Evidence for Nuclear Synthesis of Lactic Dehydrogenase in Rat Liver*

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SUMMARY

Rats were given radioactive L-leucine by intravenous injection. At times ranging from 30 sec to 4 hours after injection, livers were frozen in situ, lyophilized, and separated into nuclear and cytoplasmic fractions in nonaqueous solvents. In each of the fractions thus prepared, the following were determined: (a) concentration of free labeled amino acid, (b) specific activity of the total protein, and (c) specific radioactivity of the lactic dehydrogenase.

The injected amino acid entered both the cytoplasm and nucleus very rapidly, reaching a maximum concentration at about 1 min after injection. The level then diminished. Throughout the experimental period, the concentration of free labeled leucine was the same in the nucleus as in the cytoplasm.

The curves obtained by plotting specific activity of total protein against time after injection of the labeled amino acid were similar for the cytoplasm and nucleus. After an initial lag of less than 1 min, both curves rose rapidly for 20 min, then leveled off. The similarity between the two curves is interpreted to indicate that protein synthesis occurs in the nucleus as well as in the cytoplasm, and that the rate of protein synthesis is roughly comparable at these two sites. Such an interpretation depends upon the assumption that movement of proteins between the cytoplasm and nucleus is, in general, a slow process, an assumption for which there is considerable evidence in the literature.

The curves obtained by plotting specific radioactivity of lactic dehydrogenase against time after injection of the labeled amino acid were again very similar for the cytoplasm and nucleus. As for total protein, both curves rose rapidly for about 20 min, following an initial short lag, then leveled off. The similarity between these two curves suggests that lactic dehydrogenase is synthesized in the nucleus as well as in the cytoplasm, and that the rate of synthesis is about the same at the two loci. This interpretation depends upon the assumption that movement of the enzyme between the cytoplasm and nucleus occurs slowly.

Three independent lines of evidence indicate that the cell nucleus is capable of protein synthesis. (a) Isolated nuclei can incorporate labeled amino acids into proteins (1, 2). (b) Shortly after injection of a radioactive amino acid into an experimental animal, labeled protein can be detected in the cell nucleus as well as in the cytoplasm by radioautographic techniques (3, 4). (c) Most of the cell constituents known to be essential for protein synthesis have been identified in isolated nuclei. These include activating enzymes (5), soluble ribonucleic acid (5), messenger RNA (6), and ribosomes (7–10), which may occur as polysomes (8, 11). Whereas criticism might be leveled at each of these three lines of evidence, taken together they provide a convincing demonstration that protein synthesis is a general activity of cell nuclei.

The question may then be asked: What kinds of proteins are synthesized by the cell nucleus? In one of the early papers on protein synthesis in isolated nuclei (1), Allfrey, Mirsky, and Osawa examined this problem. After incubating isolated thymus nuclei with a labeled amino acid, they separated nuclear proteins into several fractions and measured the specific activity of each fraction. All fractions had incorporated labeled amino acid, a finding which indicated that a number of different proteins could be synthesized by the nucleus. More recently, Reid and Cole (12) examined critically the synthesis of lysine-rich histones in isolated thymus nuclei and concluded that proteins of this fraction could be synthesized by the nuclei. Studies with isolated nuclei cannot, of course, be used to assess the contribution of the cytoplasm to synthesis of a particular nuclear protein. Flamm and Birnstiel (13) have obtained evidence from pulse labeling experiments with tobacco cells that synthesis of ribosomal proteins takes place in the nucleus.

Nuclei isolated in aqueous media typically contain only proteins which are absent from, or present only in low concentrations in, the cytoplasm. These include histones, structural proteins, and a few enzymes. All such proteins appear to be bound to structural components of the nuclei, e.g. chromosomes. Isolation of nuclei in nonaqueous media, a technique originally developed by Behrens (14) and later improved by Allfrey et al. (15) and by Siebert (16), yields preparations which contain, in addition to the above proteins, a number of enzymes characteristic of the soluble fraction of the cytoplasm. Thus, all the glycolytic enzymes have been found in rat liver nuclei isolated under anhydrous conditions (16). The question then arises whether these enzymes are absent from the nucleus of the living cell, their presence in nonaqueous nuclei arising by adsorption or some other artifact, or are present in the nucleus of the living cell and lost when the nuclei are isolated in aqueous media. Con-
vicing evidence that the latter interpretation is correct has been presented by a number of investigators; some of this evidence will be discussed later.

