CYTOPLASMIC SYNTHESIS OF NUCLEAR PROTEINS

Kinetics of Accumulation of Radioactive Proteins in

Various Cell Fractions after Brief Pulses

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ABSTRACT

The synthesis of cytoplasmic and nuclear proteins has been studied in HeLa cells by ex-
amining the amount of radioactive protein appearing in the various subcellular fractions
after labeling for brief periods. Due to the rapid equilibration of the amino acid pool, the
total radioactivity in cytoplasmic protein increases linearly. The radioactivity observed in
the cytoplasm is the sum of two components, the nascent proteins on the ribosomes and the
completed proteins. At very short labeling times the specific activity of newly formed pro-
teins found in the soluble supernatant fraction (completed protein) increases as the square
of time, whereas the specific activity of the ribosomal fraction (nascent protein) reaches a
plateau after 100 sec. The kinetics of accumulation of radioactive protein in the nucleus and
the nucleolus is very similar to that of completed cytoplasmic protein, which suggests that
the proteins are of similar origin. The rate of release and migration of proteins from the
ribosomes into the nucleus requires less time than the synthesis of a polypeptide, which is
about 80 sec. The uptake of label into nucleolar proteins is as rapid as the uptake of label
into proteins of the soluble fraction of the cytoplasm, while nuclear proteins, including his-
tones, tend to be labeled more slowly. The same results are obtained if protein synthesis is
slowed with low concentrations of cycloheximide.

The kinetics of incorporation of amino acids into various fractions of the cell indicates
that the nucleus and the nucleolus contain few if any growing polypeptide chains, and thus
do not synthesize their own proteins.

INTRODUCTION

The cell has a number of compartments, each with
certain functions to perform. The nucleus contains
the DNA and is the site of RNA transcription. The
nucleolus is the site of synthesis and assembly of
ribosomes. The mitochondrion is the site of oxi-
dative phosphorylation and other specialized reac-
tions. Yet each of these cellular compartments is
composed largely of proteins. The origin of these
proteins, especially the proteins of the nucleus,
has been the subject of controversy for many
years, since the cytosol contains the ribosomes,
which are responsible for the synthesis of proteins.
Goldstein (1970) has presented an excellent re-
view of this controversy.

There are three alternatives to consider: (a) the
protein of the nucleus is formed within the nu-
cleus, (b) the protein of the nucleus is formed in
the cytoplasm and subsequently migrates into the

nucleus, and (c) certain proteins of the nucleus are formed within it and certain ones are formed in the cytoplasm.

The main line of evidence favoring the cytoplasmic synthesis of all proteins is that all or nearly all of the cell's ribosomes are in the cytoplasm. Few if any are in the nucleus (Harris, 1963; Penman, 1966; Casola and Agranoff, 1968; Rogers, 1968). Furthermore, since the drug cycloheximide inhibits protein synthesis at the level of the ribosome (Ennis and Lubin, 1964) and since it stops all protein synthesis in the cell (Warner et al., 1966) all proteins are made on ribosomes. That small amount of protein synthesis which continues is made exclusively on mitochondrial ribosomes (Galper, 1970), which are not sensitive to the drug. Finally there is now some direct evidence that some nascent nuclear proteins can be found on cytoplasmic polyribosomes (Robbins and Borun, 1967; Heady and McConkey, 1970; Craig and Perry, 1971).

On the other hand, there have been reports of protein synthesis in isolated nuclei or on isolated nuclear ribosomes (Allfrey et al., 1957, 1960; Rendi, 1960; Birnstiel and Flamm, 1964; Gallwitz and Mueller, 1969). Some of these results may be due to contaminating cytoplasmic ribosomes which bind tenaciously to the outer nuclear membrane (Holtzman et al., 1966), but for the most part the two lines of evidence are contradictory with no easy adjudication possible.

Kuehl (1967) has done a kinetic study of the distribution of newly formed radioactive lactic dehydrogenase in various cell fractions, but his data do not distinguish between nuclear synthesis of the enzyme and cytoplasmic synthesis followed by rapid migration to the nucleus. We therefore undertook to measure the distribution of radioactive protein in subcellular fractions of HeLa cells after short periods of labeling, with particular emphasis on times shorter than the average synthesis time of a polypeptide.

