THE INTRACELLULAR DISTRIBUTION OF FERRITIN FOLLOWING MICROINJECTION

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ABSTRACT

Ferritin solutions were microinjected into the ground cytoplasm of intact amebae. At several time-intervals after injection the cells were fixed and the distribution of the protein in various organelles was studied with the aid of the electron microscope. Individual molecules of ferritin were found randomly dispersed throughout the ground cytoplasm and the ground nucleoplasm. Within the mitochondria, the ferritin was localized between the outer and inner membranes. Aggregates of ferritin were found in vacuoles, some of which could be identified as food vacuoles. The findings, which provide evidence for a rapid penetration of large molecules into the nucleus, the outer compartment of the mitochondria, and the digestive vacuoles, are discussed in relation to other reports on intracellular permeability.

INTRODUCTION

The normal activities of cells require a wide variety of exchanges among the various cell components or organelles. Of the many substances which may be transferred from one compartment of the cell to another, larger molecules, such as proteins and nucleoproteins, appear to pose special problems. One may ask to what extent, at what rates, and in what direction large molecules are exchanged, whether different organelles have specific permeability differences towards large molecules, and what the mechanisms of exchange may be. The present study is concerned with the first of these questions, specifically with the extent to which large molecules can gain access to cell organelles following their introduction into the ground cytoplasm. The results indicate that several organelles are permeable to the test protein used, and suggest that the methods used may be applicable to further studies on specificity and on the mechanisms involved in such exchanges.

Three general approaches have been used to investigate the intracellular exchanges of macromolecules. These are: morphological studies, by electron microscopy; permeability studies of isolated cell components; and permeability studies of cell components within the intact cell. Morphological studies have been of value in describing the various intracellular membranes across which the exchanges of molecules must, in some way, take place. With this approach it has been possible to suggest pathways which might permit exchanges across the nuclear membrane (1).

Permeability studies of the nuclear and mitochondrial membranes have been done on isolated material. The use of such material has one major drawback: it is necessary to assume that the permeability characteristics of these organelles are not altered as a result of isolation. For this reason, experiments done on intact cells are desirable. Permeability studies of organelles within the intact cell have been concerned mainly with the nucleus. These investigations will be considered in more detail in the Discussion, but it should be

noted that, in general, the methods employed in earlier studies were not sensitive enough to permit the detection of individual molecules. Thus, in investigations where macromolecules were not detected in the nucleus, it was not possible to determine whether the nuclear membrane is strictly impermeable to these molecules, or whether penetration occurred but in amounts or at rates too low to be detected by the methods employed. This limitation was avoided in the present study by using ferritin as a test protein, the individual molecules of which could be detected by electron microscopy. Ferritin was introduced into the ground cytoplasm of intact amebae by microinjection. At various time-intervals after injection, the cells were fixed and the distribution of ferritin among the various cell organelles was determined by electron microscopy. In this way, individual molecules or particles of molecular dimensions could be localized and identified.

MATERIALS AND METHODS

The experiments were carried out on the multinucleated ameba *Chaos chaos* (*Pelomyxa carolinensis*). The culture medium for these organisms consisted of 10^{-4} M $Ca(NO_3)_2$, 10^{-5} M $MgSO_4$ and 10^{-5} M K_2HPO_4, dissolved in ion-free water. The pH was 6.8. The amebae were fed on a washed, concentrated suspension of *Paramecium aurelia*.

Nachmias and Marshall (2) have shown that these amebae can take up ferritin in pinocytosis vacuoles if it is present in the external medium. The ingested ferritin undergoes a series of changes within the vacuoles, but it does not pass across the vacuole membrane into the ground cytoplasm. To introduce ferritin into the ground cytoplasm, it was, therefore, necessary to employ microinjection. Injury to cell organelles could occur if the solution injected were such as to alter the chemical environment; therefore, a preliminary study was done on the ionic composition of the cytoplasm and the results of this study were used as a guide in preparing a solvent medium for ferritin.

