STUDIES ON PROTEIN UPTAKE BY ISOLATED TUMOR CELLS

I. Electron Microscopic Evidence of Ferritin Uptake by Ehrlich Ascites Tumor Cells

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ABSTRACT

Ferritin, added to the incubation medium of ascites tumor cells, was used as an electron microscopic marker to study the uptake of large protein molecules by morphologically intact cells. A definite uptake could be detected after 1 hour of incubation in Tyrode bicarbonate solution containing 0.04 to 13.3 mg ferritin/ml. Ferritin was found in a variety of membrane-surrounded structures, suggesting that pinocytosis and related membrane movements are occurring under physiological conditions and can account for the penetration of intact macromolecules into isolated tumor cells. Supplementation of the medium with serum albumin (33 mg/ml) increased the average amount of ferritin per cell and per pinocytotic structure. Ferritin was strongly adsorbed by fragments of lysed cells, which were readily taken up by intact cells. Besides its role as carrier, this debris appeared to stimulate membrane movements. Only rare examples were found to suggest the release of ferritin from the pinocytotic structures into the cytoplasm. Thus, the disintegration of such structures cannot be considered an obvious step towards a rapid metabolic utilization of protein by the cell. Particles of colloidal gold presented to the cell under the same conditions were not taken up to any significant extent, thus providing good evidence for a selective ingestion of particles of comparable sizes.

INTRODUCTION

It has been known for some decades that unicellular organisms and the phagocytic elements of higher species share the property of ingesting intact macromolecules and supramolecular aggregates. Morphological studies of this uptake, inspired by Lewis (24), taken up by Holter and Marshall (17), and pursued by Chapman-Andresen (6–8), Schumaker (35), Brandt (3), and Holtzer and Holtzer (21) have emphasized the importance of membrane invaginations and vesiculations in this process of ingestion. The term pinocytosis, used originally by Lewis (24) and then by Mast and Doyle (26) to describe membrane movements leading to cell drinking, was extended by Holter (18) to include movements leading to macromolecular ingestion. Bennett, furthermore, proposed the hypothesis that pinocytosis might be a transport mechanism of general importance for mammalian cells (1).

The demonstration of an uptake of unaltered

protein molecules by mammalian cells other than the well recognized phagocytes came from experiments of Coons *et al.*, using fluorescent antibody techniques (10). These authors studied the tissues of rats after the injection of various proteins into the whole animal. By exposing frozen tissue sections to specific fluorescent antibodies they could detect antigen-antibody complexes localized intracellularly, indicating a penetration of the cells by proteins. Simple labeling of albumin with fluorescein was used by Holter and Holtzer (19, 21) to compare the albumin uptake by various mammalian tissues *in vitro*. By analogy to observations made on unicellular organisms (35) and in spite of the relative inadequacy of fluorescent microscopy for localization studies on small cells, these authors postulated that albumin was taken up by pinocytotic processes. In these comparative studies, ascites tumor cells appeared to display a moderate pinocytotic activity. The uptake of specific proteins has been subsequently documented both by histochemical (38) and electron microscopic (28) observations on the tubular cells of the rat kidney. Likewise, tumor cells in culture have been shown to ingest gold particles when grown for several days in the presence of colloidal gold (16). There are no electron microscopic observations, however, on the uptake of protein by tumor cells, nor any electron microscopic demonstration of sizeable ingestion of macromolecules or supramolecular particles by tumor cells after short periods of incubation. Such data would be valuable to assess the importance of a direct protein uptake in the protein metabolism of tumor cells.

Data on the nitrogen metabolism of tissue culture cells indicate that proteins of the medium are utilized to an important extent by the growing normal (15, 27) and tumor cell (22). It has been suggested also by Busch and Greene (4) that tumor tissue might display a greater uptake of whole proteins than most normal tissues. These authors, on the basis of protein-C^{14} fixation by the tissue of tumor-bearing rats, have postulated that the protein uptake could account for doubling the weight of the tumor within 24 hours. These studies, however, do not provide conclusive evidence that proteins are taken up as whole macromolecules. Furthermore, they stand in contradiction to the relatively low degree of pinocytosis described for ascites tumor cells (21) and HeLa cells in culture (16). It appeared desirable, therefore, to clarify this point, in particular to re-examine the optical evidence of protein uptake using an iron-containing and electron-opaque protein, ferritin, as a marker. The adequacy of ferritin for studying the cellular ingestion of proteins has been well documented by the work of Farquhar and Palade (12, 13). It was felt that an electron microscopic study of ascites tumor cells, incubated in the presence of ferritin, would provide conclusive information on the occurrence, the mechanism, and the order of magnitude of this penetration process. This paper will also compare briefly the uptake of ferritin and colloidal gold. Its main purpose, however, is to serve as a basis for further studies on the quantitative aspect of protein uptake by tumor cells (33).

