

# APPEARANCE AND DISTRIBUTION OF FERRITIN IN MOUSE PERITONEAL MACROPHAGES IN VITRO AFTER UPTAKE OF HETEROLOGOUS ERYTHROCYTES

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## ABSTRACT

Mouse peritoneal macrophages have been studied *in vitro* after ingestion of treated rat, rabbit, or sheep erythrocytes. Under light microscopy, phagocytic vacuoles persist up to 24 h. Macrophages lose benzidine reactivity about 5 h after red cell ingestion, and they become prussian blue positive at 2 days. Ultrastructural studies show little or no ferritin in control macrophages not fed erythrocytes. In contrast, after red cell ingestion, ferritin is widely distributed in the cytoplasmic matrix and in some cytoplasmic granules by 48 h. The Golgi complex, pinocytotic vacuoles, endoplasmic reticulum, nuclei, and mitochondria do not contain ferritin. Between 2 and 4 days, ferritin in cytoplasmic granules increases, concomitant with decrease in the ferritin in the cytoplasmic matrix. Evidence is presented suggesting that ferritin in the cytoplasmic matrix is translocated into cytoplasmic granules by autophagy. Polyacrylamide gel studies on macrophages after uptake of red blood cells labeled with radioiron confirm that macrophages produce radiolabeled ferritin by 4 days.

Iron is stored in some nucleated mammalian cells as a component of ferritin (24). Previous studies have provided information on some of the morphologic and biochemical aspects of ferritin synthesis *in vivo* in spleen macrophages, hepatocytes, or red cell precursors (5, 6, 15-17, 30, 36, 43, 46, 47, 48, 53), or *in vitro* in HeLa cells, siderocytes, synovial cells or liver preparations (8, 14, 31, 37, 45, 46, 49). The factors that determine ferritin synthesis, storage, and breakdown within cells remain unknown. We are now studying some of these mechanisms in mouse macrophages. Macrophages seemed particularly appropriate for such studies, since they can be readily maintained *in vitro*, allowing manipulation and quantitation not possible in more complex systems. We report here our observations on the synthesis and the intracellular location of ferritin in mouse macrophages at various times after ingestion of foreign red cells.

## MATERIALS AND METHODS

### *Cultivation of Macrophages and Preparation of Erythrocytes*

Mouse peritoneal macrophages were maintained adherent to glass surfaces *in vitro* by the method of Cohn (9) in Medium 199 (iron content was 0.1 mg/liter  $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ ) containing heat-inactivated (20-40%) newborn calf serum (iron content 60  $\mu\text{g}/100$  ml). Minimal essential Eagle's medium with Earle's balanced salt solution without phenol red, 2 $\times$  concentrated (MEM), which contains no iron, was also used for the studies. Macrophages and a population of lymphoid cells were obtained from washout of the peritoneal cavity. 30-60 min after plating the peritoneal cells, nonadherent lymphoid cells were washed off the glass surface; the cell cultures thereafter consisted of 99-100% macrophages. Red blood cells were added to 1-2-day old cultures for periods ranging from 30 min to 4 h to obtain uptake of three

to four erythrocytes per macrophage, after which the cultures were washed three times to remove non-adhering cells, and then placed in fresh medium and maintained at 37°C in 5% CO<sub>2</sub>-air. Medium was changed every 2 days for the long-term cultures. Macrophage cultures up to 4 days after red cell ingestion showed no appreciable cell death. Direct counts were made of macrophages per defined area in living cell cultures under inverted phase contrast microscope; these counts showed less than 5% reduction in cell numbers. Under these conditions macrophages do not multiply. Control specimens not exposed to red blood cells were maintained under similar conditions.

Foreign red blood cells were collected and prepared as follows. Heparinized blood was collected from rabbits by cardiac puncture or from the tail vein of Sprague Dawley or BN/f Mai rats (Microbiological Associates, Inc., Bethesda, Md.). Aged red cells were obtained by washing three times in phosphate-buffered saline, storing in buffered saline first for 3–4 h at room temperature, and then overnight at 4°C for use the following day. To obtain red cells labeled with hemoglobin-<sup>55</sup>Fe, reticulocytosis was induced in a 150 g BN/f Mai strain female rat by four consecutive daily intraperitoneal injections of 0.20 ml of 1% (wt/vol) phenylhydrazine hydrochloride in 0.1 M phosphate buffer, pH 7.4. On the fifth and sixth days, 1 mCi <sup>55</sup>Fe was injected intraperitoneally as ferrous citrate-<sup>55</sup>Fe (New England Nuclear, Boston, Mass., 1 mCi/ml, 3–50 Ci/g Fe). Cells were collected 1 wk later, at which time <sup>55</sup>Fe incorporation in erythrocyte hemoglobin was adequate. Heated rat red blood cells were prepared for macrophage uptake by washing the erythrocytes with Medium 199 three times, resuspending to a packed cell: total suspension volume ratio of 1:1,000, and heating in a polystyrene test tube in a 56°C water bath for 5–7 min. A 0.1 ml sample was then added to each macrophage culture. Antibody-coated sheep red cells were prepared by incubating at 38°C for 90 min equal volumes of 1% sheep red blood cell suspension and 1:1,600 dilution of rabbit anti sheep red cell antiserum. The antiserum had an agglutinin titer of 1:3,200. Trypsinized sheep and rabbit red blood cells were prepared by incubating a 1% mixture of erythrocytes for 30 min at 37°C with equal volumes of 2.5% trypsin (Microbiological Associates, Inc.) in balanced saline.

