PROTEINS IN NUCLEOCYTOPLASMIC INTERACTIONS

I. The Fundamental Characteristics of the Rapidly Migrating Proteins and the Slow Turnover Proteins of the *Amoeba proteus* Nucleus

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ABSTRACT

By the transplantation of amino acid-^{3}H-labeled nuclei between cells and the subsequent isolation of nuclei for quantitative assay, we have confirmed that all the nuclear proteins of *Amoeba proteus* are divisible into two classes that are sharply defined by their physiological behavior. About 40% of the proteins in the nucleus rapidly migrates back and forth between the nucleus and the cytoplasm. These rapidly migrating proteins (RMP) are 25–50 times more concentrated in the nucleus than in the cytoplasm, and migration into the nucleus therefore occurs against a high concentration differential. The remaining 60% of nuclear proteins has been classified as slow turnover proteins (STP) since (as reported in a following paper) virtually all of them ultimately undergo turnover. Turnover in this context means loss of label from the nucleus, by either protein breakdown or protein migration to the cytoplasm. Isolation of nuclei in the detergent Triton X-100 results in a 20% loss of nuclear proteins but conclusions about RMP and STP were not found to be significantly affected by this loss.

Our studies show that the nuclear proteins of *Amoeba proteus* fall into two classes that are clearly definable on the basis of an unexpected in vivo behavior. One class continually migrates rapidly back and forth between nucleus and cytoplasm but is always present in much higher concentration in the nucleus than in the cytoplasm during interphase. In earlier reports (1) this class of proteins was referred to as "cytonucleoproteins," but new data make their designation as *rapidly migrating proteins* (RMP) preferable.

All the remaining nuclear proteins form a class that turns over slowly—requiring an amount of growth equivalent to many cell cycles for a complete turnover. Turnover may be due to metabolic breakdown with the disappearance of the products from the nucleus and/or movement of intact protein molecules to the cytoplasm. We call this group *slow turnover proteins* (STP). While there is no appreciable *net* shift of RMP between nucleus and cytoplasm during interphase, i.e. a dynamic equilibrium is maintained, present evidence suggests that there is, in time, considerable exchange of old for new STP within the nucleus. Histones, which in *A. proteus* constitute only a small percentage of the total nuclear protein, cannot as yet be assigned definitively to either class.

We consider it important that this new classification of nuclear proteins is based on physiological criteria—in strong contrast with the bases of most other classifications of nuclear proteins.

Whereas the earlier observations on these proteins were based on radioautographic analyses of labeled cells, the current studies are based on

methods that define in more precise quantitative terms the in vivo behavior of both classes of proteins during cell growth and division. This paper includes: (*a*) a description and evaluation of the methods; (*b*) a description of the basic experiment demonstrating the two protein classes; (*c*) a comparison of the new assays with the earlier radioautographic measurements; and (*d*) an estimate of the relative amounts of protein classes in nucleus and cytoplasm.

The ultimate objective of these investigations is to determine how these proteins relate to nuclear function, particularly to genetic replication and transcription, since we believe that the presence of these proteins within the nucleus implies that they have something to do with these two functions.

MATERIALS AND METHODS

The *Amoeba proteus* strain used in all experiments derives from a clone initiated in Berkeley, California in 1952 and maintained in a number of laboratories (with occasional recloning and redistribution) since then. The amebae were cultured according to the method described by Prescott and Carrier (5).

The ameba proteins were labeled by feeding the amebae on *Tetrahymena* that had been grown for several days on synthetic medium (2) in which amino acids-^{3}H were substituted for the unlabeled ones as follows: arginine, 7.5 μc/ml, 0.24 c/mmole; histidine, 1.7 μc/ml, 7 c/mmole; leucine, 2 μc/ml, 23 c/mmole; alanine, 7 μc/ml, 0.17 c/mmole; lysine, 10 μc/ml, 0.2 c/mmole; tryptophan, 9 μc/ml, 0.47 c/mmole; phenylalanine, 4 μc/ml, 5.7 c/mmole; isoleucine, 2.5 μc/ml, 1.3 c/mmole; valine, 2.5 μc/ml, 0.35 c/mmole; proline, 2.5 μc/ml, 5 c/mmole. All of these, except alanine and proline, are required for growth of *Tetrahymena*, and no reduction in specific activity can occur by *de novo* synthesis of these amino acids. The *Tetrahymena* were harvested, washed with inorganic medium, and fed to amebae in the usual fashion. After feeding for two or three cell generations on such food, amebae were either fasted for a minimum of 24 hr or fed unlabeled food for a minimum of 12 hr before they were used for experimental purposes. In this manner, labeled precursors were reduced to an insignificant level—or, in the popular jargon, the labeled pool was "chased." One isolated nucleus from such amebae will register 300–500 counts per minute in a windowless, gas-flow counter.

