THE CYTONUCLEOPROTEINS OF AMEBAE

I. Some Chemical Properties and Intracellular Distribution

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ABSTRACT

Autoradiographs of whole *Amoeba proteus* host cells fixed after the implantation of single nuclei from *A. proteus* donors labeled with any one of 8 different radioactive amino acids showed that the label had become highly concentrated in the host cell nucleus as well as in the donor nucleus and that the cytoplasmic activity was relatively low. When these amebae were sectioned, the radioactivity was found to be homogeneously distributed throughout the nuclei. The effect of unlabeled amino acid "chaser," the solubility of the labeled material, and the long-term behavior of the labeled material gave evidence that the radioactivity was in protein. At equilibrium, the host cell nucleus contained approximately 30 per cent of the radioactivity distributed between the two nuclei. This unequal nuclear distribution is attributed to the presence of two classes of nuclear proteins: a non-migratory one that does not leave the nucleus during interphase, and a migratory one, called cytonucleoprotein, that shuttles between nucleus and cytoplasm in a non-random manner. It is estimated that between 12 per cent and 44 per cent of the cytonucleoproteins are present in the cytoplasm of a binucleate cell at any one moment. Nuclei of *Chaos chaos* host cells also concentrated label acquired from implanted radioactive *A. proteus* nuclei.

INTRODUCTION

Cell biology has progressed to the point where there is now confidence that the basic mechanisms of how genetic information is translated into phenotypic expression will be well understood within a few months or years. The central role of messenger or informational RNA (3, 22) in the translation of the genetic code of the chromosomes into the amino acid sequences of proteins (24) is now accepted as almost proved, although we need to be alert to the fact that this view has not been proved conclusively.

Although there is justifiable optimism that we will soon comprehend in great detail how the nucleus influences the cytoplasmic condition, we are much less optimistic about the immediate prospects for an appreciation of the cytoplasmic mechanisms influencing the behavior of the nucleus (or genes). Since even hypotheses on the nature of the latter mechanisms are rare, it is increasingly necessary, in the pursuit of this knowledge, to exploit all suggestions that experimental evidence may provide. It is on the promise of one such suggestion that the investigations described here were carried out.

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Goldstein (10) reported the discovery of a class of macromolecules, presumably protein, that were believed to be in constant, non-random migration between nucleus and cytoplasm. Such material could obviously serve to convey signals from one compartment of the cell to the other and thus possesses at least one attribute expected of a cytoplasmic mechanism that could influence nuclear activity. These macromolecules might, for example, be responsive to changes in the cytoplasmic environment and upon return to the nucleus in an altered state affect the activity of particular genetic loci. The incentive furnished by Goldstein's discovery and its possible significance in the physiology of the cell has encouraged us to find out as much as possible about the characteristics of these macromolecules. Our efforts toward this end are reported in this and the following paper.

MATERIALS AND METHODS

1. *Organisms and Culture Methods*

Three species of amebae were used in our experiments: *Amoeba proteus*, *Amoeba discoides*, and *Chaos chaos*. Most of the experiments were performed with *A. proteus* and, unless indicated otherwise, it should be assumed that this was the species employed. We have maintained *A. proteus*, which derives from a clone originally established in the Zoology Department, University of California, Berkeley about 10 years ago, in continuous culture in this laboratory for approximately 3 years. The *A. discoides* cultures that we have maintained here, also for approximately 3 years, are descendants of a culture kindly furnished by Professor J. F. Danielli in London. We did not culture *C. chaos* in our laboratory but were periodically provided these animals from cultures maintained in the laboratory of Dr. J. M. Marshall of the University of Pennsylvania School of Medicine.

A. proteus and *A. discoides* were cultured according to the method of Prescott and James (19). In brief, the amebae were maintained in a medium containing 5 mg $CaHPO_4$, 6 mg KCl, and 4 mg $MgSO_4 \cdot 7H_2O$ per liter of glass-distilled water. The amebae were kept in glass petri dishes in a dark incubator at 17° ± 1°C. and were periodically fed *Tetrahymenae* that had been grown axenically in a 2 per cent proteose-peptone solution. The *Tetrahymenae* were harvested by centrifugation and washed thrice with the above salt solution to remove excess proteose-peptone, which is toxic to amebae. The ameba cultures were not completely free of other organisms but the medium in each petri dish was replaced frequently with fresh medium that had been passed through a bacterial filter; this procedure helped keep the numbers of bacteria, molds, etc. at a low level.

2. *Nuclear Transplantation Procedures*

Nuclear transplantations were performed, as described elsewhere by Goldstein (11), by the method of Commandon and deFonbrune (7) and Lorch and Danielli (15). Operations were facilitated by having donor and host cells of comparable size. In certain experiments, therefore, in which nuclear transfers were made between *A. proteus* and the much larger *C. chaos*, the specimens of *C. chaos* were cut into fragments of approximately the same size as *A. proteus*. *C. chaos* is a multinucleate ameba and the fragments, each of which contained several nuclei, behaved in an apparently normal manner.

Danielli and coworker (15) have shown that an enucleate *A. proteus* will regain normal activity upon the implantation of a normal *A. proteus* nucleus, thereby demonstrating the effectiveness of the operation. Our work has shown that binucleate amebae that are produced by the transplantation of a nucleus into a mononucleate ameba also behave normally. Binucleate cells occur naturally in our cultures, although they form a very small fraction of the total population, and can be selected on the basis of their much larger size. Natural binucleates typically divide into *three* daughter cells; two of the daughters are mononucleate and the third is a binucleate ameba. Binucleate cells created by nuclear transplantation divide in a similar manner. The descendants of the "artificial" binucleates have been followed for 4 generations and ordinarily mononucleate daughters give rise to mononucleates and binucleate daughters give rise to both mononucleates and binucleates. Trinucleate cells were also artificially created, although with less success than in the creation of binucleates, apparently because two transplantations, rather than one, were usually necessary. Although they appear normal in most respects, no attempt was made to follow such amebae through cell division.

