KINETICS OF NUCLEOSIDE INCORPORATION

INTO NUCLEAR AND CYTOPLASMIC RNA

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ABSTRACT

HeLa and conjunctiva tissue culture cells were incubated for various intervals with tritiated nucleosides and the incorporation into RNA was localized in different parts of the cell by means of autoradiography. In order to obtain quantitative measurements of incorporation from grain count data the influence of cell geometry on the absorption of the tritium β ray was considered. Relative correction factors, $\bar{E} = g/g^*$, relating an idealized grain count in the absence of absorption, g, to the actual grain count, g^*, were derived for the different cell compartments. For the average HeLa cell the factors for the nucleolus, n, non-nucleolar parts of the nucleus, N, and the cytoplasm, C, are in the ratio $\bar{E}_n/\bar{E}_N/\bar{E}_C = 2.3/1.6/1.0$. The kinetics of incorporation for cytidine and adenosine are similar. The n and N curves are characterized by a rapid rise and early saturation, whereas the C curves show an appreciable lag and no evidence of saturation for intervals as long as one generation time. Estimates of the relative amounts of RNA in each compartment were obtained from ultraviolet micro absorption measurements and used together with the kinetic data to calculate specific activities. For incubation periods of short duration the ratio of specific activities n/N for cytidine is approximately twice that for adenosine. Three hypotheses for the mechanism of ribonucleoside incorporation and RNA synthesis are discussed, and arguments favoring a transport of RNA or an RNA by-product from the nucleus and nucleolus to the cytoplasm are presented.

Protein synthesis is supposed to be controlled by RNA, which derives its specificity from DNA. Since DNA resides exclusively in the cell nucleus, and protein synthesis occurs in the cytoplasm, a corollary to this hypothesis is that RNA is transported from nucleus to cytoplasm. The evidence substantiating this concept has recently been discussed by Brachet (1).

One approach to the problem has made use of autoradiographic methods for the intracellular localization of the sites of incorporation of RNA precursors (2–9). With all the vastly different cell types studied it was found that when RNA precursors are presented to a cell they are first incorporated in the nucleus and later appear in the cytoplasm. From this the conclusion was reached that cytoplasmic RNA is of nuclear origin. On the other hand, a conclusion based on qualitative studies such as those cited is open to criticism (10) since the data can equally well be explained by assuming that the RNA's in different cell parts have different turnover rates.

The present investigation of the incorporation of the ribonucleosides cytidine and adenosine into the RNA of actively growing tissue culture cells was initiated in conjunction with experiments involving the inactivation of the nucleoli with ultraviolet microbeam irradiation (11–13). The

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kinetic studies presented here emphasize the quantitative relationships between the incorporations in different cell parts and also offer a method for estimating the effect of the irregular geometry of the tissue culture cell on the autoradiographic efficiency.

These data have enabled us to examine more closely the hypothesis of nuclear-cytoplasmic RNA transfer and furthermore to draw tentative conclusions regarding the nature of RNA synthesis within the nucleus.

Brief reports of this work have appeared previously (12, 14, 15).

MATERIALS AND METHODS

Two stocks of HeLa cells and one of conjunctiva were used. One HeLa line, originally obtained from Mr. D. DeKegel, was grown on a medium, ϕ_{10}, consisting of Hank's balanced salts solution, yeast extract, lactalbumin hydrolysate, and 10 per cent calf serum. The other cell lines, obtained from Dr. L. Castor, were propagated in Eagle's medium with 10 per cent calf serum (E_{10}).

Cells which had been growing 3 or 4 days on coverslips were used for the experiments. Incubations with ^{3}H-cytidine (1.2 mc/mg) or ^{3}H-adenosine

(4.15 mc/mg) in ϕ_{10} were performed in cuvettes in a CO_2 incubator. Incubations with the tritiated nucleosides in E_{10} were carried out in Leighton tubes.

At the termination of a period of incubation the cells were fixed in cold acetic acid–ethanol (1:3), washed in 70 per cent ethanol, treated with 1 per cent perchloric acid (PCA) at 4°C for 20 minutes, and washed for 2 hours in running tap water. Some coverslips were cut in half at this point and one half incubated for 1 hour at 37°C in 0.5 mg/ml RNase (phosphate buffer pH 7.1), treated again with cold 1 per cent PCA, and washed. The other half was incubated in buffer and treated identically. Three or four coverslips representing different durations of incubation in a labeled medium were then cemented to a microscope slide. The preparations were covered with liquid emulsion (Ilford G5 or L4), either by spreading the melted emulsion onto prewarmed slides (45–50°C) with a glass rod, or by dipping the warmed slides into a tube filled with melted emulsion, draining them thoroughly, and drying them in a horizontal position. After an appropriate exposure at 24°C the emulsion was developed in an amidol-Na_2SO_3 developer (16). The emulsion was fixed and washed, and then the cells were stained with methyl green–pyronine (Unna). Grains over the different cell parts were counted in a randomly selected sample of 50 to 100 cells.
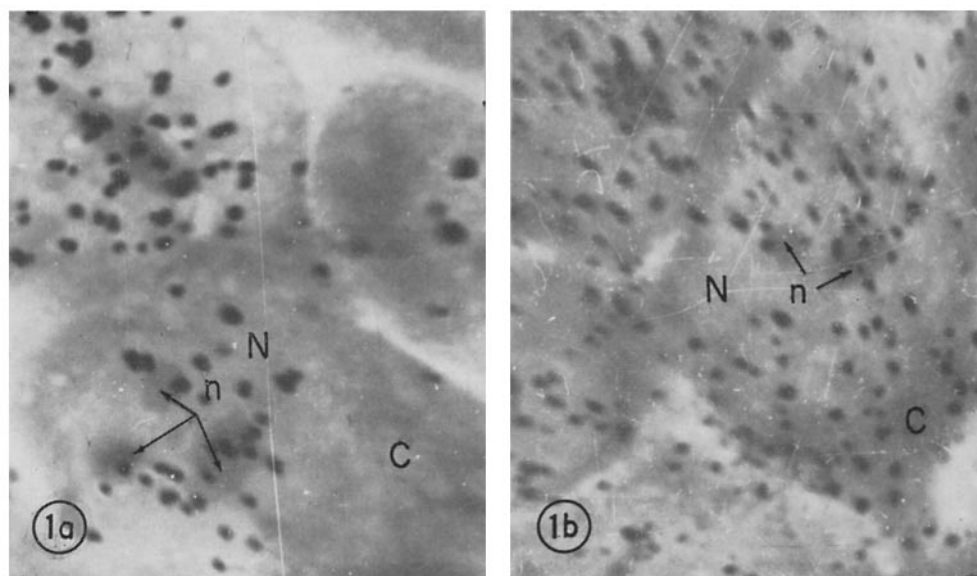