The observation of Siebert (16) that all the enzymes of the glycolytic pathway occur in approximately equal concentrations in the cytoplasm and nuclei of the cells of rat liver suggested a method for assessing the contribution of the nucleus to the synthesis of these enzymes. The method may be summarized as follows. A labeled amino acid is injected into rats, and at various times after injection the livers are taken and separated into nuclear and cytoplasmic fractions in nonaqueous media. A selected enzyme is isolated in pure form from each of these fractions, and the specific radioactivity in the enzyme from each fraction is determined as a function of time. If the enzyme chosen for study is synthesized exclusively in the cytoplasm, the radioactivity of the enzyme from this fraction should be high relative to that of the nuclear enzyme at short times after injection and should fall as the enzyme moves into the nucleus. In a like manner, it should be possible to determine whether a given enzyme is synthesized exclusively in the nucleus or in both nucleus and cytoplasm.

Such an interpretation depends on the assumption, to be discussed in detail later, that movement of the enzyme between the nucleus and cytoplasm occurs slowly. This method has been used to investigate the synthesis of lactic dehydrogenase in rat liver, and it has been found that the enzyme is synthesized in both the cell nucleus and the cytoplasm, synthesis in the cytoplasm probably being slightly more rapid than in the nucleus.

**EXPERIMENTAL PROCEDURE**

**Materials**—L-Leucine-4,5-\(^3\)H (250 mCi per mmole) and L-leucine-4,5-\(^3\)H (5.0 C per mmole) were obtained from New England Nuclear. A \(^3\)H-labeled amino acid mixture was prepared by combining a number of L-amino acids chromatographically isolated some years earlier from a bacterial hydrolysate and kindly supplied by Dr. Sidney Velick. The mixture contained the following approximate concentrations (micromoles per ml) in 0.01 M HCl: alanine, 0.8; leucine, 5.4; isoleucine, 3.5; serine, 3.8; threonine, 3.8; aspartic acid, 8.1; glutamic acid, 7.7; lysine, 3.4; arginine, 1.8; phenylalanine, 1.8; tyrosine, 0.6; histidine, 0.1; methionine, 1.8; and cysteine, 1.4. The specific activity of each amino acid in the mixture was approximately 1.5 mC per mmole. Crystallized bovine albumin was obtained from Pentex.

Crystalline lactic dehydrogenase was isolated from rat liver by the method of Gibson et al. (17). As isolated by these workers, the enzyme was homogeneous in the ultracentrifuge and on electrophoresis in the pH range 5.8 to 7.8. The preparation so obtained was checked by electrophoresis on a cellulose acetate membrane (Beckman Microzone) and found to be homogeneous in the pH range 5.0 to 7.4. At pH 8.6, two minor components appeared which represented about 8% of the total protein. These components moved anodally with respect to the main component and may have represented isozymes.

Holtzman male albino rats weighing 250 to 300 g were used in the radiisotope experiments. For preparation of lactic dehydrogenase, locally obtained male albino rats were used.

**Protein Determination**—Protein was determined by the method of Lowry et al. (18) with crystallized bovine albumin, which had been corrected for moisture, as a standard. Moisture content of the standard protein was determined by drying weighed portions 4 hours at 101° over P_2O_5. Protein determinations were always done in duplicate or triplicate.

**Enzyme Assay**—Lactic dehydrogenase was determined as described by Nisselbaum and Bodansky (19). One unit of enzyme was taken as that amount which converts 1 μ mole of NADH to NAD in 1 min at 23° and pH 7.8.

**Preparation of Antibodies**—Rabbits were given, by injection in the foot pads or under the skin on the back, 1.0 to 1.5 ml of an antigen-adjuvant emulsion prepared by emulsifying equal volumes of lactic dehydrogenase in 0.15 M NaCl and Freund's adjuvant (complete). The final emulsions contained from 1.0 to 2.4 mg of enzyme per ml. From 6 to 14 weeks following injection, the animals were bled; half of the treated rabbits gave a high antibody titer sometime within this period. Serum from these animals was pooled. The \(\gamma\)-globulin fraction was prepared from the pooled serum by the method of Nichol and Deutsch (20), dialyzed against water, and lyophilized.