3. *Procedures for Labeling Amebae*

Amebae were labeled with radioactive amino acids in one of three ways. Our data suggest that the amebae were labeled in essentially identical fashion with all three procedures. With the first method, amebae were fed radioactive *Tetrahymenae* that were labeled by the addition of a radioactive amino acid, to a final concentration of 5 to 50 μc/ml, to the standard proteose-peptone culture medium in which the *Tetrahymenae* were grown. With the second method, amebae were incubated in the absence of food organisms in a culture medium containing a radioactive amino acid usually at a concentration of 50 μc/ml. The third method was

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similar to the second, except that unlabeled *Tetrahymenae* were added with the amebae to the radioactive medium. An ameba labeled in any of these ways will be called a *directly labeled ameba.*

The radioactive materials used in these experiments were: DL-leucine-4,5-H^3 [New England Nuclear Corp., Boston, specific activity (sp ac): 3.57 c/mM]; DL-tryptophan-H^3 (Volk Radiochemical Company, Chicago, sp ac: 658 mc/mM); L-methionine-methyl-H^3 (New England Nuclear Corp., sp ac: 14.1 mc/mM); L-arginine-H^3 (Volk Radiochemical Company, sp ac: 270 mc/mM); L-phenylalanine-C^{14} (New England Nuclear Corp., sp ac: 215.1 mc/mM); DL-tryptophan-3-C^{14} (New England Nuclear Corp., sp ac: 6.15 mc/mM); L-lysine-1-C^{14} (Gift of Dr. M. Rothstein, sp ac: 0.8 mc/mM); L-methionine-S^{35} (Schwarz BioResearch, Inc., Orangeburg, New York sp ac: 1.5–5.7 mc/mM); cytidine-H^3 (Schwarz BioResearch, Inc., sp ac: 1 c/mM); carrier-free $P^{32}O_4$ (Oak Ridge National Laboratory, sp ac: *ca* 3.60 c/mM).

4. *Definition of "Chaser"*

"Chaser" is the term applied to a medium containing unlabeled precursor that was added to cell cultures following incubation of the cells in a medium containing radioactively labeled molecules of the same precursor. The chaser media contained unlabeled amino acids at 10 to 100 times the concentration of labeled precursor.

5. *Cytological Fixation*

Cytological fixation was accomplished by three different procedures. In most cases fixation was carried out with 45 per cent acetic acid followed by dehydration in a mixture of 3 parts absolute ethanol: 1 part acetic acid, and then in absolute ethanol (20). The slides were air-dried prior to autoradiography. In a similar procedure unbuffered commercial formaldehyde was used in place of 45 per cent acetic acid. Cells were flattened on a slide under a coverslip in a small drop of formaldehyde and, after a few minutes, the slides were frozen on solid CO_2. The coverslips were flicked off and the slides placed in formaldehyde at room temperature for another 10 minutes. The slides were passed through several changes of 70 per cent ethanol and, finally, into 95 per cent ethanol for 10 minutes. With a third method, which we called "lyophilization," the cells were flattened on a slide under a coverslip by the gradual removal of ameba medium with a piece of filter paper. The slides were then placed on solid CO_2 to freeze the cells, the coverslips removed, and the cells dehydrated under a vacuum in a cold desiccator.

Good cytological preservation was achieved with the acetic acid fixation, whereas with the other methods it was difficult to flatten the cells adequately without breaking them. On the other hand, evidence to be discussed below suggests that the acetic acid fixation preserves less of the total protein of the cells than the other two methods.

6. *Autoradiographic Techniques*

For most experiments autoradiography was executed by the stripping film technique (23) using Kodak AR 10 film. In later experiments, Kodak liquid emulsion NTB-2 was used (18). In a few cases a new non-aqueous autoradiographic method was used (9). It entailed placing a drop of Eastman 910 Adhesive over a specimen previously fixed on a slide and then firmly pressing the slide onto an AR 10 plate with specimen and adhesive against the film. Strong adhesion occurred in a matter of seconds. Slide and attached film were removed from the film plate by scoring around the edge of the slide with a razor blade and then carefully lifting the slide and film away from the glass backing. Storage and development were carried out by the usual stripping film techniques. No effort was made to refine the non-aqueous technique but, in spite of some deficiencies, a few good autoradiographs were obtained from leucine-H^3–labeled cells.

In many cases the slides were stained with Ehrlich's acid hematoxylin (6) following autoradiographic development to facilitate microscopic examination of the autoradiographs. The slides were stained for 4 minutes and differentiated in 0.5 per cent HCl.

7. *Counting of Autoradiographic Grains*

Autoradiographic grain counts were made at a magnification of about 1500 with a squared grid in the eyepiece. For nuclear assays, grains were counted in the emulsion over 80 to 100 per cent of the nuclear area. Cytoplasmic counts were made over randomly selected areas outside the region within one nuclear diameter of the nucleus. This latter area consists of 10 to 20 per cent of the cytoplasm and was excluded because there was often considerably more activity in the cytoplasm immediately adjacent to the nuclei in a cell into which a labeled nucleus had been grafted than in most of the remainder of the cytoplasm. The cytoplasmic activity adjacent to the nucleus could not be reasonably assayed for reasons that will be discussed later. All counts were corrected for background.

8. *Sectioning of Cells*

Amebae to be sectioned were pipetted into 45 per cent acetic acid for 1 minute and then transferred to a mixture of 3 parts ethanol: 1 part acetic acid for 10 minutes. They were then dehydrated in absolute ethanol, cleared in benzene, and embedded

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in Tissuemat. In order to facilitate location of the cells in the paraffin block, the amebae were lightly stained with eosin in the last change of ethanol. The paraffin blocks were sectioned at 3 μ and the sections mounted serially on slides. After deparaffinization and hydration, the slides were covered with AR 10 stripping film by the usual procedure.

9. *Centrifugation of Amebae*

Amebae were centrifuged in two ways. In one method, they were suspended in 0.2 M sucrose layered over 0.3 M sucrose in a centrifuge tube and centrifuged at 4°C in a microcentrifuge for 10 minutes at about 9,900 *g*. The centrifuged cells were immediately pipetted onto a chilled slide and flooded with 45 per cent acetic acid, occasionally being flattened with a coverslip during the fixation step. In the other method, amebae were suspended in ameba medium layered over 15 per cent Ficoll (13) in a centrifuge tube and centrifuged at 4°C for 20 minutes at about 15,800 *g*. These centrifuged cells were immediately pipetted into 45 per cent acetic acid, kept at 4°C for 10 minutes, placed on a microscope slide, and finally flattened with a coverslip.

