of specific sites on the cell membrane for these lectins. Variation in the degree of iodination of each of these lectins can occur.

5.4.1 The Effect of 6%, 10% and 15% FCS, insulin and Retinal Extract on the Growth Rate of L.E. Cells from 3 Different Chick Strains

Lens epithelium from strains Hy-1, Hy-2 and N were cultured as described previously. The epithelial cells were seeded and grown for 4 days in the standard medium with 6% FCS. This permitted plating of the cells to occur under the same conditions. The cell cultures were thereafter given medium containing either:

(a) 6% FCS

(b) 10% FCS

(c) 15% FCS

(d) 10µg/ml insulin + 6% FCS

The medium was changed every 2 days. Cell counting was carried out every 2-3 days as described in the Materials and Methods section. The cell numbers/plate were scored in triplicate and are plotted for

each strain in Figures 5.14-16. Figure 5.14 (strain N) represents the average of two independent experiments. Figures 5.15 and 5.16 represent the average of 3 independent experiments (strains Hy-1 and Hy-2).

In all 3 strains the growth of lens epithelium was enhanced by the presence of insulin and retinal extract when compared to the control (6% FCS). Retinal extract stimulated growth by the greatest amount. Strain differences were apparent in the response of the L.E. cells to both RE (retinal extract) and insulin. Strain Hy-1 showed the greatest increase in growth to both additives, and strain N the least response after 17 days in culture (13 days of treatment). The growth of the controls of each strain reached a plateau between 12-14 days. In both RE and insulin treated, this plateau did not occur and the cell numbers continued to increase.

Strain differences were also apparent between the growth rate of the controls and of the cultures treated with 10% FCS or 15% FCS. Strain N showed the greatest relative increase in growth compared to the control. In strain Hy-1 L.E., increasing FCS concentrations did not have an additive effect on cell number. 15% FCS treatment did not appear to stimulate growth as much as 10% FCS. Hy-2 L.E. cultures showed a relative increase in growth rate in the presence of 10% and 15% FCS, when compared to the control. This relative increase was greater than strain Hy-1 but less than strain N.

FIGURES 5.14 - 5.16.

Growth curves of LE cell cultures grown under different medium conditions. Each graph represents a different strain of chick from which the lens epithelia were derived. Dissociated LE cells were seeded into 6% FCS standard medium. After 4 days the cultures were grown in 6% FCS standard medium plus one of the following:

a. 6% FCS standard medium only.

b. plus 10% FCS.

c. plus 15% FCS.

d. plus 10µg/ml insulin

e. plus 50µg/ml retinal extract.

Mean cell counts were plotted against the appropriate age of the culture.

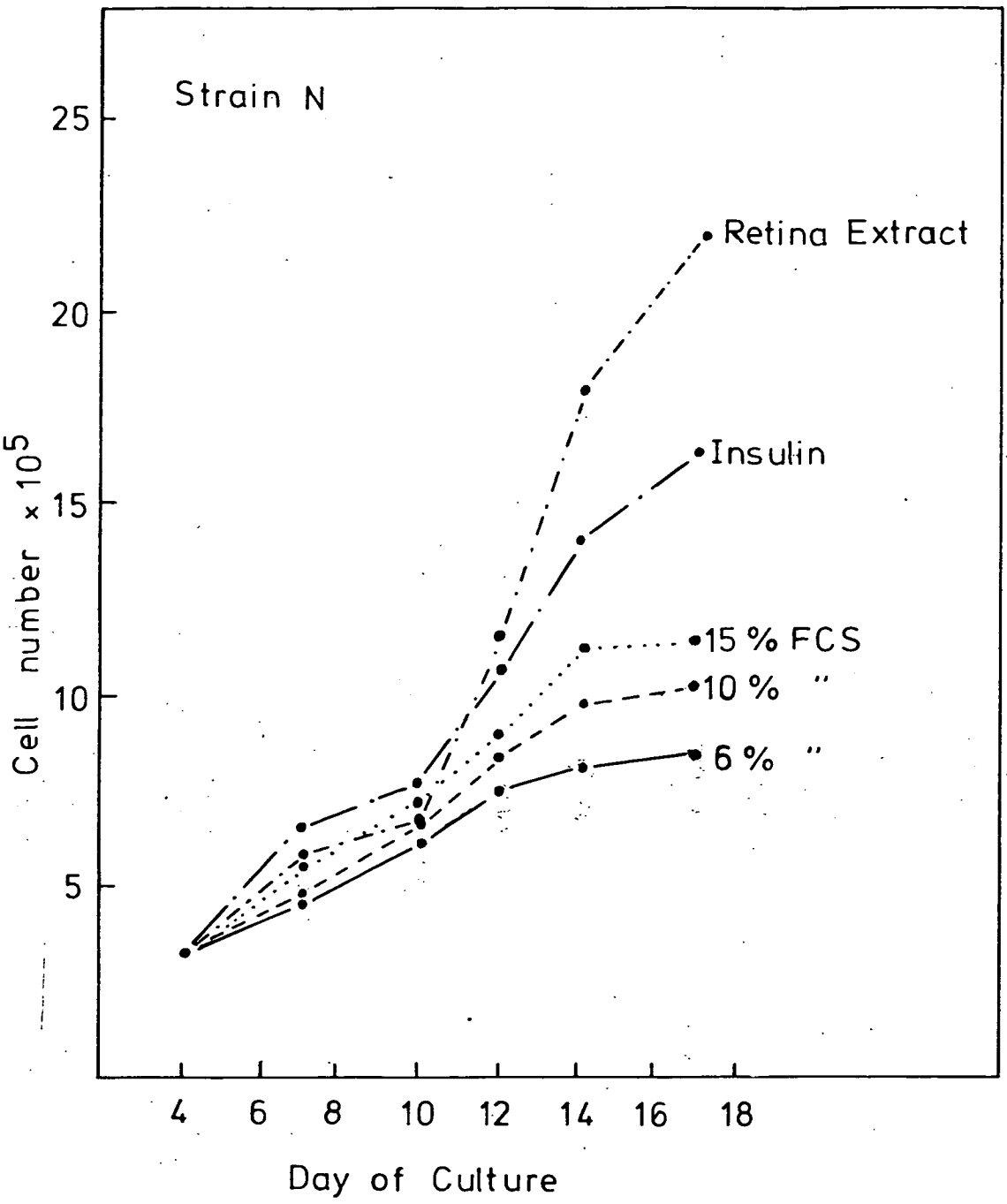

Strain N
Retina Extract
Insulin
15 % FCS
10 % "
6 % "
Cell number x 10^5
25
20
15
10
5
4
6
8
10
12
14
16
18
Day of Culture

Figure 5.14

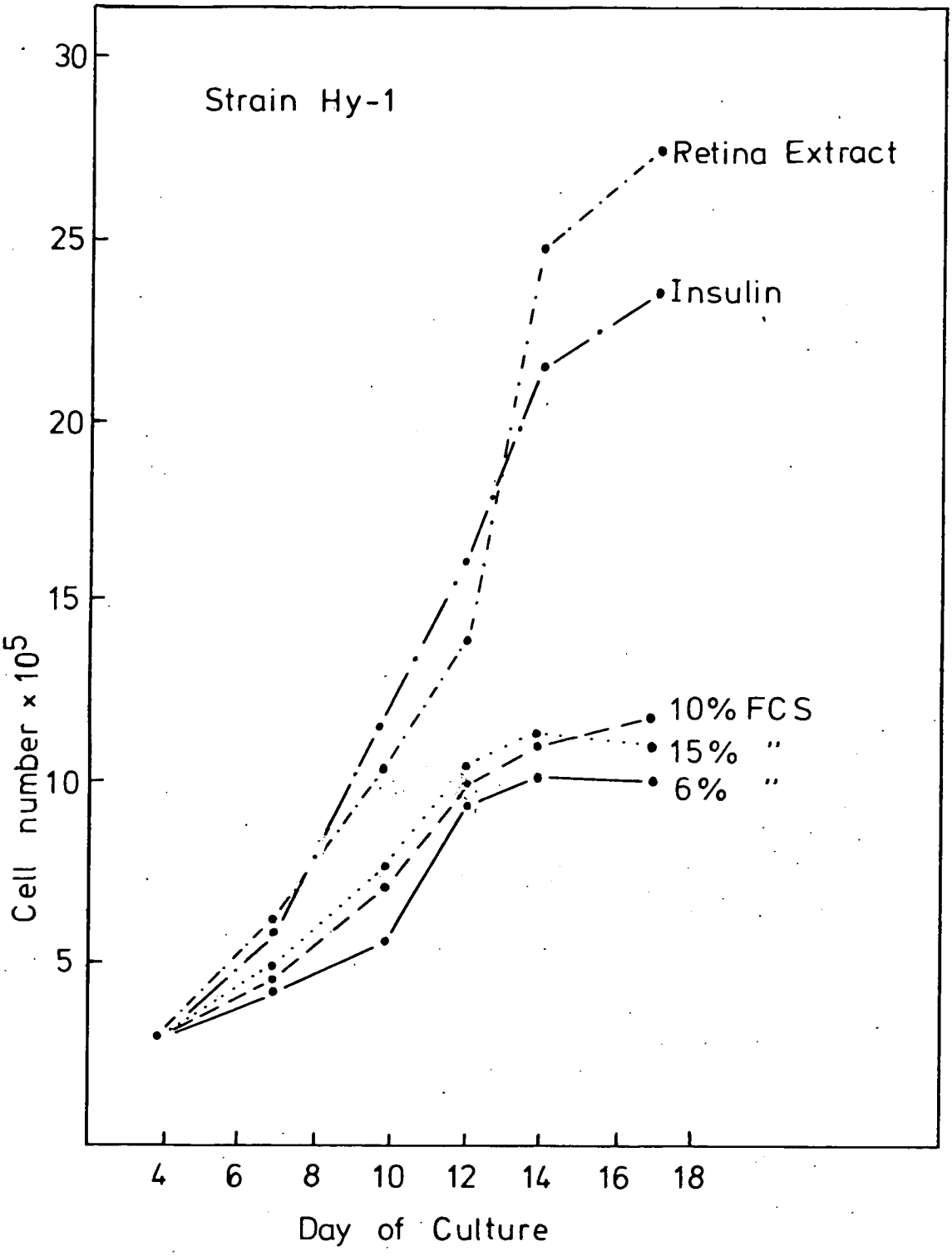

Strain Hy-1
Retina Extract
Insulin
10% FCS
15% "
6% "
Cell number × 10^5
30
25
20
15
10
5
4
6
8
10
12
14
16
18
Day of Culture

Figure 5.15

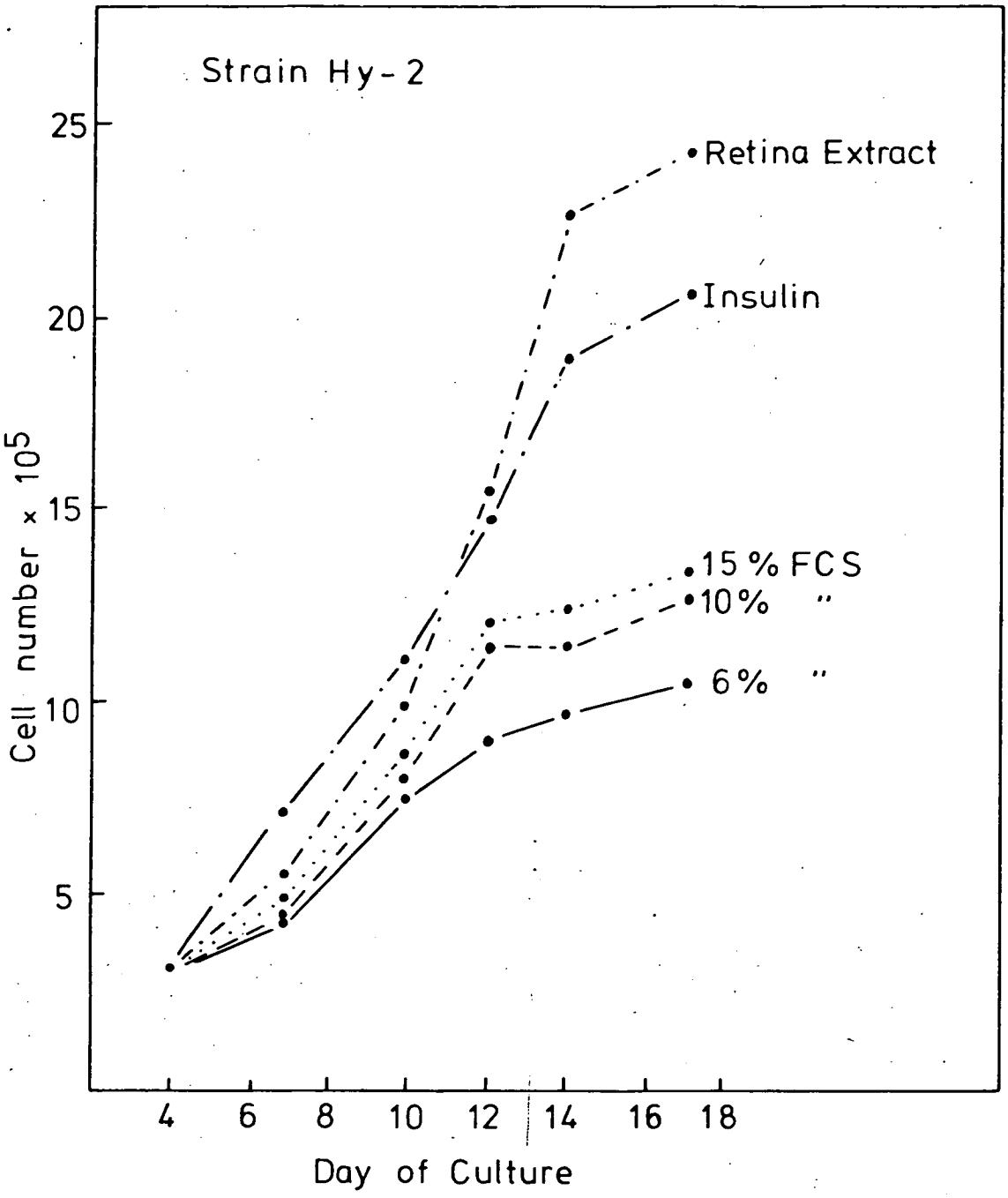

Strain Hy-2
Retina Extract
Insulin
15 % FCS
10 % "
6 % "
Cell number x 10^5
25
20
15
10
5
4
6
8
10
12
14
16
18
Day of Culture

Figure 5.16

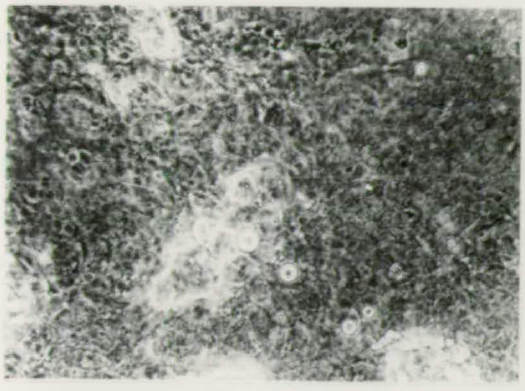
5.4.2 The Effect of 6%, 10% and 15% FCS; Insulin; and Retinal Extract on the Morphology of L.E. cultures.

Plates 5.2 and 5.3 show photographs of strain N cultures treated with 6%, 10% and 15% FCS and insulin at 14 days and 19 days of culture respectively. In both ages of cultures those treated with 6% FCS showed the most numerous lentoid formation, with accompanying bottle cells, although the lentoids were smaller than 10% FCS treated cultures. 6% FCS treated cultures also showed less multi-layering of cells than did 10% FCS treated cultures. 15% FCS treated cultures looked 'young' due to fewer lentoids when compared to the 6% FCS control of the same age. Few lentoids and bottle cells were present, but overlayering of cells did occur. A greater degree of overlayering was evident in the insulin treated cultures. Large lentoid-like structures were also present but with few accompanying bottle cells when compared to the control. Numerous mitotic cells were still visible, even after 19 days of culture (Plate 5.3 D).

