Nuclear-Cytoplasmic Relationships in Human Cells in Tissue Culture

III. Autoradiographic Study of Interrelation of Nuclear and Cytoplasmic Ribonucleic Acid*

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PLATES 1 AND 2

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ABSTRACT

The movement of ribonucleic acid (RNA) from nucleus to cytoplasm has been studied, by autoradiographic techniques, in cells of the human amnion grown in tissue culture.

Cells were exposed to cytidine-H^3 for 1 hour after which time only the RNA of the nuclei was labelled. After this 1 hour exposure the cells were placed in a medium containing an excess amount of unlabelled cytidine. Periodically, cells from this medium were fixed. Autoradiographs showed that there was a progressive movement of the label from nucleus to cytoplasm, such that after 24 hours essentially all the label was in the RNA of the cytoplasm.

A study of the incorporation of the cytidine-H^3 in deoxyribonucleic acid (DNA), in the same population of cells at the same times, indicated that the presence of excess amounts of unlabelled cytidine almost instantaneously inhibited further utilization of cytidine-H^3.

It is concluded that RNA moves from nucleus to cytoplasm as a complex polynucleotide structure.

The problem of whether or not cellular ribonucleic acid (RNA) transmits information from gene to cytoplasmic protein continues to be of considerable interest and the stimulus to much research. As part of this problem, many workers have been interested in determining whether or not nuclear RNA (nRNA), possibly the product of chromosomal activity, is a precursor of cytoplasmic RNA (cRNA), which would express genetic information through the synthesis of proteins in the cytoplasm. Brachet has admirably discussed this problem and his book (3) should be consulted for a review of the pertinent literature up to 1957.

The work reviewed by Brachet indicates that there is some controversy with regard to whether or not cRNA is synthesized by the nucleus. One can perhaps state that the disagreement seems to be primarily between biochemists and biologists, though the lines are not clearly drawn. Undoubtedly, the disagreement results from the different techniques employed: the biochemists break up cells, a procedure which is always open to question (*cf.* p. 26, Brachet (3)); the biologists deal with whole cells and this limits the precision with which they can observe the behavior of specific fractions or organelles of the cell.

The work reported here does not resolve the controversy in the terms expressed above. It does, however, lend strong support to the hypothesis of the nuclear origin of cRNA—from the whole cell, or biologist's, point of view.

Material and Methods

Human amnion cells strain A 185 21C, established in culture (17), were used throughout the experimental work reported here.

The cells were grown and maintained in a medium

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consisting of: 20 per cent adult human serum; 40 per cent tissue culture medium 199 (Microbiological Associates, Inc., Bethesda, Maryland); 40 per cent Hanks' balanced salt solution. This was called *normal growth medium.*

The tritiated cytidine (Schwarz Laboratories, Mount Vernon, New York) was available at a specific activity of 0.360 curie/mM. It was prepared for use at 1 μc./ml. of normal growth medium.

The unlabelled cytidine (Schwarz Laboratories), used throughout the experimental procedure, was made up at 5 mg./ml. of normal growth medium.

Cells being prepared for use were suspended from a stock flask with 0.1 per cent trypsin (Nutritional Biochemicals Corp., 1-300), washed and resuspended in normal growth medium. They were then planted, at a concentration of approximately 20,000 cells/ml., in a 100 mm. diameter Petri dish containing several 22 x 22 mm. coverglasses. The Petri dish was then placed in a humidified chamber, gassed with a mixture consisting of 5 per cent CO_2 and 95 per cent air, and incubated at 37°C.

Fixation was performed by removing a coverglass from the Petri dish, washing twice with Tyrode's solution and then immersing for approximately 10 to 15 minutes in absolute methyl alcohol. The preparations were then air dried.

For deoxyribonuclease (DNase) digestion, coverglasses were incubated for 2 hours at 37°C. in a solution of 0.5 mg. DNase/ml. of 0.003 M $MgSO_4$ adjusted to pH 6.7.