Macrophages containing ferritin labeled with <sup>55</sup>Fe and <sup>14</sup>C were prepared in the following manner. The cells were exposed to a portion of heated erythrocytes labeled with <sup>55</sup>Fe in MEM containing heat-inactivated newborn calf serum. At the end of 2 h, excess red cells were removed by repeatedly dipping the cover slip with attached macrophages rapidly through the meniscus of medium in a beaker. The macrophages were placed in fresh medium for 4 h, and then transferred for 1.5 h into the following medium: MEM (90% leucine free) with 10%

dialyzed newborn calf serum and labeled [<sup>14</sup>C]-leucine, 1 μCi/2.5 ml medium (Schwarz/Mann, Div. Becton, Dickinson & Co., Orangetown, N. Y., 312 mCi/mmol). After the [<sup>14</sup>C]leucine pulse the cells were rinsed twice with MEM containing 10 mM nonradioactive leucine and maintained in this leucine-rich medium plus 20% newborn calf serum.

### Cytochemistry

The presence of ferric ions was detected under light microscopy by performing the prussian blue reaction (42). Cells were fixed for 15 min in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, and then exposed for 1 h to a freshly prepared 1:1 mixture of 2% potassium ferrocyanide and 2% HCl.

The benzidine reaction to detect heme was performed on glutaraldehyde-fixed cells using a saturated solution of 3,3'-dimethoxybenzidine, washing 10 min and then counterstaining with Mayer's hematoxylin for 30 s (21). The specimens were washed and mounted in water.

The Graham-Karnovsky 3,3'-diaminobenzidine (DAB) reaction (32) was used to visualize heme by electron microscopy. This was possible since mouse macrophages normally give a negative reaction (52). The cells were fixed *in situ* in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 15 min at room temperature. After exposure to the substrate (32) for 30 min the specimens were postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.4, dehydrated through graded alcohols, and embedded in Epon (34).

### Electron Microscopy

Cells on cover slips were rinsed in isotonic saline, fixed at room temperature for 10 min in 2.5% glutaraldehyde (Fischer Scientific Co., Pittsburgh, Pa.) in 0.1 M cacodylate buffer pH 7.4, scraped off the glass surface, and then placed for 10–30 min in a cold mixture of one part of 2.5% glutaraldehyde and 2 parts of 1% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.4. The cells were then rinsed in cold saline and poststained for 15 min in 0.25% uranyl acetate in acetate buffer pH 6.3. The specimens were then pelleted in warm 2% Noble agar, dehydrated, and embedded in a Porter-Blum microtome, and viewed in a Siemens Elmiskop 1A with 50 μm condenser aperture. In most instances, unstained sections were examined to allow more reliable recognition of dispersed or sparse ferritin.

### Polyacrylamide Gel Studies

30 h after initial exposure to red cells the macrophages were lysed and treated in one of two ways for electrophoretic studies. For most experiments, cells

(number of macrophages plated  $\sim 1 \times 10^6$ ) were rinsed twice with saline buffered with 0.05 M phosphate buffer, pH 7.4, and lysed in 0.10 ml of 1% sodium dodecyl sulfate (SDS) in phosphate-buffered saline containing 10% sucrose. In other experiments macrophages ( $\sim 1 \times 10^7$  plated) in 1 ml of 0.05 M phosphate buffer, pH 7.4, were lysed by six cycles of freezing and thawing followed by pooling and homogenization with 10–15 strokes of a tight-fitting Dounce homogenizer. 10  $\mu$ g of carrier mouse ferritin were added to the lysate, and this mixture was heated for 10 min at 70°C. The precipitated proteins were removed by centrifugation at  $1,500 g \times 15$  min at 4°C. SDS and sucrose were added to the supernatant so that the final concentration was 1% and 10%, respectively.

Mouse ferritin used as a reference standard was isolated by the method of Granick (24) from pooled livers and spleens of NCS (Rockefeller Colony) mice which had been injected intraperitoneally with 0.5 ml of iron dextran (Imferon, Lakeside Laboratory, Milwaukee, Wis.) 3–4 wk previously. 20  $\mu$ g of marker mouse ferritin was added to each cell lysate to be electrophoresed. The brown color of the ferritin band was easily detected in the gel.

The cell lysates or heated supernatants were layered on 9-cm long 5% polyacrylamide gels polymerized in 5-mm internal diameter glass tubes. Both chamber and gels contained 0.1 M phosphate buffer, pH 7.0, and 1% SDS. Electrophoresis was performed by the method of Lenard (33) at 10 milliamperes per gel for 3–6 h. Gels were then cut into 0.9-mm thick slices which were incubated (three consecutive slices per vial) 16 h at 60°C in 0.75 ml toluene (Packard Instrument Co. Inc., Downers Grove, Ill.). Radioactivity was measured on a Nuclear-Chicago Mark II liquid scintillation counter (Nuclear-Chicago Corp., Des Plaines, Ill.) using a toluene:Liquiflour (1,000:42, vol/vol (New England Nuclear) scintillation fluid.

Lysates prepared by freezing and thawing and homogenization were also electrophoresed on gels which did not contain SDS. Gels containing 7% acrylamide and buffered at pH 8.9 were prepared as described by Davis (12). No sample or spacer gels were used. The chamber buffer consisted of 3.0 g tris(hydroxymethyl)-aminomethane and 14.4 g glycine per liter (pH 8.3). Current was applied at 2 milliamperes per gel for 15 min and then 5 milliamperes per gel for 45 min. The gels were then handled as described for the SDS-containing gels.