Formulation of an Injection Medium

Samples of ameba cytoplasm were obtained from mass cultures of *Chaos chaos*. The techniques for culturing and harvesting these amebae in 5- to 10-gram lots will be described in detail elsewhere. To obtain samples of cytoplasm rapidly, with minimal opportunity for changes in composition, the living cells were washed in water at 0°C and packed by centrifugation in the cold at 5000 *g* for 1 minute. The cells were not broken by this treatment, and if placed in culture medium would rapidly resume normal activity. The water was drawn off completely, and the tube, now containing only a packed mass of cells, was centrifuged at 80,000 to 100,000 *g*, at 5°C for 30 minutes. Under these conditions, the cells were broken, and the cell components were stratified *en masse*. The clear supernatant cytoplasm was withdrawn and analyzed for sodium and potassium by flame photometry, for chloride by amperometric titration, and for inorganic and labile phosphate by the Fiske-Subbarow method. These analyses were done in connection with other studies, and will be reported after further work permits a more complete treatment of the problem. For the purpose of this study, however, the results were judged sufficiently informative to permit the formulation of a medium for ferritin, which should simulate closely the normal milieu in respect to pH and to the concentrations of the major ions. The medium formulated in this way had the following composition: 0.0127 M KCl, 0.0011 M KH_2PO_4, and 0.0016 M K_2HPO_4. The pH was 7.0 to 7.1, which matched to 0.1 unit the pH determined directly on fresh samples of cytoplasm by the glass electrode. All salts were reagent grade.

Ferritin Solutions

Ferritin was prepared from horse spleen by ammonium sulfate precipitation and crystallization with cadmium sulfate (3, 4), and was further fractionated by ultracentrifugation. Solutions containing 0.25 to 0.30 per cent ferritin were prepared by dialyzing the protein against the injection medium described above. The concentration of ferritin was checked by spectrophotometry and microkjeldahl nitrogen determination.

Method of Injections

Microinjection was performed by the method of Chambers and Kopac (5). Preliminary experiments were carried out in which the volume of solution, relative to the total volume of the cell, and the concentration of ferritin within the solution were varied. It was found that ferritin solutions much more concentrated than 0.3 per cent caused local clotting of the cytoplasm. These clots were later pinched off by the amebae. With concentrations of 0.3 per cent or less no such effects were observed. A single injection, of approximately $\frac{1}{10}$ the cell volume, resulted in a final concentration of ferritin within the cytoplasm which was too low to permit ready analysis by electron microscopy, even though a few molecules could be found in almost every field in micrographs. Multiple injections of ferritin at 0.25 to 0.30 per cent concentration were, therefore, necessary. The amebae were injected either two or five times, the intervals between injections varying from 5 to 20 minutes.

Treatment of Amebae

All experimental amebae were starved for 24 hours before injection. Those which were to be fixed were also starved during the period between injection and fixation. Under these conditions the amebae did not divide during the course of the experiments. On the basis of the description of amebae in various stages of the division cycle reported by Kudo (6), it can be stated that the experimental amebae were in interphase. In order to study the effects of injection on various physiological processes, 12 amebae were injected either two or five times with ferritin solution and, instead of being starved for later fixation, were supplied with *Paramecium* and cultured in individual dishes. Normal amebae, which had not been injected, were cultured simultaneously.

Preparation for Electron Microscopy

Those amebae which were injected twice were fixed 7 minutes, 1 hour, and 24 hours after the final injection. Those amebae which were injected five times were fixed 24 hours after the last injection. The cells were fixed for 8 minutes in 1.0 per cent osmium tetroxide dissolved in veronal-acetate buffer which was adjusted to pH 8.5. Following fixation, the amebae were dehydrated in alcohol and embedded in epoxy resin. Thin sections were cut and examined with an RCA-EMU-3C electron microscope. The results of these experiments were based on the examination of 22 injected amebae.