Nuclei were isolated as follows. Individual amebae were transferred to an aqueous solution of 0.5 ml Triton X-100 (a Rohm and Haas detergent) and 1 mg spermidine-HCl per 100 ml. Disruption of the cells was accelerated by drawing the amebae in and out of a narrow-tipped braking pipette. After a cell was disrupted the nucleus was rinsed free of accompanying cytoplasm and then deposited into a small drop of 100% ethanol (for fixation) on a stainless-steel planchet. The entire procedure, except the final deposition, was followed under a dissecting microscope at approximately × 40.

To prepare the nuclei (as well as cytoplasm when required) for assay of radioactivity, the preparations on planchets were treated with concentrated formic acid to achieve maximum spreading of the material on the planchet and thus minimize self-absorption of the radiation. The radioactivity on the planchets was determined in an automatic, low-background (circa 2 cpm), gas-flow, windowless Geiger counter that assayed ^{3}H with an efficiency of 17–18%.

The transplantation of nuclei between amebae was performed according to the procedure described by Goldstein (3).

RESULTS

Reliability of the Nuclear Isolation Method

Although techniques of nuclear isolation have been in use for a long time and much has been made of experiments in which isolated nuclei have been used, it generally has been difficult—if not impossible—to determine what loss of nuclear material occurs as a consequence of the isolation. Because such information is crucial for any conclusions drawn from our experiments, we have determined by a novel method how closely the amount of protein in an isolated nucleus resembles that of protein in a nucleus in an intact cell.

We were able to compare the two kinds of nuclei, with respect to protein-^{3}H content, as follows:

ISOLATED NUCLEI: Isolated in Triton, fixed on planchets, treated with formic acid, and then assayed for radioactivity.

NONISOLATED NUCLEI: Nuclei from the same population as that above were transplanted (with no exposure to noncytoplasmic media) to nonradioactive cells; the intact cells were immediately fixed on planchets, treated with formic acid, and assayed for radioactivity.

With both procedures proteins may leak from the nuclei, but only with the isolated nuclei are they lost from the analysis; labeled proteins that leave nonisolated nuclei are retained in the cytoplasm and thus can be assayed by the regular procedure.

The data of one such experiment are given in Table I and show that there is approximately 20%

TABLE I

*Effect of Isolation on Protein Content of Nuclei**

Nonisolated nuclei:
(Protein-^{3}H-labeled nuclei in unlabeled cytoplasm)
0.2 N H_2SO_4 *in*soluble—3000 cpm/36 nuclei = 83.3 cpm/nucleus
0.2 N H_2SO_4 soluble— 343 cpm/36 nuclei = 9.5 cpm/nucleus (10.2%)
Total = 92.8

Triton-isolated protein ^{3}H-labeled nuclei:
0.2 N H_2SO_4 *in*soluble—3033 cpm/45 nuclei = 67.3 cpm/nucleus
0.2 N H_2SO_4 soluble— 182 cpm/45 nuclei = 4.0 cpm/nucleus (5.6%)
Total = 71.3
(77% of total for nonisolated nuclei)

* Fixed nuclei were extracted with 0.2 N H_2SO_4 before treatment with formic acid.

less nuclear protein in Triton-spermidine–isolated nuclei. (Since the amebae were grown on 10 different amino acids–^{3}H for at least two cell generations, we assume that the cells have reached a more or less uniform, steady-state of labeling and that the radioactivity is a measure of the protein content).

