INITIATION OF MITOSIS IN RELATION TO THE CELL CYCLE FOLLOWING FEEDING OF STARVED CHICKENS

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ABSTRACT

Cellular proliferation of newly hatched chickens was depressed by starving them for 2.5 to 3.5 days. Starvation may hold proliferative cells in different parts of the cell cycle. In order to find where in the cell cycle these cells are held, the animals were fed and the following events were measured as a function of time after the start of feeding: (1) the mitotic index, and (2) the DNA synthetic index (number of cells in DNA synthesis 1 hour after injection of H^3-thymidine). The duration of the cell's DNA synthetic period (S) was measured, permitting a more exact description of the cell cycle. Analysis of the duodenal and esophageal epithelia shows that feeding initiates cell division by stimulating cells from the G1 part of the mitotic cycle in the duodenum. In the esophagus some of the cells were either stopped or slowed down in G1, and another group of cells in G2. Feeding simultaneously stimulates both cell groups; the former moves into S, the latter into mitosis. The S period in starved animals is a little longer than that in normally fed animals but the extension can be attributed to a slightly decreased body temperature.

The interphase portion of the mammalian and avian cell cycle can be divided into three separate periods as shown by tritiated thymidine autoradiography. (1) The length of the postmitotic or pre-DNA synthetic period (G1) varies in different tissues and accounts for most of the differences in mitotic cycle duration (3–5, 9, 14). (2) G1 is followed by the DNA synthetic period (S) which is known to be of rather constant duration in the somatic cells of mammals and of birds (3, 4, 9, 11, 13, 14). (3) The post-DNA synthetic period (G2) is rather short but also variable in different tissues (3, 4, 9, 14). Mitosis (M) then follows the G2 period and completes the cell cycle.

Several studies have shown that when body cells are stimulated to proliferate, their first recognizable response is to undergo DNA synthesis prior to cell division (1, 4, 6–8, 11). Therefore, most cell populations whose mitotic rate can be stimulated are stalled or held in G1. Gelfant, investigating mouse epidermal cells, has challenged this concept by concluding that there is a population of cells fully prepared to divide but blocked in G2, until they receive an appropriate stimulus to divide (5).

This study was designed to determine where in the cell cycle cells are held up. This was done by feeding starved animals and then following the mitotic and DNA synthetic indices. Feeding after inanition was chosen as a physiological stimulus because it has considerable influence on the mitotic activity in mammals and chickens (2, 4, 10).

Two different epithelial tissues of the chicken—the rapidly dividing duodenal and the relatively slowly dividing esophageal epithelia—were chosen for analysis because different types of epithelial cell populations may not respond in the same manner to a mitotic stimulus. It is essential to know the duration of S in those cells that are being investigated, to determine any possible influence that starvation may have on the S duration and also to determine from which period of the cell cycle the cells are initiated to divide. A population of cells

initiated from G1, for example, will not cause an increase in mitotic index until at least the time needed for S and G2 have passed. If, on the other hand, a population of cells is initiated from G2, they will reach mitosis much earlier. Results of this type can be checked and verified by H^3-thymidine labeling at various times after refeeding: when a group of cells are initiated from G1, the increase in number of labeled cells will occur prior to the increase in mitotic index, whereas by initiation from G2 the mitotic activity will increase first.

METHODS AND MATERIALS

A total of forty-three newly hatched White Leghorn cockerels were incubated at 25°C under constant illumination with free access to drinking water. Following hatching, the birds were fasted for 2.5 to 3.5 days. At that time 10 of the birds were injected with H^3-thymidine, and they were killed 1 hour later. These 10 birds served as the starved controls. The remaining animals were then given food in the form of mash (Purina mouse breeder chow). All the birds ate immediately. Twenty-two of the feeding birds were killed serially from 1 to 12.5 hours later; 17 of these birds each received an intraperitoneal (ip) injection of 25 μc of H^3-thymidine (Schwarz Bio-Research Inc., Mount Vernon, New York), specific activity 6.05 c/mM in 0.2 ml distilled water. Each of these animals was killed 1 hour after injection. The other 5 feeding birds were serially killed without a prior H^3-thymidine injection.

