THE EFFECT OF THE ELECTRON-OPAQUE PORE MATERIAL ON EXCHANGES THROUGH THE NUCLEAR ANNULI

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ABSTRACT

To investigate the extent to which the electron-opaque pore material can regulate nucleocytoplasmic exchanges which occur through the nuclear annuli, experiments were performed in which polyvinylpyrrolidone (PVP)-coated colloidal gold particles (25 to 170 A in diameter) were microinjected into the cytoplasm of amebas (*Amoeba proteus*). The cells were fixed at various times after injection and examined with the electron microscope in order to determine the location of the gold particles. High concentrations of gold were found associated with the pore material at specific points adjacent to and within the pores. It is tentatively suggested that such specific accumulations could be a means of selecting substances from the cytoplasm for transport through the pores. Particles were also scattered throughout the ground cytoplasm and nucleoplasm. A comparison of the diameters of particles located in these two regions showed that the ability of materials to penetrate the nuclear envelope is a function of their size. It was estimated that the maximum size of the particles able to enter the nucleus is approximately 125 to 145 A in diameter. The regulation of exchanges with regard to particle size is thought to be dependent on the specific organization of the electron-opaque pore material.

INTRODUCTION

Although the pores of the nuclear envelope are able to serve as pathways for the nucleocytoplasmic exchanges of macromolecules, it is becoming increasingly clear that these areas are not simply spaces which permit the free diffusion of such substances between the nucleus and the cytoplasm (1–4). The material which prevents free diffusion apparently corresponds to the electron-opaque substance which can be seen, with the electron microscope, adjacent to and within the pores.

Aside from acting as a diffusion barrier, the electron-opaque material may also be operative in selecting the substances to be transported across the nuclear envelope. For example, it has been shown, using isolated nuclei, that coated colloidal gold particles can adhere to the pore material. The extent of attachment is dependent, to some degree, on the nature of the coating agent (5). Such attachment could conceivably regulate nucleocytoplasmic exchanges either directly, by selecting and accumulating specific substances for transport through the pores, or indirectly, by altering the chemical characteristics of the pores.

Another factor in addition to binding which may regulate passage between the nucleus and cytoplasm is molecular size. There is evidence suggesting that the electron-opaque material within each pore forms a specific structural element (3, 6–8).

If this were the case, the area of the pore through which exchanges can take place would be limited, thus restricting the movement of certain macromolecules.

The present investigation has two objectives: first, to determine whether substances can attach to the pore material in intact cells; and second, to determine the effect of particle size on nucleocytoplasmic exchanges. As in earlier studies (3), the methods employed involve the microinjection of PVP-coated colloidal gold particles into the cytoplasm of amebas. Examination of the cells with the electron microscope at various times after injection revealed the general distribution of the gold, and allowed measurement of the diameters of the particles located in different regions of the amebas. The results suggest that the pore material is of importance in controlling the movement of macromolecules between the nucleus and cytoplasm.

MATERIALS AND METHODS

All experiments were performed on *Amoeba proteus*. The use of mononucleated organisms facilitated binding studies, inasmuch as high concentrations of gold could be injected directly adjacent to the nucleus. Furthermore, the specific region of the nucleus which was thus exposed to the colloid could readily be located and observed with the electron microscope.

The amebas were cultured in a medium consisting of 4.8×10^{-4} M $CaCl_2$, 4.8×10^{-5} M $MgSO_4$, 1.6×10^{-4} M K_2HPO_4 and 1.2×10^{-4} M KH_2PO_4, dissolved in ion-free water. The pH was approximately 6.8. *Tetrahymena pyriformis* were supplied to the cultures as a source of food.

The amebas were starved for a period of 24 to 72 hours before being used. Starvation insured that division would not occur during the course of the experiments.

Colloidal Gold Preparations

Two different gold preparations were utilized. One contained particles which were approximately 25 to 55 A in diameter and the second was composed of particles 30 to 170 A in diameter. These preparations will be referred to as the S- and L-fractions respectively. Except for minor modifications, the S-fraction was prepared as described previously (3). The modifications were as follows: 2 ml rather than 2.5 ml of 0.6 per cent chlorauric acid were used, and the amount of 0.72 N K_2CO_3 which was added in the initial step of the preparation was reduced from 3 ml to 0.75 ml. The remaining steps, including protection with polyvinylpyrrolidone (PVP), and the centrifugation procedures were unchanged.