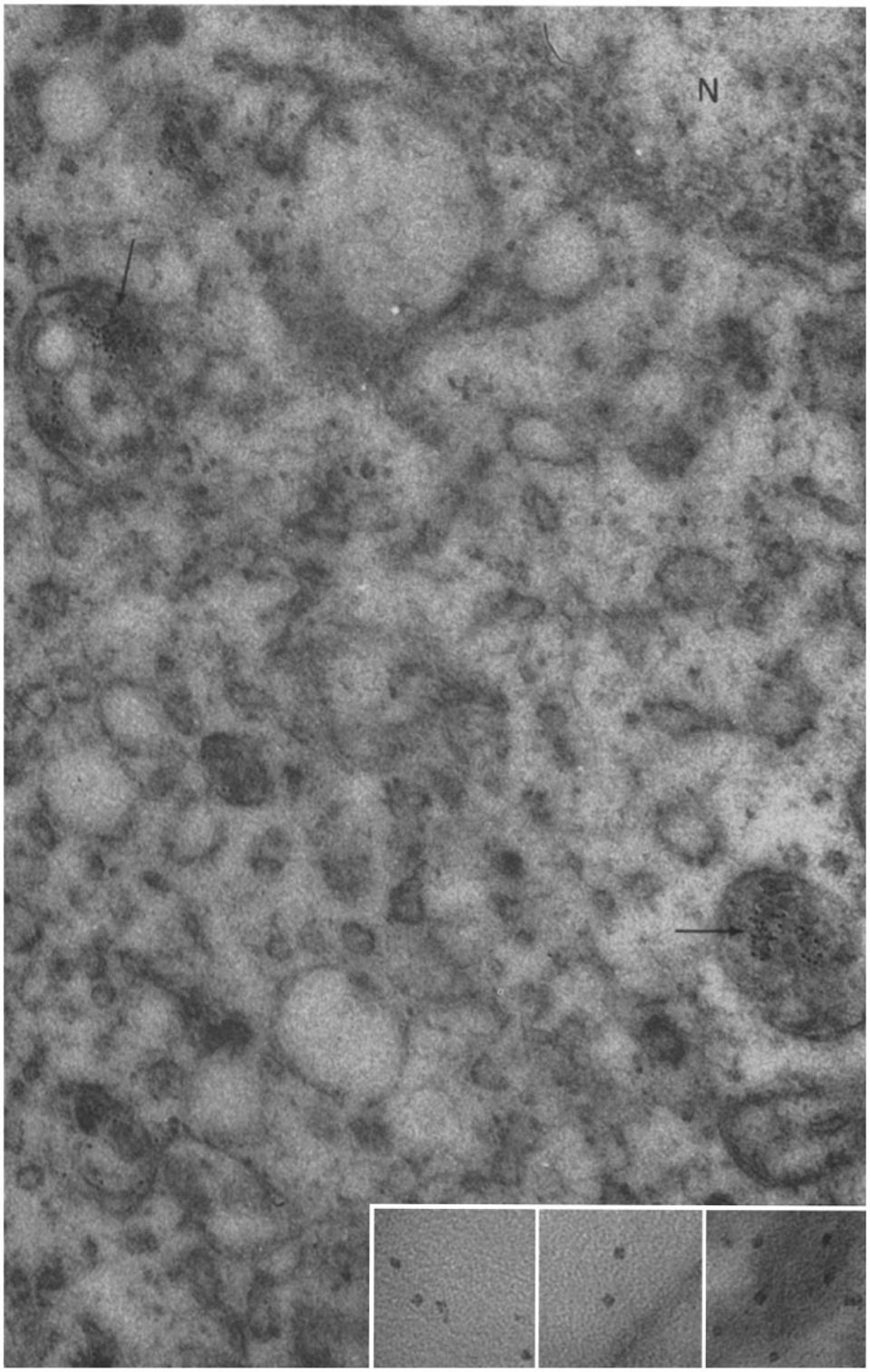
MATERIALS AND METHODS

A hyperdiploid strain of Ehrlich ascites tumor cells maintained in A strain mice was used exclusively. The tumor cells were harvested 7 to 8 days after intraperitoneal inoculation of 0.1 ml of ascites fluid. The cells were gently spun, the ascites fluid decanted, and the resulting pellet was resuspended with cold Tyrode bicarbonate solution. This suspension was counted and in all experiments 5×10^7 cells were incubated in a total of 1.5 ml. The basic incubating medium contained a bicarbonate buffer which was adjusted to pH 7.3–7.4 before use. The incubation was performed in a metabolic shaking incubator, model Dubnoff Precision, in an atmosphere of 95 per cent O_2 and 5 per cent CO_2. The pH at the termination of the incubation was determined. The Dubnoff

FIGURE 1

Ehrlich ascites tumor cell incubated for 3 hours in Tyrode's solution supplemented with 13 mg/ml ferritin. The general morphology is comparable to that of an unincubated cell. Arrows indicate 2 membrane-surrounded, ferritin-containing structures in the vicinity of the nucleus (*N*). × 80,000.

The inset shows areas taken from cytoplasmic vacuoles of a tumor cell incubated for 1 hour in the same conditions. Note the characteristic tetrad structure of the iron core of the ferritin molecule. × 360,000.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shaker was run at 110 cycles per minute and the temperature controlled at 37°C.

In each case 0.1 ml of 8×10^{-3} M glucose was added to the incubation media. The medium was supplemented with 40 μg penicillin and 50 μg streptomycin whenever the incubation period extended beyond 2 hours. In 3 experiments human serum albumin was added to the medium to a final concentration of 33.3 mg/ml.

Commercially obtained ferritin (Pentex, Incorporated, Kankakee, Illinois) was added to the incubating media, to obtain final concentrations varying from 0.004 to 13 mg/ml. The ingestion of colloidal gold was studied using Lange's solution. This gold solution precipitated in the medium, unless it had been previously stabilized with human serum albumin. Decayed solutions of Abbot's radioactive colloidal gold stabilized with gelatin gave identical results.

After incubation, the entire contents of one flask were added to 4 to 5 ml of 1 per cent OsO_4 buffered with veronal acetate (5, 30); 0.049 gm sucrose per ml was added to the fixative. After 20 minutes fixation, the cells were dehydrated in graded alcohols and embedded in *n*-butyl methacrylate. Sections were cut with a Porter-Blum microtome and picked up on carbon-coated grids (39).