RESULTS

A. *General Characteristics of Cytonucleoproteins*

1. Pattern of Labeling in Amebae Following Administration of Radioactively Labeled Amino Acids

In order to provide a basis of reference for later experiments, we examined the cellular distribution of radioactivity following the administration of leucine-H^3 to otherwise untreated amebae. Cells were labeled in the absence of food organisms for 30, 60, and 180 minutes. Autoradiographs of the fixed cells revealed that the label was distributed more or less homogeneously throughout the cell. Even in the extreme case, the grain concentration of the nucleus was only 58 per cent higher than that of the cytoplasm (Table I). This value contrasts with grain concentrations, observed following nuclear transplantations, that were at least 20 times greater over the nucleus than over the cytoplasm. (In any case, due to the absorption of tritium radiation by a thin layer of cytoplasm lying over the nucleus, grain counts tend to give an underestimate of the relative nuclear activity. However, this problem is not important in later experiments where the activities of several nuclei within a single cell are compared relative to each other rather than to the cytoplasm.)

2. Behavior of Labeled "Protein" of Radioactive Nucleus Transplanted into an Unlabeled Cell

In confirmation of the earlier work of Goldstein (10), we found that, when a nucleus from an ameba that had been fed any of a variety of radioactive amino acids was transplanted into an unlabeled ameba, the radioactivity was localized in the nucleus of the host cell as well as the transplanted nucleus within an hour after the operation (Fig. 1); relatively little radioactivity was found in the cytoplasm either immediately after the transplantation or later. It is difficult to estimate the relative activity in the cytoplasm under these circumstances because, in order to obtain an assayable grain concentration over the cytoplasm, the autoradiographic film must be exposed for such lengthy periods that the concentrations of grains over the nuclei become too dense for counting. Our estimate, which is necessarily a minimum one, is that when the host nucleus has acquired the maximum label from the grafted nucleus it has an autoradiographic grain concentration at least 20 times that of the cytoplasm. Since, as we noted in the previous section, under direct labeling conditions the autoradiograph of the nucleus displays only 1.58 times the activity of the cytoplasm *at the extreme*, the difference between host cell nucleus and cytoplasm following the implantation of a radioactive nucleus must be highly significant. We will consider later the relative *total amount* of label in nucleus and cytoplasm.

TABLE I

Relative Concentration of Radioactivity in Nucleus and Cytoplasm in Acetic Acid-Fixed Amebae Following Incubation in Leucine-H^3

Minutes of incubation in leucine-H^3	Number of cells assayed	Nuclear concentration of autoradiographic grains divided by cytoplasmic concentration of autoradiographic grains	
		Mean	Observed range
30	10	0.97 ± .09	0.50–1.52
60	9	1.16 ± .09	0.71–1.58
180	11	1.03 ± .03	0.86–1.17
Over-all	30	1.05	0.50–1.58

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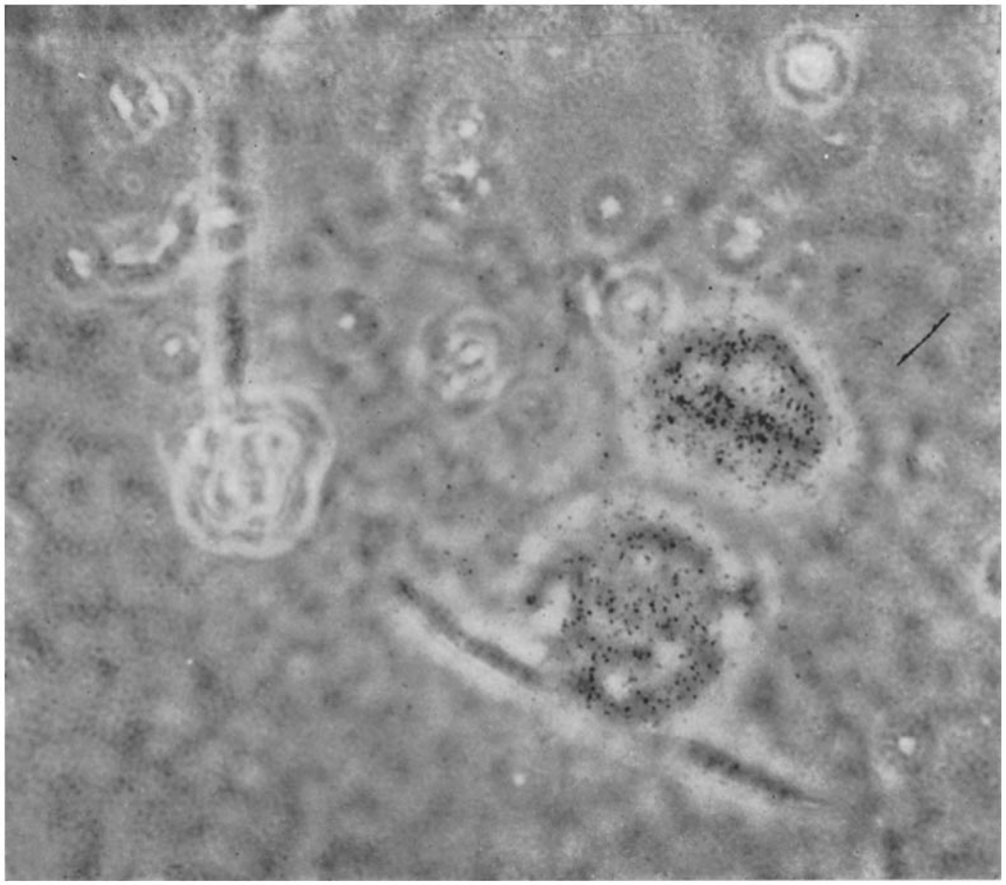

FIGURE 1 Autoradiograph of an ameba (out of focus) into which was grafted a lysine-1-C^{14}—labeled nucleus 20 hours before cell was fixed. × 300.

The simplest interpretation of these results, which is supported by additional data to be presented, is that the cell contains proteins in high concentration in the nucleus that migrate to the cytoplasm where they remain for relatively brief intervals and then return to the nucleus, presumably maintaining the shuttling process all during interphase. (In the binucleate cells in our experiments the proteins presumably shuttle back and forth between cytoplasm and both nuclei, without distinguishing between the nuclei). Because of this behavior we have decided, tentatively, to call this material "cytonucleoprotein."

3. Exclusion of Non-Polypeptide Amino Acids as the Migrating Material

In discussing the material that shuttles between nucleus and cytoplasm we have referred to it as "protein." Aside from the fact that radioactive amino acids were used as precursors in the above experiments, no evidence has yet been presented to substantiate this assumption; we will do so later. We would like, first, to describe measures taken to exclude, as the migrating material, non-polypeptide amino acids carried by the transplanted nucleus. Before describing these measures, however, we should note that the radioactive material detected in fixed cells is composed of molecules of fairly high molecular weight, since small molecules such as small polypeptides and amino acids would be extracted during the fixation of the amebae. However, the fixative-resistant radioactive material in the host cell nucleus might have been synthesized from low molecular weight radioactive precursors derived from the grafted nucleus.