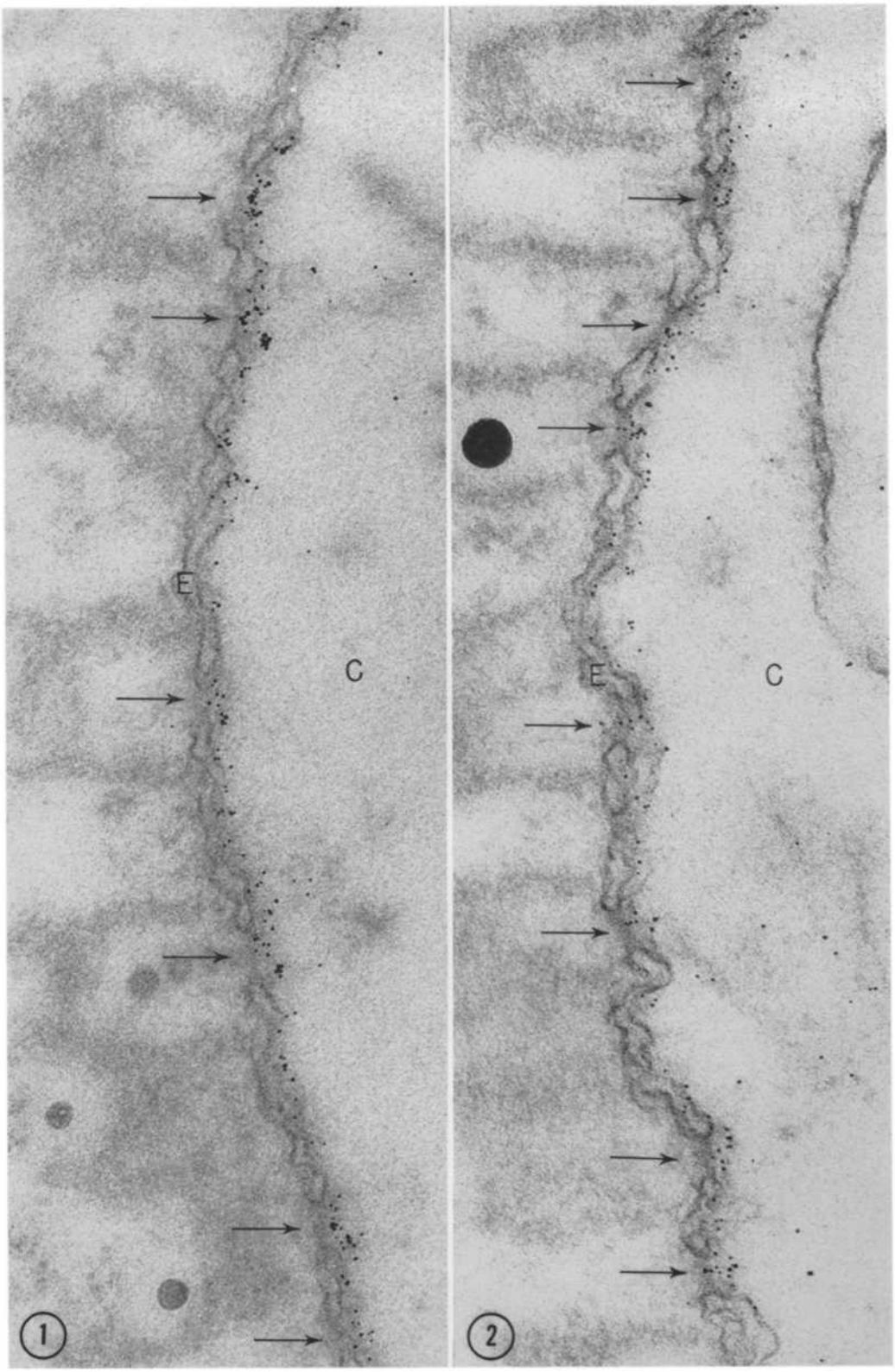
The preparation of the L-fraction involved additional modifications, and these will be considered in more detail. Six to 7 ml of 0.6 per cent chlorauric acid and 0.75 ml of 0.72 N K_2CO_3 were added to 120 ml of ion-free water. This was followed by the addition of 1 ml of an ether solution of phosphorus, which contained 4 parts of ether and 1 part of a saturated ether solution of phosphorus. The mixture was allowed to stand at room temperature for 15 minutes and was then boiled for 2½ to 3 minutes. The gold sols, which were purple at this stage, were cooled to room temperature and stabilized by the addition of 12 mg of PVP (average mol wt 15,000). Fractionation was carried out as follows: The stabilized colloid was first centrifuged at 6,500 *g* for 10 minutes, the precipitate was discarded and the supernatant was centrifuged at 26,000 *g* for 15 minutes. The precipitate obtained after this step was resuspended in ion-free water and centrifuged again at 26,000 *g* for 15 minutes. The final precipitate was collected in a concentrated state and dialyzed against a medium containing 0.0127 M KCl, 0.0016 M K_2HPO_2, and 0.0011 M KH_2PO_4 (9). The pH was 7.0 to 7.1. The apparent optical density of the sols fell within a range of 17 to 20 (λ = 500 mμ, 1 cm light path).