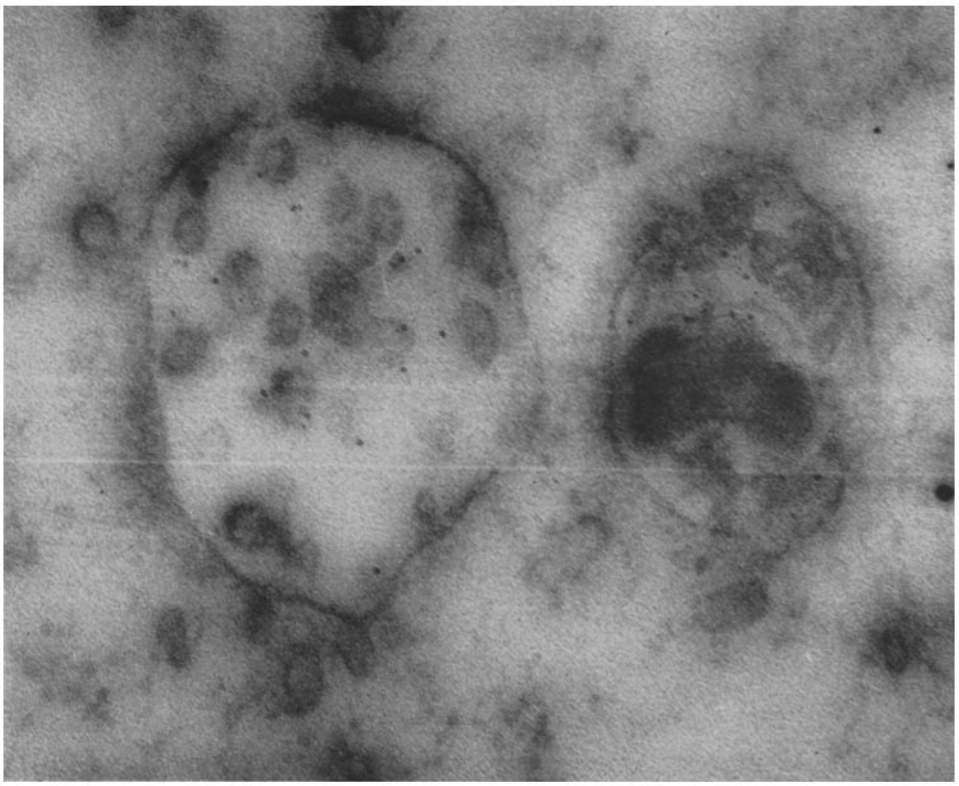


FIGURE 2

Two cytoplasmic vacuoles from a tumor cell incubated for 30 minutes in Tyrode containing 8.3 mg/ml ferritin. These structures, situated at some distance from the plasma membrane, both contain ferritin as well as smaller vesicular profiles. Ferritin uptake was commonly detected in the 30-minute samples. × 145,000.

RESULTS

Eight experiments were designed to test the ability of Ehrlich tumor cells to take up ferritin from the incubation medium *in vitro*. A ferritin uptake was detected in all experiments. At high resolutions, the electron-opaque particles revealed the tetrad structure characteristic for the iron hydroxide micelles of ferritin molecules (14) (Fig. 1). Varying the final concentration of ferritin from 0.004 to 13.3 mg/ml showed a detectable uptake

with concentrations as low as 0.04 ml/mg after 2 hours of exposure. Samples incubated for 1 and 15 minutes showed rare examples of minimal ferritin uptake, whereas 30-minute samples frequently were positive (Fig. 2). After 1 hour of incubation with 0.3 mg ferritin/ml or higher concentrations, ingestion was found with an over-all frequency of one positive section out of two. Clear evidence was found repeatedly for an increase in uptake between 1 and 3 hours. Uptake was also demonstrated under the following particular experimental conditions: 1) after intraperitoneal injection of ferritin to tumor-bearing mice, 2) after anaerobic incubation *in vitro* with various amounts of glucose, and 3) after modification of the incubation medium by various means, such as absence of glucose or addition of corticosterone (final concentration: 8.10^{-5} M). An increase in the proportion of positive cells and a much higher amount of intracellular ferritin were observed in 3 experiments in which the medium contained, in addition, 33 mg/ml human serum albumin (Figs. 3 to 5). Under these conditions, the increase in cellular ferritin resulted more from a higher ferritin content per structure than from a greater number of labeled structures. Some cells appeared to form large clear vesicles containing considerable amounts of ferritin particles (Fig. 5). More ferritin was also found on the plasma membrane.

In the rare instances where intracellular ferritin was seen after 1- and 15-minute incubation, the particles were found in moderate-size vesicles near the plasma membrane. In later stages of incubation ferritin was found in a greater variety of intracellular structures, ranging from simple vesicles to vacuoles and bodies measuring from 0.1 to 0.5 μ, with internal contents of various complexities. The same section often showed ferritin-labeled structures of several kinds distributed throughout the entire cytoplasm. However, in the sections including the nucleus, ferritin appeared more frequently in the perinuclear region than elsewhere (Fig. 1).

Ferritin appeared to be strongly adsorbed by membrane fragments of destroyed cells (Fig. 6). Ferritin-loaded debris was also taken up by the intact cell (Fig. 7), and was often observed in the immediate vicinity of cell boundaries of unusual complexity, suggesting the possibility of a high membrane activity (Fig. 3 and 4). This frequent association would be compatible with the idea that cell debris exerts a stimulating effect on membrane movements.