MATERIALS AND METHODS

Buffer Designations

Earle's, 0.116 M NaCl; 0.0054 M KCl; 0.001 M MgCl$_2$; 0.0011 M NaH$_2$PO$_4$, 0.0238 M NaHCO$_3$; 0.0055 M glucose; RSB, 0.01 M Tris pH 7.4; 0.01 M NaCl; 0.0015 M MgCl$_2$; HSB, 0.01 M Tris pH 7.4; 0.5 M NaCl; 0.05 M MgCl$_2$; SUP, 0.5% sodium dodecyl sulfate (SDS); 0.5 M urea; 0.01 M sodium phosphate (pH = 7.2).

Cell Culture and Labeling Techniques

Suspension cultures of HeLa cells (strain S3) were grown in Eagle's (1959) spinner medium supplemented with 7% horse serum. The generation time was 24 hr or less and the cells were maintained at a concentration of 2–5 × 10^5/ml. Long-term labeling of protein was accomplished by suspending 2 × 10^8 cells in 800 ml of Eagle's spinner medium supplemented with 7% horse serum and 5 × 10^{-2} μCi/ml of leucine-^{14}C for 16 hr. The labeled cells were centrifuged for 2 min at 800 g and resuspended at 5 × 10^6/ml in leucine-free medium supplemented with 7% horse serum. After a 5 min preincubation (4, 5) leucine-^{3}H was added at a concentration of 25 μCi/ml. To stop incorporation of radioactive leucine, the culture was diluted with nine volumes of Eagle's medium supplemented with ten times the normal concentration of leucine. Cell metabolism was stopped by pouring 5–25 ml samples onto crushed frozen Earle's saline (1943) supplemented with 100 μg/ml of cycloheximide and 4 mM leucine.

Cell Fractionation

Cytoplasmic and nuclear fractions were prepared according to the methods of Penman (1966) and Penman et al. (1966). The culture, on frozen Earle's solution, was centrifuged and the cells were washed twice in Earle's solution. They were resuspended in 5 ml of RSB buffer, allowed to swell for 10 min at 0°C, and disrupted by ten strokes of a stainless steel Dounce homogenizer. The nuclei and unbroken cells were removed by centrifugation at 1500 rpm for 5 min in an International PR2 refrigerated centrifuge (International Equipment Company, Needham Heights, Mass.). This pellet was washed with 2 ml of RSB and recentrifuged to give a crude nuclear pellet. The two cytoplasmic fractions were combined; Brij 58 and sodium deoxycholate were added, each to a final concentration of 0.5%. A portion was removed for determination of radioactivity and the remainder was centrifuged at 40,000 rpm for 2 hr at 4°C in a Spinco 40 rotor (Beckman Instruments, Inc., Palo Alto, Calif.) to yield a soluble supernatant fraction and a microsomal fraction. The latter fraction contains polysomes, ribosomes, and considerable other protein.

The crude nuclear pellet was resuspended in 2 ml of RSB and 0.3 ml of a detergent mixture containing 6.7% Tween 40 and 3.3% sodium deoxycholate. After vigorous mixing, the nuclei were resedimented and the detergent soluble fraction was removed. The nuclei were further fractionated according to Willems et al. (1968). The nuclei were lysed in 2 ml of HSB buffer containing 100 μg of electrophoretically purified DNase (Worthington Biochemical Corp., Freehold, N. J.). The viscous deoxyribonucleo-protein was degraded by incubation for 1–2 min at 37°C

and the resulting suspension was layered on a 16 ml 15–30% sucrose gradient in HSB which was centrifuged at 22,000 rpm for 15 min at 4°C in a Spinco SW27 rotor. The pellet, which is considered the nucleolar material, since it contains all the cell's 45S and 32S ribosomal precursor RNA, was dissolved in SUP, stirred for 1 hr, and centrifuged at 1500 rpm for 5 min to remove a small amount of insoluble debris. The entire HSB gradient was considered the nucleoplasm. After a portion was removed for determination of radioactivity, the remainder was used for isolation of basic proteins by the addition of HCl to 0.25 N for 30 min at 4°C. After centrifugation at 30,000 rpm for 1 hr at 4°C in a Spinco 30 rotor, the pellet was discarded and the supernatant was diluted with 10 vol of acetone at −20°C. The next morning, histones were collected by centrifugation at 1500 rpm and were dissolved in 0.1% SDS, 0.01 M Na phosphate, pH 7.2.