It is significant that the gold particles in both the S- and L-fractions were stabilized with PVP. This process involves the adsorption of PVP on the surface of the colloidal particles. As a result, the individual particles possessed similar chemical properties, and, therefore any variations in their distribution within the cells could be attributed to size differences rather than to chemical differences.

Microinjection and Controls

All amebas were injected once, at a point in the cytoplasm just adjacent to the nucleus. The amount of sol injected in each case corresponded to about ⅕ the volume of the cell. To study the possible physiological effects of injection, 23 control experiments were performed in which amebas were injected with either the S- or L-gold fractions and then fed *Tetra-*

FIGURES 1 AND 2 Electron micrographs of amebas injected with the 25 to 55 A gold fraction and fixed after 7 and 10 minutes, respectively. Gold particles can be seen in the ground cytoplasm (*C*) and along the outer membrane of the nuclear envelope (*E*). Gold is also present adjacent to and within the pores (arrows). × 140,000.


E
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hymena. The control amebas were observed to determine whether they were capable of normal movement, feeding and division.

Electron Microscopy

Experimental amebas which were injected with the S-fraction for study by electron microscopy were fixed for 3 minutes, 5 minutes, 7 minutes, 10 minutes, and 24 hours after injection. Organisms injected with the L-fraction were fixed after 10 minutes, 1 hour, and 24 hours. In all, 34 amebas were used.

The cells were fixed in 1 per cent buffered OsO_4, dehydrated in alcohol, and embedded in epoxy resin. The sectioning procedure varied for different experiments. In all instances, however, the nucleus was first located by examining thick sections with a phase-contrast microscope. In amebas fixed after 24 hours, thin sections (600 to 900 A) were then cut at two or three different levels through the nucleus, and electron micrographs were taken of randomly selected areas. In the short-term experiments (3 minutes to 1 hour) thin step sections were taken every 2 to 4 microns once the nucleus was located. When these sections were examined with the electron microscope, a specific region immediately adjacent to the nucleus could be found which contained an extremely high concentration of gold. This area, which was presumably the site of injection, was then photographed. All preparations were examined with a Siemens Elmiskop I at magnifications of 20,000 and 40,000.

In determining the effect of particle size on nucleocytoplasmic exchanges, it was necessary to measure the diameters of the colloidal particles in the L-fraction. The measurements were made directly from the electron micrographs and did not include the thickness of the protective PVP coat.

RESULTS

Controls

All of the 23 control amebas were able to move and feed normally following injection; furthermore, 21 of the cells ultimately divided. These results demonstrate that the normal physiological activities of the organisms were not seriously altered by the injection procedures.

S-Fraction

The general distribution of the gold particles located at the site of injection in the 3 to 10 minute experiments can be summarized as follows: first, particles were located in the ground cytoplasm in which they were randomly distributed; second, there was a high concentration of gold along the nuclear envelope, but not along any other membrane system; and third, colloidal particles were consistently found in the nucleus although the concentrations, particularly those at 3, 5, and 7 minutes after injection, were extremely low compared to the concentrations in the cytoplasm.

A more detailed examination of the nuclear envelope revealed that gold particles were associated with the electron-opaque pore material and exhibited a specific spatial distribution in relation to the pores (Figs. 1 to 3). Particles were located immediately adjacent to the pores on the cytoplasmic side of the envelope, and also within the pores. Those adjacent to the pores were frequently present in high concentrations and, in perpendicular sections, were found at any point along a plane formed by visualizing the outer membrane of the nuclear envelope as being continuous over the pore. The particles within the pores were located, in the majority of cases (185 out of 207), in the centers of the pores.