FIGURE 1

Examples of autoradiographs of ^{3}H-cytidine incorporation into HeLa cells. a, $\frac{1}{2}$ hour incubation; b, 8 hour incubation. n, nucleoli; N, nucleus; C, cytoplasm. It can be seen that the grains are confined to n and N after $\frac{1}{2}$ hour, but that after 8 hours the grains are distributed over the entire cell.

Examples of autographs of cells incubated for ½ hour and 8 hours in ³H-cytidine are shown in Fig. 1.

AUTORADIOGRAPHIC EFFICIENCY

In order to make a quantitative study of the labeling kinetics, it is necessary to know the relationship between the incorporation of labeled precursor in the various cell compartments and the grains counted on the autograph. It is sometimes assumed that the incorporation is proportional to the grain count. This would be the case if every β particle emitted by an incorporated tritium atom had equal probability of exposing the emulsion. However, if the β rays emanating from the different compartments encounter different thicknesses of cell material before reaching the emulsion and thus undergo different amounts of self absorption, then a systematic error of considerable magnitude may be made in assuming proportionality. Indeed, the low energy of the tritium β particle and the irregular geometry of a cell growing in tissue culture engender such a situation.

It is possible to observe this effect directly in a thin microscopic section cut perpendicular to the plane of the cell and emulsion. To prepare such sections the cells were grown on millipore filters, incubated for 4 hours in labeled medium, and treated as previously described for coverslips. The "sandwich" of filter, cells, and developed emulsion was then embedded in paraffin and sectioned at 3 microns. Fig. 2 shows two rather extreme examples of these preparations. In Fig. 2 a the nucleolus is bulging from the cell so that there is an extremely thin layer between it and the emulsion. A very high grain density is observed. In Fig. 2 b the nucleolus is buried beneath a 0.8 micron layer (arrows) of nucleus and cytoplasm. A very low grain density is observed. It is desirable therefore to know the three-dimensional geometry of the average cell and to derive a set of correction factors, designated by E, for the nucleolus, n, non-nucleolar parts of the nucleus, N, and the cytoplasm, C, by which the grain counts can be multiplied so as to obtain relative isotope incorporations.
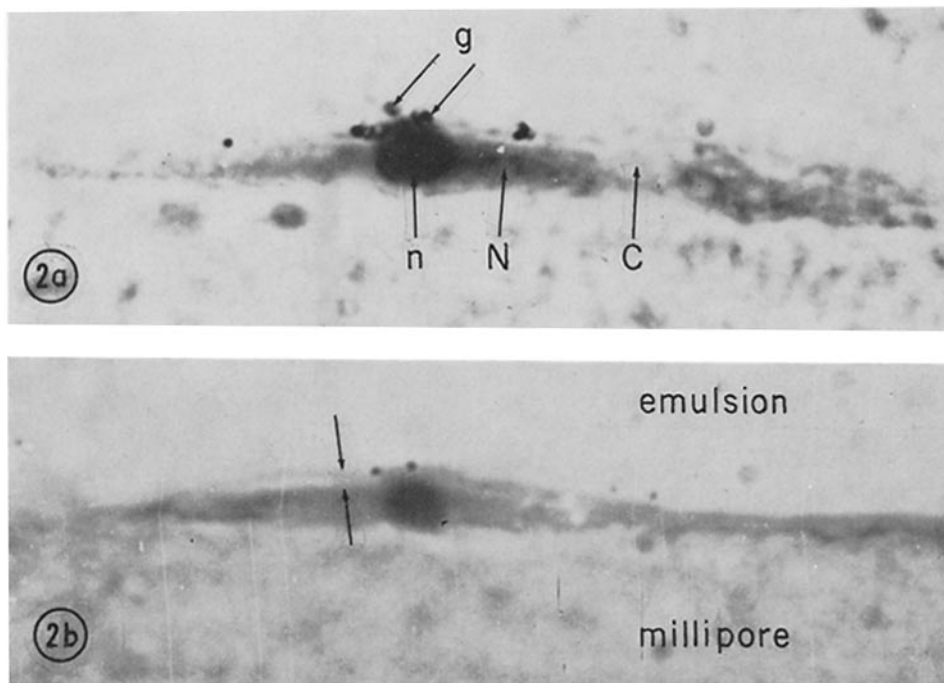
A rigorous derivation of such correction factors

FIGURE 2

Profiles of autoradiographs. Adjacent cells on the same preparation. a, cell with nucleolus practically touching emulsion; note large grain density, g. b, cell with nucleolus buried beneath 0.8 micron layer (arrows) of nucleo- and cytoplasm; note scarcity of grains.

requires a fairly precise knowledge of the interaction of the tritium β rays with matter. Unfortunately, there is a scarcity of experimental data and no generally accepted theory for the attenuation of electrons in this very low energy region (1 to 18 kev). However, for *relative* factors the problem is somewhat simplified. In this case some of the assumptions apply equally to all compartments, and consequently uncertainties associated with the calculation are diminished.