Determination of Radioactivity

Radioactivity was determined on portions of the subcellular fractions by adding NaOH to 0.5 M, incubating for 15–30 min at 37°C, and then adding trichloroacetic acid (TCA) to 10%. The precipitate was collected on glass filters and washed with 5% TCA. The filters were put in counting vials and incubated at room temperature for 15 min in the presence of 0.7 ml of 0.1 N NCS (Nuclear-Chicago, Des Plaines, Ill.) in a scintillation fluid consisting of 40 ml of Spectrafluor (Nuclear-Chicago) and 960 ml of toluene. Then, 10 ml of scintillation fluid was added and the $^3H/^{14}C$ ratio was determined.

Analysis of Intracellular Acid-Soluble Pool

The uptake of leucine into the acid-soluble pool was measured by the method of Morrill and Robbins (1967). 40 × 10⁵ HeLa cells from a spinner culture were sedimented at 800 g for 2 min and resuspended at 40 × 10⁶/ml in leucine-free medium supplemented with 7% fetal calf serum. After a 5 min preincubation, 2 μCi of leucine-^{14}C was added. At intervals during the pulse, portions were transferred to specially designed centrifuge tubes consisting of an upper reservoir with a 30 ml capacity fused to a graduated capillary tube with a precision bore having a diameter of 2.0 mm and a volume of 70 μl. These were centrifuged for 60 sec at 3000 g and the supernatant fluid was removed by suction. The centrifugation was carried out at 37°C since chilling the cells leads to erroneous determinations of the pool size because the energy-dependent transport systems are disrupted (Morrill, G., personal communication). However, in order that the acid-soluble leucine not be converted to acid-precipitable protein during the centrifugation, the capillary was filled with an identical medium, in-

cluding leucine-^{14}C, which also contained 100 μg/ml of cycloheximide.

The volume of the packed cells was measured from the calibration on the capillary tube. The volume of trapped extracellular fluid was determined to be 30% by comparison with the results which Morrill and Robbins (1967) obtained using carboxy-^{14}C-labeled inulin. The cells were resuspended in 5 ml of distilled water. Two samples of the whole cell suspension were removed. One sample was counted directly. To another sample, NaOH was added to 0.5 N, and after 15 min, 1 vol of 20% TCA. The precipitate was collected on a millipore filter and the TCA-soluble material was collected in a tube at the same time. All samples were counted in a scintillation fluid consisting of, per liter, 300 ml of Triton X-100, 40 ml of Spectrafluor (Nuclear-Chicago) and 660 ml of toluene. The data are presented as cpm/mg wet weight of cells, which is determined by correcting for the extracellular fluid present in the packed cells.

Theoretical Analysis

The theoretical analysis of the uptake of amino acids into nascent and completed protein has been presented in detail by Vol'kenshtein and Fishman (1965). In our simplified version, we make the following postulates: (a) the specific activity of the amino acid pool reaches equilibrium instantaneously (see Table II), (b) proteins are synthesized by sequential addition of amino acids (Naughton and Dintzis, 1962), and (c) during the course of the experiment (less than 10 min) newly made proteins are not degraded and a negligible amount of new ribosomes is formed.

To simplify the equations we will define a time, T, as the synthesis time, averaged over all cell proteins, of a single polypeptide chain from the formation of its first peptide bond to its release from the ribosome. From the first and third postulates, the total radioactivity in acid-precipitable protein,

$$P = N + C = kt,$$

where N is radioactive nascent protein, C is radioactive completed protein, t is time, and k is a constant. For time less than T, the amount of radioactive completed protein, C, is proportional to the product of the number of ribosomes releasing peptides and the amount of radioactivity in the released peptides, each of which is proportional to time. Thus:

$$0 \le t \le T$$

$C = at^2$, where a is another constant.