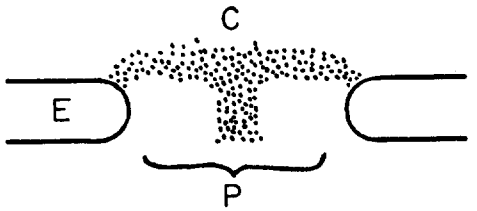
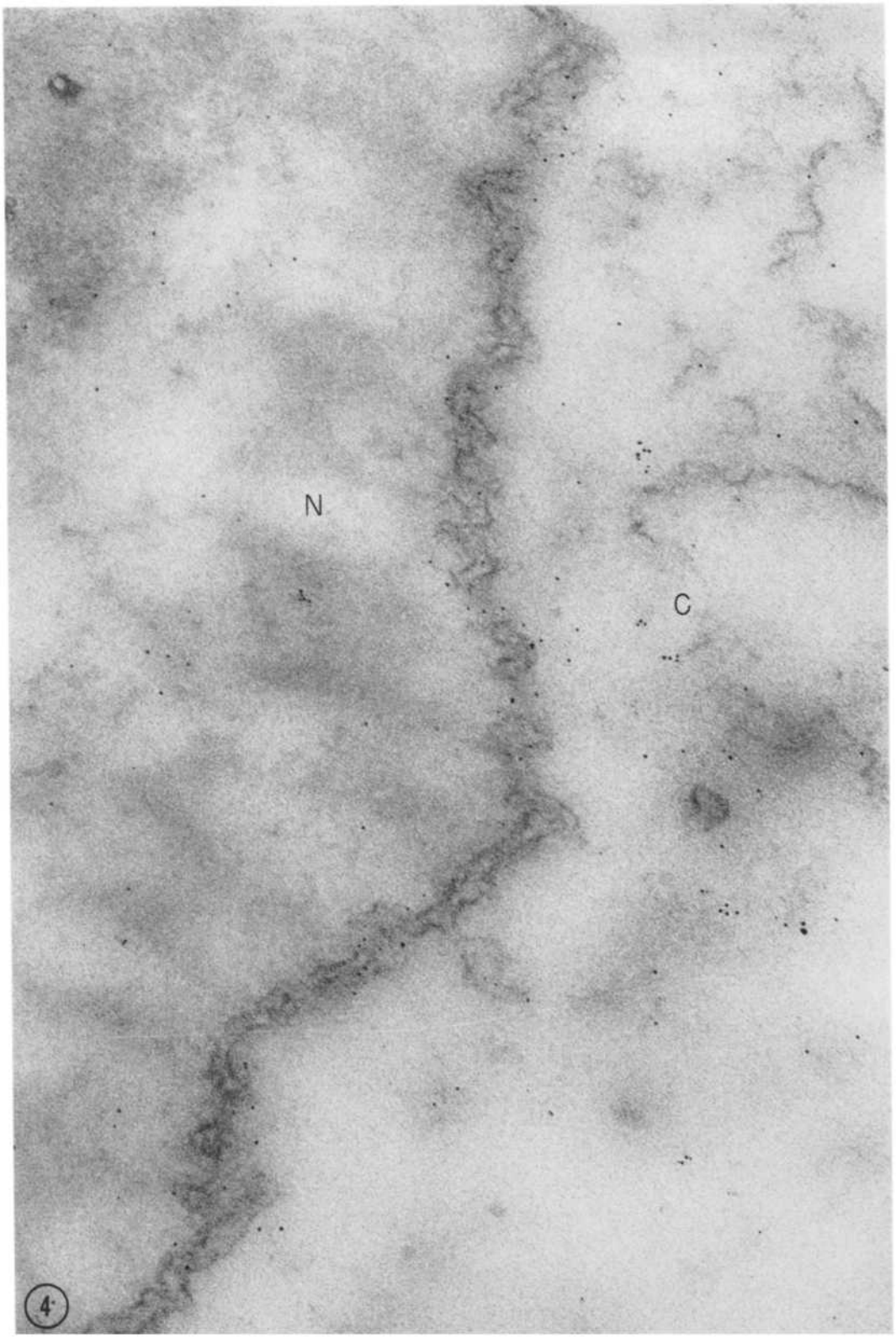


FIGURE 3 A diagrammatic representation showing the distribution of colloidal gold particles in relation to the pores. The dotted area represents the region in which gold was most commonly found. *C*, cytoplasmic side of the nuclear envelope; *E*, nuclear envelope; *P*, pore area.

In amebas fixed at 24 hours after injection, gold particles were randomly scattered throughout the nucleoplasm and ground cytoplasm (Fig. 4). The amounts of colloid in these two areas appeared to be equal. This represents a variation from the results obtained with the giant ameba *Chaos chaos* in

FIGURE 4 An ameba fixed 24 hours after being injected with the small gold fraction. Colloidal particles are scattered throughout the ground cytoplasm (*C*) and nucleoplasm (*N*). × 140,000.