Mouse ferritin isolated from mouse liver and spleen was used to stimulate antibodies in rabbits by subcutaneous injection of five 10 mg doses in complete Freund's adjuvant (BBL, Division of Bio Quest, Cockeysville, Md.) over a 2 mo period. Serum was collected 2 wk after the last injection and stored frozen. The antiserum was tested for im-

munologic specificity by immunodiffusion assay (3). Both antiserum and normal rabbit serum were tested in Ouchterlony plates against purified mouse ferritin (10  $\mu$ g protein) and a lysate of freshly collected macrophages (100  $\mu$ g protein) which had been plated 0.5 h, washed, frozen, and thawed six times.

100  $\mu$ l lysates of macrophages (50  $\mu$ g protein) which had ingested iron labeled erythrocytes 30 h previously were mixed with 10  $\mu$ l of a 1:10 dilution of rabbit anti-mouse ferritin antiserum, or with normal rabbit serum or phosphate-buffered saline for controls, and the mixtures were stored 16 h at 4°C. The mixtures were then applied to the polyacrylamide gels.

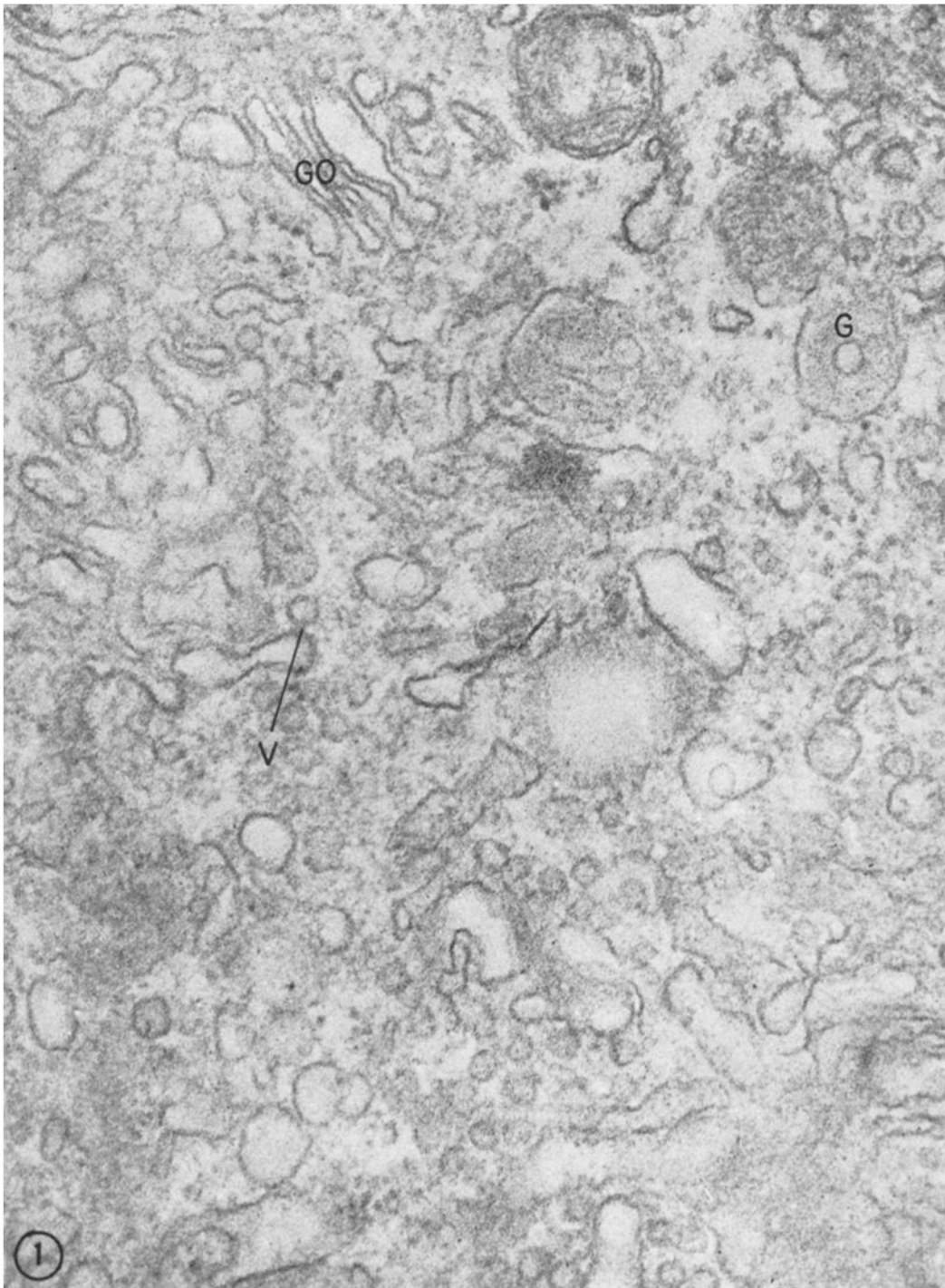
## RESULTS

### *Morphologic Features of Peritoneal Macrophages in Control Cultures*

The morphology of mouse peritoneal macrophages in vitro as well as some of the morphologic changes after phagocytosis and pinocytosis have been described in detail elsewhere (4, 9–11). After 1–2 h in culture, peritoneal macrophages become firmly adherent to glass. Under phase contrast microscopy they show an eccentric kidney-shaped nucleus with marginated chromatin. A central prominent Golgi zone is surrounded by phase-transparent pinocytic vacuoles, elongate mitochondria, and phase-opaque cytoplasmic granules. Pseudopods contain pinocytic vacuoles, and mitochondria oriented so that their long axis is parallel to the long axis of the pseudopod. Highly refractile lipid droplets accumulate with increasing time in culture near the nucleus and scattered in the pseudopods. The cell membrane of the macrophage shows a typical irregular ruffled appearance.