The amount of radioactivity in nascent peptides on ribosomes is:

$$N = P - C = kt - at^2.$$

One can relate the two constants by observing that:

$$at\ t\ =\ T, C\ =\ N\ =\ \frac{P}{2} \quad \text{and therefore,}\ a\ =\ \frac{k}{2T}.$$

Thus for $\qquad 0 \leq t \leq T,$

$$P\ =\ kt$$

$$C\ =\ \frac{kt^2}{2T}$$

$$N\ =\ kt\left(1 - \frac{t}{2T}\right)$$

and $\qquad T \leq t \leq \infty,$

$$P\ =\ kt$$

$$C\ =\ kt - \frac{kT}{2}$$

$$N\ =\ \frac{kT}{2},\ \text{a constant.}$$

The curves describing these equations are shown in Fig. 1. Note that at $t = T, C = N$, and that at $t > T$, C is parallel to P.

For our purposes the important result of the analysis is that if one measures both nascent and completed protein, the increase in radioactivity should be linear with time. Whereas if one measures only completed protein, the increase in radioactivity should be proportional to the square of the time for a short while, and then should become linear.
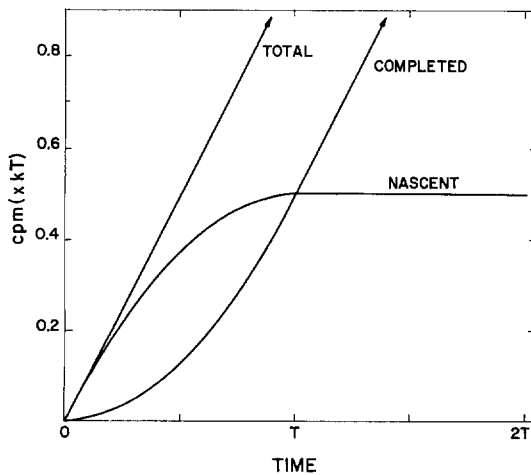

FIGURE 1 Theoretical incorporation of radioactivity into total protein, completed protein, and nascent protein. Equations describing these curves are discussed in Materials and Methods. T is the synthesis time averaged over all cell proteins, and k is a rate constant.

TABLE I

Distribution of Cellular Protein

	% leucine-^{14}C	
	Control	+ 0.5 μg /ml cycloheximide
Cytoplasm	84	83
Microsome	14	13
Soluble super-natant	60	58
Detergent wash	8	7
Nucleus	16	17
Nucleoplasm	15	16
Nucleolus	1	1

HeLa cells were labeled for one generation with leucine-^{14}C as described in Materials and Methods. All values are an average of two different experiments.

RESULTS

Distribution of Cellular Proteins

The cell fractionation techniques which have been developed by Penman et al. (1966) allow the preparation from HeLa cells of nuclear and cytoplasmic fractions with little cross contamination. As a measure of total protein, HeLa cells were labeled for one generation with leucine-^{14}C. The distribution of radioactivity in the various cell fractions is shown in Table I. The values for the cytoplasm, nucleus, and nucleolus fractions agree with those previously obtained by Warner and Soeiro (1969) using chemical determinations. Pre-incubation of the cells at a low dose of cycloheximide for 5 min (see also Fig. 4 B) does not change the distribution pattern of proteins. The difference between the cytoplasm and the sum of the soluble supernatant, detergent wash, and microsomes can be attributed to losses during isolation of the various subcellular fractions.

Equilibration of Acid-Soluble Pool

One of the major postulates of our analysis is that the pool of acid-soluble leucine equilibrates rapidly. The validity of this postulate was measured ase described in Materials and Methods. Table II shows the specific radioactivity of the total cells, the TCA-soluble, and the TCA-precipitable fractions of the cells in the presence and in the absence of a low dose (0.5 μg/ml) of cycloheximide. It can be seen in Table II that the cells actively

| | cpm /mg of cells | | | | | |
| | Control | | | + 0.5 μg /ml cycloheximide | | |
Time	Whole cell	TCA-ppt	TCA-soluble	Whole cell	TCA-ppt	TCA-soluble
(min)						
0.2	589	93	501	551	22	553
2.0	876	358	514	681	88	617
4.0	1114	683	469	726	152	573
6.2	1542	1064	482	872	253	639

The specific radioactivity of the total cells and their TCA-soluble and TCA-precipitable fractions was determined as described in Materials and Methods. The extracellular leucine-^{14}C concentration is 200 cpm/μl.

concentrate leucine-^{14}C and that the intracellular leucine pool reaches a constant activity almost instantaneously. The leucine pool found under our conditions is very small and will only support a few rounds of protein synthesis. The slowing of protein synthesis by a low concentration of cycloheximide has little effect on either the rate of equilibration or the magnitude of the intracellular pool.