Four additional starved birds were fed a different type of food; two were given dried gelatin mash (J. T. Baker, USP) and the other two were fed granular sucrose. None of these birds ate heartily; therefore, they were periodically fed by force. Five hours later each of these chickens was injected with 25 μc of H^3-thymidine, and they were killed 1 hour later.

To determine the duration of DNA synthesis in starved birds, 7 additional birds were starved for 3.5 days. Four hours before killing, each of these birds was injected ip with colchicine (1 mg/kg body wt.) in 0.2 ml distilled water. At intervals ranging from 1.5 to 10.5 hours prior to killing, each of the birds received an ip injection of 25 μc of H^3-thymidine.

Representative portions of lower esophagus, proventralis gland, and duodenum with pancreas attached were removed from all of the animals and fixed immediately in Bouin's fluid. The tissues were trimmed, embedded in paraffin, sectioned at 5 μ, mounted on slides, and autoradigraphs were prepared by the dipping technique (12). Eastman Kodak NTB-3 bulk emulsion was used for autoradiographs and the exposure time was 2 weeks at room temperature. Preparations were stained with Mayer's hemalum after autoradiographic exposure and development.

RESULTS

Measurement of S Peroid in Cell Populations of Starved Chicken

The duration of S was determined by constructing a curve of the percentage of labeled metaphase figures from data shown in Table I *versus* the time between injection of animals with H^3-thymidine and the time of sacrifice. In Table I each percentage point is based on the analysis of 25 to 50 metaphase figures from one chick. The interval in time between 50 per cent labeling on the ascending and that on the descending slope is taken as the mean S period for the particular cell population under investigation. The rationale and details of this method have been dealt with previously (3, 9, 13). Using this method, S measures 6.9 hours for the esophageal epithelium and 7.0 hours for the proventralis epithelium. The cloacal temperature of the starved chickens was 36 to 38°C, whereas in birds that were feeding, the temperatures were 39 to 40°C.

TABLE I

Percentage of Labeled Metaphases in the Proventralis and Esophageal Epithelia from Chickens Killed at Intervals between 1½ and 10½ Hrs. after Injection of H^3-Thymidine

Hrs. after injection	Percentage of labeled metaphases	
	Proventralis	Esophageal
1.5	12	6
3	95	25
4	100	72
6	100	100
7.5	84	100
9	63	100
10.5	35	52

Action of Feeding on the Cell Cycle of Duodenal Cells

The relating number of mitoses and labeled nuclei are plotted as a function of time after the start of feeding (Fig. 1). The average values are based on the analysis of 25 duodenal crypts. Fig. 1 (top) indicates that there is no significant rise in

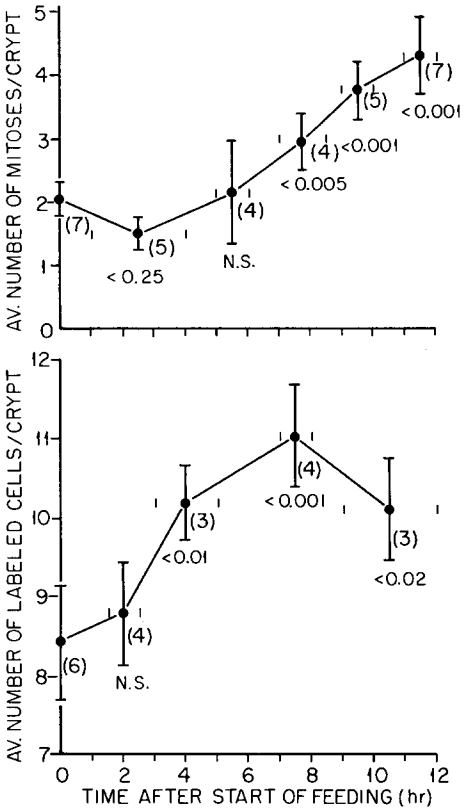


FIGURE 1 The relative number of mitoses (top) and the relative number of labeled nuclei (bottom) in the duodenal crypts plotted against time in hours after the start of feeding of previously starved animals. The average value is marked by closed circles, ●; and the number in brackets below the average is the number of animals used to determine the average. The limits of the time interval in which the animals were pooled are indicated for each point. The vertical bars indicate the standard deviation. The P level of significant difference between the control mean and each experimental mean is given below the average value for each group of pooled animals. *N.S.*, not significant.