RESULTS

Physiological and Morphological Effects of Injection

The initial effect of injecting a solution containing 0.25 to 0.30 per cent ferritin was a localized rounding of the amebae in the region injected. No clotting could be observed and movement began shortly after completion of the injection. Within approximately 2 to 4 minutes the amebae appeared to have completely recovered.

In those experiments in which the amebae were fed following multiple injections, no difference could be detected, between the injected amebae and those which had not been injected, in regard to movement, feeding, ability to divide, and rate of division. These results show that the injection procedures did not affect the normal functioning of the cells. Furthermore, electron micrographs of injected cells did not reveal any structural changes in the ground cytoplasm, nuclei or mitochondria.

Ground Cytoplasm

In all injected amebae, ferritin molecules were randomly scattered throughout the ground cytoplasm (Figs. 1 and 2). There was no indication of either precipitation or adsorption of ferritin restricted to any particular component of the cytoplasm.

Nuclei

Ferritin was consistently found within the nuclei of amebae which were fixed 24 hours after being injected either two or five times (Figs. 1 and 3). In the cells injected five times, the concentration of ferritin in the nuclei was greater than it was in those injected twice. The nuclei of doubly injected amebae, fixed after 1 hour, also contained ferritin; however, the concentration of ferritin in this organelle at 1 hour appeared to be less than that in the nuclei of corresponding amebae fixed after 24 hours. In some sections of amebae fixed 7 minutes after injection, ferritin was present in the nuclei, but often sections were obtained of nuclei in which no ferritin was found (Fig. 2). In all positive instances, whether at 24 hours, 1 hour, or 7 minutes, the ferritin was present as individual molecules, randomly dispersed throughout the ground nucleoplasm.

Nucleoli

Ferritin was not usually found in the nucleoli (Fig. 3). On occasion, a few molecules were present in the nucleolar masses, but even in these cases the concentration was not so great as that in the surrounding ground nucleoplasm. One possible explanation is that the consistency of the nucleolar material is such that diffusion into these regions occurs at a very slow rate.

Mitochondria

Most of the mitochondria of amebae which were injected five times contained ferritin. It appeared as scattered individual molecules located specifically in the space between the outer and inner membranes (Fig. 1). In those amebae which were injected only twice, ferritin was found less frequently and in lower concentration within the mitochondria, but in the same distribution at all time-intervals studied.

Vacuoles

Aggregates of ferritin often appeared in vacuoles within the cytoplasm. By feeding the amebae during the period between injection and fixation, some of the vacuoles containing ferritin could definitely be identified as food vacuoles because of the presence of recognizable organelles of *Paramecium* (Fig. 4). Since ferritin molecules do not pass from the food vacuoles into the ground cytoplasm (7), it is possible that a one-way mechanism exists whereby some proteins can enter but not leave the food vacuoles.

