Genet. Res., Camb. (1979), **34**, *pp.* 203–213
With 1 plate and 5 text-figures
Printed in Great Britain

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

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(*Received* 26 *April* 1979)

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

* In partial fulfilment of requirements for the degree of Ph.D.

0016-6723/79/2828-7670 $01.00

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 2·51 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 L14) 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

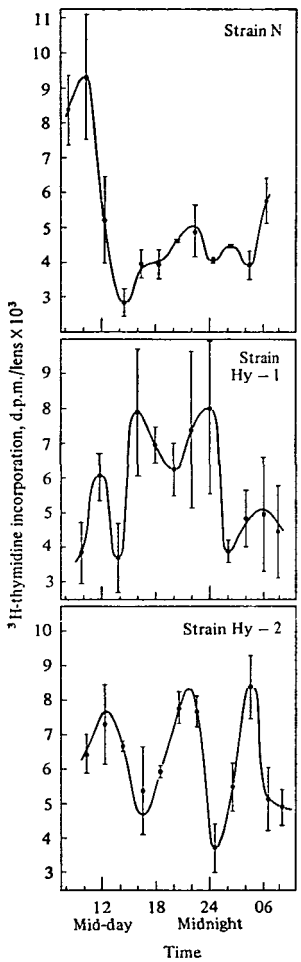


Fig. 1

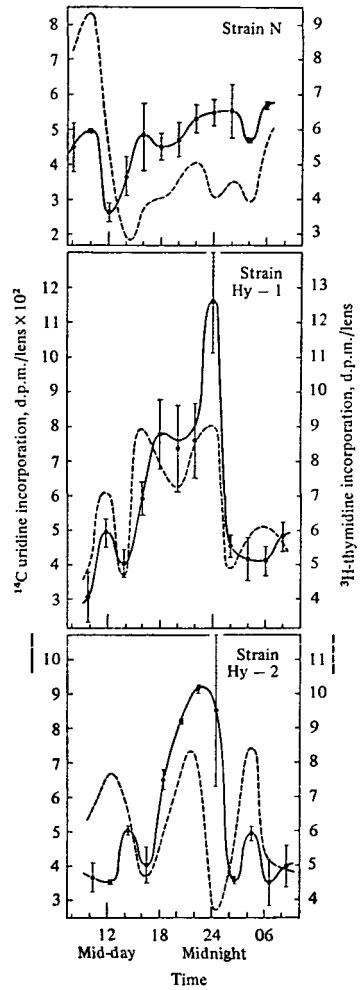


Fig. 2

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.

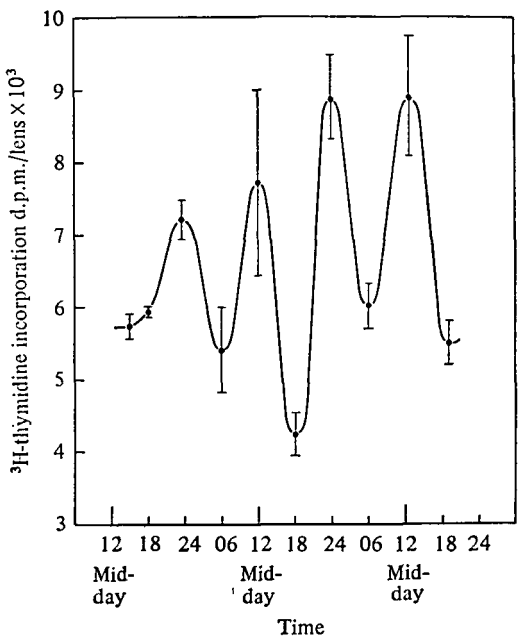


Fig. 3. Plot of the mean and standard deviation of ^{3}H-thymidine incorporation in groups of freshly explanted lenses of day-old Hy-2 chicks at approximately 6-h intervals over a 3-day period. Mean values of disintegrations/min/lens are plotted at the time of commencement of pulse labelling, and therefore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