First, we shall treat all the emitted β rays as if their trajectory were normal to the emulsion. If the compartments are considered as discs of different radii, then for each compartment the attenuation associated with non-normal trajectories is proportional to that for the normal trajectories. Thus the *relative* factors may be calculated solely on the basis of normal incidence. The only error encountered in such a treatment is that due to cross-fire, *i.e.*, to a β ray originating from one compartment giving rise to a grain over another compartment. It may be deduced from an argument analogous to that of Lamerton and Harriss (17) that when the electron energy is very low, as it is for tritium, and the compartments are larger than about 3 microns in diameter, then the cross-fire correction is negligible. In fact, for a small compartment, such as n, the correction would not exceed 20 per cent if all compartments were equally labeled. Since in our experiments n usually has about four times as many grains per unit area as the surrounding N, this error is reduced to 5 per cent or less.

Secondly, we shall use a self absorption function of the form: $\exp(-\text{const} \times \text{thickness})$. A justification for this, given in detail elsewhere (18), consists in comparing a theoretical range function, derived from the Bethe-Bloch formula and the emission spectrum of tritium, with an experimentally determined range curve (19). The theoretical curve gives the total path of the electron, which because of its irregularity is longer than the pertinent straight line distance. The experimental curve is measured with aluminum foils and a collimated beam so that it gives a shorter effective path than that encountered in autoradiography. It may be shown that for fixed cell material of density 1.3 an exponential function with the constant $\mu = 1.5$ micron^{-1} is a reasonable approximation, lying between these two extremes; furthermore, it may be shown that

the factors are not strongly dependent on the exact choice of μ.

The factors are then derived as follows: Consider a unit area of cell compartment X, of thickness $\Delta Z = Z_2 - Z_1$, where Z_1 is the surface nearest the emulsion and Z_2 is the farthest surface. Assume that the number of tritium atoms incorporated per unit area, per unit thickness of X, is a constant, K. If all the decaying tritium atoms incorporated in ΔZ produce grains, then the number of grains per unit area of X is given by:

$$(1) \qquad g_X = \int_{Z_1}^{Z_2} K \, dZ = K(Z_2 - Z_1).$$

However, from the foregoing discussion the actual number of grains measured, g_X^*, may be written as:

$$(2) \qquad g_X = \int_{Z_1}^{Z_2} K e^{-\mu Z} \, dZ.$$

The factor for X is then:

$$(3) \qquad E_X = g_X / g_X^*.$$

Normalizing the cytoplasmic factor to unity we have:

$$(4) \qquad \bar{E}_C = 1, \quad \bar{E}_N = g_N g_C^* / g_N^* g_C,$$

$$\bar{E}_n = g_n g_C^* / g_n^* g_C.$$

Measurements, using a calibrated ocular micrometer, of 50 cell profiles such as those in Fig. 2 yielded the average values of Z_1 and Z_2 shown in Table I. Taking μ to be 1.5 micron^{-1}, the factors $\bar{E}_X$ were then calculated from equations (1) to (4). These are also given in Table I.

It is found that on the average there is a 0.2 micron layer of cytoplasm between the nucleus

TABLE I

Parameters of Radioautograph Efficiency

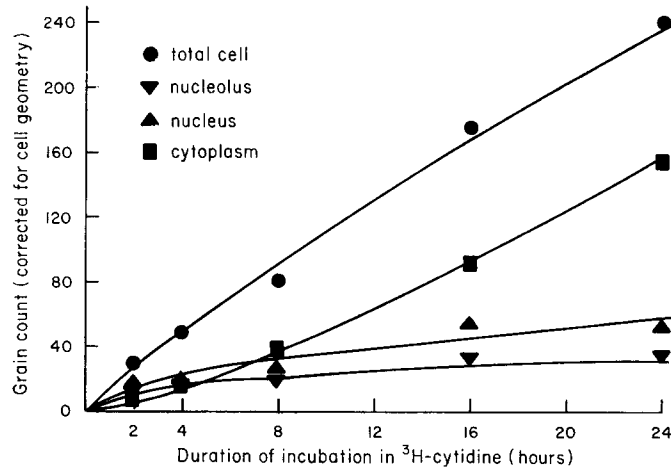| Compartment	Z_1 Minimum distance from emulsion	Z_2 Maximum distance from emulsion	$\bar{E}_X$ Normalized efficiency factor
	μ	μ	
Cytoplasm (C)	0	1.4	1.0
Nucleus (N)	0.2	2.0	1.6
Nucleolus (n)	0.4	2.3	2.3

FIGURE 3

Total incorporation of ^{3}H-cytidine into various parts of HeLa cells as a function of time. Note the decreasing *rates* of n and N and the increasing *rate* of C.

and the emulsion. Grains produced by β particles emanating from this thin, but highly efficient, layer are unintentionally counted as nuclear whereas they really belong to the cytoplasm. It is possible to express the contribution of the layer, l, in terms of the total number of cytoplasmic grains, g_C^*, the ratio of nuclear to cytoplasmic area, A_N/A_C, the thickness, ΔZ, and the factors, E. One obtains:

$$(5) \qquad g_l^* = g_C^* \left[\frac{A_N}{A_C} \frac{E_C}{E_l} \frac{\Delta Z_l}{\Delta Z_C} \right].$$