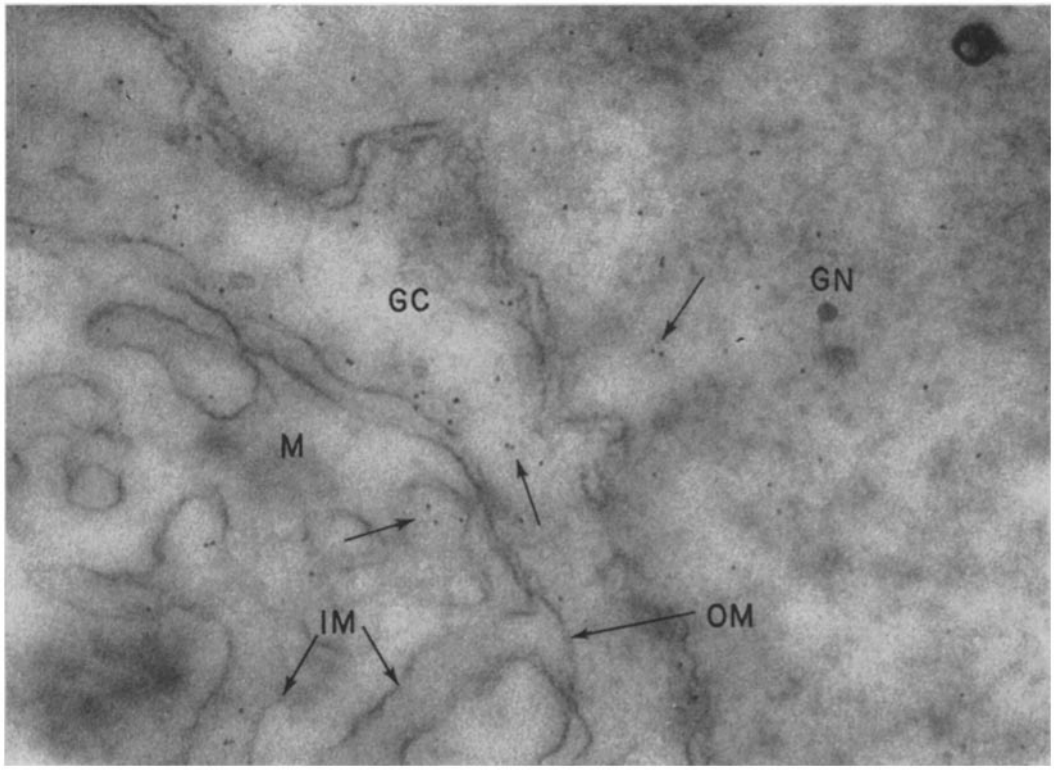

FIGURE 1

An electron micrograph of a section through an ameba fixed 24 hours after being injected five times with ferritin solution. Ferritin molecules (arrows) can be seen scattered throughout the ground cytoplasm (*GC*) and ground nucleoplasm (*GN*). Within the mitochondrion (*M*), the ferritin is localized between the outer membrane (*OM*) and inner membrane (*IM*). Magnification, 100,000.

Controls

Since the internal structure of the ferritin molecules could not always be seen, it was considered advisable to determine whether the electron-opaque particles in which no definite internal structure was observed actually represented ferritin molecules, or whether they were artifacts produced, in some way, by the injection procedures. To distinguish between these possibilities, amebae were injected five times with a control salt solution which did not contain ferritin, and fixed after 24 hours. When these results (Fig. 5) are compared to those obtained for amebae injected with salt solution containing ferritin (Fig. 1), it becomes clear that the dense particles do represent ferritin molecules.

DISCUSSION

Before drawing conclusions from the results presented, it is necessary to consider the following possibilities: first, that the results were influenced

by injury during the injection procedures; and second, that the ferritin could have been redistributed during sectioning.

It has been pointed out that no morphological changes, as observed with the electron microscope, were detected in injected amebae. Furthermore, no changes were detected in regard to movement, feeding, or division. Since these activities depend largely on the proper functioning of the nuclei and mitochondria, it can be concluded that these organelles were not detectably affected by the injection procedures.

The possibility should be kept in mind that a subtle form of injury could occur which might alter the permeability characteristics of the organelles studied but which could not be detected. However, since all available evidence indicates that following injection the amebae retain their normal structure and functions, it is felt that one can conclude, with some certainty, that the results are an indication of the type of exchanges that can occur in normal cells.

It is conceivable that, during sectioning, knife artifacts could have resulted in the redistribution of individual ferritin molecules within individual sections. Experience with such knife artifacts in amebae bearing large masses of ferritin (2) shows that their redistribution occurs in a recognizable pattern. No such pattern was observed in the material studied in this investigation. An artifact of this sort should be most readily detected in those amebae which were injected five times, and which, therefore, contained the highest concentration of ferritin. In such specimens the nuclei consistently contained ferritin, and mitochondria lying immediately adjacent to such nuclei contained ferritin in the space between the inner and outer membranes, but not within the inner matrix.