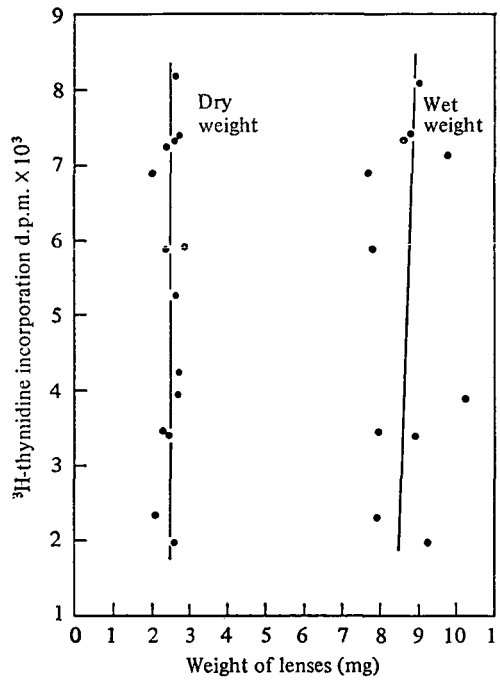
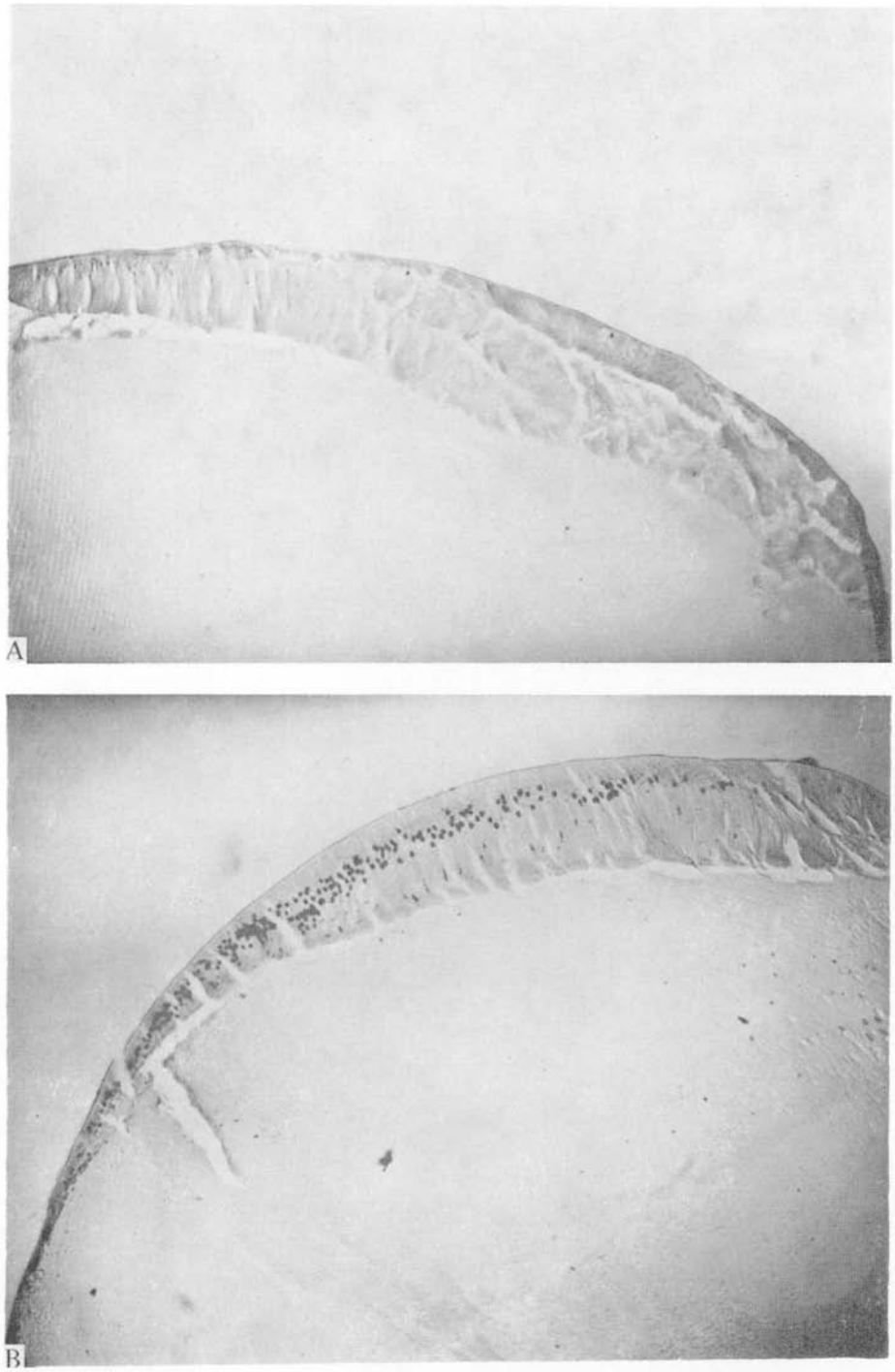


Fig. 4. The graph shows Hy-1 lens wet and dry weight plotted against ^{3}H-thymidine incorporation after a 1-h pulse. The wet weight was determined directly before incubation. The dry weight was determined after the lens was squashed on filter paper, washed and then dried. The weighing measurements were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

https://doi.org/10.1017/S0016672300019455 Published online by Cambridge University Press

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).

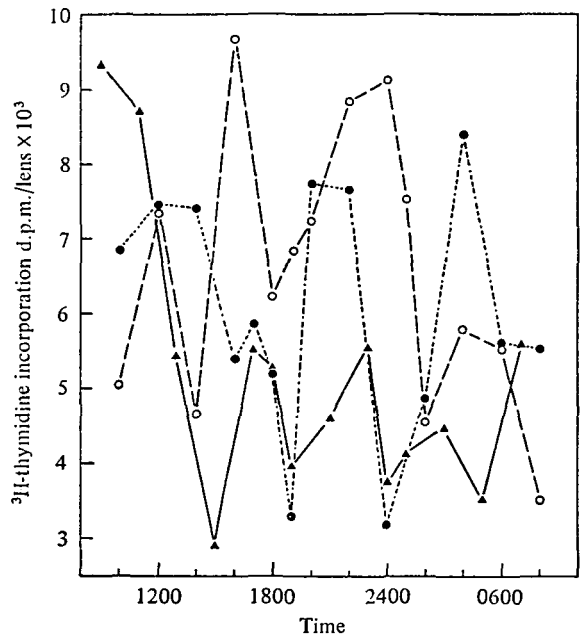


Fig. 5. Plot of the mean values of ^{3}H-thymidine incorporation (d.p.m./lens), averaged from three independent experiments, at intervals over a 24-h period. ▲——▲, Strain N; ○ – – ○, strain Hy-1; ● · – – · ●, strain Hy-2.

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.

We would like to thank Ross Poultry Ltd and the Poultry Research Centre for continuing to supply us with newly hatched chicks of the three strains; and Dr J. Jacob for advice regarding the autoradiography. This work is supported by research grants from the C.R.C. and M.R.C.

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