The data in Table I show that there is a disproportionate loss of acid-soluble protein in Triton-spermidine, and other data suggest that not all kinds of proteins are lost from the nuclei to the same extent. Further work is needed, however, to clarify what kinds of proteins are lost. For the matters considered in this paper, the loss of protein from nuclei upon isolation is of no consequence, as is shown in the following sections.

The "Basic" Observation

The discovery of proteins in back and forth migration between nucleus and cytoplasm was based on the radioautographic localization of radioactivity in a cell into which had been grafted a nucleus containing labeled protein. When such a cell was fixed a few hours after the operation, the radioactivity was found to be localized almost completely in the transplanted nucleus *and* the host cell nucleus; little or no radioactivity was detectable in the cytoplasm (Fig. 1). This suggested that there is a group of proteins in high concentration in the nucleus that continuously migrates to the cytoplasm and back into the nucleus against a high concentration differential.

This fundamental experiment has been repeated with assays of isolated nuclei and enucleate cytoplasm, and the essential conclusion of the earlier experiments has been confirmed. Data given in the following sections illustrate the nature of the confirmation. When comparing the data for different compartments of the same cell, the reader should take into account the fact that the cytoplasm is approximately 50 times the volume of the nucleus.

Evidence of "Nonmigrating" Nuclear Proteins

Although the radioactivity is concentrated in both nuclei, it was observed that there was a higher concentration of label in the transplanted nucleus. In fact, the number of radioautographic grains over the grafted nucleus averaged 2.6 times the grain number over the host cell nucleus (1). From this it was concluded that the nucleus contained a "nonmigrating" group of proteins, which—since it could be labeled with tryptophan–^{3}H—was not classifiable as histone.

If for the same experiment the activity of *isolated* nuclei is determined, the transplanted nucleus is found to average approximately six times as much activity as the host cell nucleus (Table II). Although there is considerable variation from experiment to experiment, in most experiments the *mean* ratio of activity between the nuclei lies between five and seven to one. We suspect that the variation reflects: the available supply of these proteins; differences in the stage of the life cycle of the host cell and/or the grafted nucleus; ploidy differences between nuclei; the relative volume of nuclei and cytoplasm, etc., but we have no sufficiently reliable information that would enable us to reduce the variation at present.