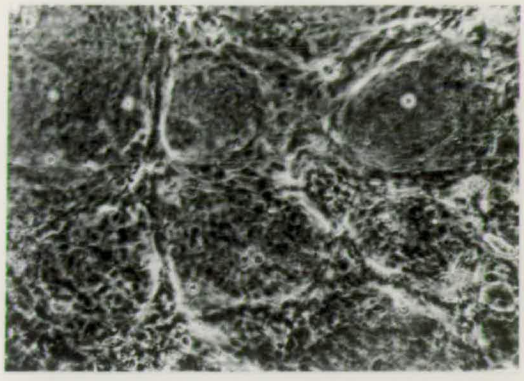
Plate 5.4 shows photographs of RE treated cultures of strain Hy-2 when compared with a 6% FCS treated control. Plate 5.4 A and B shows the difference in plating ability of cells treated with 6% FCS and RE respectively from the time of seeding. RE treated cells were rounded and did not attach firmly to the substratum. This is the reason why the other experiments reported in this section permitted all cells to plate down for 4 days before commencing treatment with medium additives. This avoided any discrepancy in the growth rate which was attributable to the relative plating abilities of the treated cells. The other photographs show the relative frequency of mitosis in the control and RE treated cultures (C and D), and the

PLATE 5.2

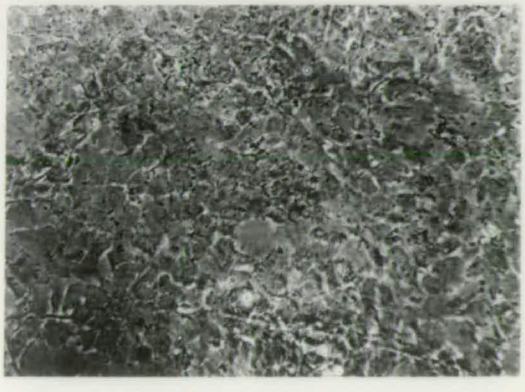
Strain N LE cultures at 14 days after seeding (A) control LE culture grown in 6% FCS standard medium; (B) LE cells grown in 10% FCS medium; (C) LE cells grown in 15% FCS medium; (D) LE cells grown in 6% FCS standard medium plus 10µg/ml insulin. 100 x magnification.



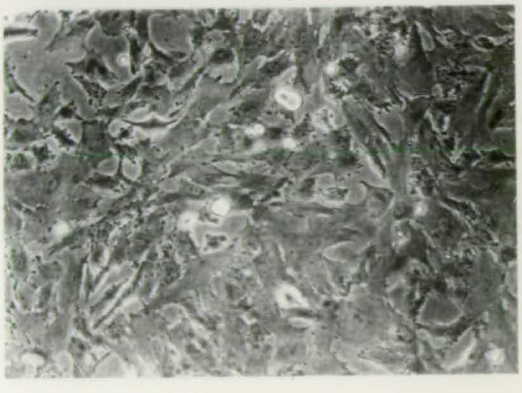
A



B



C

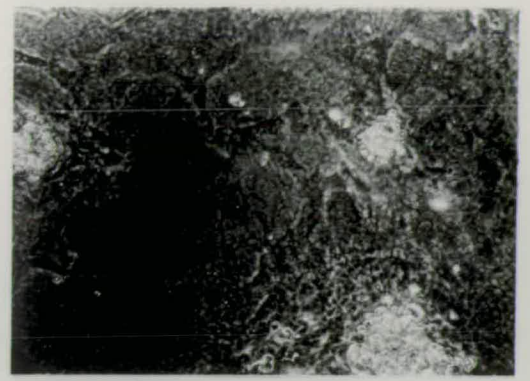


D

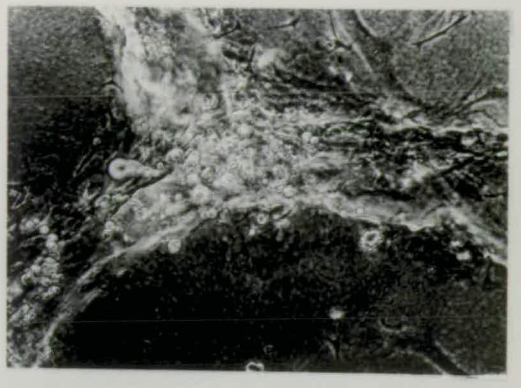
PLATE 5.2

PLATE 5.3

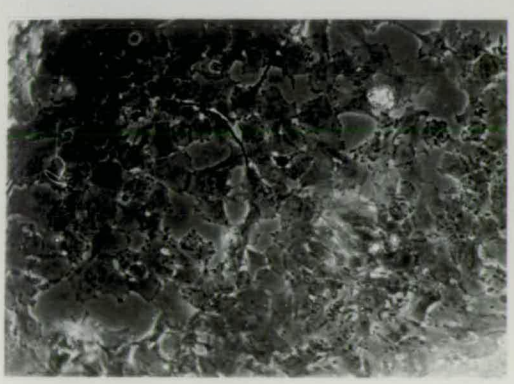
Strain N LE cultures at 19 days after seeding. (A) control LE culture grown in 6% FCS standard medium; (B) LE cells grown in 10% FCS medium; (C) LE cells grown in 15% FCS medium; (D) LE cells grown in 6% FCS standard medium plus 10µg/ml insulin. 100 x magnification.



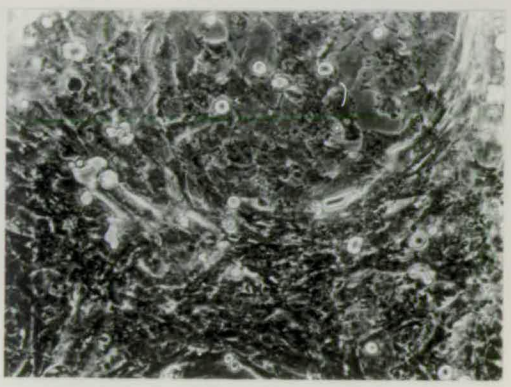
A



B



C



D

PLATE 5.3

PLATE 5.4

Photographs of strain Hy-2 LE cultures treated with 50µg/ml RE.

(A) Control LE cells grown in standard 6% FCS medium, 4 days after seeding;

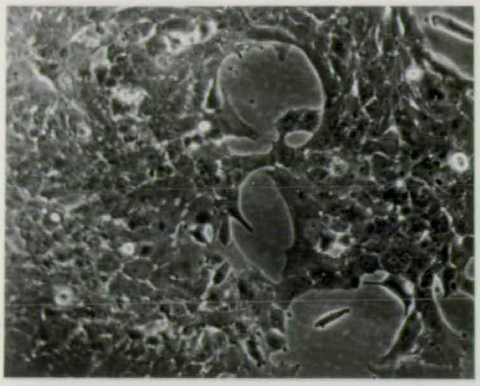
(B) LE cells grown in standard medium plus 50µg/ml RE, 4 days after seeding;

(C) LE cells grown for 8 days in 6% FCS;

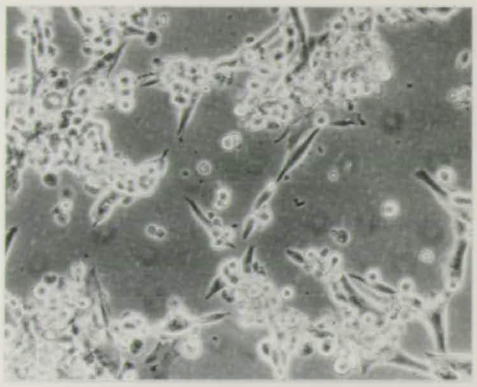
(D) Culture of the same age as (B) treated with 50µg/ml RE from 4 days after seeding;

(E) Control LE culture grown in 6% FCS standard medium for 14 days;

(F) Culture of the same age as (E) treated with 50µg/ml RE from 4 days after seeding.



A



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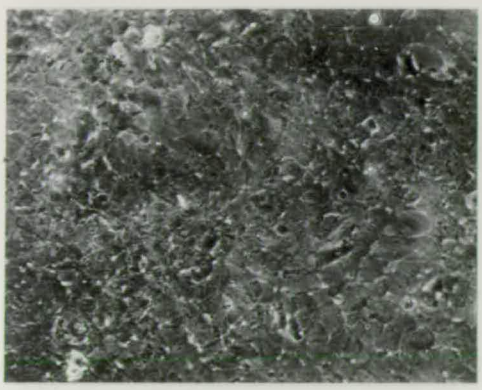

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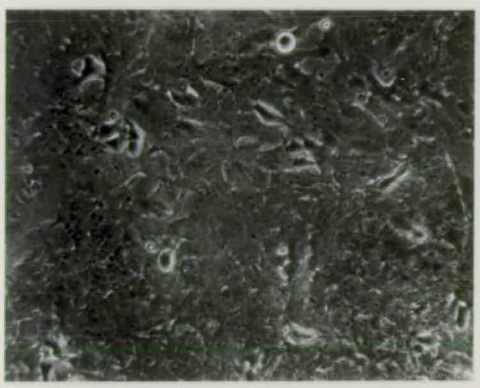

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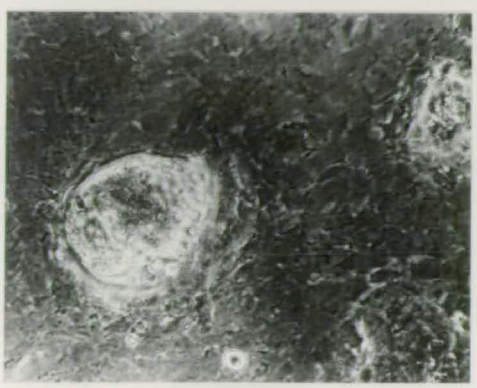

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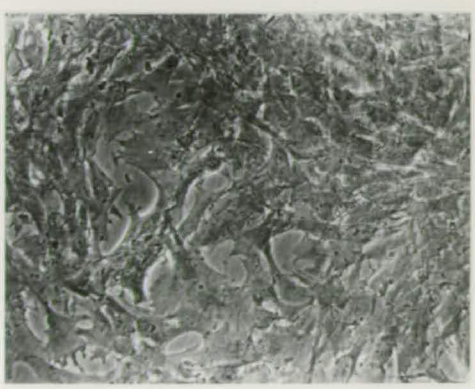

F

PLATE 5.4

complete absence of lentoids and bottle cells in the RE treated cultures. These were treated with RE from 4 days of culture.

The above morphological changes in cultures after the various treatments were similar in all 3 strains, although a single strain in each case has been illustrated.

5.4.3 Analysis of Accumulated Crystallins from L.E. Cultures after Treatment with 6%, 10% and 15% FCS; Insulin; and Retinal Extract

Cells were harvested from L.E. cultures of all 3 strains and treated with 6%, 10% and 15% FCS, insulin and RE. The cultures were 14 days old at the time of harvesting. The water soluble fraction was prepared and run on SDS polyacrylamide gels. Plates 5.5-7 show photographs of gels from strain Hy-1, Hy-2 and N respectively. Each plate shows gel lanes of 6%, 10%, 15% FCS, insulin and RE treated cultures, with a crystallin marker of total protein from day old chick lenses.

Both qualitative and quantitative differences in the accumulation of crystallin sub-units were evident in cultures treated in the various ways already described. Tables 5.2-4 give the percentage change, relative to the controls (6% FCS), of the crystallin subunits and several other polypeptides in each strain and under the various conditions.

Increasing the FCS concentration did not have a consistent effect on the pattern of crystallins accumulated in the various strains. δ_2 was increased by 10% and 15% FCS compared to the controls in both strains Hy-1 and Hy-2. However in both of these strains, 10% FCS treatment resulted in the absence of δ_1 crystallin while 15% FCS did not. Strain Hy-1 also showed a large increase in a polypeptide

PLATE 5.5

SDS polyacrylamide slab gels of the water soluble extract from strain Hy-1 cultured LE cells treated in various ways during culture. (A) 6% FCS; (B) 10% FCS; (C) 15% FCS; (D) 6% FCS plus 10µg/ml insulin; (E) 6% FCS plus 50µg/ml RE; (F) Day old chick lens crystallins as a marker.

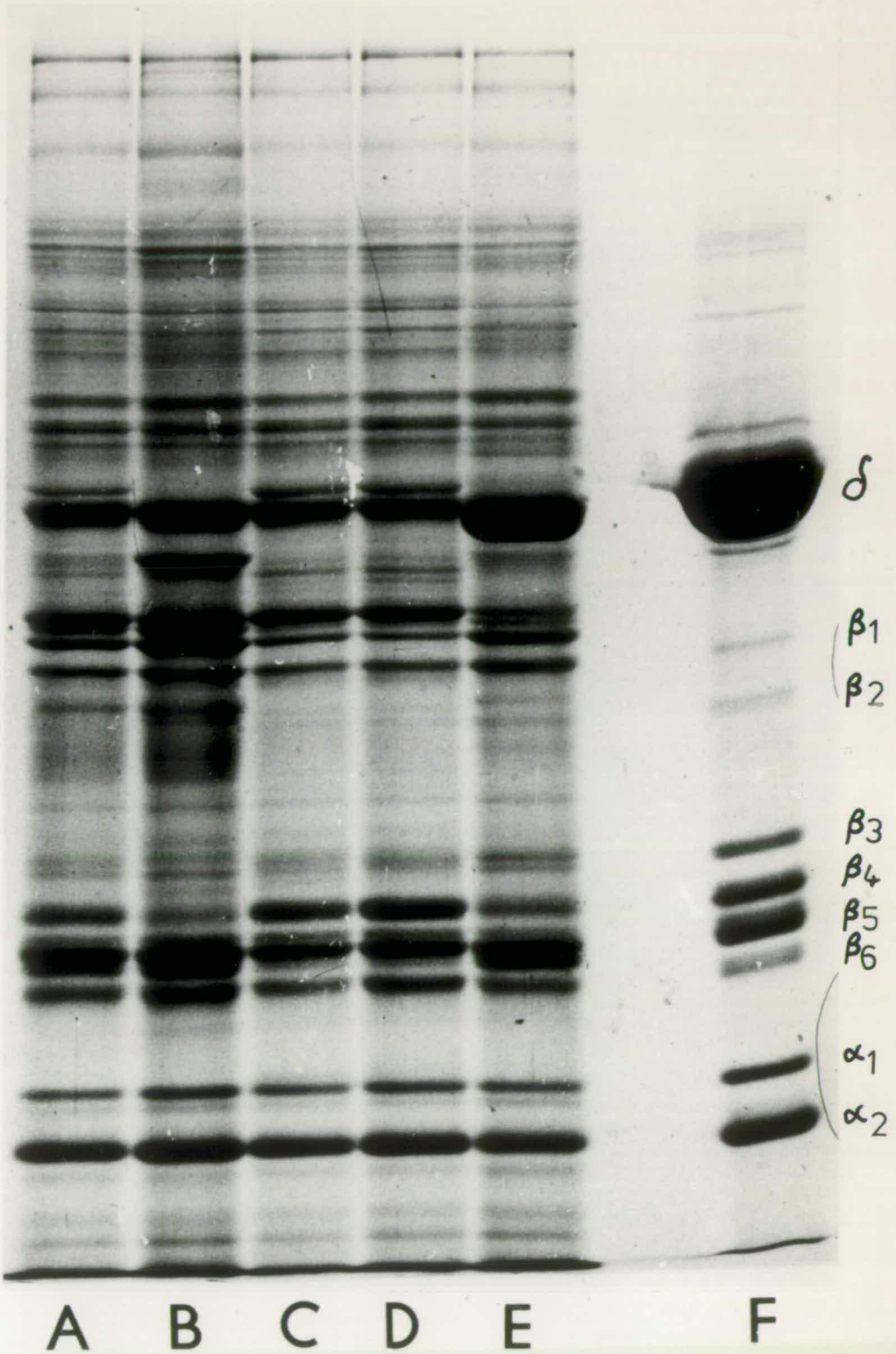

PLATE 5.5

PLATE 5.6.

SDS polyacrylamide slab gels of the water soluble extract from strain Hy-2 LE cultured LE cells treated in various ways during culture (A) 6% FCS; (B) 10% FCS; (C) 15% FCS; (D) 6% FCS plus 10µg/ml insulin; (E) 6% FCS plus 50µg/ml RE; (F) Day old chick lens crystallins as a marker.