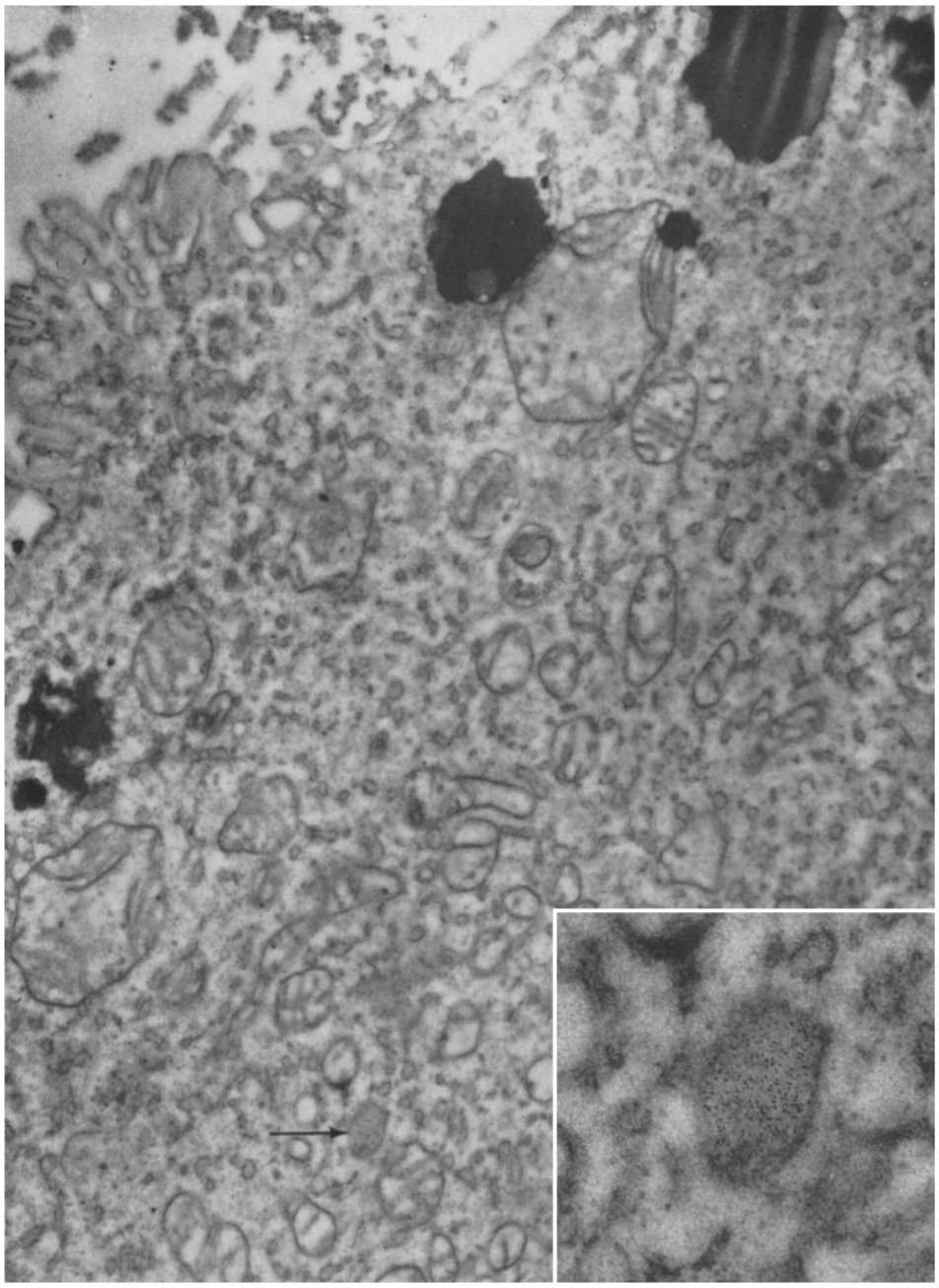
In intact cells, free cytoplasmic ferritin was a rare finding. Single granules, or aggregates seemingly devoid of a surrounding membrane, were found mainly in the perinuclear region of a few cells incubated for more than 2 hours. In cells showing obvious damage, large amounts of ferritin appeared in unstructured areas with random distribution throughout the entire cytoplasm (see text to Fig. 8). No ferritin was found in mitochondria and little on the plasma membrane of intact cells. This is in sharp contrast to the marked adsorption of ferritin to the membranous remnants of damaged cells (Fig. 6). Some nuclei contained what appeared to be isolated ferritin particles, but never in such a number as to allow a positive identification.

Several incubations were carried out with colloidal gold as an optical marker. None of them showed an ingestion comparable to that of ferritin. In intact cells a minimal uptake was found only in very rare instances (Fig. 9). Gold was occasionally seen adhering to the cell membrane. Damaged cells contained likewise a large number of colloidal gold particles, adsorbed essentially to the membrane elements of the cytoplasm, in a way similar to that of ferritin (Fig. 8). Gold was also adsorbed by cell debris, but not so much as ferritin.

DISCUSSION

The positive identification of ferritin molecules in our cell material is demonstrated by the characteristic tetrad-substructure seen at high magnification (Fig. 1). The detection of these particles in the cytoplasm of ascites tumor cells within ½ hour after exposure to ferritin can be considered strong evidence of the penetration of an intact protein. The possibility that some of the intracellular ferritin seen after incubations of 2 and 3 hours might have resulted from a *de novo* synthesis, stimulated by the presence of free iron in the medium, cannot be entirely discarded but appears very unlikely at this time.[1]

[1] It has been shown that ferrous sulfate, added to the culture medium of HeLa cells, can induce the synthesis of cytoplasmic ferritin (32). In these experiments, however, the electron-opaque ferritin particles are detected at the earliest after 4 hours' exposure. A reduction of the trivalent iron hydroxide micelles contained in ferritin is unlikely to occur in our experimental conditions. Furthermore, the iron hydrox-