By electron microscopy the Golgi complex is seen as stacks of flattened saccules and scattered vesicles in the centrosomal region. Dense cytoplasmic granules,<sup>1</sup> pinocytic vacuoles, and mitochondria are scattered in the surrounding cytoplasm; often mitochondria are also situated along the central axis of pseudopods. There is a variable but not extensive amount of rough endoplasmic reticulum. The cytoplasmic matrix contains scattered ribosomes.

<sup>1</sup> The term granule will be used as a general one to include a variety of small, round, membrane-limited cytoplasmic structures, usually light and electron opaque, including dense bodies, residual bodies, and some multivesicular bodies.



**FIGURE 1** Control specimen of mouse peritoneal macrophages maintained in culture 5 days. This centrosomal area of cytoplasm is devoid of any accumulations of electron-opaque particles compatible in size with ferritin. Shown are saccules of the multifocal Golgi complex (*GO*) surrounded by small vesicles (*V*) with moderately electron-opaque content. The cytoplasm also contains membrane-bounded granules (*G*). Cells exposed to 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, then a mixture of glutaraldehyde and 1% osmium in 0.1 M cacodylate buffer, pH 7.4 (1:2, vol:vol) and finally 0.25% uranyl acetate, pH 6.3 (see Materials and Methods). Section is unstained.  $\times 80,000$ .

In control macrophages which had not ingested red blood cells, the cytoplasm showed no or rare ferritin (Fig. 1). An occasional control specimen showed small ferritin deposits in cytoplasmic granules, perhaps reflecting previous intake of iron-containing materials (red cells?) by these cells *in vivo* or use of iron from the medium by the cell for ferritin synthesis.

#### *Light Microscope Observations on Mouse Peritoneal Macrophages after Red Cell Uptake*

Fresh, washed normal rat, rabbit, or sheep red blood cells were not taken up in appreciable numbers by mouse peritoneal macrophages. Various treatments (aging, trypsinization, coating with antibody, or heating) induced changes leading to adherence and ingestion of these heterologous red blood cells. The duration of incubation with red cells ranged from 30 min to 4 h and was in each case adjusted so that the average intake was four red blood cells per macrophage.

The phase microscope appearance of macrophages early after exposure to red blood cells in culture is shown in Fig. 2 (inset), which illustrates a macrophage which has ingested four aged rat red blood cells. Erythrocytes within macrophages could be distinguished from those merely adherent to the surface by examination at various focal planes, and by the fact that the ingested red cells showed an increased phase density as compared to those adherent to the surface. As digestion within phagocytic vacuoles progressed, red blood cells showed bizarre shapes and evidence of crenation.

The benzidine reaction was used to estimate the speed of heme degradation within phagocytic vacuoles. After the ingestion of aged or heated red blood cells, benzidine reactivity persisted in phagocytic vacuoles up to 5 h.

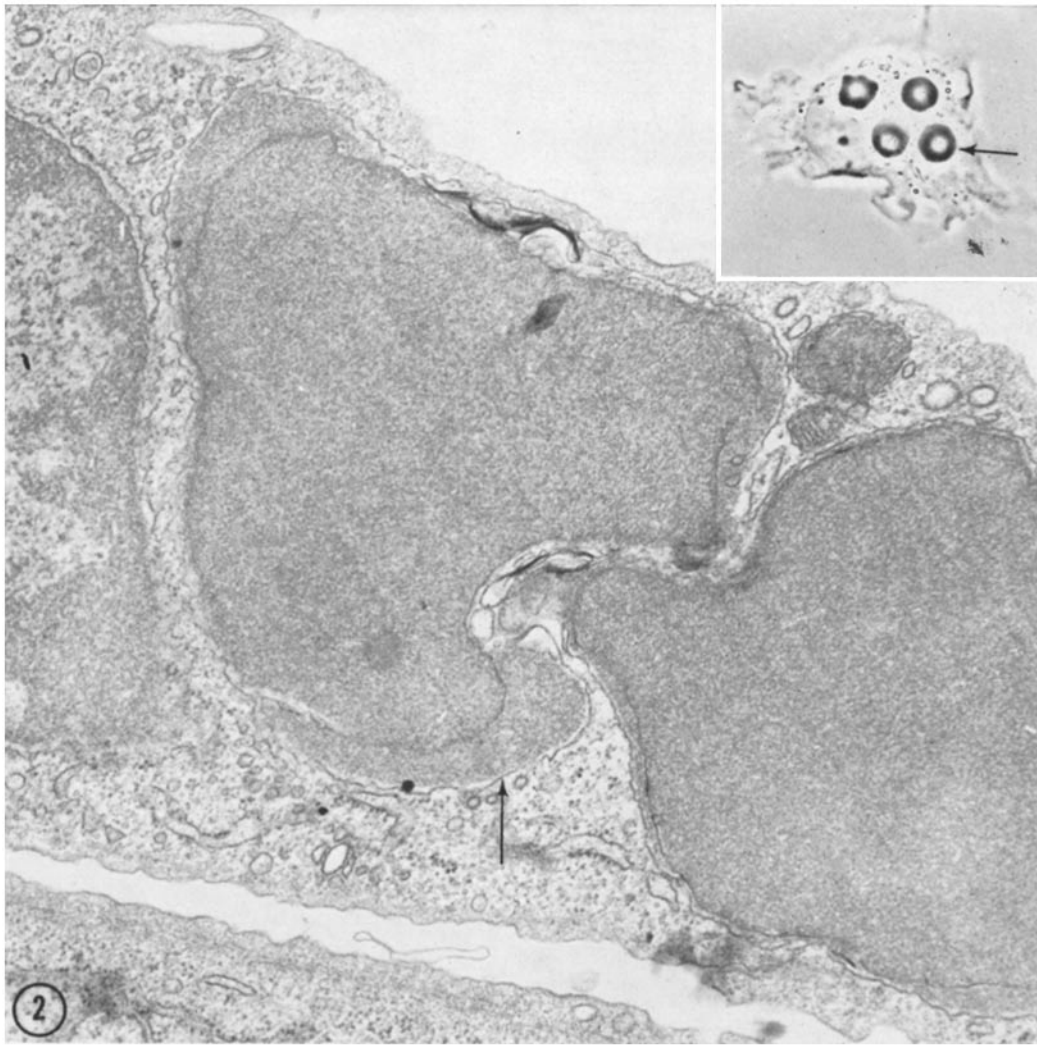
Prussian blue reactivity provided information on the appearance of ferric ions, usually found in the form of ferritin or hemosiderin in cells (42). These studies showed beginning of both diffuse and granular prussian blue positivity in mouse peritoneal macrophages 2 days after ingestion of red blood cells. Reactivity appeared more granular by 4 days. Unstained fixed preparations showed no yellow granules.