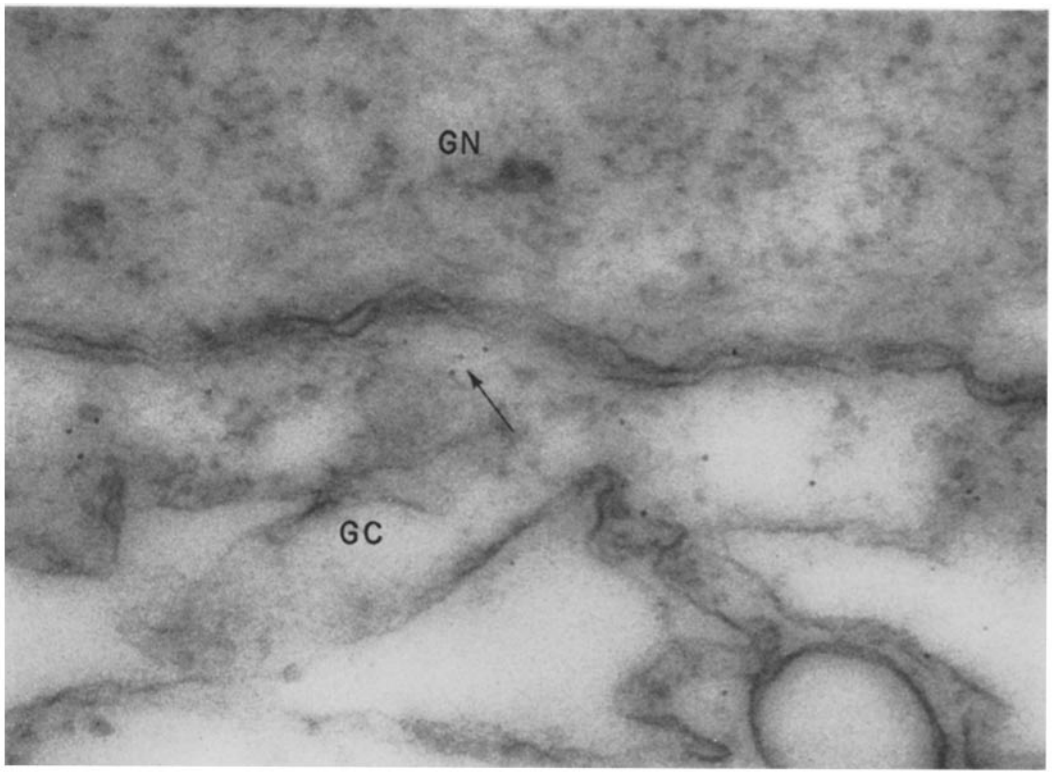

FIGURE 2

A portion of an ameba injected twice with ferritin solution and fixed after 7 minutes. In this case ferritin (arrow) can be seen in the ground cytoplasm (*GC*) but not in the ground nucleoplasm (*GN*). Magnification, 120,000.

This specific pattern of localization provides clear evidence that the ferritin molecules were not redistributed during sectioning.

It is concluded that in *Chaos chaos*, ferritin, a roughly spherical protein molecule having a diameter of 95 Angstroms (8), a molecular weight of approximately 550,000, and an isoelectric point of 4.4, can penetrate the nuclear membrane, the outer membrane of the mitochondria and the food vacuole membrane.