Kinetics of Cytoplasmic Protein Synthesis

Fig. 2 shows the accumulation of radioactivity in the total protein, completed protein, and nascent protein of a cytoplasmic extract of HeLa cells during continuous labeling with leucine-^{3}H. By comparison with Fig. 1, it is apparent that the experimental results coincide almost exactly with the theoretical analysis. This is further justification of our postulate that the soluble pool of leucine equilibrates rapidly. The main difference between Figs. 1 and 2 is that the nascent protein curve becomes level less abruptly. This is probably due to the heterogeneous size of the proteins and thus of their synthesis times. The average synthesis time, T, shown in Fig. 2 is about 80 sec. This value agrees well with the direct measurement of the synthesis time of a hemoglobin chain (Hunt et al., 1969), if one considers that the size of the average HeLa polypeptide is about three times larger (Kiehn and Holland, 1970). However, it is somewhat greater than that reported for Syrian hamster cells (Stanners, 1968; Stanners and Becker, 1971). The difference may be due to the fact that the hamster cells are growing more than twice as rapidly.

The Kinetics of Distribution of Pulse-Labeled Proteins

Fig. 3, from the same experiment as Fig. 2, shows the accumulation of acid-precipitable radioactivity in various cell fractions during labeling with leucine-^{3}H. The data are plotted as a ratio of ^{3}H to ^{14}C where the ^{14}C values, representing total protein, derive from a 16 hr incubation with leucine-^{14}C (see Table I) and serve as an internal standard to correct for small variations in yield. There is no change in the ^{14}C values during the course of the experiment. As discussed above, the incorporation into the total cytoplasmic protein is linear (Fig. 3 A), while the soluble cytoplasmic protein becomes labeled slowly at first, then at an increasing rate for about 100 sec, after which it, too, is linear (Fig. 3 B). The detergent wash of the nuclei, which include polysomes, was identical to the total cytoplasm shown in Fig. 3 A. The incorporation into total nuclear protein is shown in Fig. 3 C. It is similar to the cytoplasmic soluble protein except that the rate is somewhat slower. Both the HCL-soluble (histone) and the residual nuclear proteins are labeled with kinetics identical to that of the total nuclear material (data not shown). The nucleolar protein, Fig. 3 D, also has a curve characteristic of completed protein as in Fig. 3 B.

This is seen more clearly in Fig. 4 A, which shows a direct comparison of the kinetics of appearance of new protein in the various cell fractions. It is clear that the nucleolus is almost identical to the soluble portion of the cytoplasm while the nucleus lags somewhat but has a curve of the same shape. It seems likely that this similarity
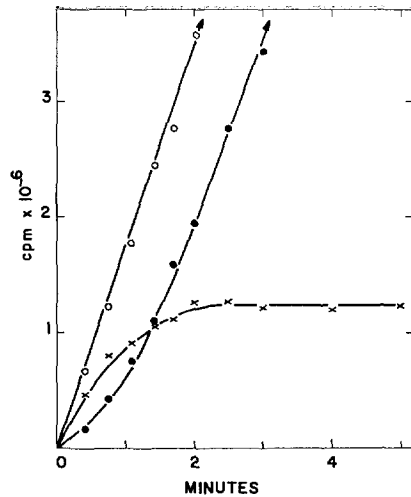
Figure 2 Radioactivity in total protein, completed protein, and nascent protein. HeLa cells, prelabeled for 16 hr with leucine-^{14}C, were labeled with leucine-^{3}H as described in Materials and Methods. At various times, samples were chilled, the cells were collected, and a crude cytoplasmic extract was prepared. A portion was saved for determination of radioactivity, and the rest was centrifuged for 2 hr at 40,000 rpm. The radioactivity of the supernatant and pellet was determined. From the ^{14}C radioactivity, 15% of the total cytoplasmic protein is found in the crude microsomal pellet; only a very small amount of this is nascent, the rest being completed ribosomal and structural proteins. Thus, the total radioactivity in nascent protein is equal to the total microsomal radioactivity minus 0.15 times the radioactivity in supernatant protein, and the total radioactivity in completed protein is equal to 1.15 times the radioactivity in the supernatant proteins. Small corrections for yield were also made using the ^{14}C radioactivity as a guide. O——O, total protein; ●——● completed protein; ×——× nascent protein.

is due to the migration of completed protein from the cytoplasm to the nucleus and the nucleolus. It indicates that there is little or no nascent protein in the nucleus and the nucleolus, because we would then expect a linear increase in radioactivity (see Fig. 1).