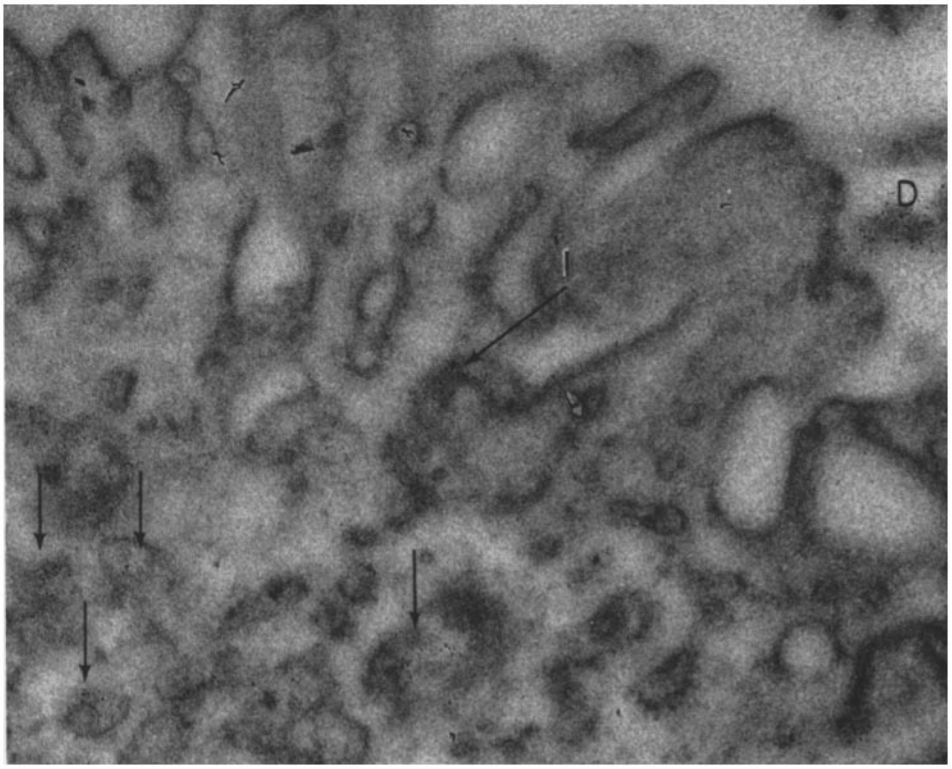


FIGURE 4

Details of Fig. 3, showing vesicular formations, invaginations, and digitations of the plasma membrane. This complex morphology strongly suggests active membrane movements. Extracellular debris (*D*), laden with ferritin, is seen in the vicinity of the plasma membrane. Ferritin is present on the cell surface, within invaginations (*I*), and within several small vesicles (arrows). × 60,000.

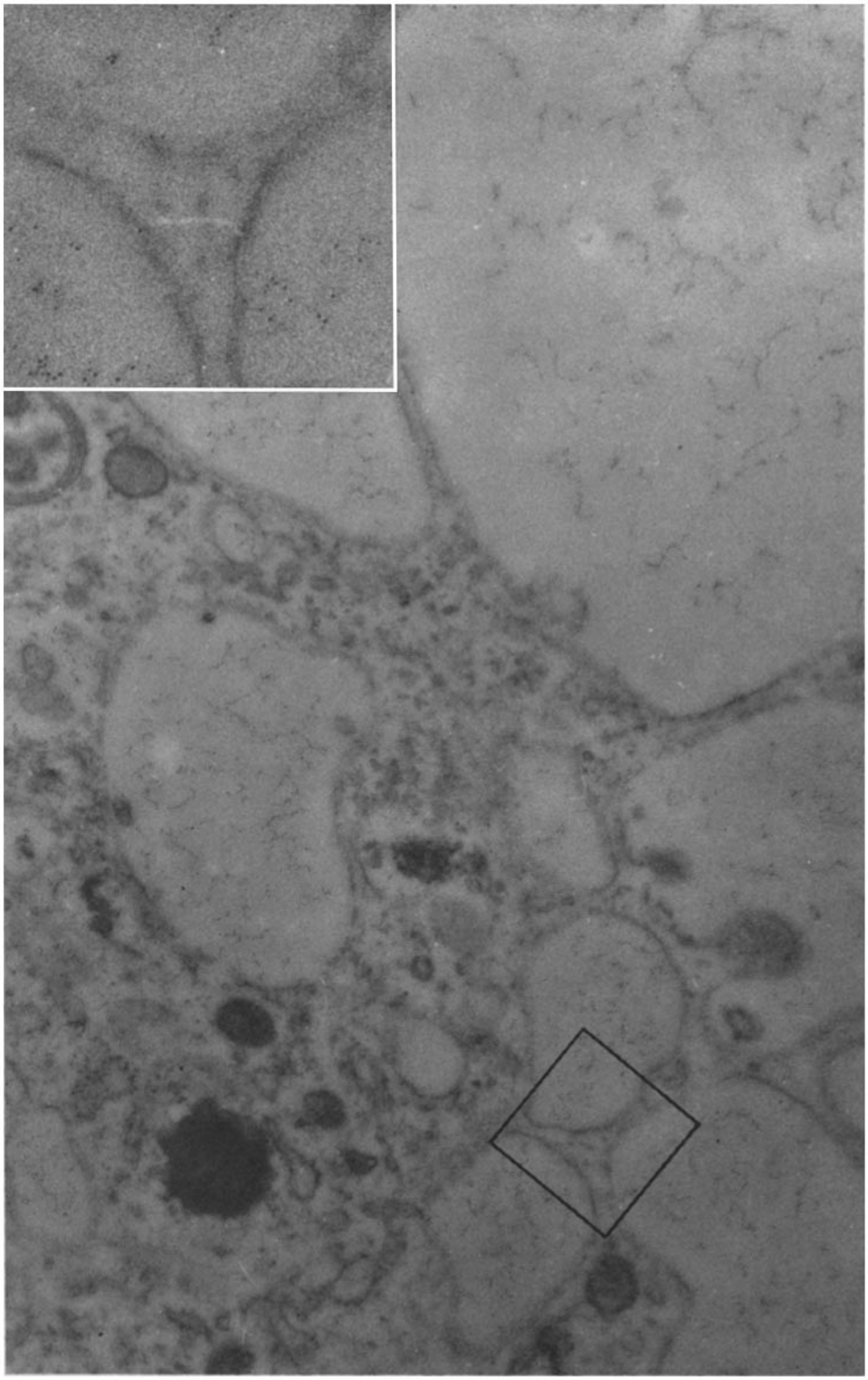
Our findings provide new support for the idea that protein uptake is a normal physiological function of tumor cells. Both the localization of ferritin within membrane-surrounded structures and the relation of many of these structures to the plasma membrane suggest that ferritin is taken up by pinocytotic vesiculations or related membrane movements. The small pinocytotic vesicles were usually outnumbered by more complex ferritin-containing structures (vacuoles of various sizes, multivesicular or dense bodies) similar to those described by Farquhar and Palade in their study of the glomeruli of nephrotic rats (12). Our time-sequence observations appear to support the concept (12) that complex inclusions result

ide freed from its protein shell would precipitate in our neutral medium as does the iron hydroxide split from iron-dextran complexes at pH 7.2 (31).