**Radioactivity Measurements**—Wet precipitates were dissolved in 0.2 to 1.2 ml (depending on the amount of precipitate) of Nuclear-Chicago solubilizer (a toluene-soluble, quaternary ammonium base supplied by Nuclear-Chicago) at room temperature. After solution was complete (1 to 18 hours), 1 ml of ethanol was added to \(^3\)C-labeled samples. All samples were then made up to a volume of 20 ml with scintillation fluid. Samples which, because of a high water content, developed turbidity upon dilution with scintillation fluid were cleared by further addition of 0.2 to 1.0 ml of Nuclear-Chicago solubilizer. The scintillation fluid had the following composition: 4.0 g 2,5-diphenyloxazole (PPO) and 0.1 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPPOP) in 1 liter of reagent grade toluene. Dry samples were wetted with water (about 0.01 ml per mg of sample) and treated in the same manner as wet precipitates.

Chromatograms were cut into 2-x-3-cm segments, which were counted separately. Segments with \(^3\)C-labeled compounds were placed directly in counting vials containing 20 ml of scintillation fluid. When aliquots of a standard solution of l-leucine-\(^3\)C were spotted on filter paper, dried, and counted in this manner, 74% of the expected counts were obtained. This figure was consistent from one trial to another and, within wide limits, was independent of the amount of labeled material applied to the paper. The loss was not reflected in a change in the external standard counts. A uniform correction (division by 0.74) was, therefore, applied to all \(^3\)C-labeled samples counted directly on paper segments. Tritium-labeled compounds were eluted from chromatogram segments for counting. Each segment was placed in a counting vial, and the following were added at 5-min intervals: 0.05 ml of H_2O, 0.4 ml of Nuclear-Chicago solubilizer, 1.0 ml of scintillation fluid, and 19 ml of scintillation fluid. The paper was removed from the vial prior to counting. When aliquots of a standard solution of l-leucine-\(^3\)H were spotted on filter paper and dried, an average of 93% of the radioactivity was recovered from the paper by this method. Counts obtained from samples eluted in this manner were corrected by dividing by 0.93.

Counting was done in a Packard Tri-Carb liquid scintillation spectrometer with automatic external standardization. Segments of chromatograms were counted for 10 min each; all other samples were counted to an error of 5% or less (95% confidence level). Counts obtained were converted to disintegrations by use of external standardization in conjunction with a quench correction curve.
Injection of Radioactive Amino Acid Solutions—Rats were
fasted for 24 to 27 hours prior to injection. Injection and
subsequent removal of the liver were done under ether
anesthesia. Each animal was given, via the femoral vein, 0.4 ml of a solution
containing 2.0 μmoles each of glycine, L-alanine, L-valine, L-isoleucine, L-proline, L-serine, L-threonine, L-aspartic acid,
L-glutamic acid, L-asparagine, L-glutamine, L-methionine, L-cysteine, L-phenylalanine, L-histidine, L-lysine, L-arginine, and
tryptophan; 0.4 μmole of L-tyrosine; 12 μmoles of NaHC03;
10 pmoles of L-leucine per ml was added. The resulting mixture
was incubated for 20 min at 37°, then cooled to 0°. A 0.2-ml
aliquot of a 10 mg per ml solution of bovine albumin in 0.05 M
NaCl was added, followed by 2.0 ml of 10% trichloracetic acid.
The suspension was centrifuged and the supernatant was
discarded. The sediment was then washed with three 2.0-ml
portions of 5% trichloracetic acid containing 0.5 μmole of L-leu-
cine per ml and resuspended in a 2.0-ml aliquot of the same
solution. The resulting suspension was heated for 15 min at
90°, cooled, and centrifuged, and the supernatant was
discarded. The sediment was washed with a 2.0-ml portion of 5%
trichloracetic acid containing 0.5 μmole of L-leucine per ml,
with a 3.0-ml portion of 95% ethanol containing 10% potassium
acetate and 0.33 μmole of L-leucine per ml, and, finally, with two
2.0-ml portions of 95% ethanol. The sediment was then
wetted with 0.05 ml of water and prepared for counting. Total protein
was determined on separate portions of each cytoplasmic and
nuclear fraction, and the results thus obtained were used to
convert the data to disintegrations per min per mg of protein.
Duplicate determinations were performed on each sample;
duplicate values agreed within 9% in all cases.