The alternative explanation for the result in Fig. 4 A could be some lag in the equilibration of the pool of amino acids in the nucleolus. Such a situation appears unlikely in view of the rapid equilibration in the whole cell as seen in Table II. However, to rule out such effects we carried out an experiment similar to that in Fig. 4 A in which the rate of protein synthesis was reduced to one

fourth by addition of cycloheximide to a concentration of 0.5 μg/ml (see Fig. 4 B). The rationale was that if the curves seen in Fig. 4 A were due to a slow equilibration of the radioactive amino acid with the nucleus, then this effect would be minimized by the slowing down of all protein synthesis. Table II shows that this concentration of cycloheximide has little effect on the uptake of leucine from the medium. It can be seen from Fig. 4 B that in the presence of cycloheximide the kinetics of labeling of nucleolar protein is still identical to that of cytoplasmic soluble protein with the nucleus lagging slightly behind. Therefore, we conclude that the nucleus does not contain nascent polypeptide chains, and thus that nuclear protein is made in the cytoplasm.

Kinetics of Migration

If nuclear protein is made in the cytoplasm, it must migrate into the nucleus. From the rate of accumulation of radioactivity in the nucleus shown in Figs. 3 and 4 it is apparent that proteins migrate very rapidly from the cytoplasm to the nucleus. To estimate just how rapidly, incorporation of leucine-^{3}H in the experiment described in Figs. 2–4 was rapidly stopped either by the addition of an excess of unlabeled leucine or by the addition of cycloheximide to 100 μg/ml. The results are shown with the dotted and dashed lines in Fig. 3. The addition of cycloheximide stops protein synthesis almost immediately and there is no change in the soluble protein. However, the chase with cold leucine permits the radioactive nascent proteins to be completed and released from ribosomes. The accumulation of radioactivity in the nucleus after a chase with cold amino acids alone is a measure of the amount of protein present as nascent peptides at the time of the chase and the amount of protein in the process of migration to the nucleus. On the other hand, the accumulation after treatment with cycloheximide is a measure of only the amount of protein in the process of migration. Because the increase in nuclear and nucleolar radioactivity after the chase is much greater without cycloheximide, there is less protein in the process of migration than there is in the form of nascent polypeptides. Therefore the time required for a protein to migrate from cytoplasm to nucleus must be short compared to the synthesis time of a protein. This is true also for acid-soluble histone proteins.
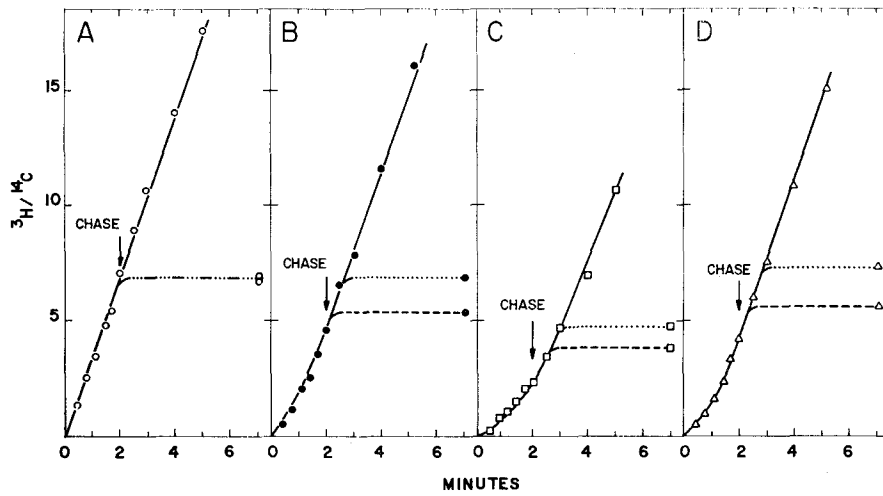
In order to estimate the rate of the migration