FIGURE 3

Tumor cell incubated for 2 hours in Tyrode supplemented with human serum albumin (33 mg/ml) and ferritin (13.3 mg/ml). Fragments of lysed cells, seen in the proximity of the plasma membrane (upper left corner), were frequently associated with a complex membrane pattern, as represented in this picture. More than 800 distinct ferritin particles were found in the cytoplasmic area shown in the figure. They were contained in a variety of structures situated throughout the cell. × 17,000.

The inset is an enlargement of a small ferritin-labeled vacuole (arrow). × 60,000.



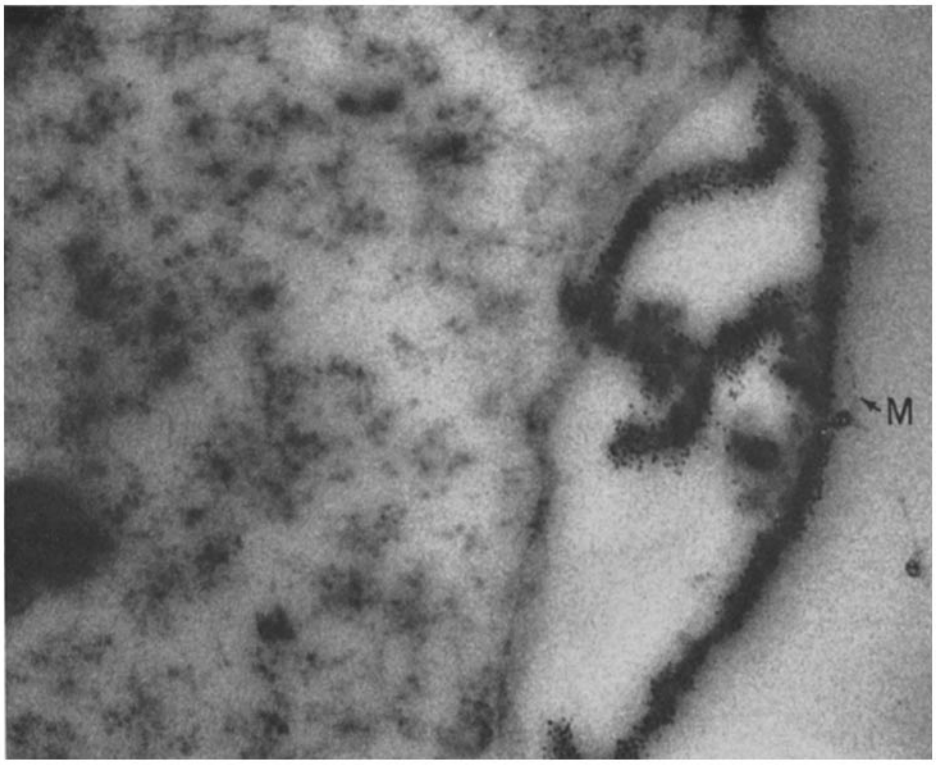


FIGURE 6

Membrane elements (*M*), derived from a disrupted cell and heavily loaded with ferritin, are shown adherent to the surface of an intact tumor cell. Note the absence of ferritin adsorption on the plasma membrane of the intact cell. Incubation: 3 hours in Tyrode containing 8.3 mg/ml ferritin. × 47,000.

essentially from the confluence and the subsequent concentration of small primary vesicles. It should be kept in mind, however, that large clear vesicles (Fig. 5) and vacuoles containing cell debris (Fig. 7) are likely to be formed by broader membrane movements.

Although a localization of free ferritin in the cytoplasmic matrix has been actively looked for, little support has been found for the view that ferritin is ultimately released from the various structures after a disruption of their surrounding membrane. Studies on the ferritin segregation in kidney cells have led to a similar conclusion (12). In the absence of further information about the degradation of ingested ferritin, it appears useful to recall data published by Coons *et al.* (10), suggesting that human γ-globulins taken up by kidney and liver epithelial cells of the rat retain their immunological reactivity for 1 to 2 days following exposure.

Although electron microscopic data are not well suited for quantitative estimations, it appears

FIGURE 5

Ascites tumor cell incubated for 1 hour in Tyrode containing 33 mg/ml serum albumin and 13.3 mg/ml ferritin. Ferritin is found predominantly in large clear cytoplasmic vesicles. In media containing a high albumin concentration, this form of vesicular uptake was found in about 15 per cent of the tumor cells. × 34,000.