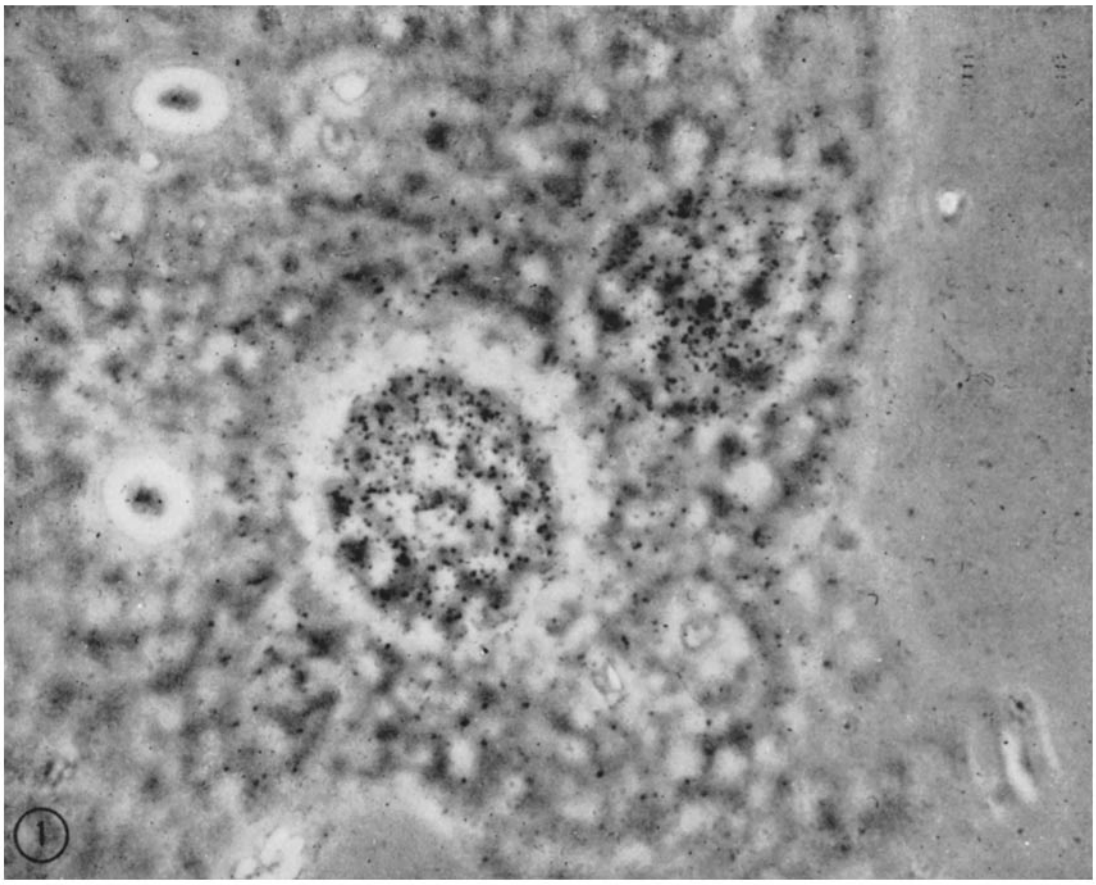

FIGURE 1 Radioautograph of a squashed ameba into which was grafted a radioactive protein-labeled nucleus approximately 20 hr before the cell was fixed. × 1000. Radioactivity seems to be almost entirely localized within the two nuclei.

To test the conclusion that there are two major nuclear protein classes—migrating and nonmigrating—Byers et al. (1) transplanted a labeled nucleus (A-2 in Fig. 2) into an unlabeled cell (A-1 in Fig. 2) and several hours later (when the distribution of radioactive protein presumably had attained an equilibrium of 2.6 parts in A-2 to 1 part in nucleus A-1) each nucleus was grafted into a new cell (B-1 and B-2 in Fig. 2). These cells (B-1 and B-2) were fixed several hours later and the number of radioautographic grains over each nucleus was determined. The ratio of activity between nuclei A-1 and B-1 was close to 1:1, and the ratio for nucleus A-2 to nucleus B-2 was over 4:1. Thus the conclusion that there are two classes of nuclear proteins was confirmed. The first host cell nucleus (A-1) presumably acquired only migrating protein label; the grafted nucleus (A-2) presumably had lost such label, thereby enriching its relative content of label in nonmigrating protein. When transplanted again (into cell B-2), nucleus A-2 now had considerably more than 2.6 times the activity of the new host cell nucleus (B-2).

This experiment has been repeated with assays of isolated nuclei, and Table III shows the data of one such experiment. (Here again, reference to Fig. 2 will help in following the description of the experiment.) For those cells assayed after the implantation of the first nucleus, the mean activity for nucleus A-2 was 6.4 times that of nucleus A-1 (Table II), whereas after the second set of nuclear transfers, nucleus A-2 averaged 11 times the activity of nucleus B-2 and the mean activity of nucleus A-1 is—*at most*—two times that of nucleus

TABLE II

Distribution of Nuclear Protein Label (without nuclear isolation)

Cell	Nuclei	cpm	Ratio	% Total
1	A-2	207		69
	A-1	26	*8.0*	9
	A-1 enucleate	68		23
2	A-2	264		72
	A-1	47	*5.6*	13
	A-1 enucleate	55		15
3	A-2	226		69
	A-1	38	*5.9*	12
	A-1 enucleate	62		19
4	A-2	199		66
	A-1	50	*4.0*	17
	A-1 enucleate	51		17
5	A-2	215		63
	A-1	27	*8.0*	8
	A-1 enucleate	101		29
6	A-2	259		67
	A-1	32	*8.1*	8
	A-1 enucleate	94		25
7	A-2	299		67
	A-1	55	*5.4*	12
	A-1 enucleate	94		21
8	A-2	330		75
	A-1	32	*10.3*	7
	A-1 enucleate	78		18
9	A-2	219		—
	A-1	49	*4.5*	—
	A-1 enucleate	Lost		—
10	A-2	192		80
	A-1	25	*7.7*	11
	A-1 enucleate	22		9

$\bar{X}$ Ratio = 6.4:1.
$\bar{X}$ Per cent in host nucleus = 11.
$\bar{X}$ Per cent in enucleate = 20.
$\bar{X}$ cpm for less active nucleus (RMP) = 38.
$\bar{X}$ cpm more active nucleus minus that of less active nucleus (STP) = 203.