Figure 3 The accumulation of acid-precipitable radioactivity in the various cell fractions during label-ing with leucine-^{3}H. Data are from the same experiment as Fig. 2. HeLa cells, prelabeled for 16 hr with leucine-^{14}C were labeled with leucine-^{3}H, and harvested at various times. At 2 min a 5 ml portion of culture was removed into a tube containing cold leucine, and another into a tube containing cold leucine plus 100 μg/ml of cycloheximide. These were harvested 5 min later. The cells were collected, fractionated into the respective subcellular fractions, and the ^{3}H/^{14}C ratio of the various fractions was determined as described in Materials and Methods. A, total cytoplasm O——O; B, soluble cytoplasmic supernatant ●——●; C, total nucleus □——□; D, nucleolus △——△; chased at 2 min with cold leucine · · · · · ; chased at 2 min with cold leucine + 100 μg/ml cycloheximide ——.
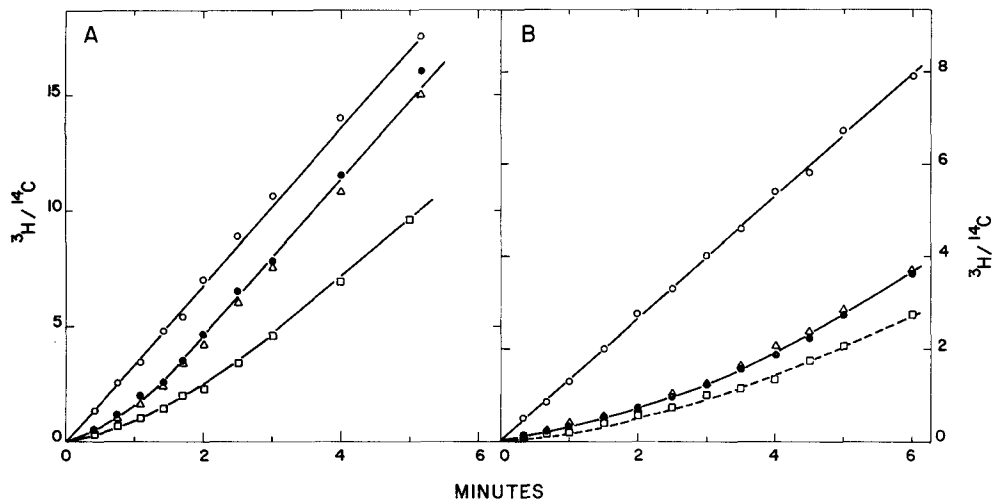

Figure 4 A, Direct comparison of the kinetics of appearance of new proteins in the various cell frac-tions. This is the same experiment as Figs. 2 and 3. B, Direct comparison of the kinetics of appearance of new proteins in the various cell fractions in the presence of 0.5 μg/ml of cycloheximide. 1 × 10^8 HeLa cells were suspended in 800 ml of Eagle's spinner medium supplemented with 7% horse serum and 2.5 μCi/ml of leucine-^{14}C for 16 hr. The labeled cells were centrifuged for 2 min at 800 g and resuspended at 3 × 10^6/ml in leucine-free medium supplemented with 7% horse serum and 0.5 μg/ml of cycloheximide. After a 5 min preincubation (4, 5), leucine-^{3}H was added at a concentration of 15 μCi/ml. Fractionation and counting procedures are the same as those described in Materials and Methods.O——O Total cytoplasm; ●——● soluble cytoplasmic supernatant; □——□ total nucleus; △——△ nucleolus.