Planimeter measurements on photographs of 20 cells gave for an average $A_N/A_C = 0.36$. When the other parameters are inserted into equation (5) we find that $g_l^* = 0.11 g_C^*$. In other words, 11 per cent of the cytoplasmic count is routinely counted as nuclear. A similar computation can be made for the layers of nucleus and cytoplasm which overlie the nucleolus, and one finds that only 1.3 per cent of the nuclear and 0.6 per cent of the cytoplasmic grain count are attributable to these layers. However, this last computation is probably not very accurate because of the fact that the nuclear activity is not homogeneously distributed within the nucleus, the nucleolar associated chromatin probably being the most active (20). Thus it is safer henceforth to consider the *nucleolus as including its associated chromatin*.
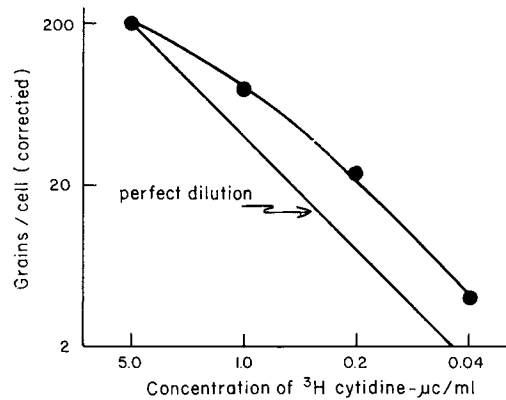
All subsequent grain counts are corrected for

FIGURE 4

Incorporation of ^{3}H-cytidine into total cell RNA as a function of concentration of labeled cytidine. Log-log scale. Note that the incorporation is proportional to the concentration of exogenous nucolesides for concentrations below 0.5 μc/ml.

these geometrical factors and expressed as g_n, g_N, and g_C, where

$$g_n = \bar{E}_n(g_n^* - 0.013 g_N^* - 0.006 g_C^*),$$
$$g_N = \bar{E}_N(g_N^* - 0.11 g_C^*), \quad g_C = 1.11 g_C^*.$$

RESULTS

The general pattern of the incorporation of cytidine into the average HeLa cell over a period comparable to one generation time is shown in
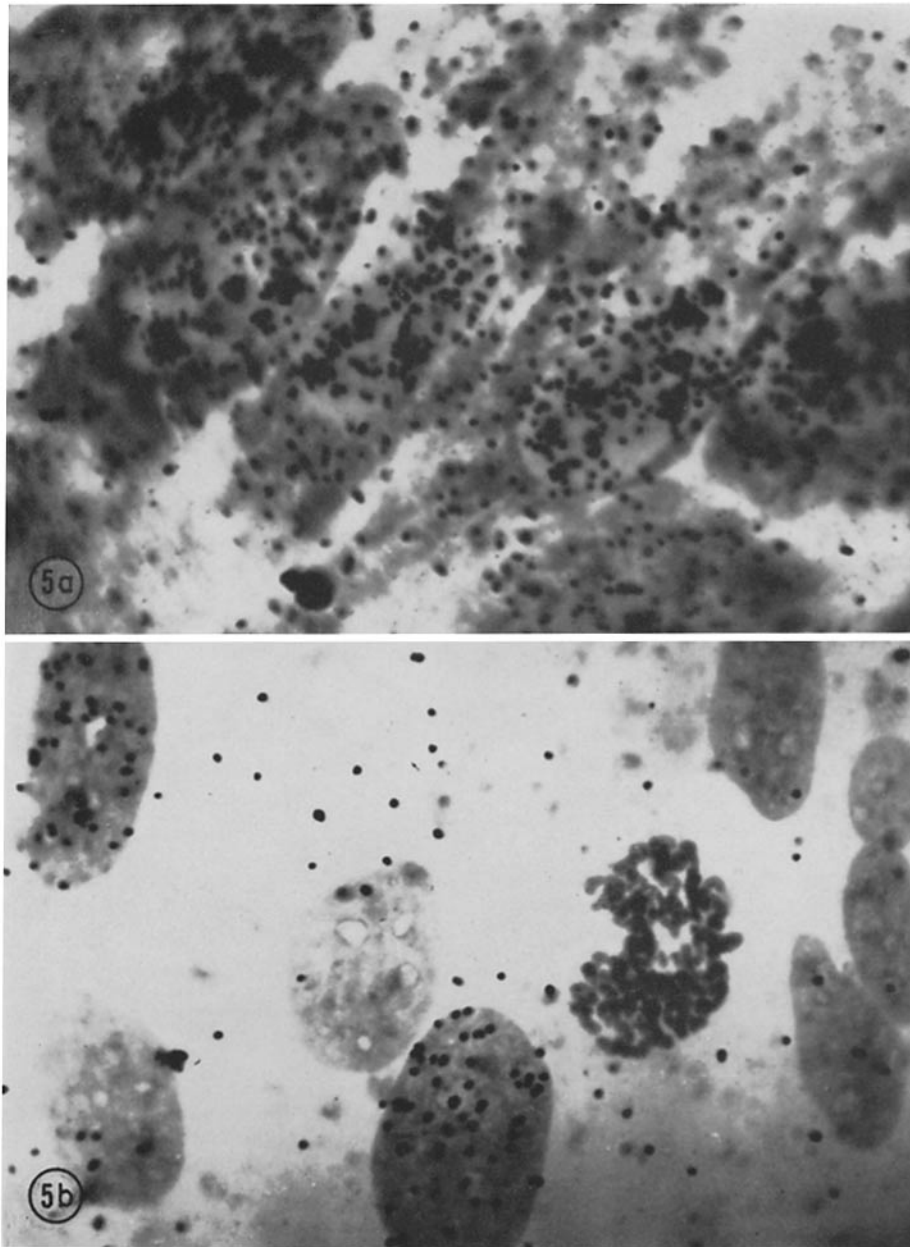
FIGURE 5

Autoradiographs of HeLa cells incubated for 4 hours in ³H-cytidine. *a*, untreated; note that all cells are labeled in all parts. *b*, RNase-treated; note that only certain nuclei are labeled, metaphase is not labeled.