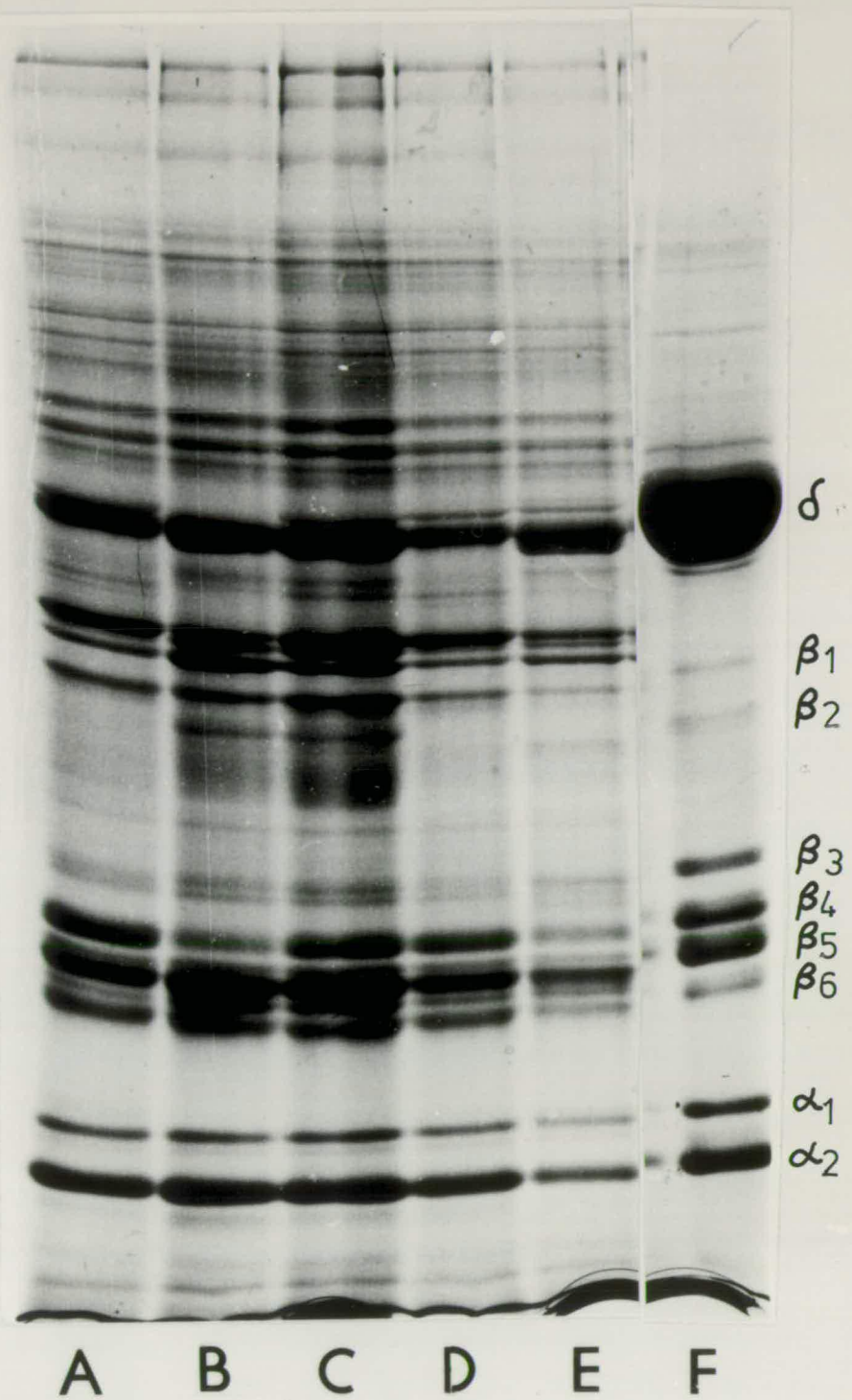

PLATE 5.6

PLATE 5.7

SDS polyacrylamide slab gels of the water soluble extract from strain N cultured LE cells treated in various ways during culture. (A) Day old chick lens crystallins as a marker; (B) 6% FCS; (C) 10% FCS; (D) 15% FCS; (E) 6% FCS plus 10µg/ml insulin. (From the same experiment). (F) Day old chick lens crystallins; (G) 6% FCS; (H) 6% FCS plus 50µg/ml RE. (F, G, and H from the same experiment).

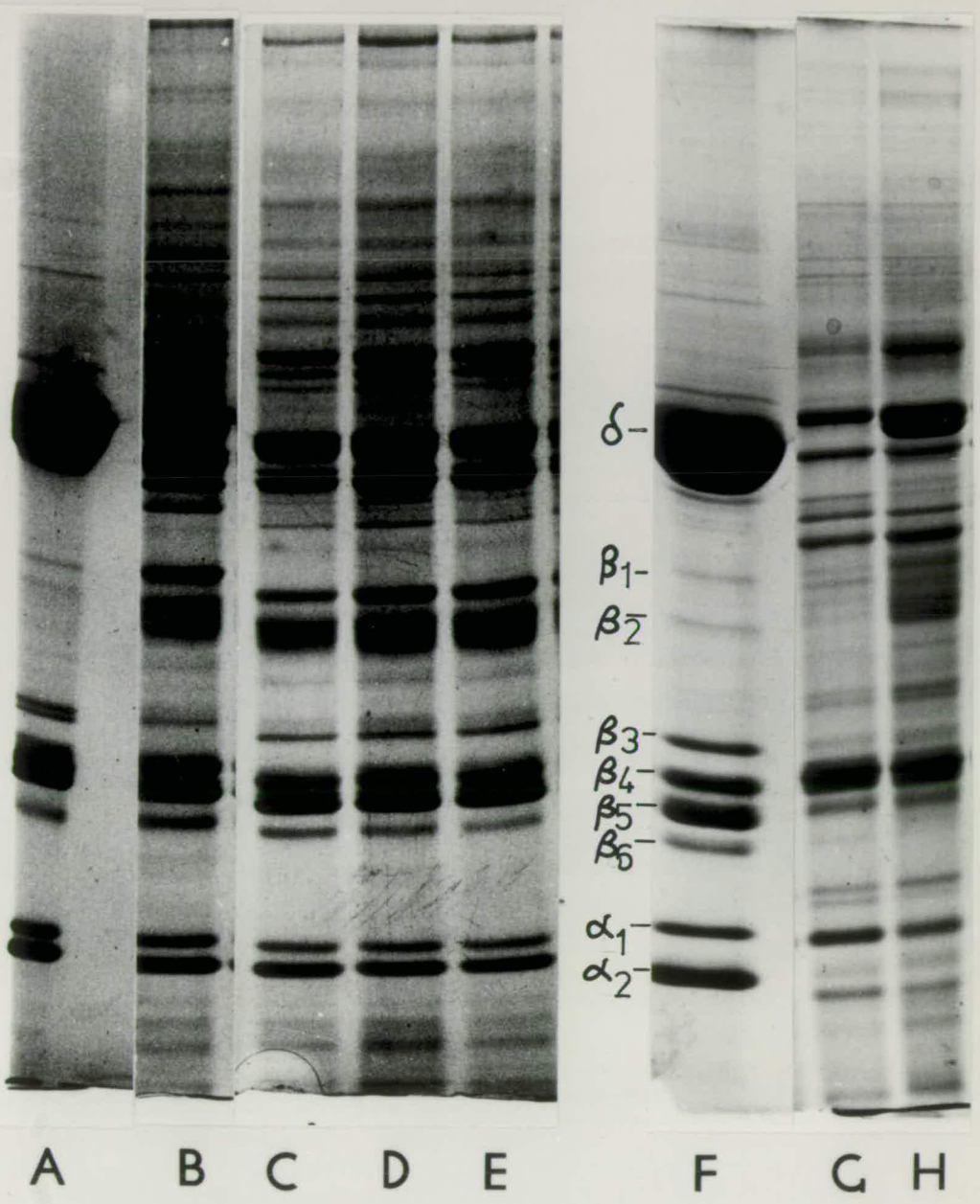

PLATE 5.7

TABLE 5.2 Strain Hy-1

Tables of the percentage change, relative to the controls (standard 6% FCS medium) of the crystallins and several other polypeptides under the various conditions indicated.

	10% FCS	15% FCS	Insulin	Retinal Extract
δ_1	0	135	164	0
δ_2	355	562	565	893
actin	212	135	112	110
β_1	247	5	71	200
β_2	400	200	200	700
β_3	133	33	33	33
β_4	302	151	190	56
β_5	78	76	84	95
β_6	96	93	133	70
α_1	187	212	212	112
α_2	63	105	118	70

TABLE 5.3 Strain Hy-2

Table of the percentage change, relative to the controls (standard 6% FCS medium) of the crystallins and several other polypeptides under the various conditions indicated.

	10% FCS	15% FCS	Insulin	Retinal Extract
δ_1	0	70	60	45
δ_2	132	138	121	157
actin	78	90	68	92
β_1	170	155	215	145
β_2	119	132	60	63
β_3	112	108	108	112
β_4	67	83	97	67
β_5	136	121	110	117
β_6	92	80	65	80
α_1	63	60	65	68
α_2	84	96	98	67

Table 5.4 Strain N

Table of the percentage change, relative to the controls (standard 6% FCS medium) of the crystallins under the various conditions of culture indicated.

	10% FCS	15% FCS	Insulin	Retinal Extract
δ_1	159	72	69	74
δ_2	450	82	107	91
actin	46	33	53	57
β_1	107	132	135	103
β_2	109	100	103	97
β_3	107	200	176	169
β_4	106	95	84	129
β_5	94	121	119	117
β_6	111	100	82	129
α_1	100	94	108	118
α_2	60	101	96	116

identified as actin (act) when treated with 10% FCS, an increase which was not evident in any other culture treatment. Actin decreased relative to the control in strain Hy-2 cultures treated with 10% and 15% FCS. The relative percentages of the β crystallins and α crystallins also changed quantitatively in all 3 strains.

Insulin treated cultures resulted in crystallin changes when compared to the controls and which differed between the strains. Hy-1 showed an increase in δ_1, δ_2, β_4 and β_6. Hy-2 showed a smaller increase in δ_2 compared to Hy-1, an increase in β_1 and a decrease in δ_1, actin and α_1. Strain N showed an increase in B_1, B_3, and β_5.

The greatest single consistent difference in the crystallins of all 3 strains was found after treatment of cultures with retina extract. δ_2 crystallin increased markedly. The greatest increase was found in strain Hy-1, where δ_2 increased with the loss of δ_1. δ_2 also increased in Hy-2, and δ_1 decreased but was not lost. Two other minor polypeptides of a slightly smaller molecular weight to crystallin also appeared in this strain. Both δ_1 and δ_2 crystallins were increased in strain N compared to the control. A decrease in α_2 crystallin was also evident in strains Hy-1 and Hy-2. However differences in the changes of the proportions of the β crystallins were not evident.

Look at Table 5-4 ?

5.5 Analysis of Intermitotic Intervals of L.E. cells from Strains Hy-1, Hy-2 and N.

Time lapse film of L.E. cultures were taken as outlined in the Materials and Methods section. At least 3 separate films of L.E. cultures from each chick strain were analysed. All cells in the field of view during filming were not necessarily scored as some cells left or entered the field during filming and others were difficult to follow due to large amounts of cell movement. The cells plotted in Figures 5.17-19 are therefore not necessarily an accurate representation of the frequency of particular intermitotic time intervals. The data does show the spread of the intermitotic intervals for each strain and gives some idea of the frequency distribution. At least 25 cells were followed in each strain and their intermitotic intervals determined.

The spread of intermitotic time intervals is clearly different between strains. Strain N shows a higher proportion of cells with longer cycle than either of the other two strains. Strain Hy-2 appears to have the least variation in the cell cycle duration.

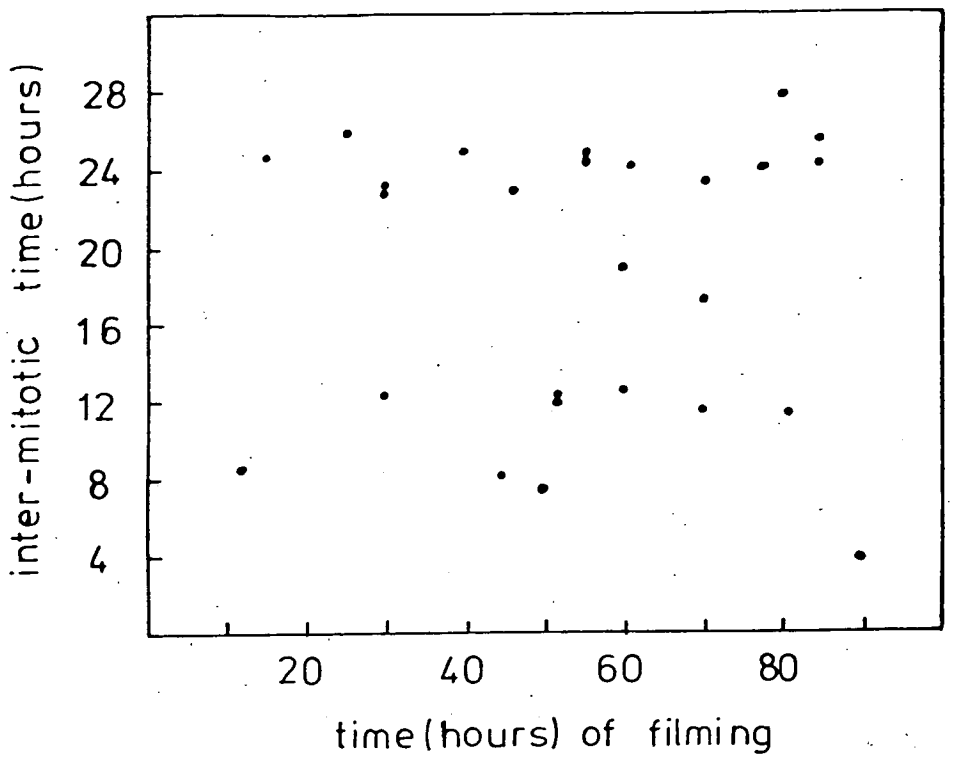

Figure 5.17 Strain N

Plots of the intermitotic intervals of LE cells in culture determined by time lapse photography. Each cell is represented by a single black dot, and is plotted against its appropriate inter-mitotic interval at the time of the initial mitosis. The horizontal axis represents the time during the period of filming. Daughter cells, when they were recorded are depicted by a vertical line joining them at the time of the initial mitosis.

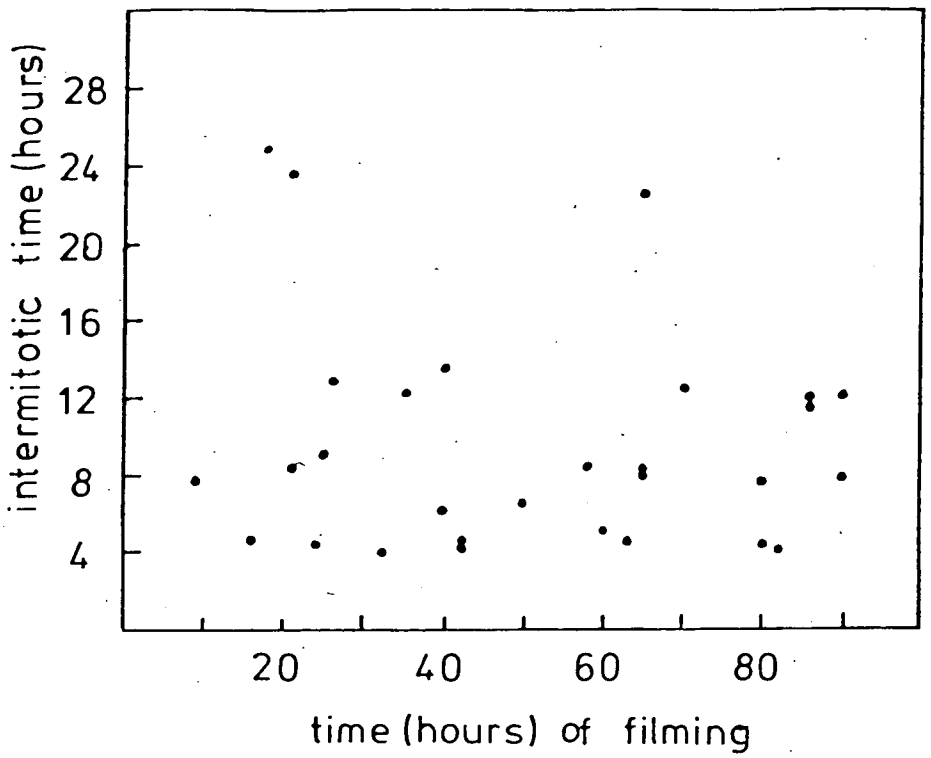

Figure 5.18 Strain Hy-1

Plots of the intermitotic intervals of LE cells in culture determined by time lapse photography. Each cell is represented by a single black dot, and is plotted against its appropriate intermitotic interval at the time of the initial mitosis. The horizontal axis represents the time during the period of filming. Daughter cells, when they were recorded are depicted by a vertical line joining them at the time of the initial mitosis.