N
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4

which the gold was concentrated in the nucleus (3). As in *Chaos*, colloidal particles were rarely seen in the mitochondria, but were frequently found in food vacuoles.

L-Fraction

In most respects, the general distribution of the large gold particles resembled that obtained with the S-fraction. Thus, in all experiments (10 minutes, 1 hour, and 24 hours) gold particles were randomly distributed in the ground cytoplasm. At the intervals of 10 minutes and 1 hour, the gold particles at the site of injection were concentrated along the outer membrane of the nuclear envelope and in the pore area (Figs. 5 and 6). No gold had entered the nucleus within 10 minutes, but particles were present in this organelle after 1 hour and 24 hours.

In determining the size of particles able to enter the nucleus, two assumptions were made: first, that all particles concentrated along the cytoplasmic surface of the nuclear envelope were available to the nucleus, and if any such particles were unable to enter the nucleus, it was not due to inaccessibility; second, that any group of particles able to penetrate the nuclear envelope would have done so within 1 hour after injection. In practice, two sets of data were obtained. In the first set, the size of particles located along the nuclear envelope in amebas fixed after 10 minutes was compared to the size of an equal number of particles found within the nucleus after 24 hours. In the second set of data, amebas fixed after 1 hour were used, and measurements of particles adjacent to and within the nucleus were made on the same organism. The data are given in Table I. Measurements were made on a total of 18 amebas and the particles measured were selected at random.

DISCUSSION

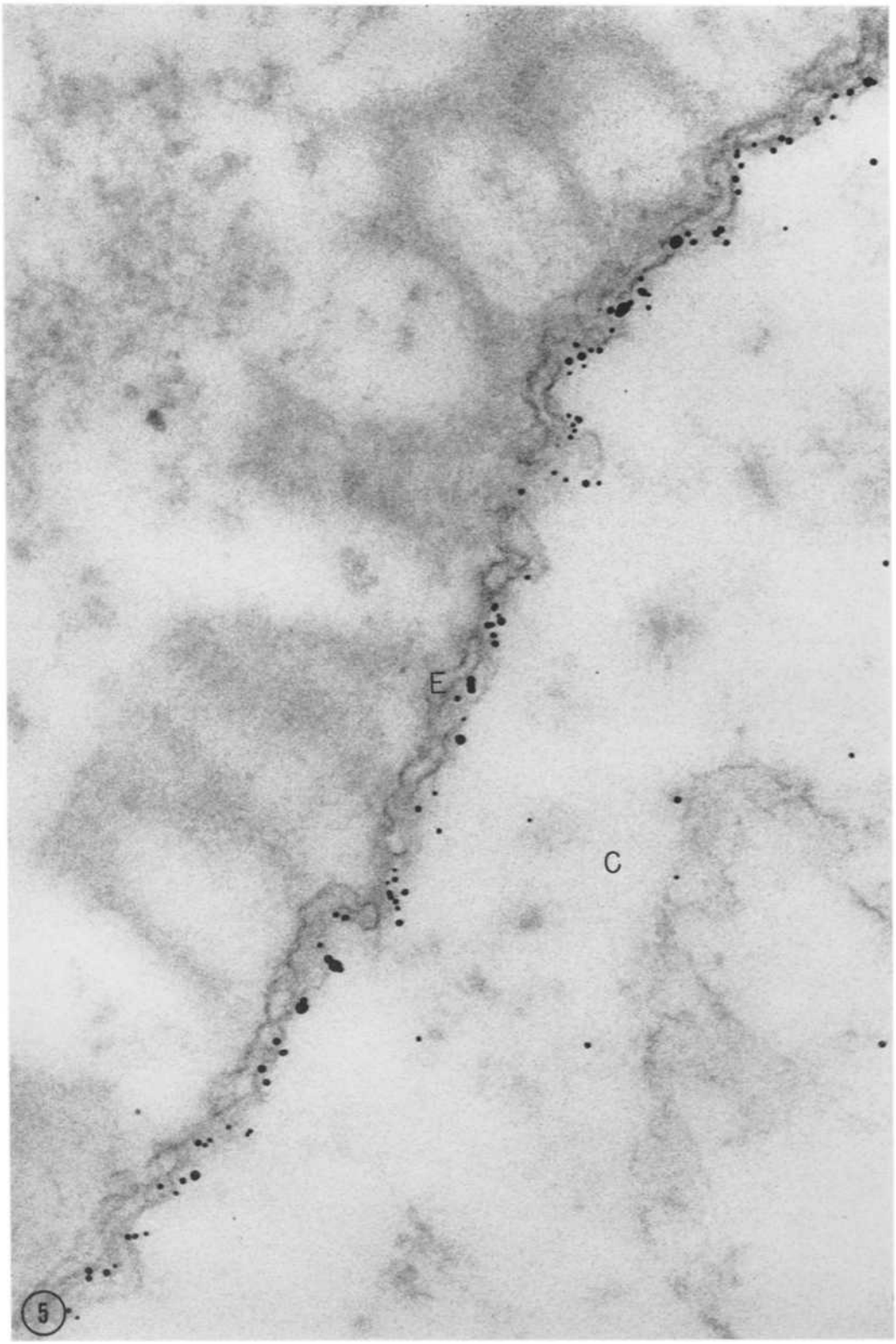
Before discussing the significance of the results, it is necessary to consider the extent to which the cells may have been injured during the injection procedures. This is of some importance since the observed gold distributions may be due in part to alterations of the nuclear envelope resulting from the injections. The possibility that the injections caused extensive damage to the nuclei is unlikely for two reasons: first, the control experiments indicate that various physiological activities which depend on the proper functioning of the nucleus are not altered by introducing colloidal gold into the cytoplasm; second, no morphological changes could be detected in experimental cells examined with the electron microscope. On the basis of this evidence, it is felt that the results do give an indication of events which are normally occurring in the cells. It should be kept in mind, however, that a subtle form of injury, not detected by the methods used, may have taken place upon injection. For example, a transitory stickiness of the pore material might result from the injections, thus influencing the degree to which gold would accumulate in the pore area.