The inset is an enlargement of a portion of Fig. 5, demonstrating a few of the many ferritin particles within the large vacuoles. × 92,000.

that isolated tumor cells do not take up ferritin as readily as glomerular epithelial cells of nephrotic rats (12). Taking the section shown in Fig. 3 as a basis for an estimate, and assuming that the ferritin distribution found in this section is representative of the cellular distribution, it can be calculated that the cytoplasm of a cell measuring 12 μ in diameter would contain 3.4×10^5 molecules of ferritin.[2]

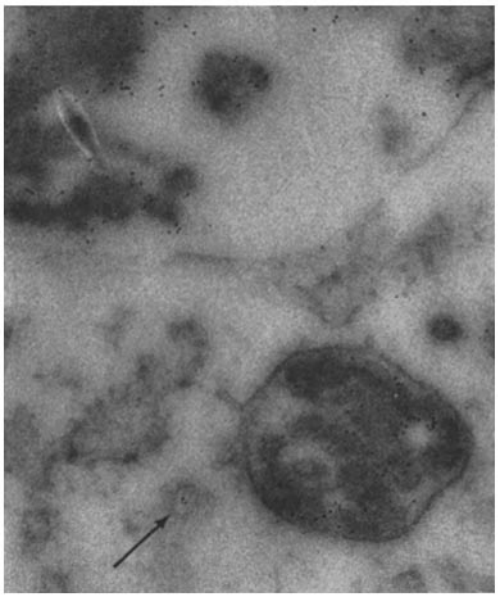


FIGURE 7

Small area of the surface of an ascites tumor cell incubated 1 hour in Tyrode with 33 mg/ml serum albumin and 13.3 mg/ml ferritin. Masses of debris intermingled with ferritin particles and situated outside the cell are visible in the upper third of the figure. The same ferritin-labeled material is also seen in a middle-size cytoplasmic vacuole. It is suggested that in this case ferritin has been taken up together with fragments of extracellular debris. The smallest type of ferritin-labeled vesicle (arrow) was prevalent after short incubations (15 minutes). The comparison between these two structures suggests the occurrence of membrane movements of different order of magnitude. × 100,000.

[2] Fig. 3 representing approximately ¼ of an equatorial cell section, 0.06 μ thick and 12 μ in diameter, contains 815 distinct ferritin particles. The ratio between the volume of the whole equatorial cell section and the total cytoplasmic volume (⅘ of cell volume) being equal to 1:105, the number of particles in the entire cytoplasm would be of the order of $4 \times 815 \times 105 = 3.4 \times 10^5$.

This number is greater than the figure usually considered to be the minimal number of molecules per cell required to elicit a pharmacological effect (9). But, as judged by other analogous instances, it appears to be smaller than the figure one would expect if the ferritin uptake were to play an important role in the protein metabolism of the cell.[3] As will be seen in a following paper (33), measurements of labeled albumin taken up in comparable experimental conditions lead to estimates of a similar order of magnitude.

The question whether *in vivo* conditions would favor greater ferritin uptake is made unlikely by the similarity of uptake observed after intraperitoneal ferritin injection into mice with an ascitic tumor. High serum albumin concentration was found to be one of the physiological conditions enhancing the uptake of ferritin. The property of protein to act as a stimulus for pinocytosis has been reported previously by Chapman-Andresen and Prescott in experiments on ameba (8) and by Farquhar and Palade in glomerular cells of the rat (12). In our experiments, albumin seemed furthermore to induce the formation of a particular type of large, clear vesicle (Fig. 5). Similar formations were also found in cells exposed for a short time to hypertonic NaCl solution (34). Treatment of cells with hypertonic NaCl and sucrose solutions has evoked interest in virology since this procedure has been shown to increase the infectivity of virus and subviral particles acting on

[3] It can be estimated that the amino acids contained in 3.4×10^5 molecules of ferritin represent less than 2 per cent of the optimal amount of free amino acids incorporated into the proteins of a single cell in 2 hours.

If leucine is used as an example, the optimal incorporation of amino acid into the proteins of ascites tumor cells has been found to be 33 μM per gram protein per hour (25). Since our data indicate that 1 gram of cell protein corresponds to 5.8×10^9 cells, the value of 33 μM/gm per hour can be converted into 3.4×10^9 molecules per cell and per hour, or 6.8×10^9 per cell in 2 hours. If it is assumed, on the other hand, that leucine represents 1/10 of the apoferritin protein (MW 460,000), the amount of leucine taken up in 3.4×10^5 molecules of apoferritin in 2 hours is of the order of 1.2×10^8 molecules. This is 1.8 per cent of 6.8×10^9. This rough estimate is based on the ferritin uptake seen in one cell section (Fig. 3). It only indicates that, for this particular cell, the amino acid yield which could be derived from the detectable ferritin is low, but perhaps not negligible.