Determination of Specific Radioactivity of Cytoplasmic and
Nuclear Lactic Dehydrogenase—Lactic dehydrogenase was
precipitated as an antibody-antigen complex after a preliminary
purification of the enzyme had been achieved according to several
steps from the method of Gibson et al. (17): A 50-ml sample of a
cytoplasmic or nuclear fraction was suspended in 2.0 ml of 0.35 M
NaCl and allowed to stand for 50 min at 0° with occasional
stirring. The sample was then placed in a bath and 1.33 ml of a 0.25 M
NaCl in 50% ethanol cooled to approximately -10° were added with stirring. The sample was allowed to remain for
50 min at -5° with occasional stirring, then centrifuged for 10
min at 10,000 X g at -5°. The supernatant was discarded; the clear
supernatant was dialyzed overnight against two changes of 0.5 M
NaCl at 0°. To each milliliter of the dialyzed enzyme solution
adding, 364 mg of (NH4)2SO4. The resulting
solution was allowed to remain for 50 min at 0°, then centrifuged for 10 min at 10,000 X g. The supernatant was discarded;
the sediment was dissolved in 1.0 ml of 0.15 M NaCl and dialyzed overnight at 0° against two changes of 0.15 M NaCl. During
the dialysis, a precipitate formed. The enzyme solution was
incubated for 2 hours at 23°, then for 24 hours at 4°; additional
material precipitated during this incubation. The solution was
centrifuged and the sediment was discarded. Further incubation
either at 23° or 4° did not result in formation of more
precipitate. An aliquot of the clear solution was assayed for
lactic dehydrogenase activity. The amount of enzyme in the
solution was determined from the relation that 420 enzyme units
= 1 mg. For each microgram of enzyme in the solution, 6 μl of
a 10 mg per ml solution of antibody in 0.067 M sodium phosphate
buffer, pH 7.8, were added. The resulting mixture was
incubated for 90 min at 23°, then for 24 hours at 4°. The antigen-
and the supernatant was discarded. Five to six separate determinations were made on each cytoplasmic
to two three determinations were made on each nuclear fraction.

Determination of Specific Activity of Total Protein—A 3- to
6-ml sample of a nuclear or cytoplasmic fraction was suspended in
0.5 ml of 0.1 M NH4OH, and 0.1 ml of a solution containing
10 μmoles of L-leucine per ml was added. The resulting mixture
was incubated for 20 min at 37°, then cooled to 0°. A 0.2-ml
aliquot of a 10 mg per ml solution of bovine albumin in 0.05 M
NaCl was added, followed by 2.0 ml of 10% trichloracetic acid.
The suspension was centrifuged and the supernatant was
discarded. The sediment was then washed with three 2.0-ml
portions of 5% trichloracetic acid containing 0.5 μmole of L-leu-
cine per ml and resuspended in a 2.0-ml aliquot of the same
solution. The resulting suspension was heated for 15 min at
90°, cooled, and centrifuged, and the supernatant was
discarded. The sediment was washed with a 2.0-ml portion of 5%
trichloracetic acid containing 0.5 μmole of L-leucine per ml,
with a 3.0-ml portion of 95% ethanol containing 10% potassium
acetate and 0.33 μmole of L-leucine per ml, and, finally, with two
2.0-ml portions of 95% ethanol. The sediment was then
wetted with 0.05 ml of water and prepared for counting. Total protein
was determined on separate portions of each cytoplasmic and
nuclear fraction, and the results thus obtained were used to
convert the data to disintegrations per min per mg of protein.
Duplicate determinations were performed on each sample;
duplicate values agreed within 9% in all cases.
tion with antibody. For samples containing low levels of radioactivity, twice the indicated amount of sample was taken; quantities of all reagents used in the isolation were then increased proportionately. Two determinations were done on each cytoplasmic sample, and one on each preparation of nuclei.