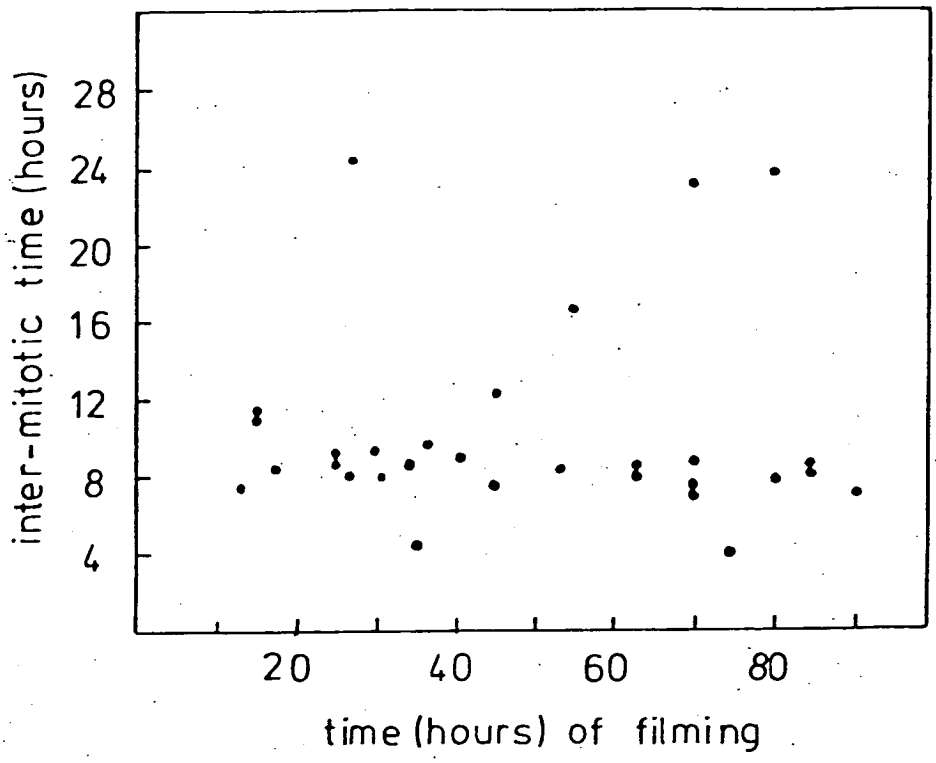

Figure 5.19 Strain Hy-2

Plots of the intermitotic intervals of LE cells in culture determined by time lapse photography. Each cell is represented by a single black dot, and is plotted against its appropriate inter-mitotic interval at the time of the initial mitosis. The horizontal axis represents the time during the period of filming. Daughter cells, when they were recorded are depicted by a vertical line joining them at the time of the initial mitosis.

CHAPTER 6. DISCUSSION

6.1 DNA and RNA Synthesis in Chick Lens Epithelium.

The culture of whole intact lenses in medium containing radioactively labelled thymidine or uridine is an indirect method of evaluating DNA and RNA synthesis respectively. This correlation is based on the fact that the labelled precursors enter the cellular pools, join the synthetic chain and become incorporated into newly synthesised DNA during S-phase in the case of thymidine or into newly synthesised RNA at the time of transcription in the case of uridine. Pulse labelling of lenses with specific precursors at different times throughout the day can therefore be used to define the patterns of macromolecular synthesis. This procedure is only valid if certain conditions are fulfilled.

(1) The specific activity of the pool of immediate precursors should be constant throughout the cell cycle, and when comparing strains should not differ. If the pool sizes of different strains are variable, the problem of differential dilution should be taken into account.

(2) The concentration of the exogenous precursor must not be a limiting factor in incorporation into the macromolecules, and therefore must represent a large proportion of the respective cellular pools.

Providing these pools increase through the cell cycle in proportion to the increase in the rates of uptake and synthesis, the amount of precursor incorporated in a short pulse is a valid measure of the rate of synthesis. However the question of pool changes throughout the cell cycle is an open one since there is insufficient data so far available to either exclude or support such changes.

No significant difference was found in ^{3}H-thymidine incorporation

into whole chick lenses when different exogenous concentrations of thymidine were used (Randall, 1977, Honours Thesis). This implies that the added precursor represents a large portion of the cellular pools, and which does not appear to be a limiting factor in DNA synthesis.

Truman et al. (1976) have demonstrated that incorporation of labelled uridine into whole lenses from chick strains Hy-1, Hy-2 and N exhibit different kinetics for different RNA classes. It is therefore unlikely that in the case of RNA, variation in pool sizes between strains is responsible for any difference in the observed rhythms.

The data obtained by autoradiography of ^{3}H-thymidine labelled chick lenses (Plate 5.1) at various times of day, suggest a variation in the number of nuclei showing DNA synthesis rather than a variation in incorporation rate which might be brought about by fluctuation of thymidine pools.

From the patterns of incorporation of both radioactively labelled thymidine and uridine into explanted chick lenses during a 24-hour time period (the data of which is presented in section 5.1) it is clear that a partial synchronisation is apparent in those cells in S-phase of the cell cycle and those synthesising RNA, and this is dependent on the time of day. When comparisons were made of the lenses of different genetic strains of chick the patterns of circadian variation were found to be strain specific. ^{3}H-thymidine incorporation was not directly related to either dry or wet weights of the lenses (Randall et al., 1979): causes for the strain differences in thymidine incorporation must be sought elsewhere.

It has been established from a variety of data that thymidine incorporation profiles are a reflection of the mitosing lens epithelial

cells. Such a direct relationship between circadian variations in DNA synthesis and mitosis has been documented for a number of larval salamander tissues such as epidermis (Scheving, Chiakulas and Abzug, 1959) and corneal epithelium (Scheving et al., 1962) and various mouse epithelial tissues (Pilgrim et al., 1963). In each case the synthesis of DNA exhibited a periodicity which correlated with mitotic rhythms over the same 24 hour period.

Variation in the periodicity and amplitude of the ^{3}H-thymidine incorporation profiles between lenses of the different chick strains under study may therefore be a reflection of the cell cycle durations in the different strains and the number of the cells in synchrony at any one time. Strains Hy-1 and N exhibit 4 peaks in the ^{3}H-thymidine incorporation profile which coincide with each other, while strain Hy-2 exhibits 3 peaks in a 24 hour period although their amplitudes differ in these genotypes. The data for Hy-1 suggests either:
1) that a proportion of cells are in rapid cycle: the highest peak possibly includes the majority of cells with a longer cycle time, or alternatively, 2) a high proportion of longer cell cycle times are staggered at regular intervals. The proportions of total counts in the first peak of N suggest that most cells have a 24 hour cycle, but a small proportion of cells are either out of phase or in more rapid cycle. In the case of Hy-2, a possible hypothesis is that the cell cycle time is more homogeneous being of the order of about 8 hours periodicity.

The rate of cell division in single L.E. cell clones in culture and the growth rate in mass culture (Eguchi et al., 1975; Clayton et al., 1976) show that the mitotic rate is intrinsic to the cells of each

of these genotypes. Furthermore, calculations based on the growth rate of single-cell clones (Eguchi et al., 1975; Clayton et al., 1976) show that there is some heterogeneity in the cell cycle duration in a population of strain N L.E. cells having an average cycle time of approximately 24 hours. From the data of Eguchi et al. (1975) Hy-1 lens epithelium was calculated to have two different but equal cell populations: one similar to N and one with a faster rate of cell division averaging 7.2 hours in duration.

Time-lapse photography was used to determine the intermitotic intervals of L.E. cultured cells from these 3 chick strains (Section 5.5). It was shown that strain Hy-2 is indeed more homogeneous with respect to intermitotic time. It can be seen from Figure 5.19 that a few cells did have a longer cycle time but the majority were approximately 8 hours in duration. The greater heterogeneity in the LE cells of strains N and Hy-1 can be seen from Figures 5.17 and 5.18 respectively. This data together with that obtained from single cell clones (Eguchi et al., 1975) has corroborated the hypotheses proposed to account for the differences in the ^{3}H-thymidine incorporation profiles. It permits a distinction between the two possibilities outlined above concerning cell cycle times in strain Hy-1. Two main populations are in evidence, one with a longer cycle and one with a shorter cell cycle in the order of 24 hours and 8 hours respectively.

From autoradiographic studies showing the distribution of ^{3}H-thymidine labelled nuclei in the L.E. at different times of day, it appears that populations of cells in synchrony occupy similar zones in the lens epithelium. It is further suggested that the central epithelium may consist of cells with a different cell cycle duration than

those nearer the periphery. In the equatorial region of the lens, mitosis ceases and the cells further differentiate to form lens fibre cells. In favour of this hypothesis is the proposal by McAvoy (1978) that a distinct lens epithelial proliferation compartment and cell elongation compartment in the rat lens is maintained by diffusion of aqueous humour via the ciliary process. He noted that lens epithelial cells immediately below the ciliary process divided most often. Proliferation factors in the aqueous humour may become less concentrated or inactivated as they diffuse, resulting in lens epithelial cells further away from the ciliary process receiving less stimulus to divide.

The G_1/G_0 phase of the cell cycle is thought to be the only variable phase of the cell cycle in chick lens epithelial cells (Mikulicich and Young, 1963). It is possible that the G_0 duration of the cells in the central region of the epithelium may be different from that in the peripheral region due to the differential exposure of these cells to factors from the aqueous humour.

The data presented above suggest that there may be two ways of increasing the number of cell divisions in fast growing animals:
(1) by decreasing the duration of the mitotic cycle, or
(2) by increasing the numbers of cells involved.
It would seem that Hy-1 may use both mechanisms while Hy-2 may rely mainly on changing the cell cycle duration. These data are therefore indicative of the genetic control of the cell cycle, such that the mitotic rate is higher in the hyperplastic strains. This being the case, then the multi-layering of the epithelium would be due to an increase in the production of stem cells rather than any change in the

recruitment of these cells for differentiation into fibres.

Interpretation of the uridine incorporation profiles is more complex as it does not only reflect the mitosing epithelial cells. In addition those undergoing differentiation are rapidly synthesising RNA (Reeder and Bell, 1965) and some RNA metabolism occurs in the differentiated fibres. The profiles reflect total RNA synthesis but this will probably only include rapidly labelled nuclear RNA and heavy ribosomal precursor as only short pulses were used.

The peaks of synchrony in uridine incorporation coincide with those of thymidine incorporation or follow 1-2 hours later (Randall et al., 1979). As more cells enter S-phase and double their DNA, RNA synthesis also increases. This pattern is therefore consistent with the idea of a gene dosage effect reported in the literature for other cell types. However, the increase in synchrony appears only to be transient falling rapidly again as cells leave S-phase. This could be attributable to a decline in RNA synthesis during mitosis which has been reported in other cell types. Circadian variations in RNA content have been reported in cell lines such as liver (Pfeiffer, 1968) and in human leucocytes (Kohler, Karacan and Rennert, 1972). In the latter case both quantitative as well as qualitative differences in RNA type following a rhythm of approximately 24 hours was reported.

These circadian rhythms have been exposed in the absence of light entraining factors, as the chicks were kept under 24 hour constant light conditions. Reproducibility of the cycle eliminates any disturbance by Man as the entraining factor. Furthermore, variations in thymidine incorporation persist for at least 3 days in whole lenses in longer term culture (Clayton et al., 1976b). The trauma of hatching

could be an entraining factor, variability in hatching time possibly contributing to the variation between lenses at any one time point. It would therefore be of interest to examine labelled precursor uptake in embryonic lenses. Variation in hormone levels has been implicated in the control of these rhythms (Bullough, 1962; Epifanova and Tchoumak, 1963; Tutton, 1973; Tutton and Helme, 1973 and Fisher, 1968). Bullough has discussed circadian mitotic rhythms in terms of waking and sleeping in relation to a high level of secretion of adrenalin while the animal is awake and active, and a low rate of secretion while asleep. Glucocorticoids (Tutton, 1973) and corticosteroids (Fisher, 1968) have also been implicated. The very short term changes reported here cannot be accounted for on this hypothesis. The lens is avascular and would be buffered against short term changes due to blood hormone levels. Translocation of hormones would need to occur through the aqueous and vitreous humors of the eye before reaching the lens. Differing hormone levels in the different strains are unlikely to account for these observed differences as Eguchi et al. (1975) have shown that the growth rate of Hy-1 lens epithelium in cell culture is intrinsically faster than that of strain N cells and which persists through three successive subcultures. Figures 5.17-19 also clearly indicate that each strain has different distributions of cell cycle times in culture, which are not under hormonal control.

As outlined in Sections 1.3 and 1.4 numerous cellular constituents display a variety of oscillatory behaviour, which either individually or in concert could be the basis of the biological clock, giving rise to circadian rhythms as discussed above. In the lens, for example, glycolysis is known to occur to a major extent attributable to the

absence of a vascular supply (Van Heyningen, 1969). The glycolytic pathway has also been shown to exhibit oscillatory behaviour in a variety of cells and cell extracts (Hess et al., 1971; Goldbeter et al. 1976). It is possible that oscillations in the energy generation of the lens could drive the observed cellular circadian rhythms.

The data presented here provide a warning concerning the importance of the time of day of comparative studies in cellular metabolism of different organisms. It stresses that the time of day of comparative studies is a more important parameter than age.

6.2 Induced Synchronisation of Cultured L.E. Cells

Synchronised cells in culture offer a powerful tool for the examination of cell cycle related events. A study was therefore carried out to see if the natural cell synchrony in whole lenses was maintained in isolated epithelial cells in culture. L.E. cells from all 3 strains of chick were found to have lost synchrony by the time they had plated (Figure 5.3). The exact time of the loss of synchrony was not established, but could be attributable to such factors as the loss of cell-cell contact, cell-fibre contact or the effect of FCS in the culture medium.

Whole lenses or isolated epithelium did not offer the same advantages as synchronous epithelial cells in culture for the type of studies to be undertaken and reported in this thesis. For example: in cell culture, protein synthesis studies could be timed to include only dividing cells and not lens fibres or those cells undergoing terminal differentiation; the cell surface of L.E. cells would be more amenable to analysis in monolayer culture; cell morphology and growth rate of cells could

be easily monitored under closely controlled culture conditions. Synchronisation of L.E. cells by artificial means was therefore undertaken. The mitotic selection and cell cycle arrest methods were adopted and are discussed fully in Chapter 4. Strain Hy-2 L.E. cells were used as much as possible for this work as it appeared that the cell population was more homogeneous with respect to cell cycle durations than the other two strains (see Section 6.1), and therefore greater synchrony may be obtained.

L.E. cells synchronised by both the mitotic selection method and the cell cycle arrest method did indeed exhibit synchronisation of the cell cycle, as judged by the incorporation of ^{3}H-thymidine at time intervals after the synchronisation procedures (Figures 5.4, and 5.5). Cells were not in complete synchrony as suggested by the trends of these incorporation profiles. If absolute synchrony was present, a more dramatic increase in ^{3}H-thymidine incorporation with a zero baseline between peaks would be expected. This appears to be very difficult to achieve, as evidence in the literature suggests for eukaryotic cells. Over 90% of cells after mitotic selection were shown to be in a narrow band of the cell cycle (Section 4.1). After 10 hours, approximately 85% cells were still in synchrony. This was even less in synchronised cells obtained by the cell cycle arrest method, being about 75% of cells in synchrony after 10 hours. This dampening in the level of synchronisation of cells has been attributed to variation in the duration of the G_1 phase for any cell population (Nias, 1968). This can also lead to the total loss of synchronisation after only 2 or 3 cell cycles. For this reason, the first period of synchronisation corresponding to a single cell cycle was only of interest because of

its maximal levels of synchronisation. It has been suggested by data from ^{3}H-thymidine incorporation studies and time-lapse photography which is discussed in Section 6.1, that the cell cycle duration of Hy-2 is fairly homogeneous, being about 8 hours long. It then follows that the population of cells under study in the first period after synchronisation is the same population of cells which is in evidence in any succeeding periods.