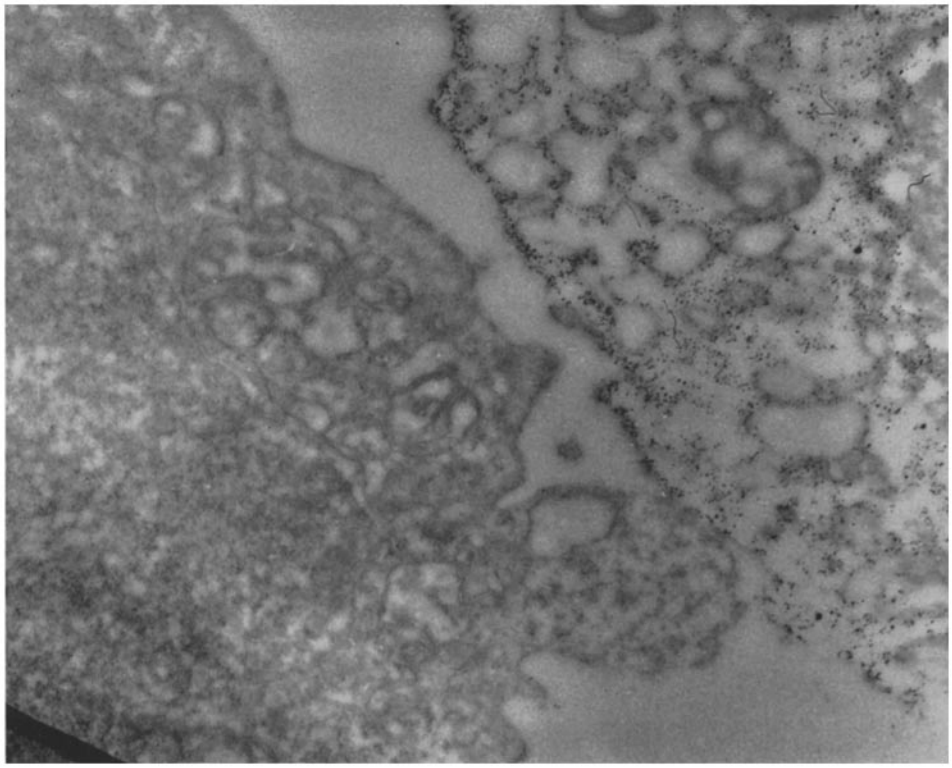


FIGURE 8

Tumor cells incubated for 30 minutes in Tyrode made hypertonic by NaCl addition (3 times the normal osmolarity in the final medium). In this case, colloidal gold was used as an optical marker. Lange's gold solution was stabilized with serum albumin (1 mg/ml) and added to the medium in a proportion of 1:1. The tumor cell to the left, though somewhat altered by the hypertonic incubation media, does not contain any visible gold. The plasma membrane of the cell to the right has been damaged and no longer acts as a barrier to the ingress of gold particles. Large amounts of gold are adsorbed to the cytoplasmic membranous components of the dead cell. Ferritin was adsorbed to damaged cells in a similar way. × 28,000.

cell monolayers (11, 23). Whether the protein uptake by tumor cells can be increase in a similar way will be discussed in a following paper (34).

The striking adsorption of ferritin on damaged cells and cellular debris can be compared to the non-specific fixation of antibodies by injured tissues, described by Holtzer (20). Knowledge of such ferritin adsorption to altered cells deserves attention in connection with the increasing use of ferritin-antibody complexes for the electron microscopic localization of specific cellular antigenic groups (29, 36, 37).

The adsorption of ferritin on extracellular debris allowed visualization of their penetration into the body of ascites tumor cells. The complex morphology of the membrane region where such ingestions were observed frequently suggested that cell fragments might stimulate membrane movements. This finding points towards the importance of cell debris—and possibly other supramolecular aggregates—as carrier for the uptake of proteins. The use of such vectors might provide a way to increase the total cellular uptake of macromolecules. These considerations are consistent with the finding that the incorporation of DNA by L fibroblasts is markedly enhanced, if the nucleic acids are presented as a protein-DNA aggregate of 0.5 μ or more in diameter (2).

It must be pointed out finally that particles of colloidal gold, even when stabilized with gelatin or

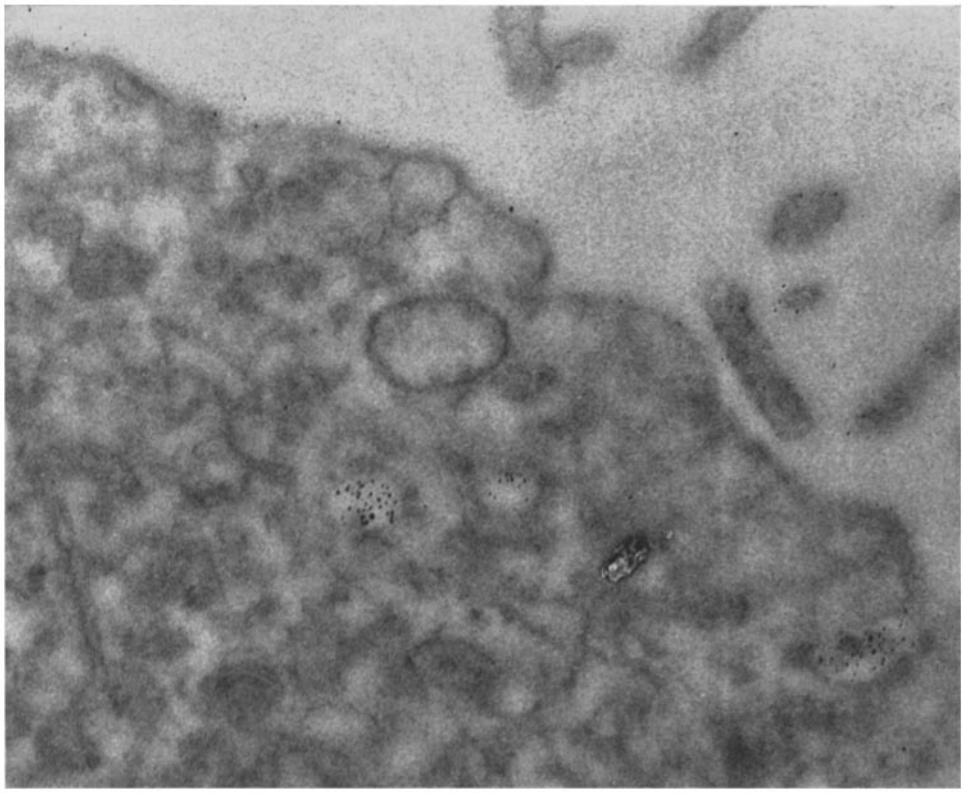


FIGURE 9

Tumor cell incubated for 30 minutes in Tyrode containing colloidal gold solution stabilized with 1 mg/ml albumin. Gold particles are visible on the cell surface, in an invagination of the plasma membrane (lower right corner), as well as within two cytoplasmic vesicles. In contrast to our observations with ferritin, the uptake of colloidal gold by intact tumor cells was a rare finding. × 55,000.

albumin, were not taken up by ascites tumor cells to any significant extent. Whether or not this discrimination is related to a different adsorption, it illustrates a certain selectivity in the pinocytotic uptake of particles of approximately similar sizes.

As this paper was being submitted for publication, Easton, Goldberg, and Green (*J. Cell Biol.*, 1962, **12,** 437) reported the uptake of ferritin-antibody complexes by Krebs ascites tumor cells. Their observation that ferritin is taken up more readily when conjugated with either immune or non-immune rabbit γ-globulin is in keeping with our view that added proteins as well as supramolecular protein aggregates may stimulate pinocytosis.

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