Labeled donor cells were incubated in chaser

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generally for at least as long as the cells were incubated in the presence of the radioactive amino acid precursor. Recipient cells were also incubated in chaser for similar periods before the operation and continuously after the operation. In addition, host cells were fed heavily with unlabeled food organisms before nuclear transplantation and the digests of the food would presumably provide further "chaser" material.

All these measures would be expected to provide an excess of unlabeled precursor material that would have effectively competed with unincorporated radioactive amino acids to reduce further labeling of proteins to an insignificant level. This seems probable but is not proven since we do not know the nature of the amino acid pools nor the nature of the immediate precursors for the labeled material we observed.

There is some further evidence on the matter of transplanted precursors. If a grafted nucleus contained a sizable pool of radioactive precursors, which presumably could be incorporated into protein after the operation, we would expect a labeling pattern between host cell nucleus and cytoplasm similar to that which is observed when amino acids are administered directly to cells that have not been operated upon (Table I). Such a pattern was never observed.

It is possible that label in the fixed cells is in amino acid complexed to transfer RNA. This possibility is excluded, however, by the observation that the radioactivity of the experimental cells is resistant to ribonuclease digestion.

The evidence presented in this section does not prove that the material that shuttles between nucleus and cytoplasm is protein. No single piece of evidence that we shall present offers conclusive proof, but other kinds of evidence will be described and the Discussion will evaluate all the data on this point.

4. Amino Acid Composition of Cytonucleoprotein

In an attempt to detect whether the cytonucleoproteins displayed any unusual content or lack of a particular amino acid, the routine nuclear transplantation experiment was performed with nuclei labeled with one of several amino acids. The amino acids used were: arginine-H^3, leucine-4,5-H^3, lysine-1-C^{14}, methionine-methyl-H^3, methionine-S^{35}, phenylalanine-C^{14}, tryptophan-H^3, and tryptophan-3-C^{14}. In every case, the results were similar: there was a relatively high concentration of activity in both host and donor nuclei, but little activity in the cytoplasm. To determine whether the results are truly identical for all precursors would require quantitative techniques we were not prepared to undertake. The results do suggest, however, that the migratory material is protein and that it probably has an amino acid composition that is typical of proteins in general.

5. Some Solubility Characteristics of the Cytonucleoproteins

As mentioned above, that the labeled material is not removed by acetic acid fixation is consistent with the view that the unextracted radioactivity in our experimental cells is in protein. It may be, however, that the migratory material actually is acid-soluble but can become bound to acid-insoluble materials upon fixation. If such binding could occur in the nucleus but not in the cytoplasm, the observed distribution of radioactivity would need to be reinterpreted. To determine whether greater proportions of acid-soluble radioactive materials were present in the cytoplasm, autoradiography was performed, by a non-aqueous method, on lyophilized cells (see Methods) 24 hours after the transplantation of a leucine-H^3-labeled nucleus into a non-radioactive cell. The usual autoradiographic picture was observed, with no evident change in the relative distribution of radioactivity between the nucleus and the cytoplasm. In a number of cases cells were also fixed with formaldehyde, since formaldehyde appears to preserve some acetic acid-soluble materials (see below). Again, the typical distribution of radioactivity was observed in cells receiving a labeled nucleus although the intensity of labeling appeared higher with formaldehyde-fixed cells than with acetic acid-fixed cells. Since no significant increase in cytoplasmic label is detectable when cells are lyophilized or fixed with formaldehyde, the view that small molecules are not involved in the labeling of host cell nuclei is further supported.

The subjective impression from the autoradiographs that more migrating labeled material of the nuclei is preserved by formaldehyde fixation than by acetic acid fixation is probably supported by Geiger counter determinations of the solubility of labeled material of intact cells that were not operated upon. Amebae were labeled by incubation in 5 μc/ml of phenylalanine-C^{14} for 18 hours, washed, and incubated in non-radioactive phenyl-

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alanine for 7 hours. Cell samples were dried on planchets and the total radioactivity was determined with a gas flow, windowless Geiger counter. One series of planchets was then extracted with 45 per cent acetic acid for 10 minutes, a mixture of 3 parts ethanol:1 part acetic acid for 10 minutes, and twice with 95 per cent ethanol for 10 minutes. The radioactivity remaining was then assayed as before. A second series of planchets was extracted with unbuffered commercial formaldehyde for 12 minutes (maximum extraction occurred in this time) and assayed for residual radioactivity. A third series was extracted with 5 per cent trichloroacetic acid at 5°C for 6 minutes (maximum extraction occurred in this time) and assayed for residual radioactivity. The results of these three extraction procedures are shown in Table II. As can be seen from the data, in most of our experiments (in which acetic acid fixation was employed) there is a possibility that some of the nuclear proteins, including the migrating cytonucleoproteins, were extracted.

TABLE II

A Comparison of the Sizes of the Acetic Acid, Formaldehyde, and Trichloroacetic Acid Soluble Pools of Radioactivity Following the Administration of Phenylalanine-C^{14} to Amebae
Figures for CPM/cell were calculated from assys of planchets containing close to 50 cells each.

Solvent (Fixative)	CPM/cell		Per cent soluble
	Total	Soluble	
45 per cent acetic acid	13.29	4.42	33.3
	14.40	5.14	35.7
	9.89	3.92	39.6
Mean	12.53	4.49	35.8
37 per cent formaldehyde	10.94	2.01	18.4
	12.82	2.60	20.3
	13.13	2.80	21.3
Mean	12.30	2.47	20.1
5 per cent trichloroacetic acid	11.87	1.94	16.3
	15.98	2.15	13.5
	9.98	1.00	10.0
Mean	12.61	1.70	13.5

6. Interspecies Migration of Cytonucleoproteins

An attempt was made to determine whether *A. proteus* cytonucleoproteins could shuttle between the nucleus and the cytoplasm of the giant ameba *Chaos chaos*. Although *C. chaos* and *A. proteus* have a number of similar features (2), they are easily distinguished by such characteristics as size and number of nuclei. It has also been shown (14) that these two species can be characterized by differences in the electrophoretic mobilities and immunological properties of their water-soluble proteins.