Fig. 3. The curve for the cytoplasm characteristically has a minimum slope at short incubation times, whereas the n and N curves have finite initial slopes which diminish appreciably within 2 hours. The relationship between the total incorporation and the external concentration of labeled nucleoside for the concentration range encountered in our experiments is shown in Fig. 4. In this experiment various amounts of ³H-cytidine at the same specific activity were diluted in E_{10}

TABLE II

Relative Incorporation of Cytidine and Adenosine into RNA and DNA

Nucleoside	Duration of incubation	No. of cells counted		Grains in nucleus of untreated cell* (per 100 cells)	Grains in nucleus of RNase treated cells* (per 100 cells)	Nucleoside in RNA	Labeled cells after RNase
		Untreated	RNase-treated				
	hr.					%	%
Cytidine	2	50	300	7,763	1,202	85	37.5
Cytidine	4	50	200	14,151	2,538	82	46
Cytidine	8	30	100	2,100	800	62	52
Adenosine	1	60	342	5,102	1,029	80	35
Adenosine	2	60	100	8,715	1,438	83.5	40

* The absolute grain numbers should not be compared for different incubation periods because different exposures of the emulsion and different isotope concentrations were used.

medium and presented to the cells for $\frac{1}{2}$ hour. It can be seen that at concentrations below about 0.5 μc/ml the incorporation is proportional to the concentration of exogenous nucleosides. At higher concentrations there is some saturation. Furthermore, in this experiment the distribution of activity in the various compartments was the same, within very narrow limits, at all concentrations (Table III).

In N the nucleosides are incorporated into DNA as well as into RNA. In order to estimate the relative incorporations into the two nucleic acids for various incubation periods, the coverslips were cut in two after fixation and one half
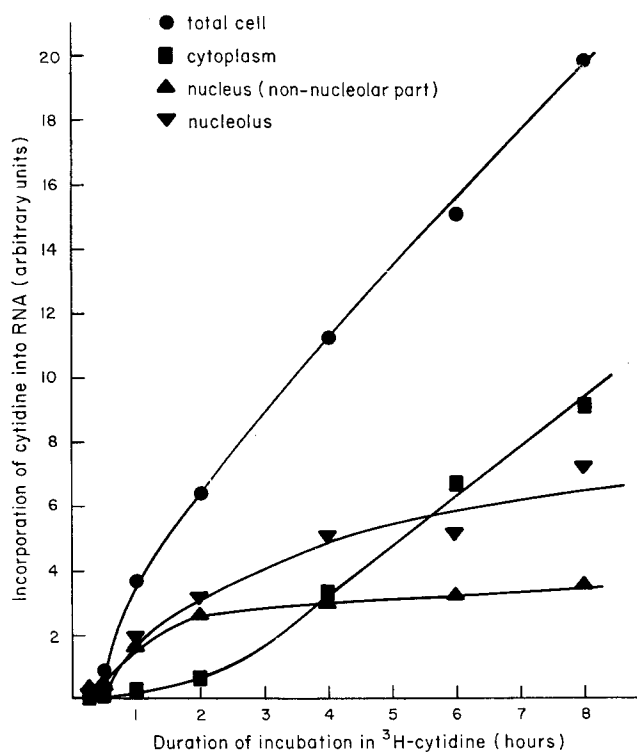

FIGURE 6

Incorporation of ^{3}H-cytidine into RNA of various parts of HeLa cells as a function of time. Note that the N curve reaches saturation at the time when the C curve attains constant slope.
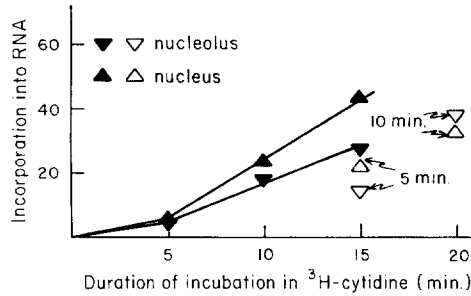
FIGURE 7

Incorporation of ^{3}H-cytidine into RNA in HeLa cells. Result of two experiments: one in ϕ_{10} medium, the other in E_{10} medium. Solid points exactly overlap when E_{10} data are fitted to ϕ_{10} at 5 minute point. Open points are from ϕ_{10} experiment and illustrate samples which are removed from the labeled medium at the indicated times and incubated for a further 10 minutes in unlabeled medium containing excess cytidine. It can be seen that the increment in activity *after* transfer to unlabeled medium is the same for 5 and 10 minutes incubation with label.
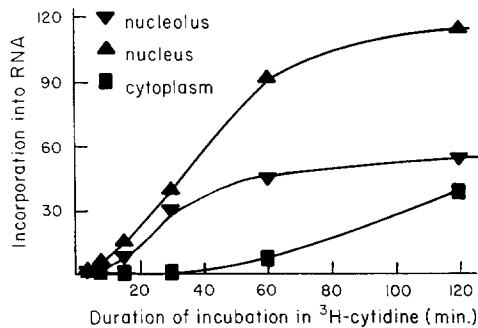

FIGURE 8

Incorporation of ^{3}H-cytidine into RNA in conjunctiva cells. Ordinates are in arbitrary units.