The ^{3}H-thymidine profiles obtained after the cell cycle arrest synchronisation procedure are characteristic in the presence of a lag period of approximately 4 hours in duration between the time of stimulation of the cells with serum and the onset of DNA synthesis. This has been discussed fully in relation to other data in Section 4.2.3.

^{14}C-uridine incorporation in Hy-2 L.E. cells synchronised by both methods of cell synchronisation also showed variation with time. Peaks in incorporation coincided with the peaks in thymidine incorporation. This further suggests a direct link between DNA synthesis and the enhancement of RNA synthesis which, as already discussed, could be accounted for by a gene dosage effect and a decrease of RNA synthesis during mitosis.

Intervals between peaks of incorporation of both ^{3}H-thymidine and ^{14}C-uridine looked very similar to those found to occur in circadian rhythms in this strain. This further suggests that these circadian rhythms are a reflection of the cell cycle of the L.E. cells in the Hy-2 chick lens.

6.3 Protein Synthesis During the Cell Cycle

Various hypotheses have been proposed (see Section 2.5.1) to account for the differential gene expression of the crystallins during ontogeny of the lens based on data from a variety of sources. These include the cell cycle and mitotic distribution (Clayton et al., 1976b; Modak et al. 1968); the rate of differentiation of lens fibres (Hanna et al., 1966); regulatory signals mediated by way of the cell surface (Clayton et al., 1976a; Okada et al., 1971); the metabolic status of the epithelial cells and adjacent tissues (Clayton et al., 1976b); and regulatory factors (McAvoy, 1978). Numerous data have implicated the cell cycle. Indeed, some data already presented (Section 5.4), concerning the addition of a retinal extract to L.E. cells, and to be discussed in the next section, do suggest correlations between the length of the cell cycle and δ crystallin synthesis. Numerous other data concerning this relationship have been reported (de Pomerai et al., 1978; Eguchi et al., 1975)(see Section 2.5.2). This correlative evidence suggested the possibility that changes in crystallin synthesis may occur during the cell cycle, such that cells with a shorter cell cycle may synthesise a different crystallin profile to that of a cell with a longer cell cycle. This hypothesis was investigated, utilising the synchronised cultures of the L.E. cells obtained by the cell cycle arrest method in order to obtain high cell yields (section 5.2.4).

An important finding from this investigation was that no qualitative differences in protein synthesis were observed at different times in the cell cycle which could be resolved by one dimensional SDS electrophoresis. The presence of contaminating asynchronous cells could however mask any qualitative differences. Nevertheless, this finding is in agreement

with much of the recent data particularly on mammalian cells which did not provide any evidence for qualitative changes in cellular protein synthesis through the cell cycle by two-dimensional SDS electrophoresis (Bravo and Celis, 1980; Milcarek and Zahn, 1978) (see Section 1.3).

Some quantitative differences were apparent at different stages during the cell cycle. δ crystallin was found to increase in synthesis by approximately 25% over the period corresponding to S-phase. α_2 crystallin was also found to increase over the same period. These were the only changes in crystallin synthesis which were evident.

As outlined in Section 2.5.2 δ crystallin synthesis has been shown to be greater in cells where the overall growth rate of the L.E. cell population under study was faster, e.g. in the 3 chick strains under study in this thesis. It is possible that cells with a shorter cell cycle synthesise more δ crystallin by having a higher ratio of time spent in S-phase relative to the rest of the cell cycle. Similarly cells which have a longer cell cycle, may spend a smaller proportion of time in S-phase relative to the rest of the cell cycle and so synthesise less δ-crystallin. This possibility however is based on the assumption that the duration of S-phase for all L.E. cells, both from embryonic and post-hatch lenses and between strains, is constant.

These data concerning an increase in δ-crystallin synthesis during S-phase were obtained using strain Hy-2 L.E. cells in culture. It would be interesting to conduct a similar study using strains N and Hy-1. The heterogeneity in the cell cycle durations of L.E. cells from these strains, however, makes the synchronisation of these cells for such a study difficult. Only if δ-crystallin did show a real increase during S-phase in cells of different cell cycle durations could

the possibility outlined above be genuine. There is also the possibility that the increase in δ crystallin synthesis observed could merely be an artefact of the synchronisation procedure attributable to biochemical upset of the cells during manipulation. It was emphasised in Section 1.3 that some of the data concerned with differential protein synthesis through the cell cycle such as histones and immunoglobulins did not agree on the timing of synthesis or indeed in some cases whether time dependent synthesis occurred at all. This could be attributable, at least in part, to the synchronisation procedures used. However extreme care has been taken to minimise this possibility in the experiments reported.

As cells terminally differentiate in the equatorial region (Figure 2.1) and elongate to form lens fibres a large amount of protein synthesis is taking place (Piatigorsky et al., 197; Milstone et al., 1975). These cells are no longer traversing the cell cycle. The greatest proportion of crystallins are therefore being synthesised in the intact lens by cells which are not cycling cells. This suggests that factors other than the cell cycle duration may be involved in the regulation of crystallin synthesis. There is always the possibility however that the cell cycle preceding terminal differentiation could in some way regulate the proteins synthesised thereafter. The chick crystallins have been shown to exhibit non-coordinate synthesis and their regulation has been shown to be sensitive to a variety of factors (Clayton et al., 1976b). Several other regulatory mechanisms may then be involved in the synthesis of each crystallin, as outlined briefly above such as the rate of differentiation into lens fibres, metabolic changes in the lens and regulatory factors.

α_2 crystallin was also shown to increase during the S-phase of the cell cycle. However α crystallin synthesis, unlike δ crystallin synthesis, has not been correlated with a short cell cycle, for example as in embryonic lens epithelium (de Pomerai et al., 1978). However in such studies α crystallin as assayed by antibodies was mainly α_1 crystallin. α_2 crystallin synthesis could therefore vary in cells with short or long cell cycle times and not have been detected in such a study.

Two other polypeptides showed an increase in synthesis greater than 20% of the averaged controls, and have been termed 'act' and 'u'. The former has been tentatively identified as actin with molecular weight of approximately 40,000-42,000 but the latter, 'u', has not been identified. It has a molecular weight of approximately 52,000-55,000. Both increased in synthesis during the period which coincided with the transition of cells from G_1 into S-phase. Riddle et al. (1979) identified a transient increase in actin synthesis following restimulation of arrested Swiss mouse 3T3 cells which coincided with entry into S-phase. Increased synthesis of other unidentified polypeptides at this same stage in the cell cycle have been reported (Ley, 1975 and Gates et al., 1978) of 50,000 molecular weight approximately. No specific function has been assigned to these polypeptides but such changes may be a general phenomena amongst all cells undergoing G_0 - S phase transition possibly involving cell shape changes at this time. If this proves to be the case then actin or any other polypeptide increasing at the G_0 - S boundary could prove to be a useful specific marker and may help to elucidate the biochemical nature of this transition.

It is interesting to note that the membrane proteins of the same cells, which exhibited a transient increase in actin synthesis, showed an increase in actin synthesis over the same period. This suggests that

actin synthesised at this time also increases its association with the cell membrane. No corresponding increase was found for the polypeptide labelled 'u' in the membrane component of these cells.

6.4 Cell Membrane Changes During The Cell Cycle.

It is a well documented phenomenon that lectin binding is found to be greatest during mitosis in many normal cell types (e.g. Shoham et al., 1974), decreasing during interphase. Transformed cells on the other hand have been found to exhibit high lectin agglutinability throughout the cell cycle and not just limited to mitosis. In the L.E. cells of the 3 strains of chick under study, differences were apparent in the relative agglutination by various lectins (Odeigah, 1977 Ph.D. Thesis; Clayton, 1978). Interpretation of these results was made difficult by the subsequent finding that a circadian rhythm in DNA synthesis was present in the L.E. cells. These lectin studies were carried out several hours after explantation of the cells resulting in the possibility that this rhythm could have influenced the results by involving more mitotic cells in one strain when compared to another. In order to establish if the differences between strains in the agglutinability of L.E. cells by lectins were real, lectin binding studies were carried out on synchronised cells. L.E. cells from all 3 strains of chick showed a greater binding of all 4 lectins (ConA, RCA, PHA and WGA) during mitosis when compared with the time period following. Lectin binding immediately after mitotic selection is comparable between strains as in all cases 90% of cells or more were in mitosis. Lectin binding at subsequent time intervals may not be directly comparable between strains because of differences in the cell cycle duration. However, by averaging lectin binding at these time points

and comparing them it is possible to get an estimate of the relative amount of lectin binding during interphase. Strains Hy-1 and Hy-2 had greater binding of lectins compared to strain N. Strain N also showed the greatest decrease in lectin binding between mitosis and 2 hours later. This data therefore suggests that there are intrinsic differences in the cell membrane configuration when strains Hy-1, Hy-2 and N are compared. Other data concerning the membrane proteins of these 3 strains analysed on SDS and IEF polyacrylamide gels and electron microscope analysis of the L.E. cell membranes (Odeigah, 1977; Odeigah et al., 1979; Clayton et al., 1976a) suggested differences between the strains. The multilayered arrangement of L.E. cells in situ in both strains Hy-1 and Hy-2 over the anterior face of the lens suggests a membrane alteration such that contact inhibition is diminished on the upper and lower surfaces of these cells compared with normal lenses (Clayton, 1975). Hy-1 and Hy-2 also display other traits which liken them to transformed cells: greater lectin binding capacity particularly during interphase and an increased mitotic rate. These traits appear to be intrinsic to the cells themselves and have arisen probably in association with high selection pressure for faster growth of the chicks themselves. Differences in the membrane configuration of L.E. cells from the 3 chick strains over and above cell cycle changes could result in differences in their response to external signals such as hormones. Such strain differences in response to external culture conditions do occur resulting in changes in the crystallins synthesised (see Section 5.4). Insulin, for example, results in such changes (Clayton et al., 1980) and is also known to exert some of its effects via the cell surface (Levine, 1966). It

has also been shown to have a greater number of binding sites on fibroblasts which divide rapidly compared with those dividing more slowly (Thomopoulos et al., 1976).

An apparent contradiction exists between the data available concerning lectin binding and agglutination studies through the cell cycle and that concerning protein synthesis studies through the cell cycle. If the cell membranes express such different sites between mitosis and interphase one would possibly expect that the synthesis of membrane protein precursors would also vary throughout the cell cycle. However this does not appear to be the case judging both from the literature and from the data presented in this thesis concerning protein synthesis during the cell cycle. This however is a general problem, not specific to lens epithelial cells. This discrepancy could be resolved by postulating changes in lectin agglutination and binding due to alteration in the configuration of already existing sites or the masking and unmasking of already existing sites. Lectins bind to the sugar moieties of the membrane glycoproteins which could change without any difference in the proteins being synthesised, by utilising enzymes already available.

6.5 Induced Changes in Growth Rate and in Crystallin Synthesis

The possibility of the cell cycle as a regulatory factor in the control of crystallin gene expression has been discussed. Numerous other factors may also be involved in controlling lens differentiation. External signals such as adjacent tissues (McAvoy, 1978); hormones e.g. prolactin and growth hormone (Wainwright, Rothstein and Gordon, 1976); and growth factors such as RE (Arruti et al., 1978) or differentiation promoting factors, e.g. lentropin (Beebe et al., 1980)

have all been shown to be capable of playing a contributory role either alone or in concert. For example, they may influence the cell cycle, which in turn regulates crystallin synthesis.

The effects of insulin, foetal calf serum and a retinal extract (R.E.) on the 3 chick strains Hy-1, Hy-2 and N were investigated. Insulin has been reported in the literature to stimulate mitosis (Piatigorsky and Rothschild, 1972) and elongation (Piatigorsky, Rothschild and Wollberg, 1973) of embryonic chick lens epithelia. FCS has also been shown to affect mitosis (Harding et al., 1971) and increase RNA synthesis in embryonic L.E. cells (Piatigorsky and Rothschild, 1971). It is not known whether insulin is responsible for the serum induced cell elongation or if the mechanism of action is similar. An increase in δ crystallin synthesis was also shown to occur simultaneously with cell elongation in embryonic L.E. cells supplemented with 15% FCS (Milstone and Piatigorsky, 1975).

A retinal extract (R.E.) isolated from bovine retinas has been shown to modulate the morphology and enhance the proliferative capacity of bovine lens epithelial cells in culture (Arruti and Courtois, 1978; and Barritault, Arruti, Whalen and Courtois, 1979). This may be of significance with respect to lens differentiation as investigators have pointed out that the retina must produce some diffusable factors which control the proliferation and the differentiation of L.E. cells during development (reviewed by Jacabson, 1966; Coulombre et al., 1963).

The effect of insulin R.E. and FCS on the growth rate of post-hatch L.E. cells from the 3 chick strains is in keeping with previous data concerning the enhancement of embryonic chick lens cells and of bovine epithelial cells in the case of R.E. Of particular interest

here, however is the differential response of the different strains to these additives. Strain Hy-1 showed a greater response than strain N with strain Hy-2 as the intermediate when insulin or R.E. was added. 6% FCS, the routine additive in the culture of these cells resulted in strain Hy-1 with the fastest growth rate and strain N with the slowest. 10% FCS, however, stimulated strain N L.E. cells to grow faster than either of the other two strains. 15% FCS did not appear to have an additive effect over 10% FCS but rather an inhibitory one. This suggests that blocking of crucial sites on the membrane may occur preventing an additive effect of the active FCS components.

The above data suggests two main points to be emphasised:
(1) FCS, insulin and R.E. all have a noticeable influence on growth rate of chick L.E. cells in culture but appear to have different mechanisms of action as judged by the growth response of these cells from 3 different strains.
(2) The L.E. cells from the 3 different strains have different sensitivities to each of these factors resulting in a different level of growth stimulation for each of these factors. This further suggests differences in the receptor sites of the cellular membranes which are likely to receive information from any external signals. Differences have already been shown in the lectin binding capacity between these strains, and Odeigah (1977) also showed membrane glycoprotein differences between those strains. The L.E. cell populations of particularly strains Hy-1 and N have been shown to be heterogeneous with respect to cell cycle time. Therefore the whole cell population may not behave in the same way.

The morphological change in newly explanted L.E. cells resulting

from R.E. treatment (Plate 5.4) suggested a change in the membranes at this time. The cells assumed a spherical appearance similar to mitotic cells with R.E., compared to a flattened appearance with adjacent cell contact in the case of the control. Arruti et al. (1978) showed that this R.E. modified the morphology of bovine lens cells in vitro making them elongate into spindle-shaped cells. However they also showed that 3 different morphologies could be obtained depending on the culture conditions (Barritault et al., 1979). Of particular interest is the observation that when these cells continued to be treated with R.E., the cell population did not divide and grow to the extent of the untreated control. However, cells which were allowed to plate in the absence of R.E., did result in growth stimulation when R.E. was added thereafter. The rounded spherical appearance of the cells did not then occur. This suggests that some adaptation of the cells to the in vitro culture conditions was somehow inhibited by R.E. presence, such as a membrane modification. At the time of seeding the cells are all dissociated as single cells. R.E. may then prevent any cellular interactions with the basement membranes, which according to Gospodarowicz et al., (1978) is the mechanism by which cell growth rate is controlled. Considering this same hypothesis, once cell contact has been made R.E. may promote greater cell contact, resulting in increased growth rate.

Most of the studies concerning R.E. have been reported utilising bovine epithelial cells. In culture, under normal conditions these cells do not form lentoids, unlike chick lens epithelial cells. In the study of R.E. reported in this thesis it was found that R.E. did not permit lentoid structures, with their associated bottle cells, to develop. This was in marked contrast to the controls, to the effect

of higher FCS concentrations and of insulin. No other evidence could be found in the literature for the induced absence of lentoid structures in chick L.E. cells *in vitro*. This phenomenon was common to all 3 strains of chick under study. Different FCS concentrations and insulin changed the frequency and size of the lentoid structures but they were always present in cultures after approximately 10 days in culture.

Lentoid structures have been shown to consist of a multilayered lens-like arrangement of elongated lens fibres with an ultra-structure similar to lens fibre cells *in vivo*. A high accumulation of crystallins has also been shown to occur in these structures (Okada *et al*., 1973). The differentiation of these lens fibres is characterised by a marked cell elongation with accompanying intensive crystallin synthesis. δ crystallin is the predominant crystallin accumulated in maturing lens fibres of embryonic chicks (Clayton, 1970). It is also very predominant in chick L.E. cultures, δ-crystallin accumulating as lentoids develop (de Pomerai, Pritchard and Clayton, 1977).