RESULTS

Isolation of Labeled Lactic Dehydrogenase

Characterization of Antigen Antibody Reaction—To 0.1 ml portions of 0.067 M sodium phosphate buffer, pH 7.8, each containing 20 μg of lactic dehydrogenase, were added 0.5-ml portions of 0.15 M NaCl containing varying amounts of the γ-globulin fraction. The resulting mixtures were incubated for 90 min at 23°, then overnight at 4°. The precipitates were sedimented and washed twice with 0.5-ml portions of 0.15 M NaCl. Protein determinations were performed on the washed precipitates; enzyme assays were done on the supernatants. Results, presented in Fig. 1, show a typical antigen-antibody reaction.

Test of Isolation Method—Lactic dehydrogenase was isolated from 50.0-mg portions of cytoplasm and nuclei as described under "Experimental Procedure." At various stages, aliquots were analyzed for total protein and specific enzyme activity. Results are presented in Table I. By reference to Fig. 1, one can calculate the amount of precipitate to be expected when given amounts of antigen and antibody react. As seen in Table I, the amount of precipitate obtained from the nuclear fraction was exactly as expected; the amount obtained from the cytoplasmic fraction was slightly higher than expected. These results indicate that both nuclear and cytoplasmic enzymes are precipitated by the antibody, and that there is little or no co-precipitation of contaminating material upon precipitation of the nuclear enzyme and only moderate amounts of co-precipitation with the cytoplasmic enzyme.

Level of Cross-contamination of Cell Fractions

Siebert has presented some evidence that no exchange of material occurs between cytoplasm and nucleus during isolation in nonaqueous solvents and that water-soluble components are not lost from the cell fractions during this procedure (16). In view of the importance of these assumptions for the present work, it was thought advisable to gather additional evidence for their validity. Since the nuclear fraction of rat liver amounts to less than 10% of the total material (22), a severe test of the separation can be made by adding a radioactively labeled cytoplasmic fraction to a preparation at an early stage and seeing how much of the radioactivity appears in the nuclear fraction.

To this end, 1.0-ml aliquots of a solution containing, per ml, 20 μC of the 14C-labeled amino acid mixture, 90 μMoles of NaCl, and 20 μmoles of sodium phosphate buffer, pH 7.0, were injected intraperitoneally into a group of rats. Four hours later the livers were taken and separated into nuclear and cytoplasmic fractions. A 3.76-g portion of the 14C-labeled cytoplasm thus obtained was added to 7.76 g of unlabeled, lyophilized liver powder after sieving, but before grinding in the ball mill. The resulting mixture was then ground and separated into a nuclear and a cytoplasmic fraction. The radioactivity in each fraction was then measured. Results are tabulated in Table II. Of the added radioactivity, 0.06% was recovered in the nuclear fraction. A reasonable assumption is that about 0.06% of the added 14C-labeled cytoplasm, or 0.0023 μg, found its way into the nuclear fraction. Part of this represents true cytoplasmic contamination of the nuclear fraction; another part, however, presumably consists of 14C-labeled nuclear material which had not been completely removed from the 14C-cytoplasm. An upper limit to the amount of cytoplasmic contamination of the nuclear fraction may be obtained if all the labeled material in the nuclear fraction.
is considered to be cytoplasmic. Since the original mixture of
\(^{14}\text{C}\)-cytoplasm and \(^{12}\text{C}\)-liver powder contained about twice as
much \(^{12}\text{C}\)-cytoplasm as \(^{14}\text{C}\)-cytoplasm, the nuclear fraction
probably is contaminated with, at most, about 0.007 g of cyto-
plasmic material, or about 14% of the total weight of the nuclear
fraction. Since unfractionated liver consists of less than 10% nu-
eli (22), the amount of nuclear contamination in the cyto-
plasmic fraction must be considerably less than this.

Results of the above control experiment could not be ex-
pected to indicate whether exchange of material between the
nucleus and cytoplasm had occurred at a step in the isolation
prior to the addition of the labeled cytoplasm, e.g. during lyophi-
lization.