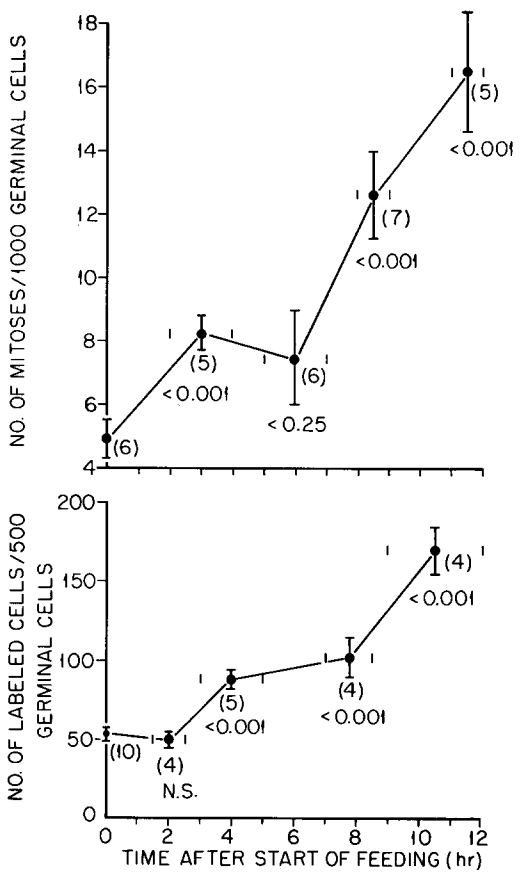


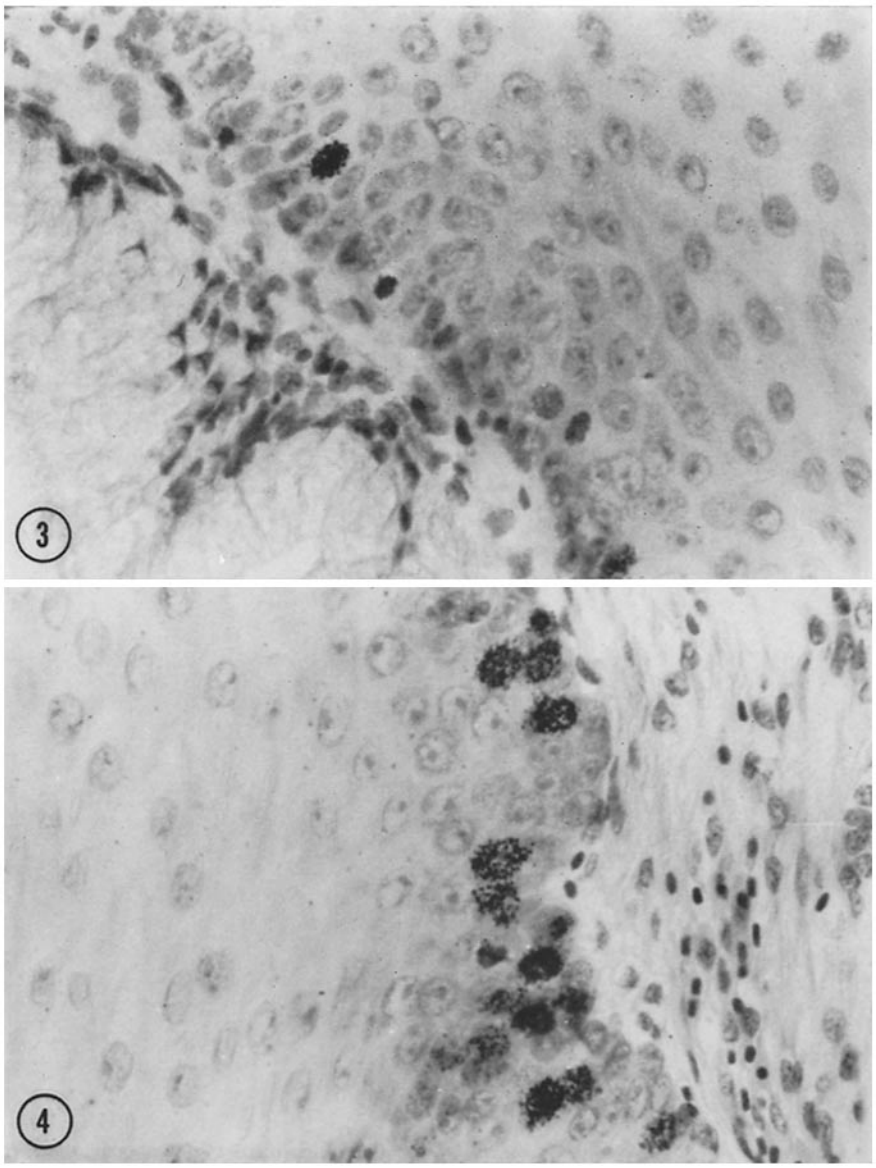
FIGURE 2 The same as for Fig. 1, except this figure shows the proliferative indices in the esophageal epithelium. Mitosis (top), labeled nuclei (bottom).