Transplantation of *C. chaos* nuclei is difficult, and therefore transfers were made only of leucine-H^3–labeled *A. proteus* nuclei into unlabeled *C. chaos*. Twenty-four hours after the operation the cells were fixed and processed for autoradiography in the usual manner. The autoradiographs revealed that the label had migrated into *C. chaos* nuclei much as was the case in earlier experiments (Fig. 2). The cytoplasmic activity, however, was higher in this instance than when a labeled *A. proteus* nucleus is grafted into *A. proteus*.

Twenty-four hours after the operation we were unable to distinguish donor and host nuclei on the basis of either grain concentration or nuclear size. Therefore, the fate of the *A. proteus* donor nucleus was followed by making similar interspecies transplantations with $P^{32}O_4$-labeled nuclei. Earlier studies (12) had indicated that P^{32} should serve as a good marker of the grafted nucleus and, indeed, it did. P^{32}-labeled donor nuclei were observed in the host cells, following a transplantation, for up to 24 hours, but not after 48 hours. It thus appears that the protein-labeled nucleus of the above experiments was present long enough to permit exchange of cytonucleoprotein, but that after 24 hours, it disintegrated or had been ejected from the cell.

In addition to the *A. proteus–C. chaos* transfers, leucine-H^3–labeled nuclei were transplanted from *A. proteus* into unlabeled *A. discoides*. The distribution of radioactivity in these "hybrids" 24 hours after the operation apparently was identical with that observed in an *A. proteus–A. proteus* transplantation. Whether this interspecies migration is significant is uncertain, because these two strains do not differ in any marked way, particularly with respect to their protein components (14).

It is probable, in the light of these experiments,

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that cytonucleoproteins do not have rigid species-specific characteristics and it is possible that molecules of this type have a widespread occurrence. Three factors, however, urge caution in the construction of generalities: (*a*) the species involved are *relatively* closely related; (*b*) there is present in the cytoplasm of the *A. proteus–C. chaos* "hybrids" a higher concentration of radioactivity than is the case with an intraspecies transplantation; and (*c*) the apparent loss of the *A. proteus* nucleus from the *C. chaos* host raises the question as to whether the exchange of cytonucleoprotein label occurs without some alteration of the molecules.

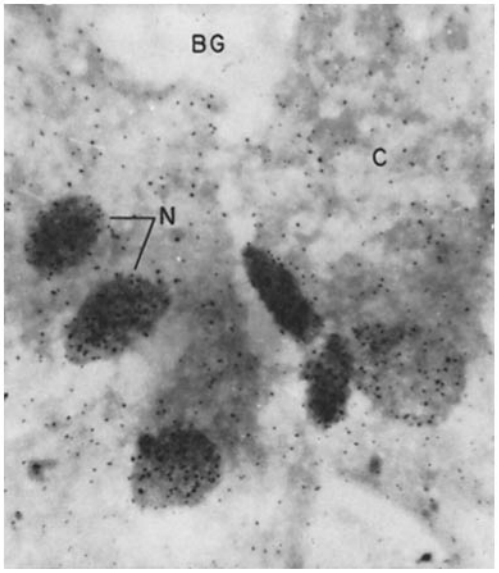

FIGURE 2 Autoradiograph of part of a *Chaos chaos* cell fixed 19 hours after the implantation of leucine-H^3–labeled *A. proteus* nucleus. The nuclei (*N*), which were probably all from *C. chaos*, show significantly higher activity than the cytoplasm (*C*). *BG* is background. Stained with Ehrlich's acid hematoxylin. × 200.

B. *Evidence for the Presence of Two Major Classes of Labeled Proteins in the Nucleus*

Data in the following paper (5) show that there is a *net* transfer of radioactive cytonucleoproteins from a grafted labeled nucleus to the host cell nucleus until an equilibrium is established approximately 4 to 5 hours after the transplantation. At equilibrium the host nucleus always contained less than 50 per cent of the total radioactivity of the two nuclei, regardless of the amino acid precursor used (Table III). In addition, similar equilibrium distributions are found whether donor and host nuclei are from sister cells or from cells selected from the culture at random (last 2 lines of Table III). This excludes the possibility that the inequality was due to variations in nuclear volume, since sister cell nuclei are similar in size. We conclude, therefore, that, at equilibrium after the transfer of a protein-labeled nucleus, the host cell nucleus contains approximately 30 per cent of the total activity of the two nuclei, although the data in Table III are really derived from autoradiographic grain *concentration* determinations.

TABLE III

Distribution of Radioactivity at Equilibrium in the Nuclei of a Binucleate Cell Created by Implantation of a Labeled Nucleus

Donor cells were incubated in 50 μc radioactive amino acid/ml for 24 hours and then in chaser for 24 hours.

Label in donor nucleus	Number of cells	Host nucleus share* of nuclear activity
		per cent
DL-tryptophan-H^3	14	27.8 ± 2.2
L-arginine-H^3	19	30.0 ± 2.9
L-methionine-H^3‡	17	29.6 ± 1.8
DL-leucine-H^3 (random)§	24	30.0 ± 1.6
DL-leucine-H^3 (sisters)§	13	27.1 ± 2.1

* The host nucleus share is expressed as the concentration of autoradiographic grains over the host nucleus divided by the combined concentrations of the donor and host nuclei.

‡ In the methionine series, the donor nuclei appeared consistently smaller than the host nuclei for reasons that are undetermined.

§ Leucine-H^3–labeled nuclei were transferred between cells selected at random and between sister cells of a single division.

The unequal distribution between host and donor nuclei suggested that we were observing two general classes of labeled nuclear proteins: (*a*) the migrating cytonucleoproteins, and (*b*) a class of non-migrating proteins. To determine whether two general classes of proteins truly exist in the nucleus, several variations on the basic nuclear transplantation experiment were performed in an attempt to answer the following

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questions. Will the cytonucleoproteins, in the course of their shuttling activity, distribute randomly (and equally) between identically treated nuclei in the same cell? Will the distribution of cytonucleoproteins between a transplanted and a non-transplanted nucleus differ from that between identically treated nuclei? If there is a greater inequality in distribution between non-identically earlier from a cell selected at random from a stock culture; S. III, the host contained its own nucleus and one grafted 24 hours earlier from the host cell's sister of the previous division; S. IV, the host cell, which earlier had been enucleated, contained two sister cell nuclei that had been implanted a few minutes before the implantation of a labeled nucleus. In the fifth series (S. V) of experiments, a