Analysis of the crystallins accumulated in these lentoid-free cultures after R.E. treatment showed a pronounced accumulation of δ-crystallin in all 3 strains but particularly in strain Hy-1 when compared with untreated control cells or any other treatment used. There appears to be a conflict in results in the case of R.E. treated cultures between an increase in δ-crystallin synthesis and the absence of lentoid structures. Piatigorsky *et al*. (1973) showed by cell elongation inhibitory experiments using colchicine treatment of embryonic L.E. in culture that δ-crystallin synthesis and δ-crystallin mRNA synthesis were independent of cell elongation. Therefore it is possible to uncouple biochemical differentiation, in terms of crystallin

accumulation, from morphological differentiation.

However, there is the possibility that these factors change the rate of maturation of these cultures. A single time point of analysis would therefore not obviate such differences as lentoids may develop at a later stage. Successive time points of analysis would need to be invoked in order to resolve this possibility.

Proximity to the retina has been shown to be necessary for the induction of the lens (Coulombre et al., 1963). As already mentioned, some diffusible factor is necessary in the normal early development of the embryonic lens when δ-crystallin accumulation is high and the growth rate is high. Similar events have been induced by R.E. in vitro. However promotion of δ-crystallin synthesis only and not lens fibre formation appears to occur. This suggests that there may be other factors involved in the morphological differentiation of L.E. cells. Beebe, Feagans and Jebeno (1980) have recently described the presence of a factor they have termed lentropin from the vitreous humour which promotes lens fibre differentiation. Yammamoto (1976) had also suggested the presence of such a factor stemming from the posterior chamber of the eye and which promotes fibre differentiation. A factor which stimulates cell division has been shown to be present in the aqueous humour (Reddan, Weinsieder and Wilson, 1979). Extracts from various ocular tissues including iris, pigmented epithelium, choroid and the vitreous body were found to elicit similar proliferation responses and morphological changes on bovine epithelial cells as with retinal extract.(Barritault et al., 1979). This led to the proposal that all of these extracts may involve only a single factor common to all of these tissues, but this is uncertain.

Cell division and lens fibre differentiation can occur in chick L.E. *in vitro* cell culture, isolated from other ocular tissues, in the presence of 6% FCS and a defined growth medium. This suggests that some analogous factors must be present in serum.

It is therefore possible that several of the factors described above, including insulin and those present in serum, may act in concert regulating lens epithelial cell proliferation and subsequent differentiation into lens fibre cells. It is likely that the cell division - cell elongation transition occurring at the lens equator, coinciding with the position of the ciliary process, is the region where numerous factors may be involved in the differentiation process.

The effects of R.E., insulin and FCS has further demonstrated that the crystallins can be regulated in a non-coordinate fashion as previously reported (Clayton *et al.*, 1976a). The differences between the strains in response to any one of the additives with respect to both growth rate and crystallin synthesis emphasises two things:

1) the importance of external signals in regulation of differentiation, and,

2) the importance of the competence of the cells to respond to these signals and which can vary between cells of different strains.

CHAPTER 7. CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK

7.1 Conclusions

7.1.1 A circadian rhythm in DNA synthesis is present in the lens epithelium of whole chick lenses. A similar rhythm in RNA synthesis is also evident. These rhythms have been shown to be a reflection of the mitosing epithelial cells and are strain specific. These differences occur as a result of the composition of the whole lens epithelium population with respect to the duration of the cell cycle. These results suggest that the cell cycle duration in L.E. is under genetic control.

7.1.2 This Circadian rhythm is not maintained in dissociated L.E. in culture and so could not be used as a source of synchronous cultures. However synchronous cultures of L.E. can be induced in vitro utilising the mitotic selection method and a method involving cell cycle arrest followed by restimulation back into the cell cycle. Strain Hy-2 L.E. cells offered the best opportunity for synchronisation and subsequent biochemical studies because it contained predominantly cells of one intermitotic duration.

7.1.3 Protein synthesis studies of these synchronised cells led to the conclusion that no qualitative differences were apparent in the synthesis of cytoplasmic proteins at different times during the cell cycle of L.E. cells. Quantitative changes were found in several instances. δ and α_2 crystallin synthesis increased at a time corresponding to S-phase. This suggests that cells with a shorter cell cycle, and therefore spending relatively more time in S-phase, would synthesise more δ and α_2

crystallins. Evidence is available correlating increased δ crystallin synthesis with cells of short cell cycle. The conclusion was made that, pending further studies, the cell cycle could be involved in the regulation of crystallin synthesis in conjunction with other controlling factors.

Two polypeptides increased in synthesis at a time corresponding to the end of G_1 and the beginning of S-phase. One of these has been identified as actin. These may prove useful markers for G_1-S phase transition in the study of the biochemistry of this important cell cycle event.

7.1.4 In contrast to the absence of significant changes of membrane polypeptides during the cell cycle, major differences were apparent in the lectin binding capacity of L.E. cells during the cell cycle. This dichotomy may be resolved by suggesting changes in the fluidity of the membrane resulting in different configurations of the membrane glycoproteins between mitosis and interphase. Lectins have more than one binding site and so result in a crosslinking of receptors. The configuration of these sugar moieties of the membrane glycoproteins may be of considerable importance for lectins to bind.

Strains Hy-1 and Hy-2 showed greater lectin binding than strain N both during mitosis and interphase. This observation taken together with the data concerning the higher proliferative capacities and abnormal cellular behaviour in vivo resulting in lens epithelium hyperplasia, suggests that the L.E. cells of strains Hy-1 and Hy-2 exhibit properties associated with transformed cells. This may be a direct result of the high selection

pressure for faster growth exerted on strains Hy-1 and Hy-2 chickens.

Differences in the membrane receptors of L.E. cells between the different chick strains under study could result in differences in their response to external signals such as growth factors and hormones.

7.1.5 The effect of insulin, foetal calf serum and retinal extract on the crystallins synthesised in cultured L.E. cells provides further evidence that the crystallins are regulated in a non-co-ordinate fashion. Differences in the response of L.E. cells from the 3 different chick strains to the same external stimuli, suggest that the receptors involved in the transmission of such external signals differ between the strains. These differences could be analogous to those found for differential lectin binding between the strains.

The observation that retinal extract treated L.E. cultures exhibited a large increase in δ crystallin synthesis but an absence of lentoids led to the conclusion that biochemical differentiation in the form of crystallin synthesis could be dissociated from morphological differentiation. RE promotes biochemical differentiation in chick L.E. cells and somehow prevents morphological differentiation occurring. Some other factor may be necessary in greater concentrations to promote morphological differentiation. Such a factor has been documented. From the work presented it is concluded that numerous factors are involved in concert in the regulation of lens epithelial cell proliferation and their subsequent differentiation

into lens fibre cells. These include: the cell cycle and its associated circadian rhythm; the genetic make-up of the cells themselves; serum factors such as hormones and growth factors; and factors from other ocular tissues.

7.2 Recommendations for Future Work

The underlying basis of the circadian rhythm present in the chick lens is an interesting problem. However the magnitude of the problem is perhaps evident from section 1.3, which attempted to review some of the cellular components exhibiting oscillatory behaviour and so could possibly play some part in the basis of the rhythm. Every epithelial tissue so far examined for the presence of a circadian rhythm has exhibited one. However few tissues have offered such an ideal study system as the epithelium in the lens. Other tissues exhibiting circadian rhythms and which have been studied in detail include the liver and the gut epithelium. Such studies are complicated by the greater complexity and heterogeneity of these tissues and the close contact with the vascular supply. The lens offers a system without these disadvantages which could prove to be of value in what could be a very complicated set of inter-relationships.

This thesis has reported experiments with synchronous cultures stemming from primary cultures. The fact that the cells used were primary cultures was a major drawback in this study because of the time and effort involved in obtaining such cultures. It would therefore be of great value in the study of chick L.E. cells for a cell line to be established which could be maintained without the necessity of continuing subculture to maintain the cells in the cycling state. Under the present normal culture conditions lentoids develop simultaneously with

a plateau in the cell growth rate. It would be worthwhile to investigate a growth medium which maintained these cells in a cycling state. Such a factor as RE, isolated from chick retina, might make this possible providing its effects were well characterised. Homogeneous cultures with respect to cell cycle duration could possibly be derived for different intermitotic times. Studies such as those reported in this thesis could then be taken a step further and cells with different intermitotic intervals compared to see if such observations as cell cycle dependent protein synthesis is a real and general phenomenon.

External signals to L.E. cells appear to play an important role in the regulation of cell proliferation and differentiation. The mechanism of action of these signals is of great importance in the understanding of such fundamental problems as the cell cycle and differentiation. Comparative investigations of genetic differences between strains have proved to be a useful tool in the dissection of biological processes. Genetic modifications in the L.E. cells of the 3 chick strains examined in conjunction with known regulatory signals such as RE and lectin binding studies, could help to illuminate the regulatory mechanisms involved.

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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.

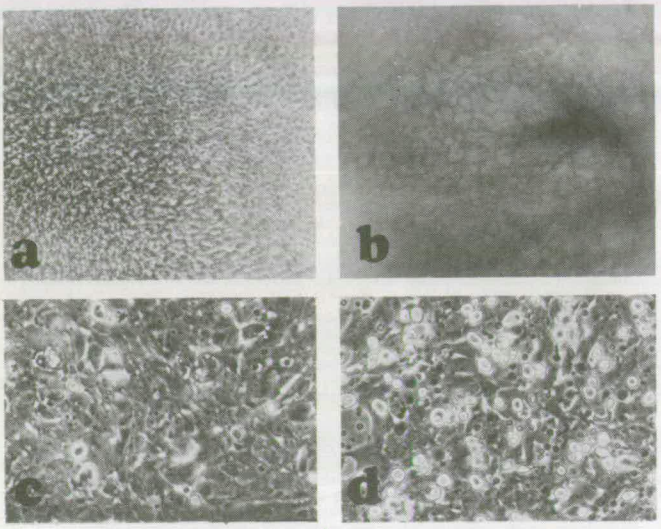


Fig. 1.

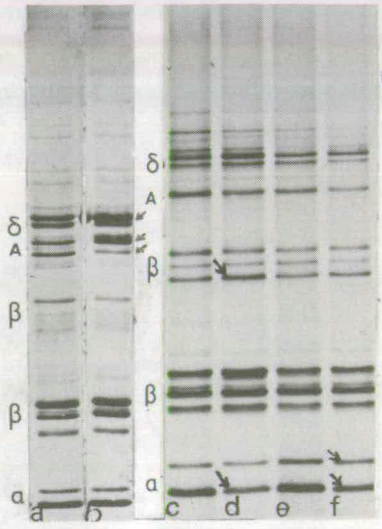


Fig. 2.

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
β2					8.6	4.2	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)

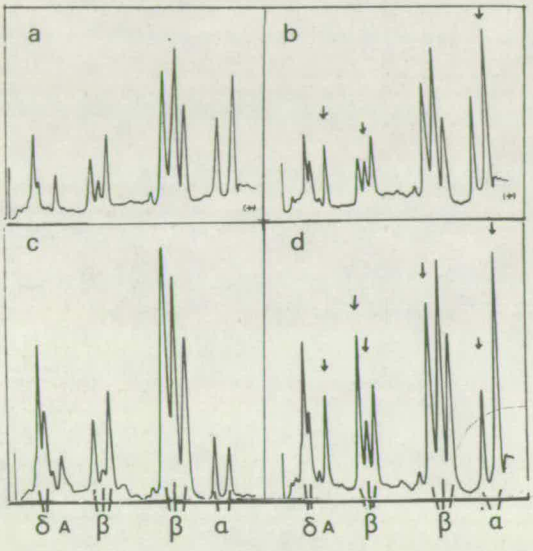
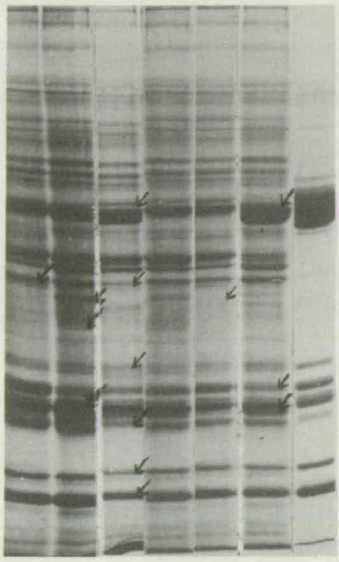


Fig. 3.



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.

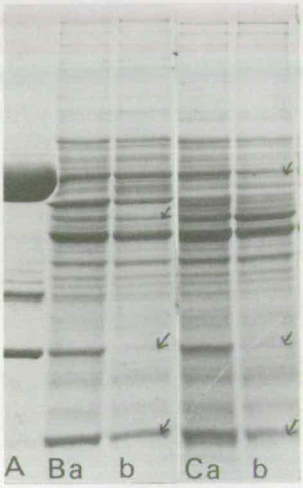


Fig. 5.

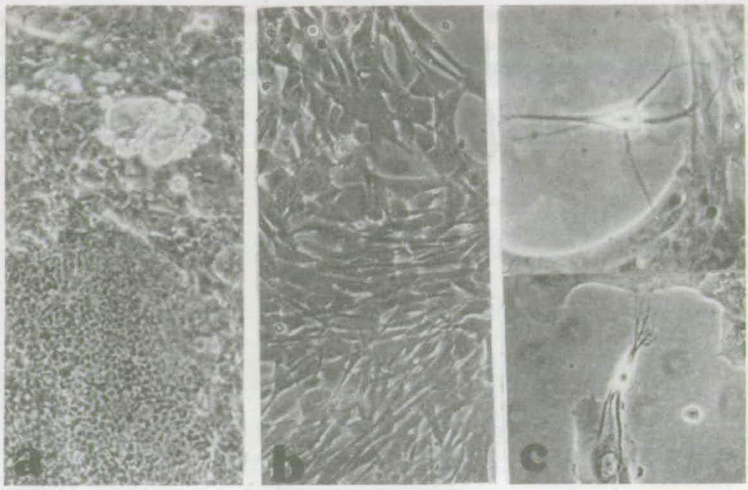


Fig. 6.

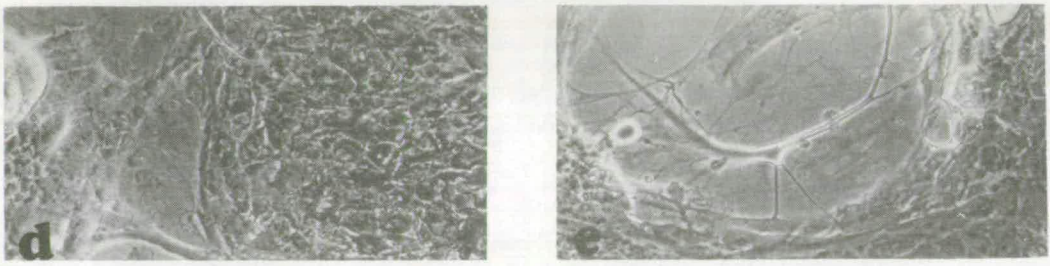


Fig. 6.

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.

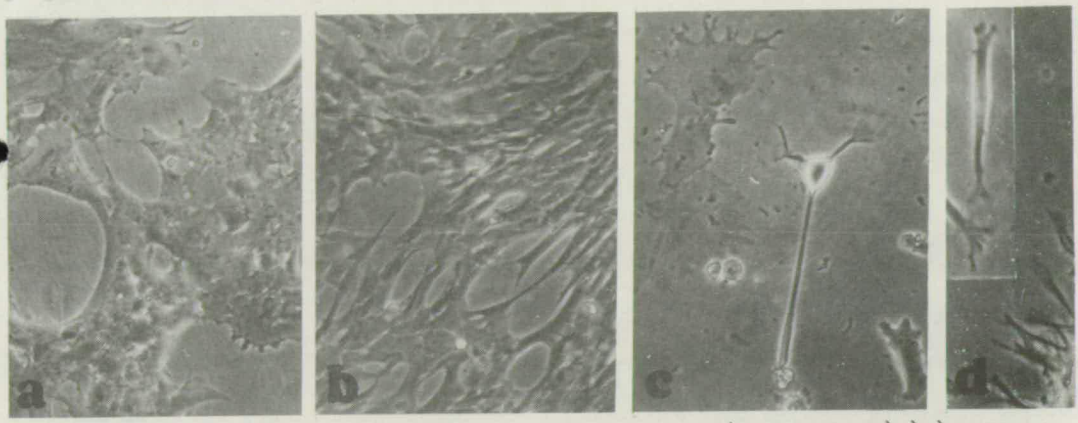


Fig. 7. Lens epithelium cells in secondary culture. a) control. b)c)d) cells in cultures derived from MNNG treated primary culture. b) fusiform cells. c) neurone like cell and epithelial cells. d) bipolar cells from edge of culture, inset, two bipolar cells.