mitotic activity until 7.75 hours after feeding. The mitotic rate then appears to increase throughout the remainder of the experiment. The low value at 2.5 hours indicates that feeding did not initiate an immediate increase in the number of dividing cells. The DNA synthetic index curve (Fig. 1, bottom) suggests an increased response as early as 2 hours after feeding; however, the increase in the synthetic index is not statistically significant until 4 hours, and the increase then continues at a significantly higher level than in the starved controls for the remainder of the experiment, except perhaps at 10.5 hours. The 10.5-hour value suggests that the peak of the DNA synthetic activity is passed; in fact, the P level < 0.02 shows only a probable statistical difference with respect to that in the starved controls.

Comparison of the two curves in Fig. 1 shows that the cells are stimulated to enter DNA synthesis about 4 to 6 hours before the rise in the mitotic activity.

Action of Feeding on the Cell Cycle of Esophageal Cells

The same type of curves as seen in Fig. 1 for the duodenum were constructed for the esophageal cells (Fig. 2). The average values are based on the analysis of 1000 germinal cells for the mitotic index and 500 germinal cells for the DNA synthetic index. These curves suggest a difference in response be-



FIGURES 3 and 4 Photomicrographs showing the action of feeding on the DNA synthetic index of the esophageal epithelium in a starved chicken (Fig. 3) and in a chicken that has been feeding for 8 hours (Fig. 4). Notice the increased number of labeled nuclei in the fed animal. × 535.

tween the two cell populations. Contrary to the duodenal cell population, the esophageal cell population shows an earlier increase in mitotic activity (Fig. 2, top). At 3 hours, one finds a highly significant increase in mitotic index. The curve then appears to dip slightly at 6 hours, but the dip is neither significantly different from the 3-hour value nor from the control value. The values at 8.5 and 11.5 hours are then greatly increased over the control values.

Fig. 2 (bottom) shows an apparent lag of at least 2 hours before there is a significant increase in the DNA synthetic index (at 4 hours). The synthetic index then continues to rise throughout the

remainder of the experiment, with no suggestion of a drop at the 10.5-hour interval as was seen in the duodenum.

The action of feeding on the synthetic index of the esophageal epithelium is illustrated in the photomicrographs Figs. 3 and 4.

The synthetic index of the birds fed gelatin or sucrose showed no increase over that of starved controls during the 5-hour feeding period prior to H^3-thymidine injection and killing 1 hour later.

DISCUSSION

At the onset it is important to discuss the duration of S, as this information is necessary to the interpretations that follow.

The S period has previously been measured for several cell populations of newly hatched chickens with body temperatures of 40.5°C, and the duration of S was found to be 5 to 6 hours (4). The 6.9- and 7.0-hour S values for the starved chickens with a body temperature of about 37°C (the same body temperature as for most mammals) are equal to the S values reported for most mammalian somatic cells at 37°C (3, 4, 9 11, 13). Temperature consideration may account wholly for the difference in S values between starved and fed chickens; in any case, the effect of starvation on the S period cannot be very large. Under the experimental conditions (feeding of starved chickens), the actual S period probably is a little more than 5 hours.