#### *Electron Microscope Observations on Mouse Peritoneal Macrophages after Ingestion of Heterologous Erythrocytes*

Various types of fixation and staining were evaluated to determine the best way to detect clearly ferritin deposition within the cells. There was no appreciable difference in demonstration of ferritin after osmium fixation alone as compared to fixation in the glutaraldehyde-osmium system. Exposure to uranyl acetate after fixation did not alter the appearance of ferritin. Ferritin was most readily demonstrated in unstained sections in which contrast of the normal cytoplasmic structures was low, allowing the iron core of ferritin to be easily detected. The ultrastructural uniformity of the ~6 nm ferritin core allowed it to be distinguished from occasional scattered nonspecific precipitate or beam contamination.

The ultrastructural appearance of a macrophage within the first few hours after ingestion of rat red blood cells is shown in Fig. 2. Apparently unaltered erythrocytes were present in phagocytic vacuoles. Ultrastructural studies using the diaminobenzidine reaction to detect heme reactivity showed that during the process of intravacuolar digestion phagocytic vacuoles were markedly positive (Fig. 3). As digestion proceeded, reaction product within vacuoles diminished. At 24 h after red blood cell uptake, apparent remnants of erythrocytes were observed in cytoplasmic vacuoles. Rare to moderate numbers of cytoplasmic ferritin particles were present. By 48 h after ingestion, digestive vacuoles were no longer found in the cytoplasm, but cytoplasmic granules were increased in size and number.

Between 48 and 60 h after phagocytosis of red blood cells, there were large numbers of ferritin particles dispersed in the cytoplasm (Fig. 4). Small deposits of ferritin were also present within cytoplasmic granules. The lipid droplets in the cytoplasm frequently showed ferritin at their periphery (Fig. 8). Ferritin particles were not observed in the nucleus, mitochondria, Golgi components, pinocytic vacuoles, or endoplasmic reticulum. By 72–96 h (Fig. 5) after erythrocyte uptake, the amount of ferritin in granules increased markedly over that seen at 48–60 h. There was some aggregation of electron-opaque particles in cytoplasmic granules; these aggregates might represent hemosiderin as well as ferritin.



**FIGURE 2** Mouse peritoneal macrophage within 4 h after ingestion of treated red blood cells (arrow) which lie within cytoplasmic vacuoles. The cytoplasm is filled with small vesicles and strips of endoplasmic reticulum. No cytoplasmic granules are visible. Section is stained with uranyl acetate and lead citrate.  $\times 30,000$ . Inset shows phase microscope appearance of a mouse peritoneal macrophage 2 h after ingestion of four aged rat red blood cells (arrow). The cell margin has a typical ruffled appearance. Mitochondria and refractile lipid droplets are seen in the cytoplasm.  $\times 2,000$ .

*Morphologic Evidence Suggesting Autophagy as the Mechanism for Transfer of Cytoplasmic Ferritin to Granules*

56–96 h after ingestion of treated heterologous erythrocytes, the morphologic findings suggested that cytoplasmic ferritin was transferred to granules in bulk by various types of autophagic

processes. The autophagic processes appeared to be of two major types: (a) invaginations of membranes of pinocytic vacuoles and granules, and (b) a focal encircling of cytoplasmic ferritin deposits by cytomembranes.

In a majority of the cases cytoplasmic ferritin was incorporated into pinocytic vacuoles and granules by invaginations, ranging from tiny droplets the size of pinocytic vesicles (Figs. 11, 12)