For ribonuclease (RNase) digestion, coverglasses were incubated for 2 hours at 37°C. in a solution of 0.4 mg. RNase/ml. of distilled H_2O adjusted to pH 6.5. (DNase and RNase were crystalline preparations obtained from Worthington Biochemical Corp., Freehold, New Jersey.)

The usual stripping film techniques for autoradiography were employed (see *e.g.* reference 10).

EXPERIMENTAL PROCEDURE

If amnion cells are exposed to tritiated cytidine for 1 hour, fixed immediately thereafter, and then treated with deoxyribonuclease (DNase), one finds that all the residual radioactivity of the cell is in the RNA of the nucleus. Thus we have an RNA-labelled nucleus in unlabelled cytoplasm, and under favorable circumstances, one should be able to trace the course of the label if the cells are removed to a non-radioactive medium following the brief exposure to tritiated cytidine.

The experiments were conducted in the following manner:

1. Cells were grown for 2 to 3 days on several 22 x 22 millimeter coverglasses in one large Petri dish containing normal growth medium.

2. Following this incubation, normal growth medium was exchanged for a normal growth medium containing 1 μc./ml. cytidine-H^3 and the cells were incubated in this for 1 hour.

3. Following this 1 hour incubation:

(*a*). Some coverglasses were fixed at once (these are called 0 hour specimens in later discussions).

(*b*). Some coverglasses were placed in normal growth medium.

(*c*). Some coverglasses were placed in normal growth medium containing 5 mg. cytidine-H^1/ml.

4. Coverglasses from group (*b*) and group (*c*) were fixed at periodic intervals.

5. Following the completion of the series of fixations, each of the coverglasses was cut in two. One-half was digested with RNase and the other half with DNase.

6. The half coverglasses were then mounted—cells up—on standard microscope slides and coated, in the dark, with autoradiographic stripping film. Incubation under film for 5 to 7 days was followed by standard development of the film.

RESULTS

A. DNase-Digested Cells:

The cells described in this section were treated with DNase before autoradiography. These cells digested with DNase were demonstrated to have all residual activity localized in RNA (or a cell component that was completely removed by digestion with RNase).

Fig. 1 shows cells that were fixed at 0 hour (immediately upon removal from 1 hour incubation with cytidine-H^3). As is readily discernible, all the label is localized in the nuclei of the cells. (Not as clearly evident is the fact that a majority of the activity is localized in the nucleoli.)

Since all the activity at 0 hour is localized in the RNA of the nucleus, it was reasoned that if cells were placed at this time in a medium containing a great excess of unlabelled cytidine, all the radioactivity at any later time—wherever localized in the cell—could be accounted for as having been in the RNA molecule derived from nRNA.

Fig. 2 shows cells that were incubated in excess unlabelled cytidine[1] for 8 hours following removal from the labelled medium. It is evident that the radioactivity is now almost uniformly distributed throughout the cell.

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[1] The excess unlabelled cytidine was present at a concentration of 5 mg./ml., which was approximately 7×10^3 times the concentration of cytidine-H^3 initially present in the medium.

Fig. 3 shows cells incubated for 24 hours in the medium containing excess unlabelled cytidine. In these cells, virtually all the radioactivity is now in the cytoplasm with little or no radioactivity in the nucleus (see figure legend).

This type of observation clearly suggests that labelled RNA is moving from nucleus to cytoplasm and moving as a more or less intact molecule.

B. RNase-Digested Cells:

One assumption inherent in the above conclusion is that the presence of excess unlabelled cytidine removes from metabolic availability—by dilution—any residual pool of label present when the cells are withdrawn from the medium containing cytidine-H^3. Furthermore, it is assumed that any labelled cytidine liberated by the breakdown of nRNA is also effectively diluted out by the excess unlabelled cytidine. To confirm this view, one may look at the incorporation of cytidine-H^3 into deoxyribonucleic acid (DNA) during DNA synthesis in the same population of cells. Since DNA synthesis is a discontinuous process, one can detect the presence of a pool of labelled precursor by observing how many nuclei become labelled (in DNA) at any particular interval.

The cells described below were treated with RNase before autoradiography. In those cells digested with RNase all the residual radioactivity is in DNA.