FIGURE 9

Incorporation of ^{3}H-adenosine into RNA in HeLa cells. Upper, in E_{10} medium; lower, in ϕ_{10} medium. Ordinates are in arbitrary units.

were treated with RNase. This treatment removes all the pyronine staining from the cytoplasm and nucleoli and leaves only methyl green-stained nuclei, a certain percentage of which incorporate label. Cells from the two halves representing a 4 hour incubation in ^{3}H-cytidine are shown in Fig. 5. The fraction of the nuclei which are significantly labeled after RNase varies with incubation time from about one-third after 2 hours to more than one-half after 8 hours. When grain counts were made on nuclei from the two alternate halves, the values shown in Table II were obtained. The percentage of cytidine label which is incorporated into RNA decreases from
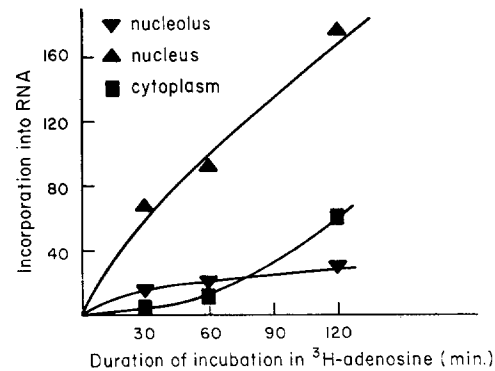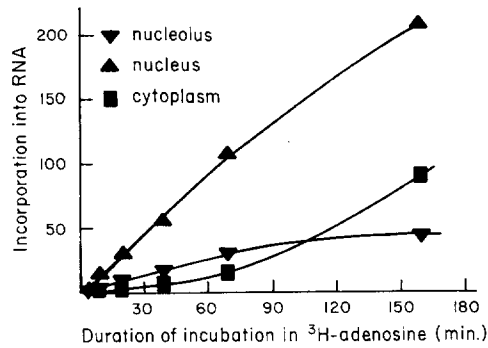
about 85 per cent after 2 hours to about 60 per cent after 8 hours. For adenosine the percentage of label in RNA after short incubation periods seems a bit lower.

Using these data we may express the N values so that they represent only incorporation into RNA. Fig. 6 shows data representing the average of three separate cytidine experiments. Again the striking difference between the C curve and those for n and N is observed. In these experiments incorporation into N almost reaches a steady state at 2 hours.

To look closer at the details of early incorporation, the kinetics were run over shorter time ranges (Figs. 7 to 9). As can be seen by comparing Figs. 7 and 8, the general form of the curves for HcLa and conjunctiva cells is the same. However, the relative saturation levels of n and N are different in the two cells and may possibly reflect differences in the relative contents of n and N RNA's. It can be seen from Fig. 7 and by com-

TABLE III

Distribution of Nucleosides

Nucleoside	Experiment	Concentration of ^{3}H-nucleoside	½ hour incubation per cent of activity			1 hour incubation per cent of activity		
			n	N	C	n	N	C
		$\mu c/ml$						
Cytidine	Concentration experiment (Fig. 4), four separate incubations	0.04	33	65	4			
		0.2	34	64	2			
		1.0	39	61	0			
		5.0	36	62	2			
Cytidine	ϕ_{10} experiment (Fig. 6)		34	64	2	48	46	6
Cytidine	Previously published experiment (13)					41	54	5
Adenosine	ϕ_{10} experiment (Fig. 9, lower)		18	77	5	15.5	75	9.5
	E_{10} experiment (Fig. 9, upper)		21	72	7	20	72	8

paring the upper and lower parts of Fig. 9 that there is no difference in the pattern of incorporation whether the incubations are carried out in Eagle's medium or in the richer ϕ_{10} medium, which contains yeast extract. There is a higher total incorporation in E_{10} for a given ^{3}H-cytidine concentration, but the relative amounts in each compartment are the same for both media.

The following features are consistently observed: (a) the rate of incorporation of labeled nucleosides into the RNA of the cytoplasm is absolutely zero at zero incubation time, and (b) there is a brief lag of about 5 to 10 minutes before n and N reach their maximum incorporation rate.

The second point reflects the time necessary to fill the precursor pools of n and N. This is further substantiated by the transfer experiments shown in Fig. 7. Samples of cells were removed from the labeled medium at 5 and 10 minutes and transferred to unlabeled medium containing excess cytidine for an additional 10 minutes. It is seen that the increment in label during this second 10 minutes is only slightly higher for the 10 minute removal than for the 5 minute removal, suggesting that the pool was practically filled at 5 minutes.

In comparing the intracellular distribution of activity after short incubations in cytidine medium with that found in adenosine incubations, a striking difference is noted (Table III). It may be seen that, relatively to the incorporation in N, more than twice as much cytidine as adenosine is taken up in the nucleolus after ½ hour incubation, and more than three times as much after 1 hour.

This point will be taken up later in terms of specific activities.

In order to obtain an estimate of the total amount of RNA in n, N, and C, ultraviolet absorption measurements were made on individual cells. Sample populations of HeLa cells were treated exactly as in the autoradiographic experiments (except for the incubation in labeled medium) and then mounted in glycerin. A series of photographs at 257 mμ of preparations treated with PCA, DNase, RNase, and both nucleases are shown in Fig. 10. Also shown are representa tive densitometric traces made so as to intercept n, N, and C.