Change of radioactive proteins after chase

	Cold leucine alone	Cold leucine + 100μg/ml cycloheximide
	cpm	cpm
Total cytoplasmic protein	-1×10^5 (−3%)	-1×10^5 (−3%)
Completed cytoplasmic protein*	$+1 \times 10^6$ (+45%)	$+3.5 \times 10^5$ (+17%)
Nascent protein	-1.2×10^6 (−97%)	-3.2×10^5 (−25%)
Nuclear protein	$+1.9 \times 10^5$ (+92%)	$+1.1 \times 10^5$ (+52%)
Nucleolar protein	$+1.8 \times 10^4$ (+70%)	$+8.1 \times 10^3$ (+37%)

* Calculated as described in the caption to Fig. 2. Data are obtained from Fig. 3 which is the same experiment as Fig. 2.

of proteins from the cytoplasm into the nucleus or nucleolus, it is necessary to express the data in Fig. 3 in terms of the amount of change in radioactivity after the chase. This is shown in Table III. The first line of the table shows that the chase with cold leucine is effective even without the help of cycloheximide. The next lines show that the same amount of radioactivity which is chased from nascent protein is found in completed protein. In the absence of cycloheximide, all of the nascent protein is chased from the polysomes. In the presence of cycloheximide one-fourth of the nascent protein is chased, probably due to the few seconds required for the drug to function fully. Presumably only the nearly finished nascent chains would be completed in the presence of cycloheximide, and they have twice the radioactivity of the average nascent chain. Thus cycloheximide probably becomes effective with about one-eighth of a synthesis time, or 10 sec. By comparing the two columns it becomes apparent that 10^4 cpm of nucleolar polypeptide and 8×10^4 cpm of nuclear polypeptide has been frozen on the cytoplasmic polysomes by the addition of cycloheximide. It is possible to make a rough estimate of the time required for a protein to move from the cytoplasm to the nucleus or the nucleolus. By the time the chase was added, at 2 min, the incorporation curves have become linear, and the rate of incorporation into nucleus and nucleolus is 2×10^5 and 2×10^4 cpm/min, respectively. Thus the amount of peptide entering the nucleus and the nucleolus after the cycloheximide treatment (last two lines of right column) represents less than 0.5 min of synthesis. The important conclusion is that the transport time of a protein going into the nucleus is much less than the time required to synthesize it.

This analysis has assumed that, like protein synthesis, the transport of proteins within the cell does not occur at 0°C. Since it takes 15–60 min to complete an experiment and to fractionate the cells, any migration during that time would completely overshadow that in the brief periods we have studied. The fact that we could detect some protein migrating to the nucleus after cycloheximide treatment substantiates this assumption, but clearly migration of proteins at 0°C may affect our quantitative estimates.

DISCUSSION

The results presented above show that most of the nuclear and nucleolar proteins of HeLa cells are synthesized in the cytoplasm on ribosomes and migrate to their site of utilization. In this respect, our results agree with those obtained for histone biosynthesis by Robbins and Borun (1967). On the other hand, our data are not sufficiently sensitive to rule out all protein synthesis within the nucleus. Probably the synthesis of 10% or 20% of the total nuclear protein would go undetected.

Our results do clearly show that the migration of protein to the nucleus is so rapid that most of the kinetics studies carried out in the past were inadequate to resolve the site of protein synthesis. Clearly one must study times which are short in comparison with the synthesis time of the polypeptide.

Our data provide more questions than answers about the mode of migration of proteins into the nucleus. Two general modes of migration can exist: free diffusion and active transport. If we consider the diffusion from a cell of radius 7 μ into a nucleus of radius 3.5 μ, then, using the three-dimensional diffusion equation for a spherical shell (Adam and Delbrück, 1968), the calculated average mean diffusion time is less than 1 sec for a globular pro-

tein of mol wt 50,000. This estimate, of course, ignores many important factors such as the nuclear membrane and the viscosity of the cytosol. Still, the number obtained from Table II for the time of transport, 0.5 min, is sufficiently larger than the crude theoretical value of 1 sec, so that free diffusion is within the realm of possibility.

On the other hand, since the rate of diffusion is proportional to the absolute temperature, we would expect that at 0°C it should continue at almost 90% of the rate at 37°C. The data in Table III indicate that migration of protein to the nucleus has been shut down by chilling the cells, because we detect proteins migrating to the nucleus after cycloheximide. This weak evidence then suggests that there is some catalytic aspect to the intracellular migration of proteins. Further support for this notion derives from the isolation by Ishibashi (1970) of a mutant strain of adenovirus whose capsid proteins are not transported from the cytoplasm to the nucleus. Clearly this important question requires further study.

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