Fig. 4 shows cells fixed at 0 hour (immediately upon removal from medium containing cytidine-H^3). One can see that approximately $\frac{1}{3}$ of the nuclei are radioactive. Figure 5 shows cells fixed 8 hours after removal from the label, but incubated in normal growth medium *without* added unlabelled cytidine. Here approximately $\frac{2}{3}$ of the nuclei are labelled, indicating that there is indeed residual cytidine-H^3 available for incorporation into DNA. Figure 6 shows cells after 24 hours incubation in the medium *without* unlabelled cytidine, and here *all* the nuclei are labelled.

If, however, one looks at the cells that are incubated in unlabelled cytidine following the 1 hour exposure to cytidine-H^3, one finds virtually no change in the number of nuclei labelled at any time as compared to the number labelled at 0 hour. Table I summarizes the data on this point. (The changes that appear to occur in the percentage of nuclei labelled in those instances of incubation with unlabelled cytidine, if significant, could be accounted for on the basis of mitoses. That is, those cells that were synthesizing DNA—hence incorporating label—at 0 hour are unlikely to have been ready for mitosis at 2 hours (unpublished experiments). However, other cells that have already passed the DNA doubling stage—hence not incorporating label—would be dividing and thus increasing the proportion of unlabelled cells.)

TABLE I

Difference between Presence and Absence of Unlabelled Cytidine on the Number of Nuclei that Became Labelled in DNA with Time

0 hour values for column marked "*with* unlabelled cytidine" are the same as 0 hour values for the opposite column. Each time interval represents duplicate counts on each of a pair of samples.

Time	*Without* Unlabelled Cytidine		*With* Unlabelled Cytidine	
	No. Labelled/Total		No. Labelled/Total	
hrs.		*per cent*		*per cent*
0	17/57	29.8		
	36/114	31.6		
	23/59	39.0		
	34/110	30.9		
	Total 110/340	32.4		
2	18/58	31.1	19/55	34.5
	46/116	39.7	36/113	31.9
	25/55	45.5	17/61	27.8
	50/151	33.1	15/103	14.6
	Total 139/380	36.5	87/332	26.2
8	37/58	63.8	21/51	41.2
	92/169	54.5	25/113	22.1
	44/63	69.8	21/52	40.3
	57/116	49.2	39/121	32.2
	Total 230/406	56.7	106/337	31.4
24	59/61	96.8	23/58	39.7
	114/120	94.2	33/116	28.4
	59/63	93.7	15/59	25.4
			45/112	40.2
	Total 232/244	95.1	116/345	33.7

DISCUSSION

The evidence presented here is considered to indicate strongly that nRNA is a direct precursor of cRNA. Perhaps the "molecule" of nRNA is

altered to some extent upon passage to the cytoplasm, but clearly at least some part of a polynucleotide structure is transmitted intact. The conclusion drawn is dependent upon another assumption; that is, the evidence indicating there is a dilution of the cytidine-H^3 pool for DNA synthesis can be taken to mean that the pool of cytidine-H^3 for RNA synthesis is similarly affected. That this assumption is probably valid is indicated by three points:

1. When cells removed from cytidine-H^3 after 1 hour incubation are placed in a medium *not* containing excess unlabelled cytidine, the RNA labelling continues to increase with time such that 24 hours later the cells are much more heavily labelled in RNA than at 0 hour. Furthermore, there continues to be considerable labelling of nuclear RNA—which does not occur when excess unlabelled cytidine is present;

2. Since cytidine-H^3 appears initially to be a suitable precursor for both DNA and RNA, it appears valid to assume that the presence of unlabelled cytidine would be effective in diluting the precursor pool for both nucleic acids;

3. The similarity in the amount of total cell RNA labelling at 0 hour and at 24 hours (preliminary unpublished data) would appear to be quite coincidental if our assumption were not valid.