In an earlier report it was shown, using isolated oocyte nuclei, that PVP-coated gold particles can bind to the electron-opaque material located within and adjacent to the pores of the nuclear envelope (5). The present results suggest that attachment to the pore material can also occur in intact amebas, since the PVP-coated gold particles were concentrated along the nuclear envelope and at points within and adjacent to the pores. Similar concentrations of gold were not observed along any other membrane systems.

The observation that gold particles are located specifically in the centers of the nuclear pores confirms previous results obtained with the giant ameba *Chaos chaos* (3). These findings can most easily be explained by assuming that the material within each pore forms a tubular element, and that the passage of macromolecules is restricted to the lumen of the tube.

The results obtained with the large gold fraction indicate the degree to which the pore material can regulate the exchanges of particles of different size. These results show that gold particles up to approximately 85 A in diameter can readily enter the nucleus. Particles ranging from 89 to 106 A in

FIGURE 5 A section through an ameba injected with the 30 to 170A gold fraction. The cell was fixed 10 minutes after the injection. Gold particles are present in the ground cytoplasm (*C*) and along the nuclear envelope (*E*). × 140,000.


E
C
5

diameter were also able to penetrate the nuclear envelope, but in limited numbers. Most significant, however, is the fact that particles larger than 106 A (110 to 170 A in diameter) were almost entirely excluded from the nucleus.

It has been pointed out that the measurements of particle diameter do not include the PVP-coat. In attempting to measure the thickness of the coat, a number of methods were employed, including shadow casting, negative contrast staining, and determinations of the distance between particles packed on Formvar-coated grids. In no instance could an appreciable difference be detected between the over-all size of coated particles and that of non-coated particles which were used as controls. Although no absolute values could be obtained, the results do indicate that the PVP-coat is not extensive. Thus, an estimate for the hydrated coat thickness of approximately 10 to 20 A is probably a reasonable one. Using this value, and assuming that the PVP-coat remains intact following injection (see Addendum), the maximum size of particles able to enter the nucleus would be about 125 to 145 A rather than 106 A. The fact that this figure is considerably less than the diameter of the pores, estimated at approximately 640 A (10), indicates that the electron-opaque pore material fills a large portion of the pore area.

Even below the size at which particles of colloidal gold are excluded from the nucleus, the dimensions of these particles are significant in determining the rate of penetration. This is demonstrated by the fact that no particles in the L-fraction were found in the nucleus after 10 minutes, whereas the particles in the S-fraction had penetrated the nuclear envelope within 3 minutes.

In considering the present results, it should be kept in mind that the factors regulating the nucleocytoplasmic exchanges of small molecules and even certain macromolecues could be quite different than those suggested by experiments with colloidal gold. For example, the electrical studies of Kanno and Loewenstein (11) indicate that ions are able to diffuse freely through the pores of amphibian oocyte nuclei, apparently unaffected by the pore material. It has been shown, on the other hand, that PVP and bovine serum albumin cannot freely diffuse into the nuclei of similar cells (1).