B-1. (We have assumed, for computation purposes only, that the more radioactive member of the A-1, B-1 pair is nucleus A-1. Since any variation—due to assay errors, biological factors, etc.—would produce some inequality, it appears unlikely that a 2:1 ratio can be considered to be significantly different from a 1:1 ratio. This must be assumed because when the nuclei are isolated we can not distinguish A-1 from B-1).

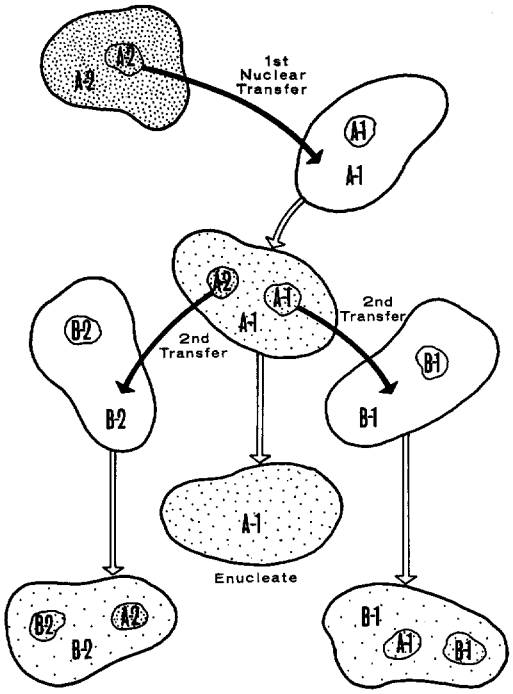

FIGURE 2 Scheme of nuclear transplantations used to derive the data in Tables II and III. For the first transfer, A-2 was grafted into cell A-1. The next day, nucleus A-2 was grafted into cell B-2, and nucleus A-1 was grafted into cell B-1. These two new host cells were either fixed whole immediately for the assays shown on Table II or the nuclei were isolated from them the next day for the data given in Table III.

These data, then, confirm the existence of two major groups of nuclear proteins: (*a*) a relatively rapidly migrating group which equilibrates rapidly between nucleus and cytoplasm;[1] this is the group that appears as labeled material in the initial host nucleus (A-1); and (*b*) a seemingly nonmigrating group that remains in the original grafted nucleus (A-2) and that becomes—when A-2 is again transplanted to B-2—a higher proportion of the *labeled* nuclear protein, thereby producing a ratio between

[1] Goldstein, L., and D. M. Prescott. 1967. Proteins in nucleocytoplasmic interactions. II. Turnover and changes in nuclear protein distribution with time and growth. Manuscript in preparation.

TABLE III

Distribution of Nuclear Protein Label after "Triple" Transfer (with nuclear isolation)

Cell	Nuclei	cpm	Ratio
11	A-2	191	*17.4*
	B-2	11	
	A-1	21	
	B-1	13	*1.6*
	A-1 enucleate	53	
12	A-2	103	*6.9*
	B-2	15	
	A-1	17	
	B-1	8	*2.1*
	A-1 enucleate	124	
13	A-2	259	*12.3*
	B-2	21	
	A-1	15	
	B-1	8	*1.9*
	A-1 enucleate	Lost	
14	A-2	157	*11.2*
	B-2	14	
	A-1	22	
	B-1	8	*2.8*
	A-1 enucleate	90	
15	A-2	180	*10.6*
	B-2	17	
	A-1	10	
	B-1	14	*1.4*
	A-1 enucleate	47	
16	A-2	152	*6.9*
	B-2	22	
	A-1	9	
	B-1	18	*2.0*
	A-1 enucleate	Lost	
17	A-2	218	*11.5*
	B-2	19	
	A-1	7	
	B-1	16	*2.3*
	A-1 enucleate	57	

$\bar{X}$ Ratio "hotter" pair = 11.0:1.
$\bar{X}$ Ratio "cooler" pair = 2.0:1.
($\bar{X}$ Ratio after 1st transfer, Table II = 6.4).
$\bar{X}$ cpm in nuclei A-1 plus B-1 = 27.
$\bar{X}$ cpm in nucleus A-2 minus B-2 (STP) = 163.
(Nuclei for this set of cells isolated a day after the analyses were performed on the cells of Table II.)

nuclei A-2 and B-2 greater than that between A-2 and A-1.