That the phenomenon of nuclear synthesis of cRNA is probably universal, is evident from two other pieces of work, descriptions of which appeared in short notes during the preparation of this manuscript. The experimental procedures were very similar. Woods and Taylor (15, 15 *a*) carried out this work on plant root tips and Zalokar (16) executed some remarkable experiments with *Neurospora*. The general conclusions reached are similar to those expressed here.

There are a number of lines of evidence which are claimed to discredit any view that nRNA is a precursor of cRNA. The "older" evidence against this view was chiefly:

1. The "specific activity time curves" for various RNA fractions are not consistent with the hypothesis that nRNA is a precursor of cRNA (*cf.* Barnum *et al.* (2));

2. The nucleotide composition of cRNA differs from that of nRNA (*cf.* Elson *et al.* (5)).

Since the publication of Brachet's book (3) other work has appeared which also argues against the hypothesis. Weill and Ledig (13), who studied the specific activities of different cell fractions over a long time course, claimed that nRNA could not be a precursor of cRNA since 3 days after the administration of a labelled precursor, the specific activity of cRNA which had been lower than that for nRNA was now higher than that for nRNA. Osawa and coworkers (11, 7) also studied the specific activities of various cell fractions—in this case nuclei and cytoplasm were fractionated even further than in earlier work—and came to the conclusion that, though there was some similarity between one nuclear fraction and one cytoplasmic fraction with respect to nucleotide composition, the specific activities were such as to preclude the one being the precursor of the other.

All of the above experiments remain open to question for one or more of the following reasons:

(*a*). In the process of breaking up cells, some substances may leak out from one or more fractions or some fractions may adsorb materials not normally in association in a living cell. (See for example Kay *et al.* (8)).

(*b*). Many workers (Allfrey and Mirsky (1), Vincent (12), Osawa *et al.* (11), Logan (9), and Kay *et al.* (8)), have shown that nRNA may be composed of at least two different fractions and there is no reason to believe that these two fractions are themselves homogeneous. If there are many RNA fractions, they may have different specific activities; their specific activities may be different at different physiological states; and the different RNAs may move to the cytoplasm at different rates.

(*c*). A related objection is that it may be fallacious to study specific activity of different fractions at the same instant of time. That is, if nRNA is a precursor of cRNA then one should compare nRNA at time X with cRNA at time Y, time Y being the moment at which the nRNA, examined at time X, should have appeared in the cytoplasm. This undoubtedly is a difficult task but probably could be executed in a system such as we have employed.

(*d*) It is possible that, as with *Acetabularia* (4)[2], there is some independent RNA synthesis in the cytoplasm. However, our experiments (unpublished) with enucleate amnion cells indicate that RNA is not synthesized in the cytoplasm. These non-nucleate cells are capable of amino acid in-

[2] Recent critical work of Naora, *et al.* (*Exp. Cell Research*, 1959, **16**, 434) suggests that there is really no net synthesis of RNA in enucleate *Acetabularia*.

corporation into protein but are unable to incorporate various RNA precursors into RNA.

None of the above criticism, of course, is applicable to the work reported here, although subtleties such as indicated in (*b*) above would not be detected. However, McMaster-Kaye and Taylor (10) have been able to make some of these distinctions in work on whole cells.

Some of the discrepancies described may be due to the mechanisms described by Herbert (6). He studied the incorporation of adenosine-8-C^{14} diphosphate into homogenized rat liver and found that the nucleus was responsible for the incorporation of the label into the interior of the RNA molecule, while a cytoplasmic fraction was responsible for incorporation or exchange of end groups of the RNA molecule.

During the course of pollen grain growth, it has been observed by Woodard (14) that the amount of RNA, as measured by azure B binding, is greatest in the cytoplasm at a time when the RNA content of the nucleus is at a minimum. This is taken to mean that nRNA cannot be a direct precursor of cRNA. What was not discussed by Woodard is the fact that the techniques employed measure only the localization at any moment of time and therefore it is impossible to establish where the substance is being synthesized. That is, it is possible that the nucleus does synthesize the cRNA and that passage to the cytoplasm is virtually instantaneous. As Woodard suggests, clearer interpretations might come from a "combined use of autoradiography and microphotometry," although even from our results using autoradiography alone it appears certain that at least a good portion of the cRNA comes from the nucleus. A combined technique may clarify the interpretation of the behavior of different RNA fractions of the cell and a beginning toward this end has been made by McMaster-Kaye & Taylor (10).