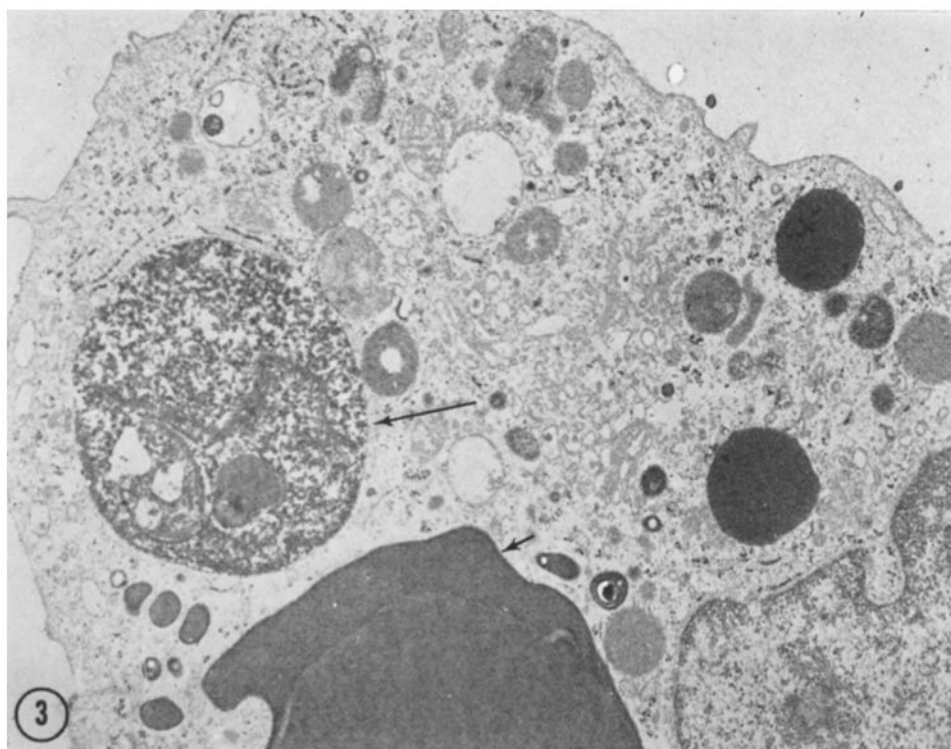


FIGURE 3 Mouse peritoneal macrophage 1 h after ingestion of heated rat erythrocytes; fixed and exposed to diaminobenzidine to detect heme. Reactivity is shown in vacuoles containing undigested red blood cells (short arrow) and in one vacuole containing more advanced stages of digestion (long arrow). The latter type of vacuole in control specimens shows very few remnants which are not electron opaque. Stained preparation.  $\times 19,500$ .

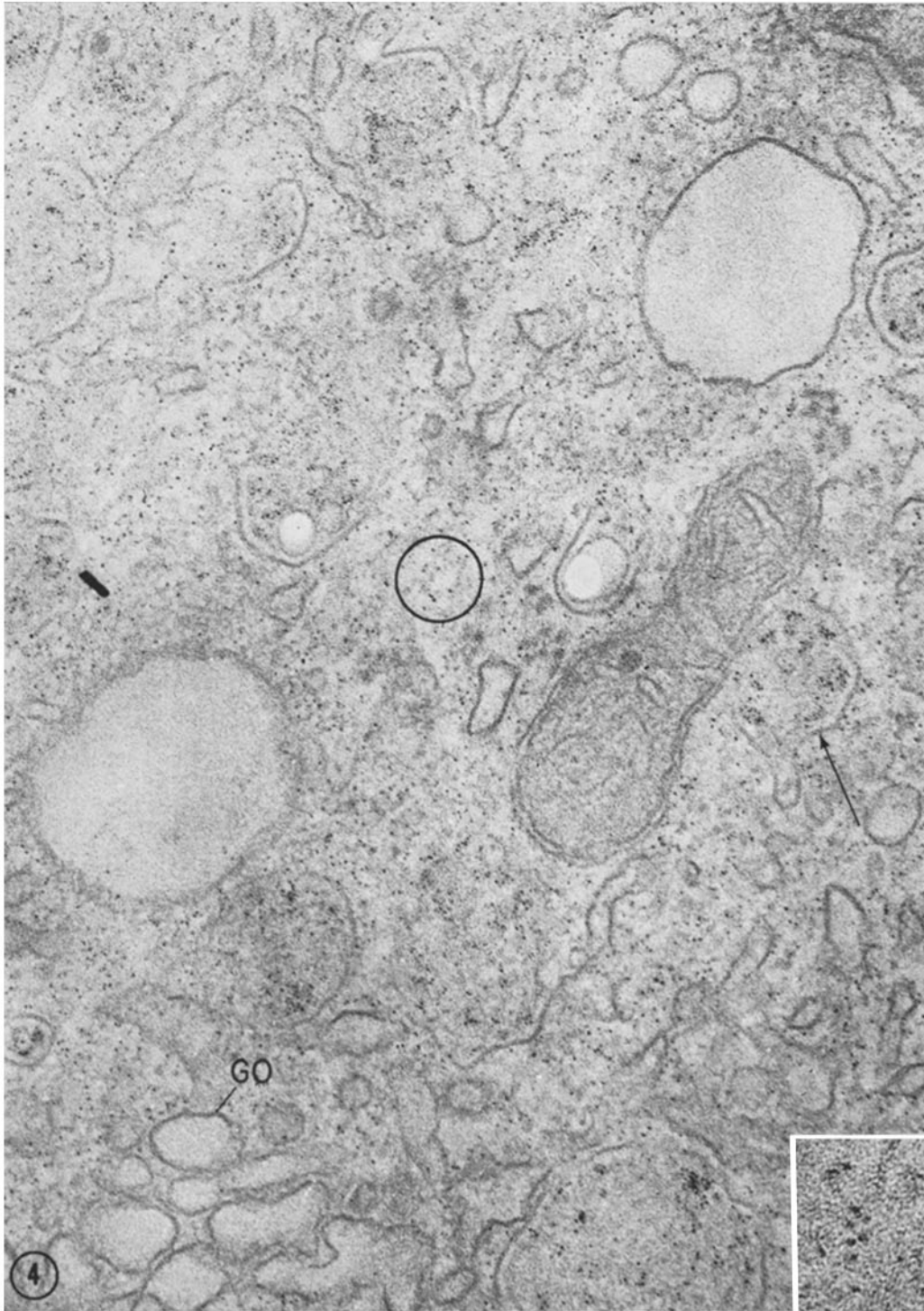
to large pouches a micron or more in size (Figs. 9, 10). Pinching off to small invaginations then could produce multivesicular bodies containing ferritin within the internal vesicles (Fig. 11). The separation of large invaginations from the limiting membrane of the vacuole, or forming granule, could produce the type of structure illustrated in Figs. 9 and 10, namely a large vacuole containing a sphere of ferritin-laden cytoplasm. Less frequently the deformed invaginating vacuoles showed narrow, irregular protrusions, similar to those seen in certain drug effects (Figs. 13, 14).