Although these latter proteins have been referred to as "nonmigrating," other experiments to be described in a following paper show that the label in these proteins also leaves the nucleus but at a relatively slow rate. Thus, as our newer data indicate, it is more reasonable to call the latter group slow turnover proteins (STP) and the other group rapidly migrating proteins (RMP). The STP correspond to the group called nonmigrating proteins by Byers et al. (1), and the RMP, as mentioned earlier, correspond to the group they called cytonucleoproteins.

A comparison of the values in Table II with those in Table III supports the view that STP label leaves the nucleus very slowly but that RMP label equilibrates rapidly. We expect the mean value for the combined activity of nuclei A-1 and B-1 *and* cytoplasm B-1 after the second transfer to equal the mean value—38 cpm per nucleus—for nucleus A-1 of the first transfer (Table II). The mean value of second transfer nuclei A-1 and B-1 is 27 cpm (Table III); if we add an estimated (maximum) value of 20 cpm for cytoplasm B-1 (see below), the total is 47—which is reasonably close to the predicted 38. To obtain the value for STP the activity of the less radioactive nucleus is subtracted from that of the more radioactive nucleus. This gives a mean value of 203 cpm per nucleus for the first transfer (Table II) and 163 cpm per nucleus for the 2nd transfer (nucleus A-2 minus nucleus B-2 in Table III). Since the nuclei (A-1 and A-2) in Table III were transplanted to cells B-1 and B-2 a day after nucleus A-2 was grafted into cell A-1 (data of Table II), these data are compatible with an expected loss of circa 20% of STP label per day.[1]

Amount of RMP in the Cytoplasm

Byers et al. (1) indirectly and Goldstein (4) directly estimated the amount of cytoplasmic RMP, but it is now clear that their estimates were distorted by deficiencies in quantitation of radioautographs, by fixation artifacts, and probably by irregularities in the geometry of preparations. Therefore, a reestimation of the content of cytoplasmic RMP by the more direct and reliable means now available was performed.

The correct interpretation of the following experiments is based in part on the knowledge that RMP are distributed between nucleus and cyto-

plasm in a binucleate cell as they are in a mononucleate cell (4). That is, the concentration of RMP remains constant in each compartment but the distribution of *label*, if one grafts a radioactive protein-labeled nucleus into an unlabeled nucleate cell, is that expected of a tracer. The results of all our recent experiments, although not concerned with this matter directly, accord well with the conclusion that the concentration of RMP in a given, single nucleus and in the cytoplasm is not affected appreciably by the number of nuclei in the cell.

Since the distribution of RMP is unaffected by the number of nuclei, the content of RMP may be estimated from experiments similar to that illustrated in Fig. 1 and Tables II and III. The activity of the enucleate donor (A-1) of the first recipient cell is compared to that of the nuclei, and we find (Table II) that the cytoplasm has almost twice as much RMP (20% of the cell total) as the less radioactive nucleus (11% of the cell total). Most of our data, however, indicate that the cytoplasm may have between one and two times the amount of RMP present in the nucleus and we therefore assume, for simplicity of calculations, unless we have a direct measure, that the amount of RMP in the cytoplasm is equal to the amount of RMP in the nucleus.

Since the *A. proteus* nucleus is approximately 2% of the cell volume, the nuclear *concentration* of RMP (assuming that ⅛–½ of the cell total is in the nucleus) must be 25–50 times greater than the cytoplasmic concentration.