An average value of absorbance was calculated from traces through about ten cells in each category. The absorbance, A, due to RNA was then calculated two ways: as PCA minus RNase, and as DNase minus both nucleases. The results are shown in Table IV. Since the average thickness of each compartment, t, is known from Table I, the relative concentrations can be calculated from the relation $A/t = $ (constant)(concentration). The relative areas were measured by planimetry and the relative amounts were calculated from: amount = (constant)(A)(area). These calculated values of concentration and amount are also given in Table IV. Since the sample of cells measured was small and only an average absorbance trace through each cell was made, these values are only rough estimates. However, they are sufficiently accurate to point out that for RNA the steady state cytoplasmic compart-
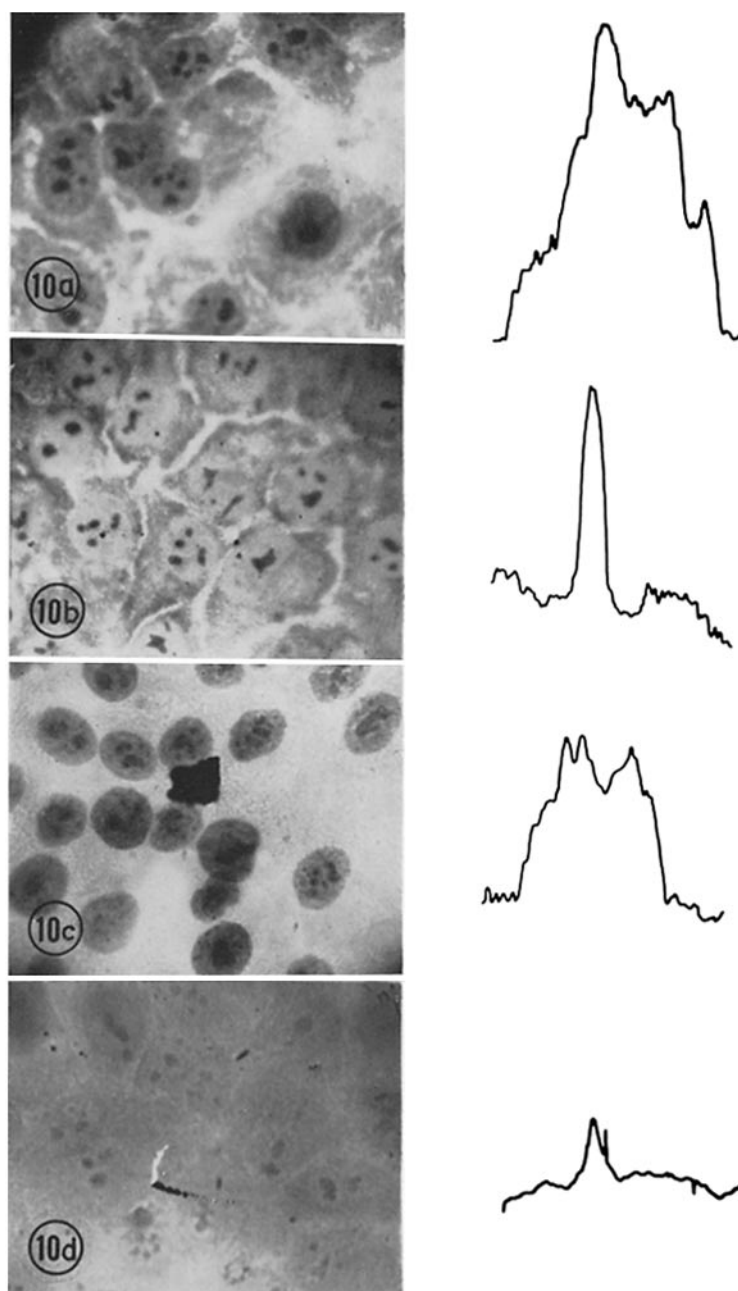
FIGURE 10

Left, examples of 257 mμ ultraviolet micrographs of HeLa cells. *a*, 1 per cent PCA at 5°C, 20 minutes; *b*, DNase-treated; *c*, RNase-treated; *d*, both nucleases. Right, sample densitometric traces across a cell in each category. Traces were made so as to pass through *C*, *N*, and *n*.

TABLE IV

Amounts and Concentrations of RNA

Compartment	Relative absorbances*			Relative concentration of RNA‡	Total amount of RNA§
	PCA minus RNase	DNase minus both nucleases	Average of first two columns		
Nucleolus (n)	0.35	0.32	0.33	5.2	0.33
Nucleus (N)	0.06	0.06	0.06	1.0	1.0
Cytoplasm (C)	0.18	0.20	0.19	4.1	8.9

* Normalized for equal N.
‡ From average thickness values $n:N:C = 1.9:1.8:1.4$.
§ From average area values $n:N:C = 1:17:47$.

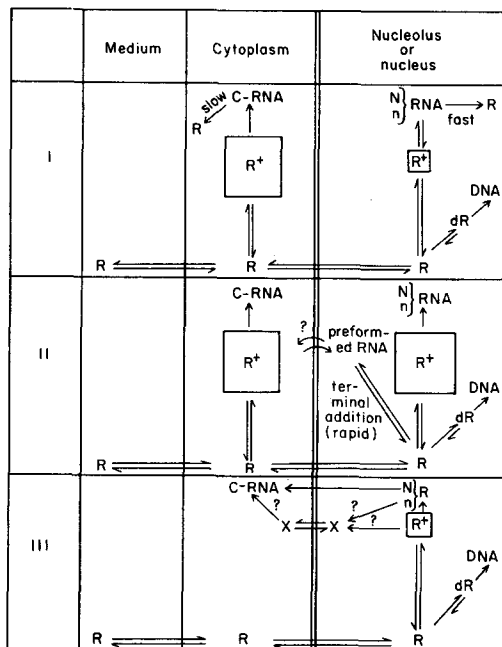FIGURE 11

Hypotheses for mechanism of ribonucleoside (R) incorporation (see text).

ment is 6 to 7 times larger than the two nuclear compartments combined.