The other major type of autophagy appeared to involve the encirclement of cytoplasmic ferritin deposits by cytomembranes derived from the endoplasmic reticulum (Fig. 6). The inclusion of Golgi or other vesicles in this type of autophagic vacuole led in some instances to multivesicular bodies in which ferritin was seen in the matrix, but not in the internal vesicles (Fig. 7).

These profiles suggestive of autophagy cannot be taken as conclusive, since careful examination of serial sections might have revealed them to be invaginations without pinching off, or incomplete encirclement with cytomembranes. Control cells that had not ingested erythrocytes showed occasional invaginations of pinocytotic vacuolar membrane, but none of the other signs of autophagy were present.

#### *Polyacrylamide Studies on Macrophages that had Engulfed Rat Red Blood Cells*

Electrophoresis in polyacrylamide gels containing 1% SDS was performed on lysates from macrophages which had first ingested rat erythrocytes labeled with  $^{55}\text{Fe}$  and then 4 h later were exposed for 1.5 h to medium containing  $[^{14}\text{C}]$ leucine. The lysates revealed a single peak of  $^{55}\text{Fe}$  activity which was somewhat closer to the origin than was the



**FIGURE 4.** Mouse peritoneal macrophage 56 h after the ingestion of aged rat blood cells. Deposits of ferritin (circled area) are scattered throughout the cytoplasm. Ferritin is also present within cytoplasmic granules, in some cases in small clusters (arrow). The mitochondrion, the Golgi saccules (*GO*), and the various types of vesicles and vacuoles in the section do not show any ferritin particles. Section is unstained.  $\times 80,000$ . Inset shows higher magnification of ferritin particles, one with a tetrad appearance of the core.  $\times 200,000$ .