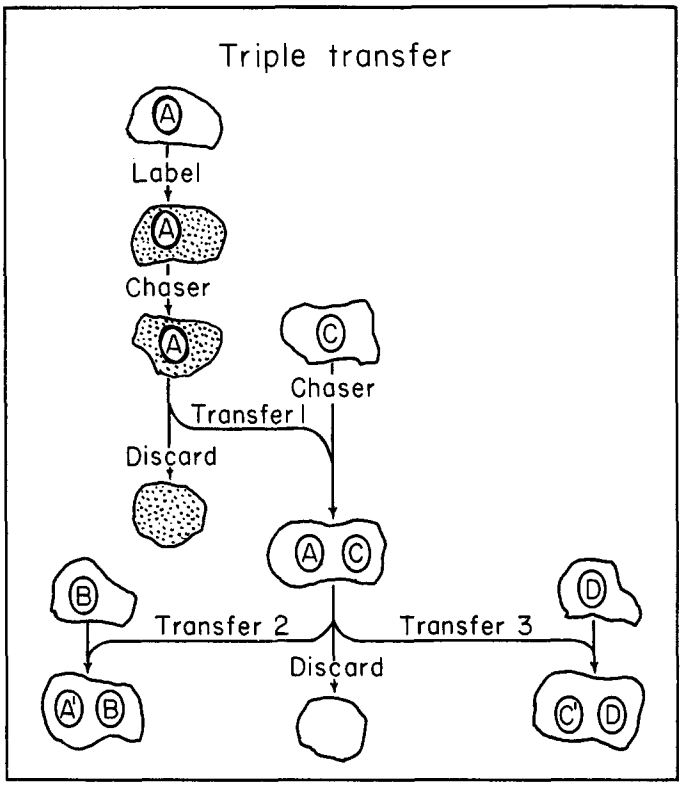

FIGURE 3 Diagram of experiment described in the text. Nucleus from a directly labeled cell (*A*) is grafted, after period in chaser, into unlabeled cell (*C*), which had been preincubated in chaser. After time for equilibrium to be established, nucleus *A* is grafted into unlabeled cell *B* and nucleus *C* is grafted into unlabeled cell *D*. Grafted nuclei *A* and *C* are designated *A'* and *C'*, respectively, in their new hosts.

treated nuclei, is the inequality sufficient to account for the 70 per cent donor nucleus: 30 per cent host nucleus distribution observed after the usual experiment (Table III)?

Five types of experiments were performed to answer these questions. The first four series involved transplanting a nucleus from a cell uniformly labeled with leucine-H^3 into an unlabeled *binucleate* host cell. The binucleate host differed in each series: S. I, the host was a "natural" binucleate found in a stock culture; S. II, the host contained its own nucleus and one grafted 24 hours radioactive nucleus was grafted into an unlabeled mononucleate host and after 24 hours' incubation both the donor and host nuclei were grafted into new mononucleate hosts and allowed to incubate another 24 hours prior to fixation (see Fig. 3).

The distributions of radioactivity between the nuclei of interest in each of the five series are given in Table IV. It is impossible to distinguish a grafted nucleus from a non-grafted one unless the former originated in a directly labeled cell and, consequently, contains label in the non-migratory proteins as well as the cytonucleoproteins; *e.g.*,

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nuclei A and A' in Fig. 3. Therefore, in comparing the distribution of activity between the two host nuclei of the first four series, we are limited by not knowing the origin of either nucleus in the fixed cells. Thus, in every series of experiments described in this section the data have been averaged by assuming that the more radioactive nucleus of the two host nuclei is in a different class from the less radioactive one. Since this assumption clearly is unwarranted in series S. I, at least, and since one could not expect a perfect 50:50 distribution even under the best circumstances, the 54.6 per cent: 45.4 per cent distribution given in column $H/(H + h)$ of Table IV is taken as a base line for equal distribution in these experiments. Variance ratio tests (4) show that the variances of series S. II to S. V are not significantly greater than that of S. I and the greater difference in activity between nuclei in each of series S. II to S. V than between the nuclei in S. I *may* be due to experimental effects, but these latter differences are not shown to be statistically significant. These data indicate, however, that: (*a*) the distribution of radioactive cytonucleoproteins between two host cell nuclei approaches equality when neither of the nuclei has been transplanted (S. I); (*b*) the inequality in radioactive content between two nuclei that have received only cytonucleoprotein label (S. I to S. IV and S. V, $C'/(C' + D)$) is always substantially less than between a donor and host nucleus if the donor came from a directly labeled cell (as in S. VI and S. V, $A'/(A' + B)$).

TABLE IV

Distribution of Leucine-H³-Labeled Nuclear Proteins Following Various Nuclear Transplantation Sequences Described in Text

Experimental values represent percentages.

Operation series	Number of cells	Distribution of radioactivity
Trinucleate cells:		$H/(H + h)$*
S.I	14	54.6 ± 1.2
S.II	11	58.7 ± 1.8
S.III	17	58.1 ± 1.1
S.IV	9	58.6 ± 1.9
Binucleate cells:		$A'/(A' + B)$‡
		$C'/(C' + D)$‡
S.V.	16	80.1 ± 1.7
		62.6 ± 1.5
S.VI§	24	$A/(A + C)$‡
		70.0 ± 1.6

* Gives proportion of host nuclear activity in more radioactive of two host nuclei. H, autoradiographic grain concentration over more radioactive host nucleus; h, autoradiographic grain concentration over less radioactive host nucleus.

‡ See Fig. 3 for explanation of symbols. The letter designations are arbitrary in the case of C' and D because there is no basis for determining the origin of the more active nucleus in each cell (see text).

§ Taken from 4th line of Table III.

Several conclusions can be drawn from the above data. First, the cytonucleoproteins apparently distribute randomly between nuclei that have not been subjected to transplantation operations. Support for this conclusion comes from data on two particular cells similar to those in series S. I, except that the host cell was a natural trinucleate in one case and a natural quadrinucleate in the other. The host nuclear label was distributed in the proportion of 0.35 to 0.33 to 0.32 in the trinucleate and 0.27 to 0.26 to 0.24 to 0.23 in the quadrinucleate. Second, there is a possibility that the transplantation of a nucleus in some manner prevents radioactive cytonucleoproteins from distributing equally between a nucleus that has been grafted and one that has not [as in S. II, S. III, and S. V, $C'/(C' + D)$]. Third, the inequality in distribution of radioactivity between grafted and nongrafted host nuclei (S. II, S. III) and between donor and host nuclei [S. V, $C'/(C' + D)$] when it is known that only migratory proteins are labeled is not great enough to account for the inequality in the distribution of radioactivity between a donor and a host nucleus when the donor nucleus comes from a directly labeled ameba. We can conclude finally, from this third point, that the donor nucleus probably contains radioactivity in a *nonmigratory* class of proteins as well as in the cytonucleoproteins. Consistent with this final conclusion is the observation that the later host nuclei acquire proportionately smaller shares of the total nuclear label when a nucleus from a directly labeled donor is serially transplanted into unlabeled hosts (*cf.* S. VI and S. V, $A'/(A' + B)$). We would expect such a decrease of label in succeeding host cell nuclei if all the non-migratory label remains with the donor at each step and if a fraction of the cytonucleoprotein is left behind in each host.