The duodenal results indicate that the increase in the mitotic index occurred about 5 to 6 hours after that of the DNA synthetic index. This is taken as evidence that initiation of proliferative activity is due to a stimulation of cells that are held in G1. These G1 cells then enter S and eventually divide about 5 to 6 hours later. In the duodenum there was no evidence for division initiation in a population of cells held in the G2 period. Our duodenal data cannot exclude the possibility that a small population of cells were held in G2 and that these cells could be stimulated to divide after a lag of 5 or more hours. The possibility of having such a 5-hour lag period in the duodenum seems unlikely because in the esophagus a G2 cell population is stimulated to divide in 3 hours or less (Fig. 2).

An increase in mitotic activity in the esophagus occurs so early that it cannot be explained on the basis of cells coming all the way from G1; therefore, these esophageal cells must have come from either G2 or the latter part of S. Two points indicate that the increased number of dividing cells did not come from S: (1) There is a suggestive but not significant dip in mitotic activity at 6 hours, hinting that an appreciable number of cells are not coming from S; and (2) the duration of S is not appreciably affected by starvation, suggesting that if cells once enter S they proceed through this period at a nearly normal rate. These points taken together indicate that the initial rise in mitotic index is due to cells that were held in G2. That the synthetic index increases by at least 3.75 hours after feeding suggests that there was another population of esophageal cells held in G1 and they eventually also will influence the mitotic index. Indeed the increase in mitotic index at 8.5 and 10.5 hours is presumably due to this cell population which was stimulated from G1 following feeding. Judging from the mitotic index, this population appears to be much larger than that of cells initiated to divide from the G2 period.

Under our experimental conditions, we conclude that the duodenum has only one population of stalled cells and these are held in G1, and feeding, therefore, initiates eventual cell division by stimulating cells from this part of the mitotic cycle. The esophageal results indicate that one group of its cells were held in G1 and another group of cells were held in G2. In the esophagus, feeding stimulates (almost simultaneously) both cell populations; the G1 cell group start DNA synthesis and the G2 cell group start mitosis.

Our esophageal data lead us, therefore, to agree with Bullough and Laurence (2) and with Gelfant (5) that some cell populations have a group of cells that are held in an extended G2 period, and upon appropriate stimulus these cells are initiated to divide in a short time-interval. However, neither our present data nor those of Gelfant permit us to say whether these cells are slowly but steadily passing through the G2 period or whether they are a unique group of cells which would not automatically divide without an appropriate stimulus. In any case, it is clear, in the two cell populations that we examined, that the majority of cells were held in G1.

Why the duodenum and esophagus do not respond in the same manner following the feeding of starved chickens is not known. Perhaps, the morphological design of a stratified epithelium (esophagus) allows inhibitory substances, as proposed by Bullough and Laurence (2), to accumulate in the immediate environment of the

proliferating cells. In the single-layered epithelium of the duodenal crypts, the proliferating cells present a free surface to the intestinal lumen. Thus, the morphological design of this single-layered population might not permit the accumulation of the inhibitory substances.

In general, we know little about the mechanism by which a supply of food stimulates cells to divide, or about the duration of time between stimulus and the proliferative response. The lag between consumption of food and the actual stimulus to the cell must be shorter than 3 hours in the esophagus and less than 4 hours in the duodenum, for in these times one sees a statistically significant effect on cell proliferation. These values reflect the maximum lag period, and it seems likely that the actual lag period is much shorter. In other cell systems that are capable of being experimentally induced to proliferate, the lag period shows considerable variation from 1 hour, in the ear epidermis of starved mice cultured *in vitro*, to more than 12 hours, in regenerating liver or the scratched lens epithelium (1, 6–8, 11).

That the animals which were fed gelatin and sucrose did not show an increase in synthe ic index suggests that a complete diet is necessary to stimulate proliferative activity. This observation agrees with the earlier findings of Leduc (10). Also, it appears that the physical action of food passing over the esophagus was not enough to stimulate noticeable proliferative activity.

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