In conclusion, evidence has been presented which suggests that PVP-coated gold particles are able to attach to the pore material in intact amebas. Although the exact function of attachment is not known, it is interesting to speculate that it could be a means of selecting macromolecules for exchange across the nuclear envelope. A second factor which seems to affect both the ability of particles to enter the nucleus and their rate of entrance is molecular size. The maximum size of particles able to penetrate the pores is estimated to be about 125 to 145 A in diameter. Regulation with regard to particle size seems to depend on the specific arrangement of the electron-opaque material within the pores.

Addendum: The estimate of the maximum size of particles able to enter the nucleus is based partially on the assumption that the PVP adsorbed to the gold particles is not displaced by some component of the cytoplasm following injection. If such displacement were to occur, the over-all effective diameters of the colloidal particles could be greatly altered.

To experimentally determine the relationship between the gold particles and the protective agent after injection, the following procedure was employed: first, gold sols were prepared and protected with ferritin, making it possible to visualize both the colloid particles and the stabilizing agent with the electron microscope; second, the ferritin-gold particles were injected into the cytoplasm of amebas, and the cells were fixed after 1 hour. Subsequent examination with the electron microscope showed that the gold particles present in the cytoplasm were almost always associated with one or more ferritin molecules (Fig. 7), indicating that the ferritin molecules remain bound to the gold following injection. This experiment does not prove that PVP also remains bound to the gold after injection, but it does provide a basis for making such an assumption.

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FIGURE 6 A portion of an ameba injected with the large gold fraction, and fixed after 1 hour. Colloidal particles are concentrated along the nuclear envelope (*E*). By this time, a few particles have entered the nucleus (*N*). *C*, ground cytoplasm. × 140,000.

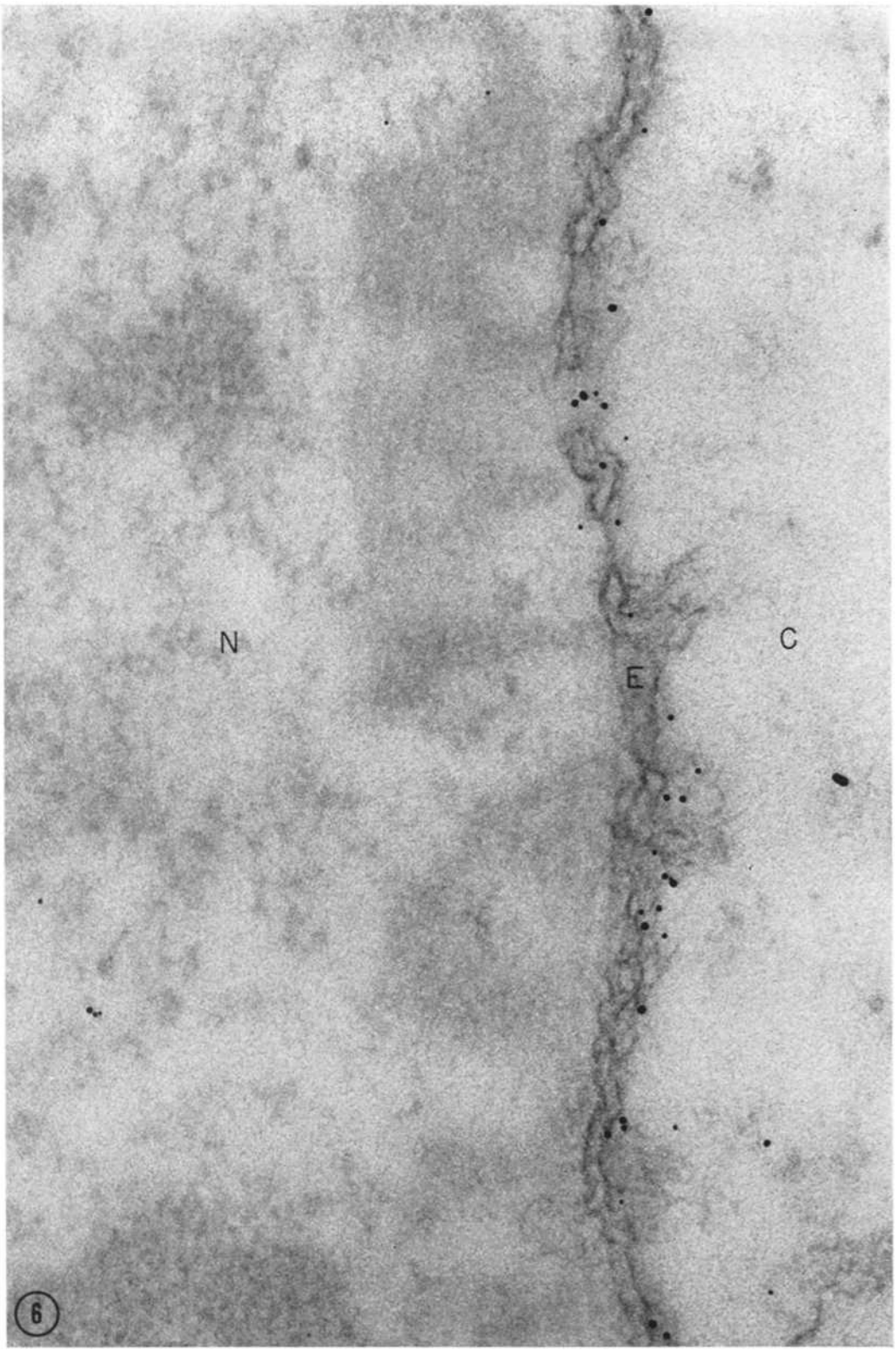
N
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TABLE I