DISCUSSION

Once again the rapidly migrating proteins (RMP) are shown to move back and forth continuously between nucleus and cytoplasm; moreover, they do so against a 25- to 50-fold concentration differential in one direction. Since the regulation of genetic activity very probably occurs via some feedback from cytoplasm to nucleus, it is tempting to think that the nonrandom movement of RMP is somehow involved in this regulatory process. That the behavior of RMP has not been observed in other cellular systems is probably due to the unavailability of adequate methods for the detection of this activity.[2]

The behavior of the slow turnover proteins (STP) will be taken up in subsequent papers, but we should note here the probability of a much greater complexity in the study of these proteins than in the study of RMP. While the RMP may be (at least functionally) a relatively homogeneous group, the group of STP probably is composed of several protein types, such as: histone, structural proteins of the nucleolus, proteins of nascent ribosomes, proteins of the nuclear envelope, etc. The deficiencies of the earlier quantitative studies (1, 4) are probably due in part to the fact that STP are more soluble in acetic acid than are other nuclear proteins; acetic acid was part of most fixatives used in the earlier work but was not used here. If—as we have found[3]—some of the STP have a greater solubility in acetic acid than other proteins, then the ratio of protein-^{3}H between grafted nucleus and host cell nucleus for cells subjected to acetic acid fixation will be lower than the true value. Thus, Byers et al. (1) observed a 2.6:1 ratio, whereas from our observations the ratio was generally near 6:1. This may be something of a blessing in disguise, since classification of subgroups of STP may be facilitated by differences in solubility of the various components; some studies in this direction are in progress.

The difference between the ratios observed by Byers et al. (1) and those of our experiments may also be due to the possibility that much of STP is in the most central part of the nucleoplasm surrounded by an outer "shell" of nucleoli found in the ameba nucleus. Under such conditions analyses of radioautographs of sectioned or squashed preparations would yield a lower than true ratio because in either circumstance the outer regions of nucleus are represented out of proportion to their share of the nuclear volume. Since we disrupted *whole* nuclei with formic acid to extremely thin layers, our analyses are without geometric distortions and reflect more closely the true amounts of the various proteins.

The basic conclusions drawn by Byers et al. (1) regarding the *A. proteus* nuclear proteins are confirmed, but quantitative features now have been amended. When a nucleus with protein-^{3}H is grafted into an unlabeled cell and the distribution of RMP has reached equilibrium, the ratio of radioactivity between the grafted nucleus and the host cell nucleus is shown by present techniques to be about 6:1. If the cytoplasm contains approxi-

[2] Recent work of A. Zetterberg (*Exptl. Cell Res.* 43:526 (1966)) indicates that some proteins in mouse fibroblasts behave in a similar fashion.

[3] Prescott, D. M., and L. Goldstein. Unpublished experiments.

mately as much RMP as either nucleus and we have a ratio of 6:1, we can say:

> The cell with the grafted nucleus has 1 unit of RMP-^{3}H in each nucleus and 1 unit of RMP-^{3}H in the cytoplasm. It follows, therefore, that when the nucleus with protein-^{3}H was transplanted it had 5 radioactive units of STP and 3 of RMP and that, in the cell from which the grafted nucleus came, the original, preoperative distribution was 5 parts STP-^{3}H in the nucleus, 3 parts RMP-^{3}H in the nucleus, and 3 parts RMP-^{3}H in the cytoplasm. Thus, about 40% of the proteins in the nucleus is the rapidly migrating kind.

These values are all subject to some variability but the order of magnitude is certainly correct.

In subsequent papers the question of how much STP is present in the cytoplasm will be considered, but a completely satisfactory answer does not seem imminent.

Byers et al. (1) estimated that the concentration of RMP in the nucleus was between 30 and 240 times greater than in the cytoplasm, and Goldstein (4) estimated that the RMP were approximately 80 times more concentrated in the nucleus. These values reflect in part the technical deficiencies already mentioned, as well as the indirect nature of the analyses. Our data show that the RMP are between 25 and 50 times more concentrated in the nucleus than in the cytoplasm—probably closer to 50 times more concentrated most of the time.

The largest source of error in our analyses may be the 20% loss of nuclear protein that occurs upon isolation of nuclei in the Triton solution. This loss would appear to be serious in view of the evidence (Table I) that not all kinds of protein are lost to the same extent. For the kinds of analyses performed here, however, this loss appears not to be consequential, since we find that the distribution of protein determined without isolating the nuclei (Table II) is similar to the distribution determined with isolated nuclei.

The turnover, localization, and migration of acid-soluble nuclear proteins (including histones) have proven to be complex matters and will be considered in a subsequent paper.

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