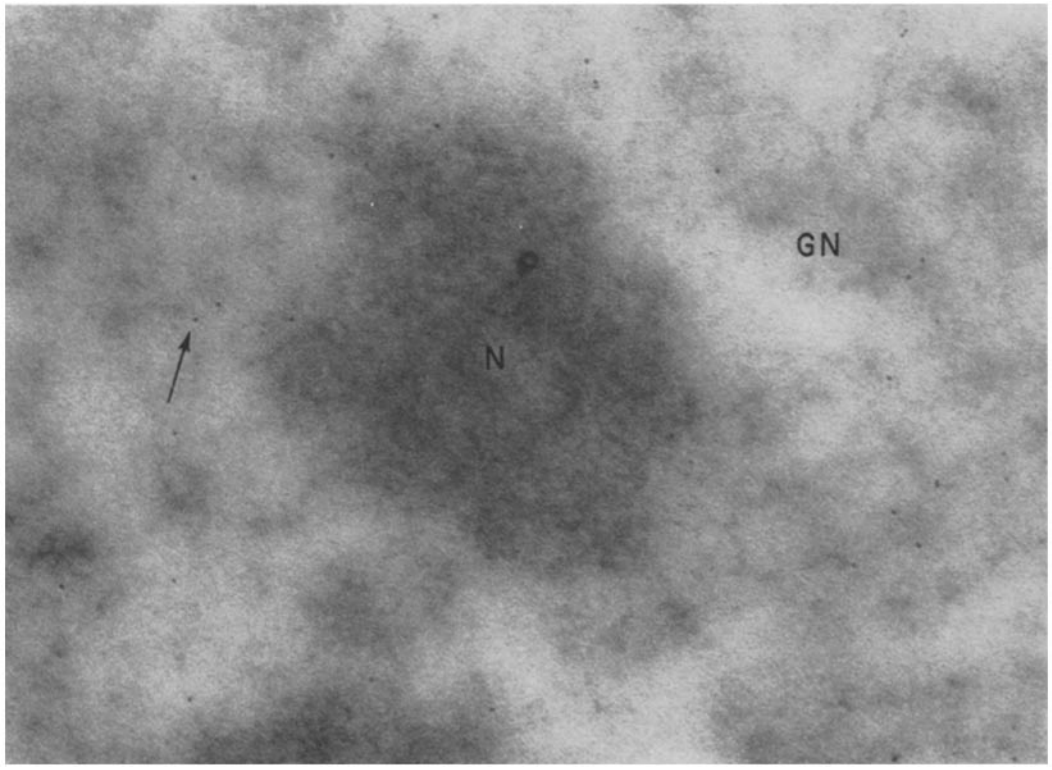

FIGURE 3

Part of the nucleus of an ameba which had been injected five times. Ferritin molecules (arrow) are scattered throughout the ground nucleoplasm (*GN*), but none are present in the nucleolus (*N*). Magnification, 94,000.

According to Brachet (9), the nuclear membrane in amebae is permeable to ribonuclease. Goldstein (10) has found that a substance, believed to be a protein, can pass from one nucleus to another in amebae, and even concentrate in the nuclei. The findings of this study concur with those of Brachet and of Goldstein, and indicate that nuclear permeability is not limited to smaller molecules such as ribonuclease.

Harding and Feldherr (11) microinjected solutions containing various concentrations of polyvinylpyrrolidone and serum albumin into frog oocytes. From the resulting volume changes of the nucleus, observed 3 minutes after injection, it was concluded that molecules having a molecular weight of 40,000 or larger do not freely penetrate the nuclear membrane. Feldherr and Feldherr (12) injected fluorescein-labeled gammaglobulin into *Cecropia* moth oocytes and could not detect any of the globulin in the nucleus after 10 minutes, again suggesting that the nuclear membrane in oocytes acts as a barrier to the penetration of macromolecules. There are three possible explanations which might account for the discrepancy between these results and those obtained in the present study: first, the permeability characteristics of the nuclear membrane in amebae may be different from those of oocytes; second,

the different results may be due to the fact that macromolecules of different sizes, shapes, and charges were used; and third, it is possible that some penetration of macromolecules into the nuclei of the oocytes had occurred, but that the methods employed were not sensitive enough to detect such substances in low concentrations.