DISCUSSION

Several interpretations for the distinct difference in form between the incorporation kinetics of n- or N-RNA and of C-RNA may be offered (Fig. 11): I. Incorporation into all compartments is independent. C has a slower turnover rate and a much larger pool of endogenous precursor, R^+, than n or N, and the observed difference is due to different turnover rates and different pool equilibration times. This hypothesis implies that R^+ is more complex than a nucleoside and is *not* able to pass from the cytoplasm into the nucleus. II. Incorporation into all compartments is independent. The size of the C, n, and N pools of R^+ are nearly the same. The nucleus (nucleolus) has a second and relatively rapid incorporation mechanism (*e.g.* terminal addition onto "transfer RNA") which is responsible for the early preferential incorporation into n and/or N. III. Nucleosides are not directly incorporated into RNA in the cytoplasm. Either all the RNA is made in the nucleus and transported to the cytoplasm as such, or some complex intermediate, X, is made and then transported to C.

The alternatives all contain a scheme for the conversion of nucleosides to DNA precursors. The incorporation into DNA is slow as compared with N-RNA in spite of the fact that the DNA content is at least four times that of N-RNA. Furthermore, in experiments in which the incubation with labeled precursor was followed by an incubation with excess unlabeled precursor the ratio of incorporation of cytidine into DNA/ RNA increases after transfer to the unlabeled medium (20). This is explainable by all the above hypotheses if we assume that there is virtually no turnover of DNA cytidine and that there is either a turnover of N-RNA or a transport of it to other parts of the cell.

Hypothesis I is favored by Harris (10), who performed experiments with cultured connective tissue cells in which he labeled cells for short periods with nucleoside and then measured the kinetics of nuclear and cytoplasmic fractions during a chase with excess unlabeled precursor.

Although he obtained a decrease in nuclear label and an increase in cytoplasmic label, he argues that the data do not indicate a nucleus to cytoplasm transfer because the increase in cytoplasmic activity is greater than the loss in n plus N, and that the increase occurs before any appreciable diminution in the nucleus. However, here the geometry considerations discussed earlier may be applicable. The thinner cytoplasm is on the average 1.6 and 2.3 times as effective in producing grains from a given ^3H concentration as N and n respectively, and hence would show a deceptively higher activity. As the cytoplasmic activity increases, the activity in the cytoplasmic layer overlying the nucleus also increases and hence might give rise to an artifactitious lag in the nuclear decline.

Hypothesis II has been proposed by Vincent and Baltus (21) to account for the RNA fraction from nucleoli of starfish oocytes, which initially has an abnormally high cytidine specific activity. In our experiments we have also noted that the nucleoli, *relative to N*, have a higher incorporation of cytidine than of adenosine. At $\frac{1}{2}$ hour, when the cytoplasmic incorporation is still negligible, the specific activity ratios calculated from Tables III and IV are: cytidine, $n/N = 1.7$; adenosine, $n/N = 0.8$. Harris (10) also reported that more cytidine than adenosine was incorporated by the nucleolus. On the other hand, we have not isolated the individual labeled nucleotides after hydrolysis and we know from macro scale experiments on HeLa cells (22, 23) that the cytidine label goes into both pyrimidine bases and the adenosine label into both purine bases. Hence, it is impossible to decide whether our data reflect differences in base composition or differences in in metabolic pathway. The answer to this question awaits a clean separation of n and N fractions followed by nucleotide analysis. Since the mature oocytes studied by Vincent are not growing and possibly *not* synthesizing ribosomes, whereas the HeLa cells are growing rapidly and are committed to double their ribosomes every 24 hours, the two systems should be compared only with caution.

Hypothesis III has been proposed previously (12, 13) to account for experiments which showed that when the nucleolar (or nucleolus-associated chromatin) activity was reduced to a negligible amount, the cytoplasmic activity was diminished by more than 60 per cent. It cannot be established now whether the RNA moves from nucleus to cytoplasm as an intact molecule or whether a complex precursor, X, produced at the nucleolus or in conjunction with a nucleolar by-product, moves to the cytoplasm.

From a quantitative standpoint the nearly reciprocal relationship between the n or N curves and the C curve also argues for a nuclear-cytoplasmic transfer of RNA. Such a reciprocity could otherwise occur only through a fortuitous combination of rate constants if the three systems were completely independent. Theoretical relationships for the activity in various compartments were derived for a model system similar to type III, and it was shown (12) that they mimic rather well the experimental kinetic curves.

Prescott (24), working with nucleate and anucleate fragments of ameba, concluded that there is a nuclear-cytoplasmic transfer of RNA in this protozoan; and Edström, making a delicate microelectrophoretic separation of the RNA bases from n, N, and C in spider oocytes showed that n-RNA had the same base composition as C-RNA (25). Both these experiments led support to the type III mechanism.

Within the nucleus the two RNA systems, n and N, appear to be independent. However, this statement is made with the reservation stipulated earlier that the nucleolus-associated chromatin activity is being counted as nucleolar. Indeed, from the data of Pelling (26) and some of our own observations (20) it is possible that all the early activity is associated with chromatin, *that associated with the nucleolus being especially active*. If we were able, in our system, to make a clear distinction between activity in the nucleolar substance and activity in the associated chromatin, perhaps the true n-RNA curve would become a sigmoid one similar to that for C-RNA. However, our data do show that a system whereby all the RNA is synthesized in regions remote from n (*i.e.* in N) and then transported to n, as suggested by some authors (27, 28), does not exist.

In conclusion, then, the kinetic studies presented here, as well as the experiments in the literature dealing with pulse labeling and chasing, allow, on a qualitative level, several alternative hypotheses. Experiments of a more refined nature tend to emphasize hypothesis III as a major mechanism, although they do not exclude the possibility that the other systems are also operating. Certainly the heterogeneity of RNA, *i.e.*

transfer, ribosomal, and possibly also "messenger" RNA, as well as the possibility of alternate incorporation mechanisms (29–31), might lead one to expect more than one system of RNA synthesis.

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