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Explanation of Plates

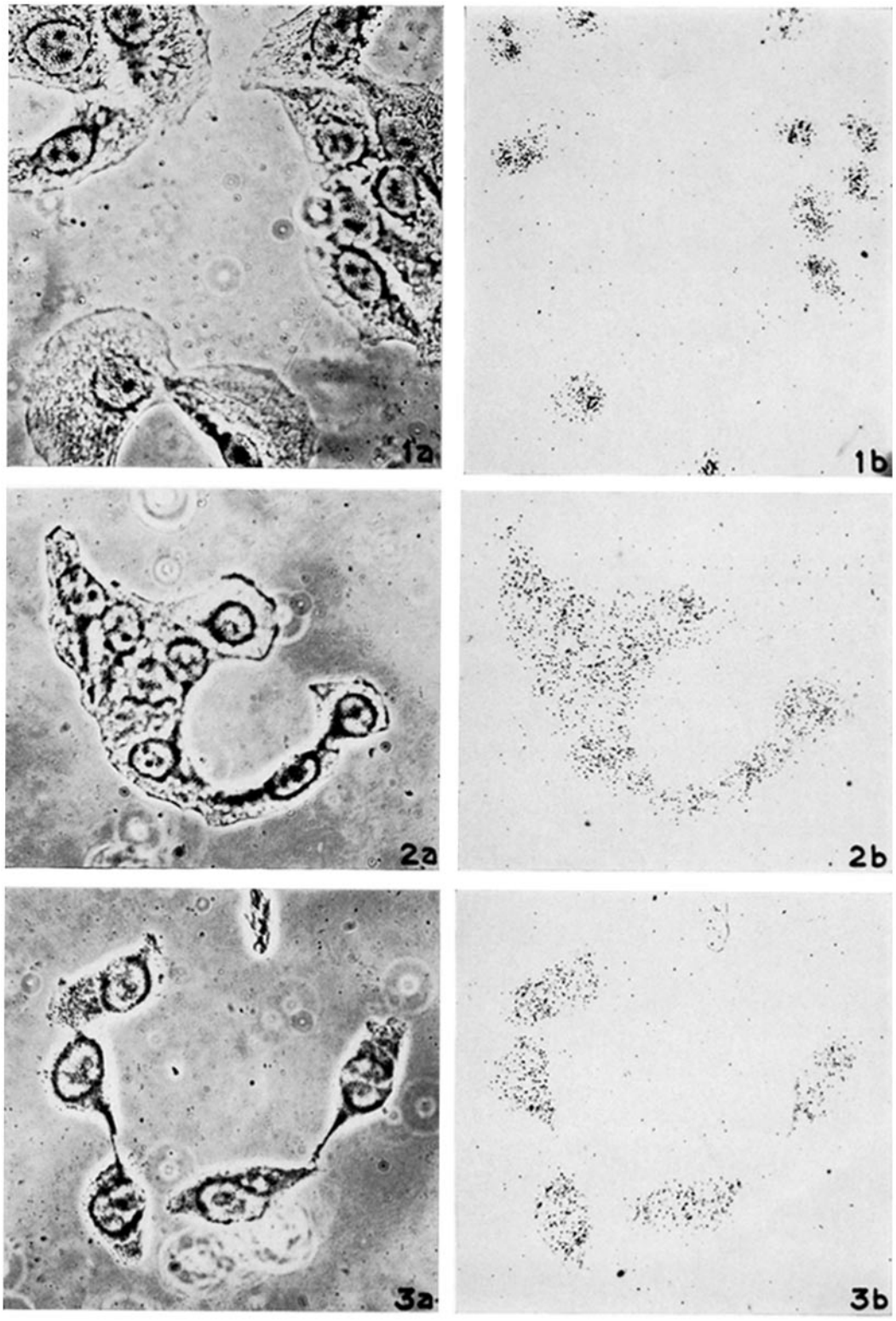
Plate 1

Figs. 1 to 3. On the left are phase contrast photomicrographs of fixed amnion cells. On the right are the corresponding brightfield photomicrographs of the autoradiographs of these cells. These cells were digested with DNase following the treatments described in the text and below. The residual labelling is entirely within RNA. (Nuclei are 10 to 15 microns in diameter.)