Accumulation of Injected Leucine in Cytoplasm and Nucleus

A difference between the specific radioactivity of an enzyme
isolated from the nucleus and that from the cytoplasm following
injection of a labeled amino acid can be interpreted in terms of
the site of synthesis of the enzyme only if something is known
about the rate at which the injected amino acid reaches the
two loci. The concentration of labeled leucine in the cytoplasm
and the nucleus was, therefore, determined as a function of the
time after injection of the radioactive amino acid. Results are
presented in Figs. 2 and 3. Because of the alkaline hydrolysis
step used in the determination of the amino acid, leucyl soluble
RNA as well as free leucine is measured. As seen in Fig. 2, the
injected amino acid had reached a high concentration in both the
nucleus and the cytoplasm in the shortest time period examined
(30 sec). Furthermore, the concentration of labeled amino
acid was, within experimental error, the same in the nucleus as
in the cytoplasm for all time periods.

These results establish that any difference in the specific
activity of a given protein in the cytoplasm and nucleus reflects a
difference in the rate of synthesis of that protein and not a differ-
ential availability of labeled amino acid at the two sites of
synthesis.

Nuclear and Cytoplasmic Protein Synthesis

Total Protein—The specific activity of the total nuclear and
cytoplasmic proteins at various times following injection of
radioactive leucine is given in Figs. 4 and 5. After a short lag
(Fig. 4), the specific activity rises rapidly in both cytoplasmic and
nuclear proteins, then levels off about 20 min after injection (Fig. 5). In Fig. 6, the specific activity of the cytoplasmic protein divided by the specific activity of the cytoplasmic protein plus the specific activity of the nuclear protein has been plotted against time after injection. At short times after injection, the specific activity of the cytoplasmic protein is slightly higher than that of the nuclear protein; at longer times, the specific activities approach one another.

If it is assumed (see "Discussion") that movement of proteins between the cytoplasm and nucleus occurs relatively slowly, then it may be concluded that protein is synthesized in the cell nucleus as well as in the cytoplasm and that the rate of synthesis may be slightly higher in the cytoplasm than in the nucleus.

Lactic Dehydrogenase—The specific radioactivity of nuclear and cytoplasmic lactic dehydrogenase as a function of time after injection is plotted in Figs. 7 and 8. The curves have a form similar to those for total protein, and again the curves for the nucleus and cytoplasm rise initially at nearly the same rate, permitting the conclusion that lactic dehydrogenase is synthesized both in the cytoplasm and in the nucleus. A comparison of the curves for lactic dehydrogenase with those for total protein shows that the rise in specific activity of total protein at short times after injection is more than twice as rapid as the rise in specific activity of lactic dehydrogenase and that, at longer times after injection, the specific activity of the total protein begins to fall while the specific activity of the lactic dehydrogenase is still rising. These observations are consistent with a slow turnover of lactic dehydrogenase as compared with total liver proteins.

It can be seen from Fig. 6 that at short times after injection the specific activity of the cytoplasmic lactic dehydrogenase is slightly higher than that of the nuclear enzyme, whereas at longer times the specific activities approach one another. This is the result one would expect if lactic dehydrogenase synthesis in the cytoplasm were slightly faster than in the nucleus and movement of the enzyme between cytoplasm and nucleus were slow.

**DISCUSSION**

If valid conclusions concerning the site of synthesis of a particular enzyme are to be drawn from the method described in this paper, several conditions must be fulfilled. (a) Nuclear and cytoplasmic fractions must be separated without loss or exchange of the enzyme being studied. It is well established that large amounts of soluble protein are leached from the nucleus during isolation in aqueous media (16, 23). Therefore, nuclear and cytoplasmic fractions have been prepared in nonaqueous solvents. Siebert has presented evidence that no exchange of material occurs between the nucleus and cytoplasm during isolation of these fractions in nonaqueous solvents (16). The results presented above have furnished additional evidence for the validity of this assumption. In this connection, it is interesting that nuclei isolated in aqueous media have in several instances been reported to lack DNA polymerase activity (24, 25) whereas nuclei isolated in nonaqueous media contain considerable amounts of this enzyme (24, 26). Since, a priori, DNA polymerase would be expected to be localized in the nucleus, it seems likely that the enzyme may be lost from nuclei upon isolation in aqueous media, but is retained when nuclei are prepared in organic solvents. (b) The enzyme under investigation must be isolated in pure form from cytoplasmic and nuclear fractions. To accomplish this without using unduly large amounts of material, the well known specificity of the antigen-antibody reaction has been utilized. The data presented in Fig. 1 and Table I indicate that lactic dehydrogenase can be precipitated specifically and quantitatively from both the nuclear...
and cytoplasmic fractions as an antibody-antigen complex. (c) The injected amino acid must reach the nucleus and cytoplasm at the same time or, alternatively, allowance must be made for the difference in arrival time of the labeled amino acid at the two sites. It is shown under "Results" that the former condition is fulfilled in this experimental system. (d) Movement of the enzyme under study between the cytoplasm and nucleus of the living cell must be sufficiently slow that a difference in rate of synthesis at the two sites will be reflected in a measurable difference in the specific activity of the enzyme from the two sites. Based on the available information in the literature, this condition is fulfilled. This point will be discussed in some detail.