6 months after injecting saccharated iron. Richter (17) also identified small amounts of ferritin in the nuclei and mitochondria of macrophages. The mitochondria of erythroblasts contain ferritin (18), the concentration being particularly high during certain illnesses. Since the history of the cells studied in these investigations was

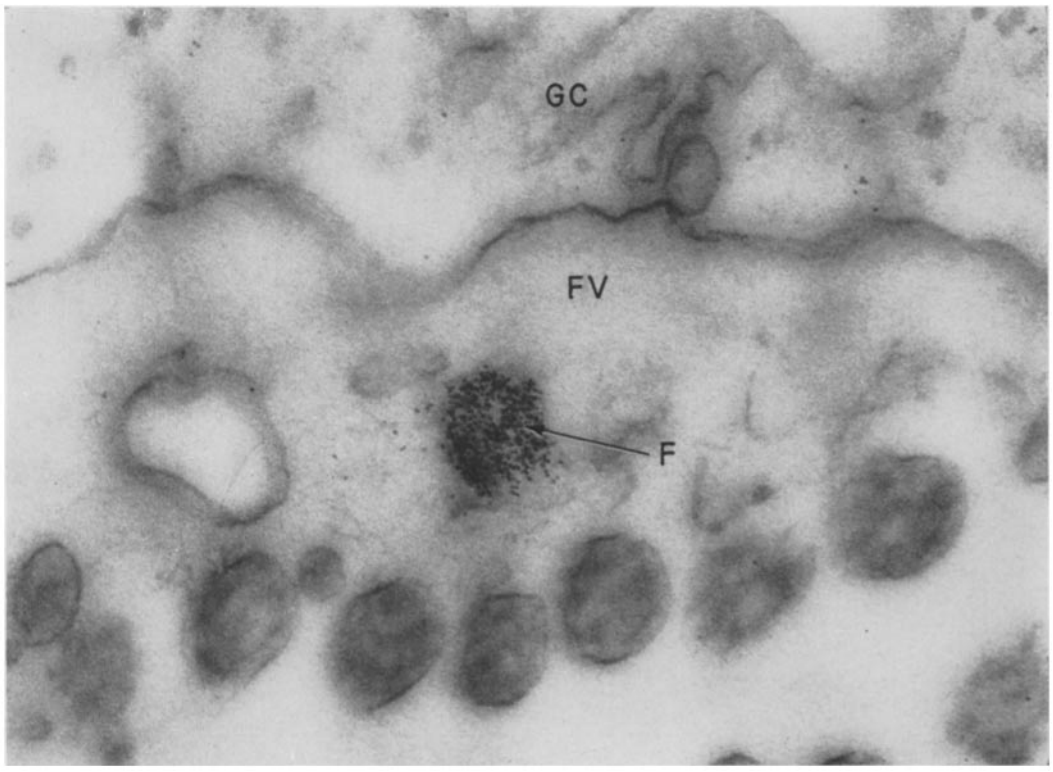

FIGURE 4

A micrograph showing an aggregation of ferritin molecules (*F*) within a vacuole (*FV*). A portion of the ground cytoplasm (*GC*) is also visible. This ameba was injected 5 times and fixed after 24 hours. By the presence of undigested organelles of *Paramecium*, which are not shown here, this vacuole was identified as a food vacuole. Magnification, 90,000.

With the aid of electron microscopy, the distribution of ferritin has been studied within mammalian somatic cells which normally contain this protein. In liver cells, Kuff and Dalton (13) found ferritin distributed in the ground cytoplasm, but stated that none is present in the nuclei or mitochondria of liver cells. Similar results were obtained by Bessis and Caroli (14). Kerr and Muir (15) found ferritin in both the nuclei and mitochondria of liver cells. Richter (16) found large aggregates of ferritin in the nuclei of liver cells

not known, the significance of the results in relation to the permeability of the nuclear membrane and the membranes of the mitochondria cannot be determined. It is possible, in the cases in which ferritin was found in the nuclei and mitochondria, that it did not enter by penetrating the membranes of these organelles, but instead was incorporated during the processes of nuclear division and mitochondrial formation. It is also possible, under certain conditions, that the nuclei may synthesize ferritin (16).

The distribution of foreign proteins in somatic cells has been studied by the use of antigen-antibody staining techniques, and also with labeled proteins. The results of these experiments, which have been summarized by Holtzer and Holtzer (19), are too varied to permit any general conclusions to be drawn concerning the permeability of the nuclear membrane in somatic cells.