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C. *Localization of Cytonucleoproteins and Non-Migrating Proteins of the Nucleus*

1. Localization Within the Nucleus

Since our observations on the distribution of radioactivity were made on fixed *whole* cells, we were unable to determine the intranuclear localization of the cytonucleoproteins and the non-migrating proteins. Are these proteins primarily intranuclear or are they associated with the nuclear envelope? If intranuclear, are the labeled proteins distributed homogeneously or not?

FIGURE 4 Three-micron section of cell that had received leucine-H^3–labeled nucleus *ca.* 4 hours before fixation. Portion of cell shows both host and donor nuclei. Stained with Giemsa. × 500.

Ten amebae, into each of which a leucine-H^3–labeled nucleus had been grafted 4–5 hours earlier, were fixed, embedded, and sectioned at 3 μ (see Methods). After autoradiographic exposure and development, all of the serial sections were carefully examined. We observed (Fig. 4) that the radioactivity was distributed throughout both the donor and host cell nuclei in all of the sections and that, as far as bright-field microscopy reveals, the label is homogeneously distributed throughout the nucleoplasm, nucleoli, and nuclear envelope. Furthermore, the radioactivity was found to be distributed in a proportion of approximately 80 per cent in donor nucleus to 20 per cent in host nucleus. The deviation from the expected 70 to 30 per cent distribution we consider to be due to minor technical details and therefore conclude, for the present, that both the cytonucleoproteins and the non-migrating nuclear proteins are uniformly distributed throughout the nucleus.

2. Localization Within the Cytoplasm

Careful examination of the autoradiographs of cells into which a radioactive protein-labeled nucleus had been grafted reveals that there is often a gradient of radioactivity of diminishing intensity from the nuclear envelope into the cytoplasm. Under our usual experimental procedures the gradient diminishes to a level of radioactivity not much above background within the distance of approximately one nuclear diameter from the nucleus. Several interpretations of this observation can be imagined, but as yet we do not know the basis for the activity gradient.

3. Localization Within Centrifuged Cells

Amebae into which radioactive protein-labeled nuclei were grafted were centrifuged prior to fixation (see Methods) to provide a clue to cytoplasmic localization of the cytonucleoproteins. Centrifugation causes stratification of the cytoplasmic contents of the cell and we hoped to detect, after the fashion of Zalokar (25), specific layers displaying radioactivity. At a maximum of about 15,800 *g* (higher forces would break up amebae) for 20 minutes, at least all light microscope detectable structures, *e.g.*, granules, mitochondria, vacuoles, and nuclei, should stratify, according to Singh (21).

The autoradiographs of the centrifuged amebae revealed no concentration of radioactivity in any region of the stratified cytoplasm of 17 cells. If the cytonucleoproteins are associated with any cytoplasmic structures, the structures, since they are not stratified, are probably smaller than those detectable by the light microscope.

D. *The Amount of Cytonucleoproteins Present in the Cytoplasm*

We have noted that the cytonucleoproteins are more *concentrated* in the nucleus than in the cytoplasm, but what is the relative *amount* of cytonucleoprotein in each compartment? It is difficult, if not impossible, to obtain direct estimates by autoradiographic grain counting of the cyto-

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plasmic share of these proteins, for three reasons: (*a*) the assay of autoradiographic grains in the radioactivity gradient adjacent to the nucleus cannot be done on a sampling basis and would require an extremely tedious and unreliable counting of all the grains in the autoradiograph, although geometry considerations seem to make any accurate determinations impossible; (*b*) to obtain an autoradiographic grain density in the cytoplasm sufficiently great to count accurately, the grain density of autoradiographs of the nucleus of the same cell must be too dense for precise assay; (*c*) we do not know that all the radioactivity that migrates to the cytoplasm is in cytonucleoproteins; some may be in proteins that do not return to the nucleus.

Indirect estimates of the cytoplasmic share of the cytonucleoproteins that may bypass the first two of the above difficulties can be made by calculations from the data in Table IV. If we note how much the proportion of A' to $(A' + B)$ in S. V deviates from an expected proportion, we can calculate that the amount of cytonucleoprotein label of the cell present in the cytoplasm is 12 per cent or 44 per cent on the basis of two different extreme assumptions as to the influence of the nuclear transplantation operation on the distribution of cytonucleoproteins between a grafted nucleus and a non-grafted nucleus. (See Addendum for calculations of these values.) Support for the lower value is found in an experiment presented in the following paper (5). Leucine-H^3–labeled nuclei were grafted into unlabeled hosts that were fixed and autoradiographed at various intervals after the operations. After combining the grain counts over the donor and host nuclei in each host cell, the average combined concentration over the nuclei of eight cells fixed in 10 minutes or less after the operations was 21.0 ± 3.6 grains/81 μ^2, whereas the average for the nuclei of 65 cells fixed between 30 minutes and 24 hours after the operations was 24.1 ± 5.3. Although the grains counted in this case represent both cytonucleoproteins and nonmigratory nuclear proteins, the data suggest that considerably less than 44 per cent of the total cytonucleoprotein label is lost to the cytoplasm during the course of migration. Since all of these calculations have been made for a binucleate cell, it is probable that, whichever figure for the cytoplasmic share of the cytonucleoproteins is correct, the value will be higher for a normal mononucleate cell.

The above calculations also can be used to estimate the relative concentration of cytonucleoproteins in nucleus and cytoplasm. Since the two nuclei occupy approximately 3 per cent of the cell (17): if the cytoplasm contains 12 per cent of the cellular cytonucleoproteins, the concentration is approximately 240 times greater in a nucleus than in the cytoplasm; if the cytoplasm contains 44 per cent of the cellular cytonucleoproteins, the concentration is approximately 30 to 50 times greater in the nucleus than in the cytoplasm. Our necessarily crude estimates taken from direct autoradiographic grain counts (see section A, part 2) showed that the nuclear concentration of cytonucleoprotein had to be at least 20 times that in the cytoplasm.