There is a considerable body of literature concerning movement of various substances across the nuclear membrane. A study of this literature permits the following generalizations. (a) Whereas the nuclear membrane may serve as an effective barrier to the free diffusion of some small molecules, others appear to move between the cytoplasm and nucleus with little hindrance. (b) Macromolecules, in general, move across the nuclear membrane slowly, or not at all. Since the present study is concerned with the rate of movement of macromolecules (proteins) between the nucleus and cytoplasm, only evidence bearing on the latter point will be considered.

A difference in the concentration of a non-particle-bound protein in the nucleus and cytoplasm may reflect an inability of that protein to cross the nuclear membrane. In regenerating rat liver and Novikoff hepatoma, the concentration of DNA polymerase in the nucleus has been shown to be appreciably greater than in the cytoplasm (24). The enzyme can be readily extracted with dilute sucrose solution, indicating that it is not bound or adsorbed to some component in the nucleus. Since, over the short distances involved, free diffusion would have eliminated this concentration gradient in a very short time, it may be concluded that the nuclear membrane is an effective barrier to the free diffusion of this enzyme. Under certain conditions, a similar situation can be observed for some of the glycolytic enzymes. In normal rat liver the concentration of each of the glycolytic enzymes is about the same in the nucleus as in the cytoplasm (16). At short times following partial hepatectomy, however, the nuclear concentrations of some of the glycolytic enzymes are found to be increased relative to their concentrations in the cytoplasm (27). Such an effect would not be expected if the nuclear and cytoplasmic enzymes were in rapid equilibrium with one another. Among the enzymes showing such a nuclear-cytoplasmic concentration differential was lactic dehydrogenase, the enzyme examined in the present paper.

A second method for assessing the permeability of the nuclear membrane to large molecules consists of introducing the molecules into the cytoplasm by microinjection and looking for them in the nucleus. Harding and Feldherr injected solutions of polyvinylpyrrolidone (mol wt 40,000) and bovine serum albumin into the cytoplasm of amphibian oocytes (28). Both of these substances were capable of causing nuclear volume changes, indicating that the nuclear membrane serves as a barrier to their free diffusion. Feldherr has injected polyvinylpyrrolidone-coated colloidal gold particles 25 to 170 A in diameter into Amoeba cytoplasm and followed the appearance of the particles in the nucleus with the electron microscope (29). Ten minutes after injection, few gold particles were found in the nucleus; an appreciable concentration was there 1 hour after injection.