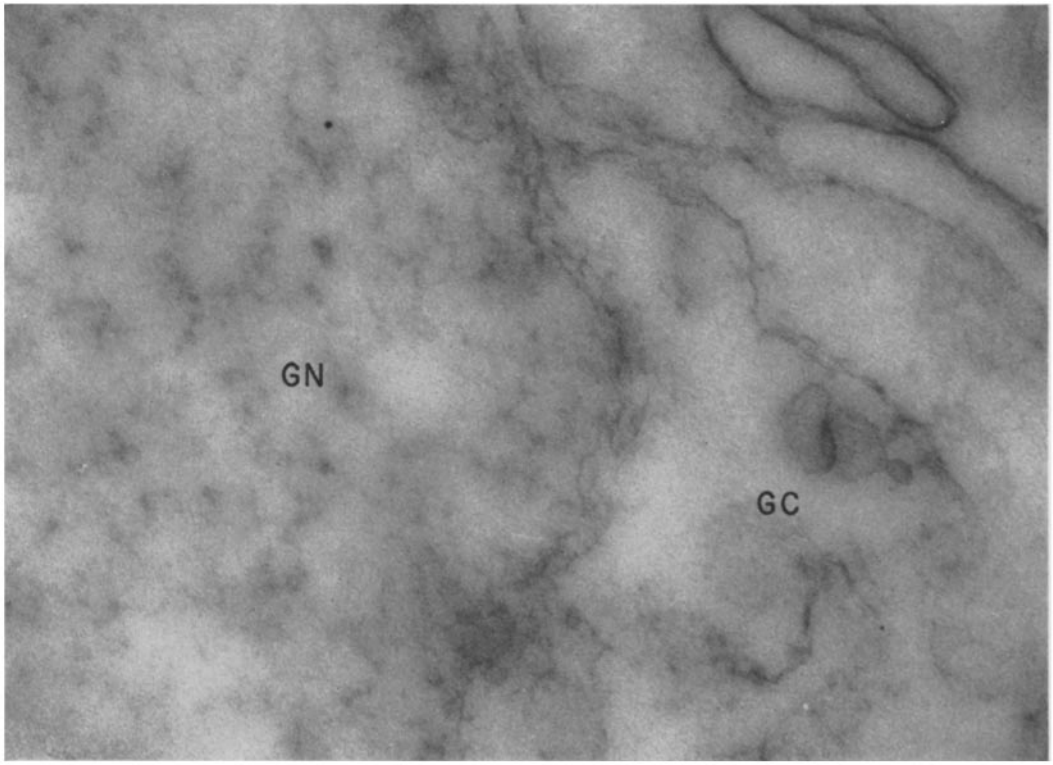

FIGURE 5

A section through an ameba injected five times with salt solution which did not contain ferritin, and fixed after 24 hours. No dense particles resembling ferritin can be seen in either the ground cytoplasm (*GC*) or ground nucleoplasm (*GN*). Magnification, 100,000.

The results reported here indicate that the test protein used could gain access to at least three cell components from the ground cytoplasm. The nature of the evidence presented does not permit any conclusions regarding the specific mechanisms involved. It would seem from previous studies (20, 21) that the so called "pores" of the nuclear membrane represent one possible pathway for macromolecules to enter the nucleus. It might be expected that if the ferritin did enter through the "pores" the concentration of this protein would be higher in corresponding areas of the nucleus, especially in amebae fixed a short time after injection. No such local concentration was detected. However, calculations of the diffusion rate of ferritin, under conditions which might reasonably exist in these experiments, indicate that movement of the molecules away from their point of entrance would be too rapid to permit such a concentration to be built up. Consequently, the mechanisms by which ferritin passes from the ground cytoplasm into the nucleus remain conjectural. Similarly, the mechanisms by which ferritin enters the mitochondria and the food vacuoles remain to be analyzed experimentally. Further studies with ferritin and other "electron-opaque" particles of varying size and chemical composition should permit such an experimental analysis.

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