Figs. 1 *a* and 1 *b*. These cells were exposed to cytidine-H^3 for 1 hour and then fixed immediately. Note the complete localization of the label within the nuclei and particularly high activity within the nucleoli.

Figs. 2 *a* and 2 *b*. These cells were exposed to cytidine-H^3 for 1 hour and placed in a medium containing a high concentration of unlabelled cytidine for 8 hours. Note the presence of label in the cytoplasm, though the nuclei are still somewhat more heavily labelled.

Figs. 3 *a* and 3 *b*. These cells were exposed to cytidine-H^3 for 1 hour and then placed in a medium containing a high concentration of unlabelled cytidine for 24 hours. These cells were selected for photography because they are somewhat rounded up and this results in the nucleus forming most of the thickest part of the cell. Therefore, if the activity were uniformly distributed throughout the cell, by far the greatest activity should be evident over the nucleus. This is clearly not the case. In fact, considering the presence of overlying cytoplasm, the nuclei probably have virtually no activity at this time.



(Goldstein and Micou: Nuclear-cytoplasmic RNA)

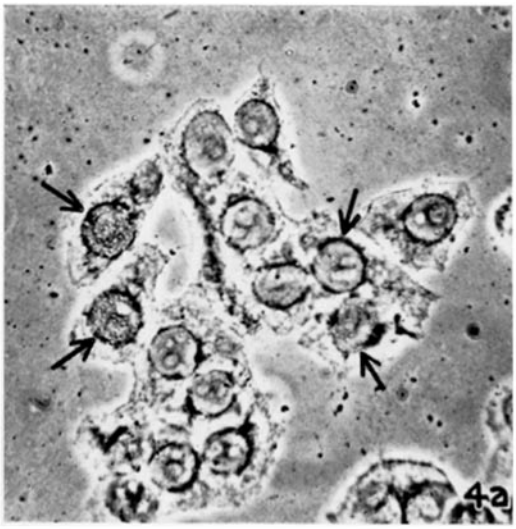
PLATE 2

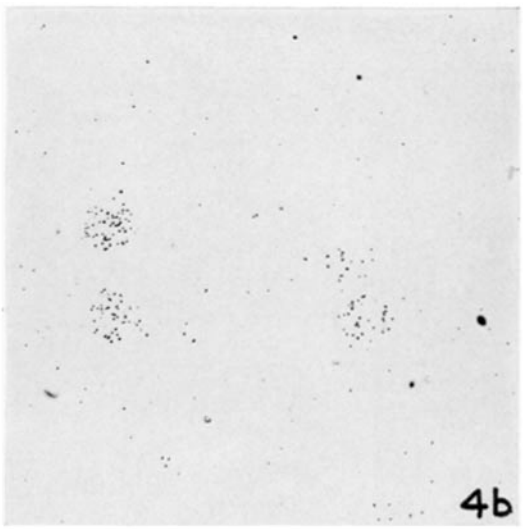
FIGS. 4 to 6. On the left are phase contrast photomicrographs of fixed amnion cells. On the right are the corresponding brightfield photomicrographs of the autoradiographs of these cells. These cells were digested with RNase following the treatments described in the text and below. The residual labelling is entirely within DNA. Arrows point to labelled nuclei. (Nuclei are 10 to 15 microns in diameter.)