DISCUSSION

We have examined a number of properties of the cellular material that we have chosen to call, for the present, cytonucleoprotein. The primary features that describe cytonucleoproteins are that they are present in the nucleus at a concentration at least 20 times to perhaps as much as 240 times the concentration in the cytoplasm and that these proteins shuttle continuously between nucleus and cytoplasm in interphase amebae. These two features, which we believe to be unique, at least in combination, served as diagnostic aids in subsequent attempts to characterize the cytonucleoproteins.

We believe the shuttling material to be protein for a variety of reasons: it is labeled in our experiments with radioactive amino acids; the label, once incorporated, is not lost by dilution with unlabeled amino acids administered as "chaser"; and the solubility properties that we have thus far studied resemble those of a large polypeptide or protein. Since these are properties of the labeled material in fixed cells, it may be argued that our experimental material is low molecular weight peptides or even amino acids that, when introduced by nuclear transplantation, are preferentially incorporated into proteins of the host cell nucleus and do not shuttle as we have described. In the following paper (5), however, we report that the material continues to show the same behavior for at least 4 cell divisions after the initial nuclear transfer, and it is highly unlikely that low molecular weight labeled precursors would be present or retain such a specificity for so

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many cell generations. We would expect macromolecules, however, to persist, and the only macromolecules that could display all the properties herein described are proteins.

We have no information respecting the homogeneity of the cytonucleoproteins; perhaps they compose one group of molecules in the sense of a crystalline protein, but it is just as probable, from our data, that there are many proteins in the cytonucleoprotein class. Our labeling experiments do show, however, that there is a distinctly different class of proteins that are primarily localized in the nucleus but that *do not* migrate to the cytoplasm. We know little about the nonmigrating proteins, but their presence is experimentally useful for they serve as reference points for some of the assays we can make on the cytonucleoproteins. Thus, we have labeled the nuclear proteins with 8 different radioactive amino acids and found that the proportion of radioactivity in cytonucleoproteins to the radioactivity in the nonmigrating proteins is the same with all precursors. This suggests that the two classes are similar in gross composition. Fast-green staining (1) indicates the presence of histones in the nuclei of formaldehyde-fixed cells, but it is possible that they would be extracted by our usual acid fixation procedure (8, 16). Since histones typically contain no tryptophan, or only traces (16), the fact that tryptophan-labeled proteins migrate and give ratios similar to other amino acids suggests that both the cytonucleoproteins and the nonmigratory proteins contain non-histone components.

Whether there is a class of proteins that does not eventually migrate out of the nucleus is in some doubt. Prescott (18) has shown that, by amputating approximately half the cytoplasm of a radioactive protein-labeled ameba approximately every 36 hours and permitting regeneration between amputations, it was possible, after 30 amputations, effectively to deplete the nucleus of all protein label. It is not clear from Prescott's experiments whether the loss of nuclear label was due to migration into the cytoplasm or protein turnover. In any event, for present purposes we may conclude that during one normal cell cycle there is a substantial portion of nuclear proteins that does not migrate.

The evidence that *A. proteus* cytonucleoproteins continue to show their unique characteristics when transplanted to other "species" suggests that these proteins are similar in the three species tested. The fact that there is more cytoplasmic label in such hybrids, however, may reflect a heterogeneity in the class of cytonucleoproteins, with only the fraction that continues to be more concentrated in the host nuclei being compatible to both donor and host species.

Efforts to pinpoint localization of the labeled proteins within the nucleus or within the cytoplasm have failed at the light microscope level. It may be that the cytonucleoproteins are not preferentially associated with any cellular structure. If this proves to be true, we should look to special properties in the nuclear membrane to account for the marked difference in concentration on either side of that membrane.

ADDENDUM

Calculation of the Cytoplasmic Share of Cellular Cytonucleoproteins

An estimate of the cytoplasmic share of the cytonucleoproteins may be made from a comparison of the distributions of radioactivity in the cells containing nuclei A and C and A' and B in Fig. 3. We see in Table IV that $A:C$ (S.VI) = 70:30 and $A':B$ (S.V) = 80:20. From the $A:C$ proportion, ignoring for the moment the cytoplasmic label, one can make several predictions about the *expected* $A':B$ distribution of activity, and from the deviation of the *observed* $A':B$ ratio from the expected, one can calculate the amount of cytoplasmic cytonucleoproteins.

In performing the calculations several assumptions must be made. We assume that the total amount of non-migratory label in A' will be the same as in A. We also assume that the distribution of labeled cytonucleoproteins between A' and B will be in the same proportion as between A and C although the *total* amount of label will differ. The amount of labeled cytonucleoproteins carried by nucleus A into the cell with nucleus B will depend on the manner in which those proteins distribute between nuclei A and C. There are two possibilities: (*a*) the cytonucleoproteins distribute, on the average, equally between the nuclei giving a 50:50 distribution; or (*b*) they distribute in the proportion of 60:40 as is suggested by some of our data (see $C'/(C' + D)$), Table IV: S.II, S.III, S.IV, and S.V.

If we assume that the distribution is 60:40, the radioactivity in the cell with nuclei A and C will be distributed as follows:—

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	Relative Units of Radioactivity		
	Nucleus A	*Nucleus C*	$A/(A + C)$
Non-migratory protein	25	0	
Cytonucleoprotein	45	30	0.6 (assumed)
	—	—	
Total (observed)	70	30	

In the next transfer, nucleus A would carry 25 units of non-migratory label and 45 units of cytonucleoprotein label into the cell with nucleus B. The predicted $A':B$ would then be:—

	Relative Units of Radioactivity		
	Nucleus A'	*Nucleus B*	$A'/(A' + B)$
Non-migratory protein	25	0	
Cytonucleoprotein	$\frac{0.6(45 - X)}{}$	$\frac{0.4(45 - X)}{}$	0.6 (assumed)
Total (calculated)	$52 - 0.6X$	$18 - 0.4X$	
Observed ratio	80	20	

where X is the value for relative amount of cytonucleoprotein label in the cytoplasm.

From the observed value of 80:20 for $A':B$

$$\frac{52 - 0.6X}{18 - 0.4X} = \frac{80}{20}$$

$$X = 20$$

The cytoplasmic share of cytonucleoproteins is then 20/45 or 44 per cent. If we assume that the cytonucleoproteins distribute such that $A:C = A':B = 50:50$, the cytoplasmic share of cytonucleoproteins becomes 12 per cent. These values apply to binucleate cells; we can not determine, without additional information, what the value would be for a mononucleate cell.

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