The Size Distribution of Colloidal Gold Particles Located Adjacent to and within the Nucleus

Particle size	10-Minute and 24-Hour data		1-Hour data		Combined results	
	Adjacent*	Within‡	Adjacent	Within	Adjacent	Within
A						
30–43	31	24	14	12	45	36
47–64	181	250	89	144	270	394
68–85	157	166	102	91	259	257
89–106	60	21	30	7	90	28
110–170	33	1	19	0	52	1
Totals	462	462	254	254	716	716

* Particles adjacent to the nuclear envelope in 10-minute postinjection cells.
‡ Particles within the nucleus in 24-hour postinjection cells.

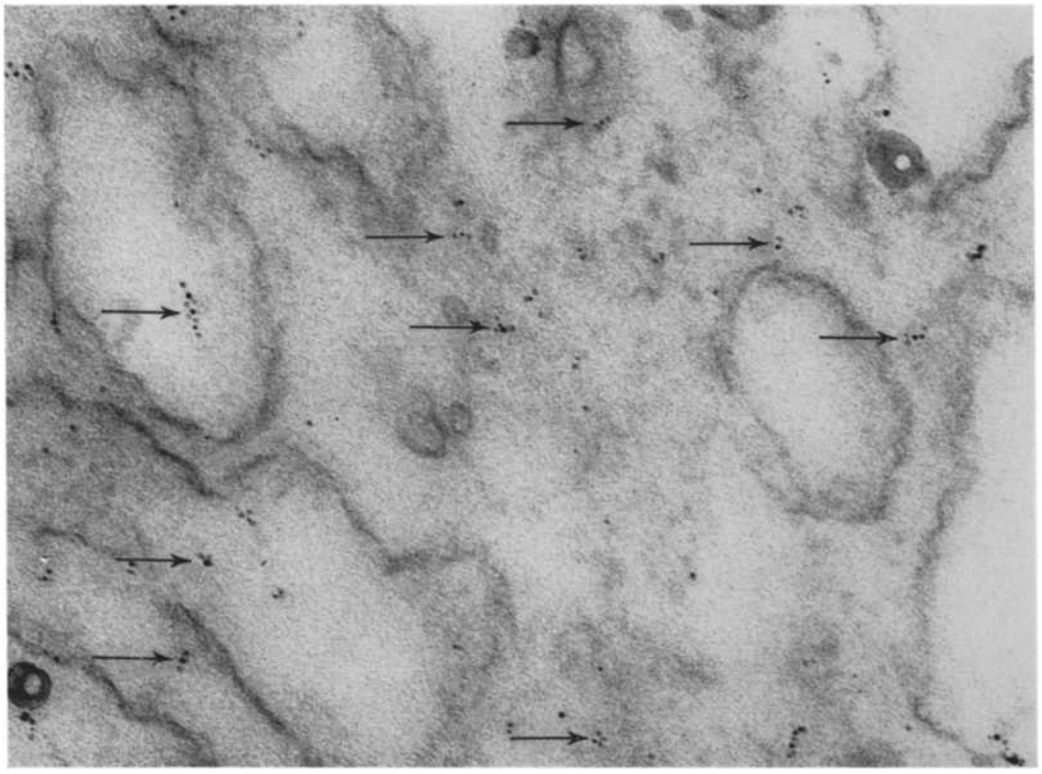

FIGURE 7 An electron micrograph of an ameba injected with ferritin-gold particles (arrows) and fixed after 1 hour. The close association between the gold (the denser particles) and the ferritin can be seen. × 140,000.

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