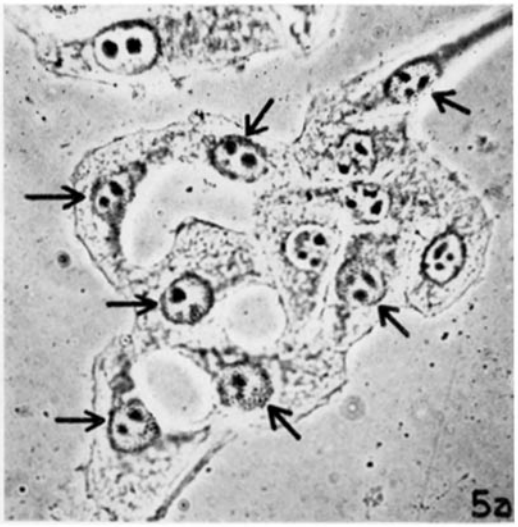
FIGS. 4 *a* and 4 *b*. These cells were exposed to cytidine-H^3 for 1 hour and then fixed immediately. Approximately 30 per cent of the nuclei are labelled.

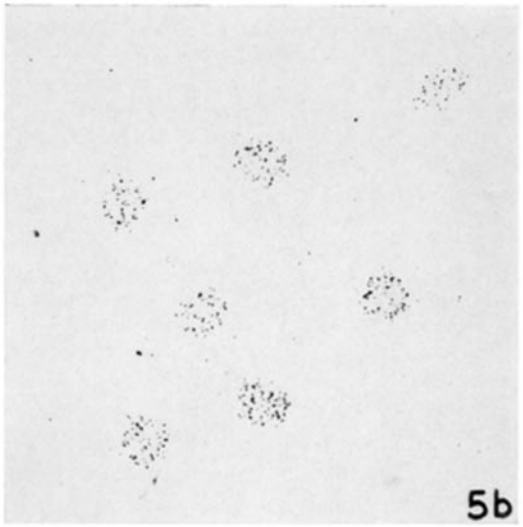
FIGS. 5 *a* and 5 *b*. These cells were exposed to cytidine-H^3 for 1 hour and then placed in a medium *without* added unlabelled cytidine for 8 hours. Approximately 60 per cent of the nuclei are labelled.

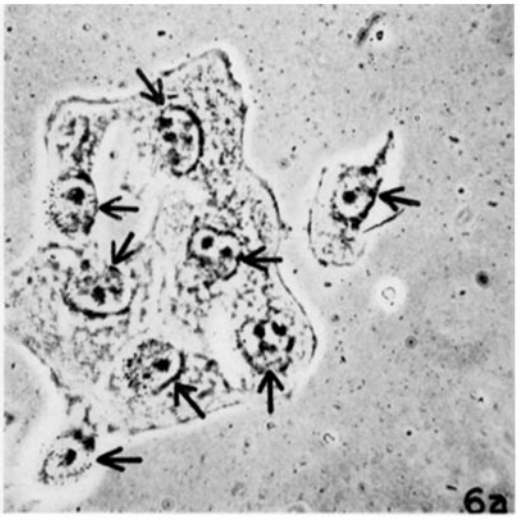
FIGS. 6 *a* and 6 *b*. These cells were exposed to cytidine-H^3 for 1 hour and then placed in a medium *without* added unlabelled cytidine for 24 hours. All of the nuclei are labelled.

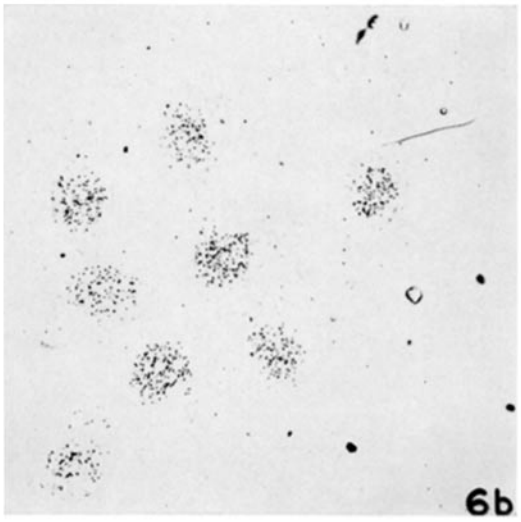












(Goldstein and Micou: Nuclear-cytoplasmic RNA)