Feldherr and Feldherr injected fluorescein-labeled rabbit γ-globulin into immature oocytes of the Cecropia moth (30). Ten minutes after injection the fluorescent protein had diffused to fill the entire cytoplasm but had not entered the nucleus. A method similar to that discussed above, but which does not necessitate the use of the very large cells required for microinjection studies, depends on the ability of some cells to phagocytize large molecules. Kruse and McMaster injected into mice various serum proteins and egg albumin which had been tagged by coupling the proteins to highly colored dyes (31). The dye-protein complexes, which were rapidly taken up by reticuloendothelial cells throughout the body, were localized in the cytoplasm; even at long times after injection they were not detected in the nuclei. Schiller, Schayer, and Hess injected fluorescein-conjugated bovine albumin into rats and observed that after 12 hours the cytoplasm of the phagocytic Kupffer cells was strongly labeled, whereas the nuclei were devoid of fluorescence (32). In contrast to these studies is that of Coons, Leduc, and Kaplan (33). These authors injected solutions of egg albumin, bovine albumin, and human γ-globulin into mice and at various times after injection determined the intracellular localization of the injected proteins by means of the fluorescent antibody technique. The injected proteins appeared in the nuclei as well as the cytoplasm of various types of phagocytic cells. Ten minutes following injection, the shortest time period for which data are given, egg albumin and γ-globulin were found in both the cytoplasm and nuclei of cells of the liver and kidney. It would seem from the description in the text and the published photomicrographs, however, that the concentration of injected protein was substantially higher in the cytoplasm than in the nucleus at this short time. At longer times after injection, all of the antigen taken up by the epithelial cells of the liver and kidney originally became localized in the nucleus. Ribonuclease, histones, and protamines, when added to the extracellular medium, are taken up by a number of different types of cells (34-37). Entrance of these proteins into the cell does not seem to occur via phagocytosis, however; presumably, direct penetration of the cell membrane is involved. Alterations in the staining properties of the cell nucleus indicate that these substances also pass across the nuclear membrane. Although no quantitative studies on the rate of movement into the nucleus have been reported, it appears that, in some instances, these proteins appear in the nucleus a few minutes after their addition to the extracellular fluid. Thus, the histones, protamines, and RNAse probably provide an exception to the rule that movement of macromolecules across the nuclear membrane occurs slowly. These are unusual proteins, however. All are highly basic and have a low molecular weight, all are cytotoxic, and, in contrast to most proteins, all readily penetrate the cell membrane. This class of proteins is exceptional and does not negate the generalization that macromolecules move slowly across the nuclear membrane.

Pulse-labeling experiments provide an example of still another technique which can be exploited to estimate the rate at which macromolecules pass across the nuclear membrane. It has been shown in a large number of studies that the initial labeling of RNA with radioactive precursors takes place in the nucleus and that there is a time lag before the label appears in the cytoplasm (38). For the cells of higher organisms, this lag ranges from 10 min to a number of hours in duration. It is possible that part of the lag represents time required for the RNA to be
released from the site of synthesis on the chromatin. It has been shown with HeLa cells and hamster fibroblasts, however, that upon breakdown of the nuclear membrane during mitosis labeled nuclear RNA is rapidly distributed throughout the cytoplasm (38).

On the basis of the studies quoted above, it seems justifiable to conclude that, if a given amount of a macromolecule were found greater in the cytoplasm than in the nucleus, the rate of synthesis possibly being somewhat greater in the cytoplasm. Because the nuclei comprise less than 10% of the total mass of the liver cells (22), the over-all contribution of the nucleus to the synthesis of liver lactic dehydrogenase is small.

A current hypothesis holds that ribosomes become attached to molecules of messenger RNA and initiate protein synthesis while the RNA molecules are incomplete and still attached to the DNA template. After release from the DNA template, the mRNA1 molecules are presumed to migrate, together with attached ribosomes, to the cytoplasm, where protein synthesis continues. In a system where this type of mechanism operated, synthesis of a given protein would occur both in the cytoplasm and the nucleus. The rate of movement would have resulted in a much different labeling pattern than the one actually observed. Therefore, lactic dehydrogenase must be synthesized in both the nucleus and the cytoplasm, the rate of synthesis possibly being somewhat smaller in the nucleus than in the cytoplasm. If the rate of synthesis of total protein is about the same in the nucleus and cytoplasm, coupled with the observation that cytoplasmic polysomes (11), the hypothesis that translation of a given strand of messenger RNA begins in the nucleus and continues in the cytoplasm, suggests that the synthesis of many proteins may be divided between the cytoplasm and nucleus. If this could be substantiated, it would provide support for the hypothesis that translation of a given strand of messenger RNA begins in the nucleus and continues in the cytoplasm.

The observation that the movement of labeled amino acid into the nucleus is not appreciably hindered by the nuclear membrane might be explained in two alternative ways. (a) In thymus nuclei, a system for active transport of amino acids across the nuclear membrane has been demonstrated (41, 42). Such a mechanism may also be operative in rat liver nuclei. (b) Amino acids may move from the extracellular space into the nucleus via the channels in the endoplasmic reticulum and thus avoid the necessity of passing through the cytoplasm and the outer layer of the double nuclear membrane.

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REFERENCES


1 The abbreviation used is: mRNA, messenger ribonucleic acid.
Evidence for Nuclear Synthesis of Lactic Dehydrogenase in Rat Liver
LeRoy Kuehl


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