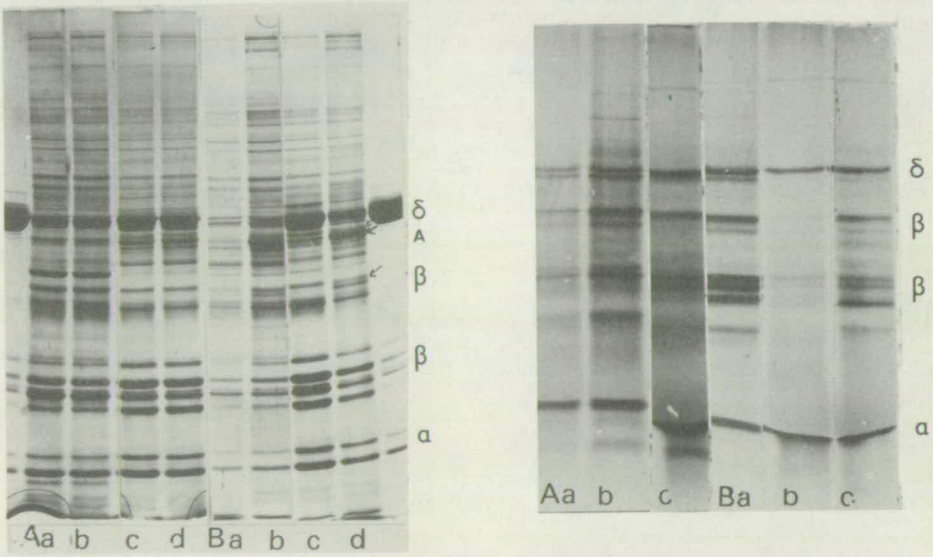


Fig. 8. Proteins from LE control cells and cells after MNNG treatment at 7 days. A) Db. B) Hy-1. Aa) Ba) 9 days, control. Ab) Bb) 9 days, MNNG treated. Ac) Bc) control, 24 and 21 days respectively. Ad) Bd) MNNG treated, 24 and 21 days.
Fig. 9. mRNA translation products from MNNG and control cultures: 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

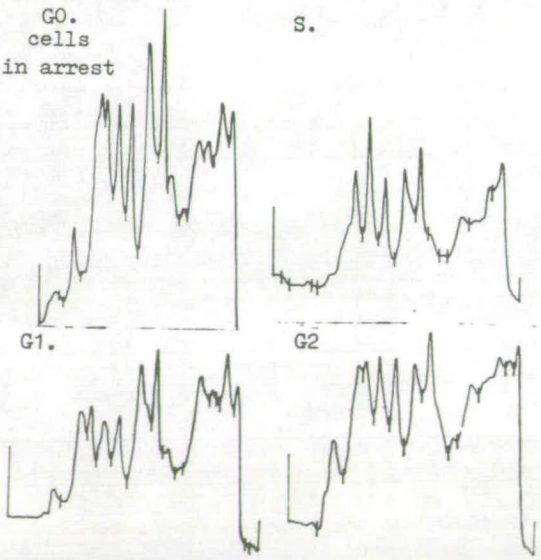


Fig. 10. Densitometer trace from fluorograph of PAG i.e.f. analysis of pulse labelled crystallins from synchronised LE dells. The majority of cycling cells were determined to be in G_1, S, G_2/M, by pulse labelling with ^{3}H thymidine as described in Randall, Truman and Clayton (1979).

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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Genetic differences of DNA and RNA synthesis in the epithelium of the lens of the chick

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SUMMARY

The genetically unrelated chick strains Hy-1 and Hy-2, which have been strongly selected for growth rate, both exhibit hyperplasia of the lens epithelium. These two strains and a control strain N, not selected for growth rate, were compared with respect to incorporation of ^{3}H-thymidine and ^{14}C-uridine by freshly excised lenses in culture at different times throughout a 24-h period. The levels of incorporation of label into the lens cells were found to vary according to the time of day. The pattern of diurnal variation in both thymidine and uridine incorporation was found to be strain specific. Hy-1 and Hy-2 showed a greater degree of synchrony than did normal (N) lenses, and the frequency of the peaks of incorporation was also higher. Autoradiography confirmed that only lens epithelium incorporates thymidine during culture and that the number of labelled nuclei depends on the time of day when the lenses were explanted. These data point to genetic control of the cell cycle.

1. INTRODUCTION

Two genetically unrelated strains of chick, Hy-1 and Hy-2, have been rigorously selected for high early growth rate over a period of several years: Hy-1 is an inbred strain and Hy-2 the F-1 between two inbred strains. Both show hyperplasia of the lens epithelium and a variety of abnormalities of cellular properties when compared with a strain (N) not selected for rapid growth. In Hy-1 and Hy-2 the lens epithelium contains an excessive number of cells as compared to normal and it forms a multi-layered structure across the anterior face of the lens between the capsule and fibre body. The morphological arrangement of these epithelial cells suggests that they are deficient in contact inhibition on their upper and lower surfaces and shows that they have a tendency to differentiate into fibre cells within the layers (Clayton, 1975). An investigation of epithelium from Hy-1 has shown it to be deviant from normal. Abnormalities in cell culture conditions include the mitotic rate, which is approximately double that of normal cells, cell behaviour, a tendency for precocious fibre differentiation (Eguchi, Clayton & Perry, 1975; Clayton *et al.* 1976*a*) and DNA, RNA and protein metabolism. The regulation of the rate of synthesis of each class of RNA is under genetic control as indicated by uridine incorporation studies, and differs

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markedly between strains (Truman *et al.* 1976). Similarly quantitative differences between strains in the rate of crystallin synthesis and qualitative changes in the membrane composition were found (Clayton *et al.* 1976*b*).

The epithelium of the lens of the eye has been found to exhibit diurnal rhythms with respect to mitosis in the rabbit (von Sallmann & Grimes, 1952) and the rat (von Sallmann & Grimes, 1966) and diurnal variations in the rate of DNA synthesis and mitosis have been found in other epithelial tissues, of, for example, the mouse (Pilgrim, Erb & Maurer, 1963; Potten *et al.* 1977) the rat (Bystrenina & Podderingina, 1976) and humans (Fisher, 1968; Schell *et al.* 1977). Diurnal rhythms have also been observed in RNA content in cell lines such as liver (Pfeiffer, 1968) and in human leucocytes (Kohler, Karacan & Rennert, 1972).

It was observed that when explanted lenses from the three strains were set up in culture, they showed an apparent cyclic pattern of synthesis according to the time lapsed after explantation which appeared strain specific. Clayton *et al.* (1976*b*) suggested that the levels of synthesis could be explained if they were related to the stage of the cell cycle and if the strains differed in the periodicity of the cycle and its sensitivity to modification.

This paper reports that diurnal variation in DNA and RNA synthesis is intrinsic to the chick lens and that the periodicity of the cycle is related to the genotype. This therefore provides a basis for genetic investigations of the regulation of the cell cycle.

2. MATERIALS AND METHODS

(*a*) *Chicks*

Day-old chicks of the Hy-1 and Hy-2 strains and of the normal control strain (*N*) were obtained from Sterling Poultry Products Ltd, Ratho, Midlothian and the Poultry Research Centre. All chicks were kept under constant light conditions prior to experimentation.

(*b*) *Culture of lenses*

Eyes were removed from chicks after decapitation and the lenses were dissected out under sterile conditions and explanted into minimal essential medium (MEM) with Hanks salts with 25 mM (HEPES), Gibco Biocult, Glasgow, Scotland. The lenses were immediately transferred to 2 ml culture medium M199 + 10% foetal calf serum and 200 iu/ml penicillin (Gibco, Biocult), and incubated for one hour at 37 °C in 5% CO_2 in air. The medium was labelled either with ^{3}H-thymidine alone (100 μCi/ml) or double labelled with ^{3}H-thymidine (100 μCi/ml) and ^{14}C-uridine (5 μCi/ml). The maximum time between decapitation of the chick and the start of the radioactive pulse was 25 minutes. Radioactive chemicals were obtained from the Radiochemical Centre, Amersham, England.

(*c*) *Determination of precursor incorporation*

After labelling, lenses were washed in cold phosphate buffered saline (PBS) (Takeichi, 1961), and transferred singly to a 2 cm square of Whatman 3 mm

chromatography paper. The lens capsule was punctured and the lens squashed firmly onto the paper which was then transferred to 5% TCA. The non-absorbent surface of polythene-backed paper (Benchkote, Whatman) was used directly beneath the chromatography paper to prevent the loss of any material. Each paper carrying squashed lens material was washed twice separately in 5% TCA then communally in absolute ethanol and ether. After drying the papers were placed in vials with a scintillant containing 12·5 g PPO and 0·75 g dimethyl POPOP in 2·5l toluene and counted on a liquid scintillation spectrometer (Intertechnique). Scintillation chemicals were obtained from Koch-Light Laboratories Ltd. It was verified that incorporated precursor was adequately washed from the squashed lenses by labelling lenses for varying periods from zero-time to 90 min. The plotted results of disintegrations/min/lens against time showed a smooth curve which passed through the origin.

(*d*) *Autoradiography*

After one hour incubation in ^{3}H-thymidine as outlined above, lenses were washed in cold PBS, fixed, embedded in paraffin wax, sectioned at 10 μm, and the sections were stained with haemotoxylin and eosin. Nuclear emulsion (Ilford L4) was applied by the dipping method (Messier and Leblond, 1957) and exposed at 4 °C for several days (as indicated in figure legends) in sealed containers containing dessicant. Slides were then developed using Kodak D19. Photographs were taken using the Carl Zeiss Ultraphot.

3. RESULTS

(*a*) *Thymidine incorporation*

When freshly excised chick lenses were incubated in medium containing ^{3}H-thymidine for 1 h, the level of incorporation of radioactivity into DNA which was obtained varied according to the time of day (Fig. 1). The fluctuations of incorporation found over the period of 24 h were greater than the standard deviations of the observations on the individual lenses made at one time. When comparisons were made of the lenses of different genetic strains of the chick the pattern of diurnal variation was found to be strain specific. The number of cells engaged in DNA synthesis is greater (as judged by the amplitude of the peaks), as is the synchrony (as confirmed by autoradiography, e.g. Plate 1), in the strains with hyperplasia of the lens epithelium (Hy-1 and Hy-2) than in lenses of more normal morphology (N). The statistical significance of the differences between the maxima and minima were evaluated by Student's *t* test. All three peaks of incorporation of strain Hy-2 are statistically highly significant. The results with Hy-1 are difficult to interpret as the variance is greater than in the other two strains and may mask the cycle to a certain extent, but pooled data (Fig. 5) indicates 4 peaks within a 24-h period. Because of the limited number of observations within a 24-h period, and because each observation is of one hour duration, the precise times of maxima and minima of incorporation can only be estimated by interpolation, as has been

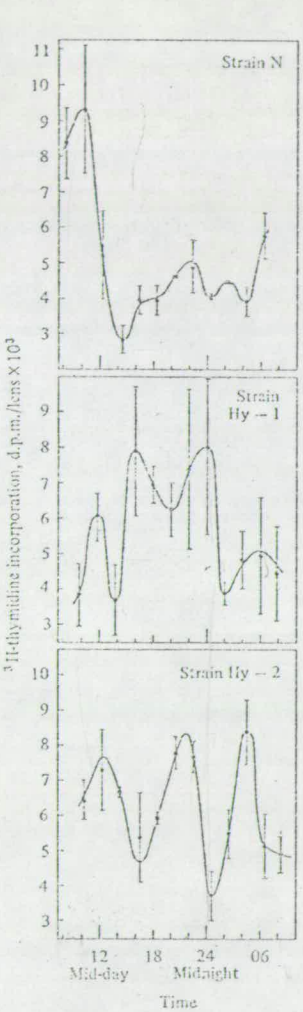


Fig. 1

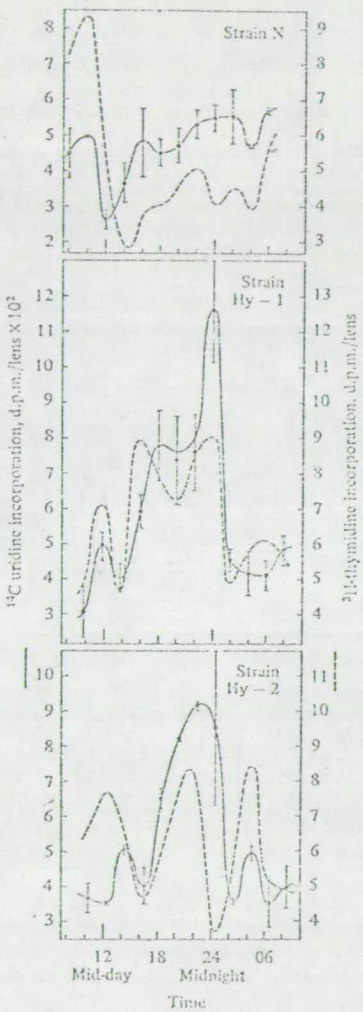


Fig. 2

Fig. 1. Plot of the mean and standard deviation of ^{3}H-thymidine incorporation in groups of freshly explanted lenses of the three strains of day-old chick (N, Hy-1 and Hy-2) at 2-h intervals over a 24-h period. Mean values of disintegrations/min/lens are plotted at the time of commencement of pulse-labelling and therefore correspond to the following hour. The length of the bar represents twice the standard deviation.

Fig. 2. Plot of the mean and standard deviation of ^{14}C-uridine incorporation of groups of freshly explanted lenses of day-old chicks at 2-h intervals over a 24-h period. Superimposed is the ^{3}H-thymidine incorporation profile for the same chicks (dotted line) to show the relationship with the ^{14}C-uridine incorporation. Each lens was pulse labelled for 1 h. Mean values of disintegrations/min/lens are plotted at the time of commencement of pulse-labelling and correspond to the following hour. The length of the bar represents twice the standard deviation.

done to some extent in Fig. 1. The patterns of incorporation are reproducible in different batches of chicks and were consistent in three independent experiments under similar conditions.

Longer term monitoring of thymidine incorporation using less frequent intervals shows that statistically significant diurnal variation persists for at least 4 days after hatching (Fig. 3).

(*b*) *Uridine incorporation*

Freshly explanted lenses incubated in medium containing ^{14}C-uridine for one hour also exhibited partial synchrony and a diurnal rhythm in incorporation of the RNA precursor which was characteristic for each strain (Fig. 2). As in thymidine incorporation, synchrony is greater in the strains with hyperplasia of the lens epithelium than in the control strain (N). All the maxima of incorporation are statistically significant.

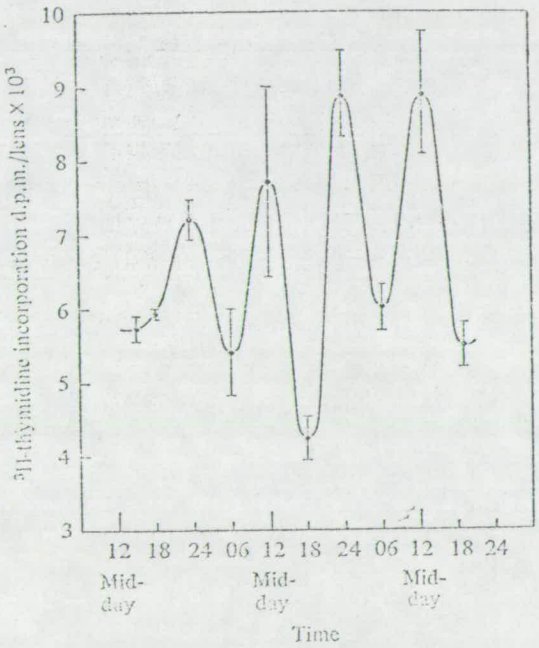


Fig. 3. Plot of the mean and standard deviation of ^{3}H-thymidine incorporation in groups of freshly explanted lenses of day-old Hy-2 chicks at approximately 6-h intervals over a 3-day period. Mean values of disintegrations/min/lens are plotted at the time of commencement of pulse labelling, and therefore correspond to the following hour. The length of the bar represents twice the standard deviation.

A definite relationship exists between thymidine and uridine uptake of the same lenses (Fig. 2). The frequency of synchronous bursts over 24 h are the same in both profiles. However the uridine incorporation maxima appear to occur slightly later than the thymidine maxima in Hy-1 and Hy-2.

(c) *Autoradiography*

Freshly excised lenses of the 3 strains were incubated for one hour in medium containing ^{3}H-thymidine. The times of labelling were chosen from the thymidine incorporation profiles (Fig. 1) to correspond to a maximum and minimum for each strain. Autoradiography of these lenses has confirmed that only the lens epithelium incorporates thymidine during a one hour pulse in all three strains. The results are consistent with the incorporation data in that the level of precursor uptake varies with time. Lenses labelled at a time coincident with maximal thymidine incorporation as judged by a previous experiment showed a greater number of labelled nuclei than lenses labelled at a time corresponding to a minimal level (Plate 1).

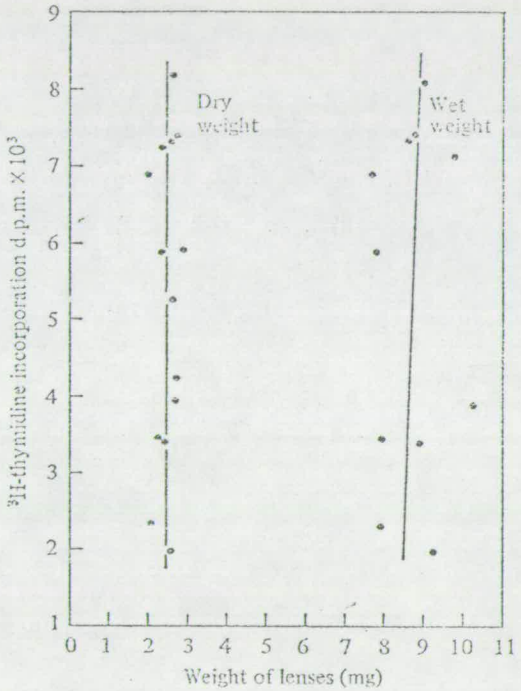
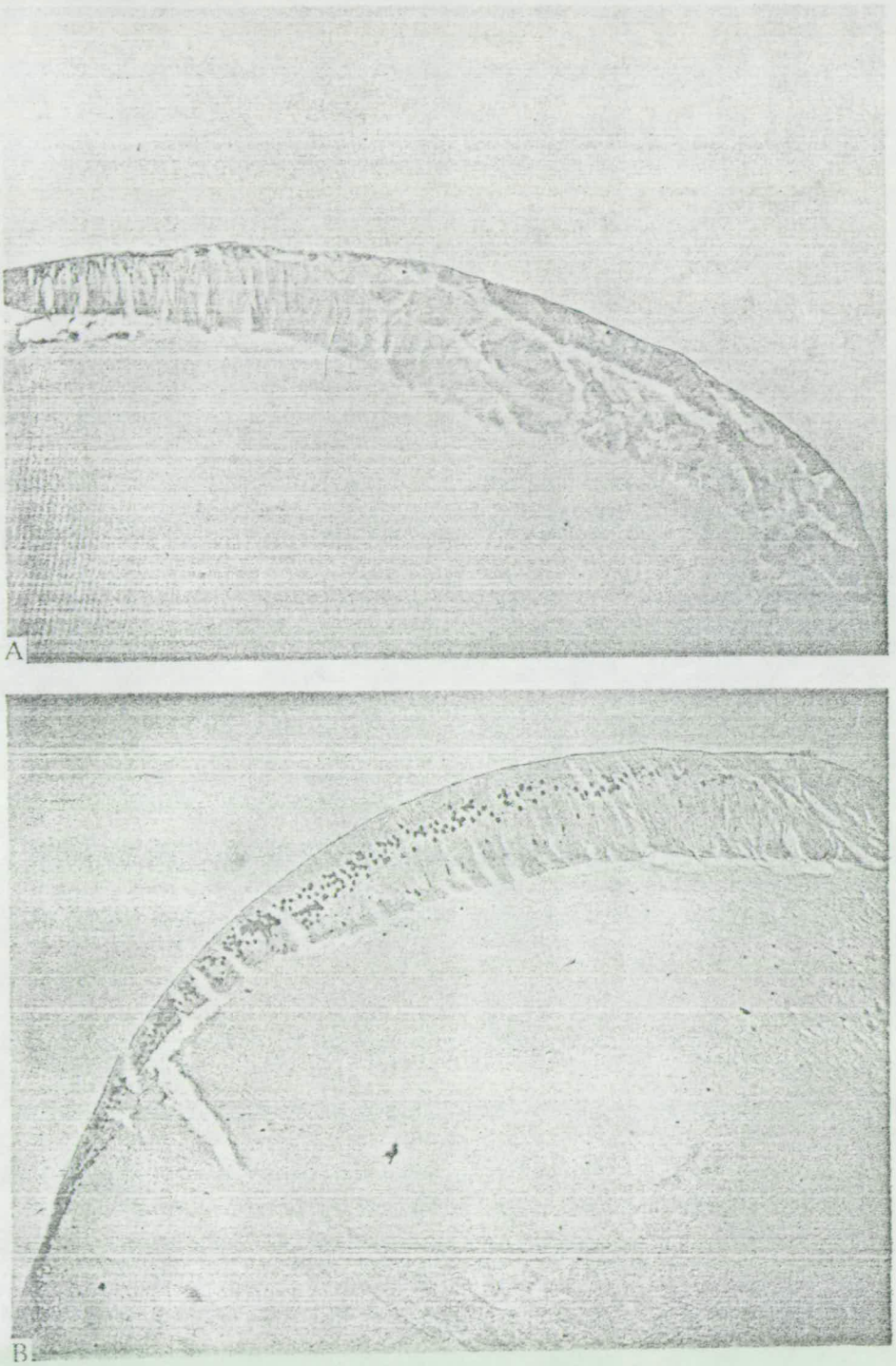


Fig. 4. The graph shows Hy-1 lens wet and dry weight plotted against ^{3}H-thymidine incorporation after a 1-h pulse. The wet weight was determined directly before incubation. The dry weight was determined after the lens was squashed on filter paper, washed and then dried. The weighing measurements were accurate to 0·01 mg.

4. DISCUSSION

Pulse labelling of lenses throughout the day can be used to define the pattern of macromolecular synthesis. Providing the precursor pools increase through the cycle in proportion to the increase in the rates of uptake in synthesis, the amount of precursor incorporated in a short pulse is a valid measure of the rate of synthesis. However the question of pool changes throughout the cell cycle is an open one since there is insufficient data so far available either to exclude or support such changes.



Autoradiographs showing incorporation of ^{3}H-thymidine in day-old Hy-1 chick lenses. Freshly explanted lenses were labelled for one hour at varying times of day, embedded, stained and sectioned then dipped in photographic emulsion. They were exposed for 4 days. A was labelled between 14.10 and 15.10, a minimum on the ^{3}H-thymidine profile of Fig. 1. B was labelled between 23.30 and 00.30, a maximum peak in Fig. 1.

Synchrony in mitotic figures in different regions of the lens epithelium from the labelled nuclei has established that thymidine incorporation profiles are a reflexion of the mitosing lens epithelial cells. Moreover the data obtained by autoradiography imply a variation in the number of nuclei showing DNA synthesis rather than variation in incorporation rate which might be brought about by fluctuation of thymidine pools. (Plate 1).

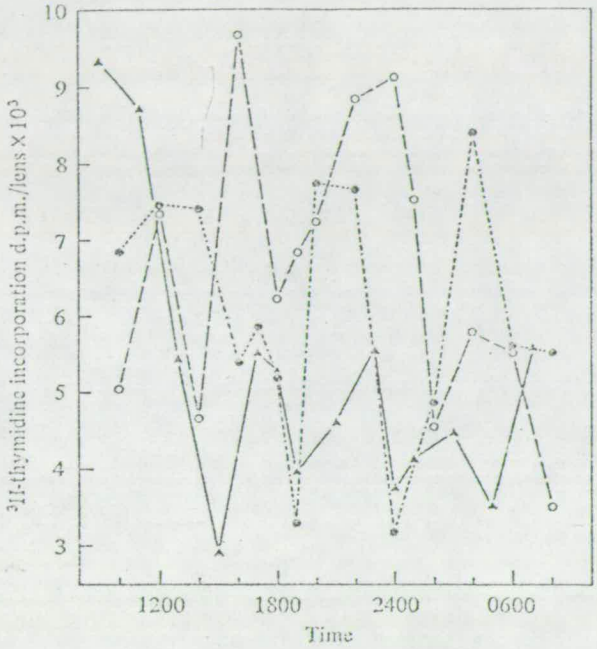


Fig. 5. Plot of the mean values of ^{3}H-thymidine incorporation (d.p.m./lens), averaged from three independent experiments, at intervals over a 24-h period. A——A, Strain N; ○ – – ○, strain Hy-1; ● · – – · ●, strain Hy-2.

Fig. 4 shows that thymidine incorporation has no relationship to either dry weight or wet weight of the lenses: causes for the strain differences in thymidine incorporation must be sought elsewhere. The rate of cell division in single-cell clones in culture and the rate of growth in mass culture (Eguchi *et al.* 1975; Clayton *et al.* 1976) show that the mitotic behaviour is intrinsic to the cells of each genotype. Furthermore, calculations based on the growth rate of single-cell clones (Eguchi *et al.* 1975; Clayton *et al.* 1976) shows that while there is some heterogeneity in a population on N cells, with an average cycle time of 24 h, Hy-1 has two different, but equal cell populations, one similar to N, and one with a high rate of cell division, with an average cycle time of 7·2 h.

The minor fluctuations (peak 4 in Hy-1 and peaks 2, 3 and 4 in N) are seen to be real when the incorporation data from 3 repeat experiments are superimposed (Fig. 5) or averaged (Table 1). The data for Hy-1 suggests either (1) that a high proportion of cells must be in rapid cycle: the third peak, which is the highest, possibly includes the majority of cells with a longer cycle time, or alternatively

(2) a high proportion of the longer cycles are staggered at regular intervals. The proportions of total counts in the first peak of N suggest that most cells have a 24-h cycle, but a small proportion are either out of phase or in more rapid cycle. In the case of Hy-2, a possible hypothesis is that all cells are highly synchronized with a reduced cycle time of about 8 h periodicity.

The data suggest that there may be two ways of increasing the numbers of cell divisions, in fast growing animals by decreasing the duration of a mitotic cycle, or by increasing the numbers of cells involved. It would seem that Hy-1 may use both mechanisms, while Hy-2 may rely mainly on changing the cycle. These data are therefore indicative of the genetic control of the cell cycle, such that the mitotic rate is higher in the hyperplastic strains. This being the case, then the multi-layering of the epithelium would be due to an increase in the production of stem cells rather than any change in the recruitment of these cells for differentiation into fibres. It has been suggested (Clayton, 1975) that Hy-1 and Hy-2 retain, for a prolonged period, the high rate of cell division which normally characterizes embryonic chick lens cells but declines after 11 days of incubation. The time of onset of multilayers in the embryo agrees with this suggestion (McDevitt & Clayton, 1979).

If the cell cycle duration is strain specific as this data suggests, the question then arises as to which phase of the cycle is under the genetic control. Evidence indicates that G1 is the only phase of the cell cycle of lens epithelium which can undergo arrest (Mikulicich & Young, 1963; Prescott, 1968). The regulation of the cell cycle might therefore be expected to be exerted at the level of the G0–G1 transition.

Taking into account that the actual periodicity of the peaks of maximum synchrony in thymidine may be masked both by the duration of the observation and by the limited number of observations in a 24-h period, a pattern appears to emerge from the data with respect to the time interval between maxima. This interval approximates to 4 h or multiples thereof in the different strains. This is being further investigated. If this is the case this data supports the cell-cycle model of Klevecz (1976) involving a sub-cycle Gq which has a duration equal to the period of the cellular clock and of which quantitized generation time is an expression.

Interpretation of the uridine incorporation profiles is more complex as it does not only reflect the mitosing epithelial cells. In addition, those undergoing differentiation are rapidly synthesizing RNA (Reeder & Bell, 1965) and some RNA metabolism occurs in the differentiated fibres. The profiles reflect total RNA synthesis (Fig. 2) but this will probably only include rapidly labelled nuclear RNA and heavy ribosomal precursor as only short pulses were used.

It has been observed that rRNA is much more rapidly labelled in Hy-1 than in control (N). In Hy-1, rRNA is labelled within an hour and is in the process of being degraded by the time the rRNA in N becomes labelled (Truman *et al.* 1976) This could therefore contribute to the differences in the uridine incorporation profiles between these strains.

The peaks of synchrony in uridine incorporation coincide with those of thymidine incorporation or follow 1–2 h later (Fig. 2). As more cells enter S-phase and double their DNA, RNA synthesis also doubles. The pattern is therefore consistent with the idea of a gene dosage effect reported in the literature for other cell types.

These diurnal rhythms have been exposed in the absence of light entraining factors, as the chicks were kept under constant light conditions. Reproducibility of the cycle eliminates any disturbance by Man as the entraining factor. Furthermore variations in thymidine incorporation persist for at least three days in whole lenses in longer term culture (Clayton *et al.* 1976*b*). The trauma of hatching could be an entraining factor, variability in hatching time possibly accounting for the variation between lenses at any one time point. It would therefore be of interest to examine labelled precursor uptake in embryonic lenses.

Table 1. *Area of each peak from Fig. 5 expressed as a percentage of the total*

Strain	Peak no.	% of total
N	1	50·6
	2	14·8
	3	18·8
	4	15·9
Hy-1	1	21·9
	2	19·6
	3	38·8
	4	20·0
Hy-2	1	47·0
	2	24·5
	3	28·5

Variation in hormone levels has been implicated in the control of these rhythms (Bullough, 1962; Epifanova & Tchoumak, 1963; Tutton & Helme, 1973; Tutton, 1973). Bullough has discussed circadian mitotic rhythms in terms of waking and sleeping in relation to a high level of secretion of adrenalin while the animal is awake and active, and a low rate of secretion while asleep. The very short term changes reported here cannot be accounted for on this hypothesis. Furthermore the lens is avascular and would be relatively buffered against short term changes due to blood hormone levels. Differing hormone levels in Hy-1 and N strains cannot account for the differences we have observed, since the growth curves of lens epithelium in cell culture show that Hy-1 cells have an intrinsically higher growth rate than N cells which persists through three successive subcultures (Eguchi *et al.* 1975).

If the diurnal rhythms discussed are indeed intrinsic, biochemical oscillations might be the basis of the cellular clock. The activity of enzymes has been observed to vary with time (Klevecz & Ruddle, 1968) as have other cellular constituents, for example cyclic nucleotides (Abell & Monahan, 1973; Marks & Grimm, 1972) and non-histone proteins (Allfrey *et al.* 1973).

The data presented here provide a warning concerning the importance of the time of day of comparative studies in cellular metabolism of different organisms. It stresses that the time of day is a more important parameter than age.

Investigations of genetic modifications may be expected to illuminate the processes whereby cell division is regulated and its relationship with cellular differentiation. Comparing strains such as Hy-1 and Hy-2, selected for high growth rate, with normal strains and determining the nature of the genetic control of the cell cycle could provide a new attack on the problem of cell reproduction *in vivo*.

The relative proportions of the different crystallins synthesized in lens fibre cells, whether derived from lens epithelium or trans-differentiated from neural retina is affected by the age of the embryo, from which the cells are obtained, δ-crystallin being predominant at earlier stages and β-crystallins tending to replace δ-crystallin later in development. At all ages, Hy-1 synthesizes more δ-crystallin than the corresponding N cells. We have therefore proposed that a high δ-crystallin content is related to a short mitotic interval (de Pomerai & Clayton, 1978; Clayton, 1979). If this is confirmed, it would indicate that mitotic traverse time can act as a regulator of crystallin synthesis.

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