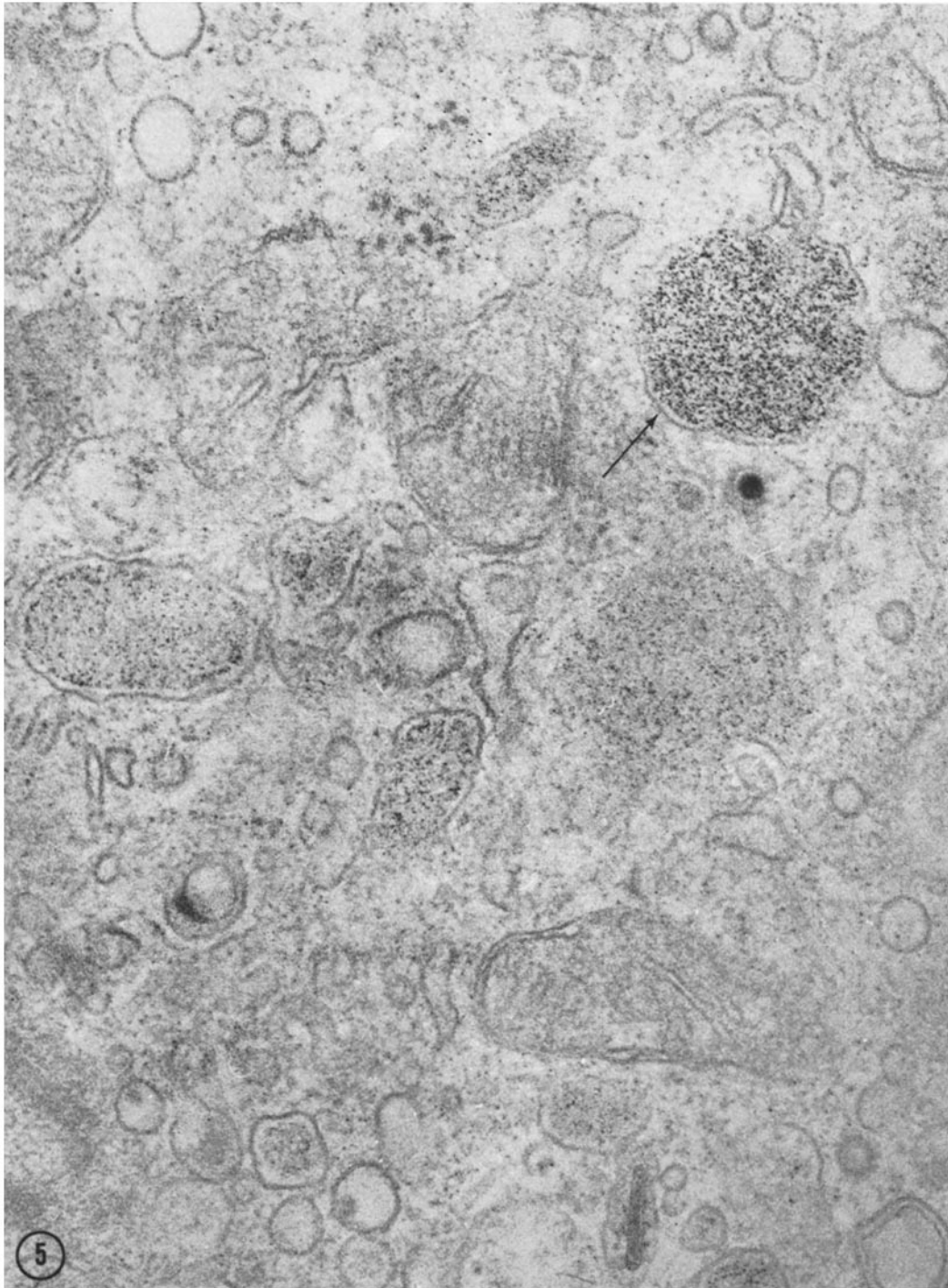


FIGURE 5 Mouse peritoneal macrophage 5 days after the ingestion of trypsinized rat red blood cells. The cytoplasmic content of ferritin appears less than that of the 2-3 day specimen (compare to Fig. 4), whereas granules (arrow) are now heavily labeled with isolated ferritin particles and aggregates, possibly hemosiderin. Mitochondria and vesicles do not show ferritin. Section is unstained.  $\times 80,000$ .

marker mouse ferritin (Fig. 17A and legend).  $^{14}\text{C}$  activity was localized both in this iron peak and in a broader peak that had migrated farther into the gel (Fig. 16A). Since ferritin is not precipitated by heating at 70–80°C (24), in contrast to the behavior of many other proteins, some of the macrophage lysates were heated for 10 min under these conditions and the supernatants were examined in gels. There was no diminution in the  $^{56}\text{Fe}$  peak and the coincident  $^{14}\text{C}$  peak nearest the origin.

Electrophoresis on lysates performed in the absence of SDS showed a peak containing both  $^{56}\text{Fe}$  and  $^{14}\text{C}$ , with the approximate mobility of mouse ferritin isolated from liver and spleen.

The specificity of the rabbit antimouse ferritin antiserum was demonstrated by immunodiffusion (3). In tests against mouse ferritin and lysates of macrophages that had not taken in red cells in vitro (100  $\mu\text{g}$  protein) (Fig. 15), a precipitin band was formed against mouse ferritin, but not against the normal macrophage extract. When normal rabbit serum was substituted for the antiserum, no precipitin bands were demonstrated.

Macrophage lysates obtained 30 h after ingestion of erythrocytes and subsequent pulse labeling with [ $^{14}\text{C}$ ]leucine were mixed with rabbit antimouse ferritin antiserum before electrophoresis (see Materials and Methods). When the lysate-antiserum mixture was used in the gel system, the amount of  $^{56}\text{Fe}$  and  $^{14}\text{C}$  in the ferritin region was markedly diminished and an increased amount of both isotopes was found at the origin (Fig. 16B and 17B). The migration of the second broader  $^{14}\text{C}$

peak positioned farther into the gel was not affected by specific antiserum. Mixing of the labeled macrophage lysates with normal rabbit serum did not result in retardation of the putative ferritin peak containing  $^{56}\text{Fe}$  and  $^{14}\text{C}$ .

## DISCUSSION

Most of the total body iron is in hemoglobin, with varying amounts also distributed in storage depots in macrophages and other cells, in body fluids, associated with serum proteins such as transferrin or albumin, or in labile iron pools (44). Mechanisms exist so that normally iron is recycled from effete red blood cells to red cell precursors, and in certain demand situations, i.e., after hemorrhage, storage iron becomes available for hemoglobin production. Much data has been accumulated on the kinetic aspects of iron incorporation into red cells and into storage depots (40, 44), but precise definition of the cell processes responsible for iron storage and mobilization remains obscure.

Previous studies demonstrated that ferritin particles may be found in the cytoplasmic matrix of spleen macrophages after feeding large quantities of iron-containing compounds to animals (47). The changes induced by overloading normal animals with iron compounds may, however, be different from those that occur in normal iron processing from hemoglobin. Muir and Golberg studied an in vivo system in which accumulations of subcutaneous macrophages were studied at a local site after injection of iron dextran (36). From analysis of biopsies at timed intervals after injection, these

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FIGURE 6 Area of cytoplasm from mouse peritoneal macrophage 2 days after ingestion of trypsinized rat red blood cells. A focal area of cytoplasm which contains abundant ferritin is surrounded by layers of cytomembranes (arrow). Section is unstained.  $\times 80,000$ .

FIGURE 7 Portion of cytoplasm from mouse peritoneal macrophage 56 h after ingestion of trypsinized rat red blood cells. Cytomembranes (arrow) appear to have partially surrounded an area of cytoplasm which contains ferritin and vesicles, probably of Golgi origin. The vesicles contain no ferritin. Portion of a cytoplasmic granule (G) containing ferritin is seen nearby. Section is unstained.  $\times 80,000$ .

FIGURE 8 Lipid droplet (L) in cytoplasm of a mouse peritoneal macrophage 2 days after ingestion of trypsinized rat red blood cells. Accumulations of ferritin particles are present at the periphery of the droplet. Unstained section.  $\times 80,000$ .

FIGURE 9 Pinocytic vacuole (PV) in the cytoplasm of a mouse peritoneal macrophage 56 h after the ingestion of aged rat red blood cells. Inside the vacuole is a sphere of membrane-bounded cytoplasm containing ferritin particles and vesicles. Serial sections would be required to determine whether this intravacuolar cytoplasm is an invagination or has pinched off to produce an autophagic vacuole. Most of the vesicles and endoplasmic reticulum profiles in the surrounding cytoplasm do not contain ferritin, but a few do. Section is unstained.  $\times 80,000$ .

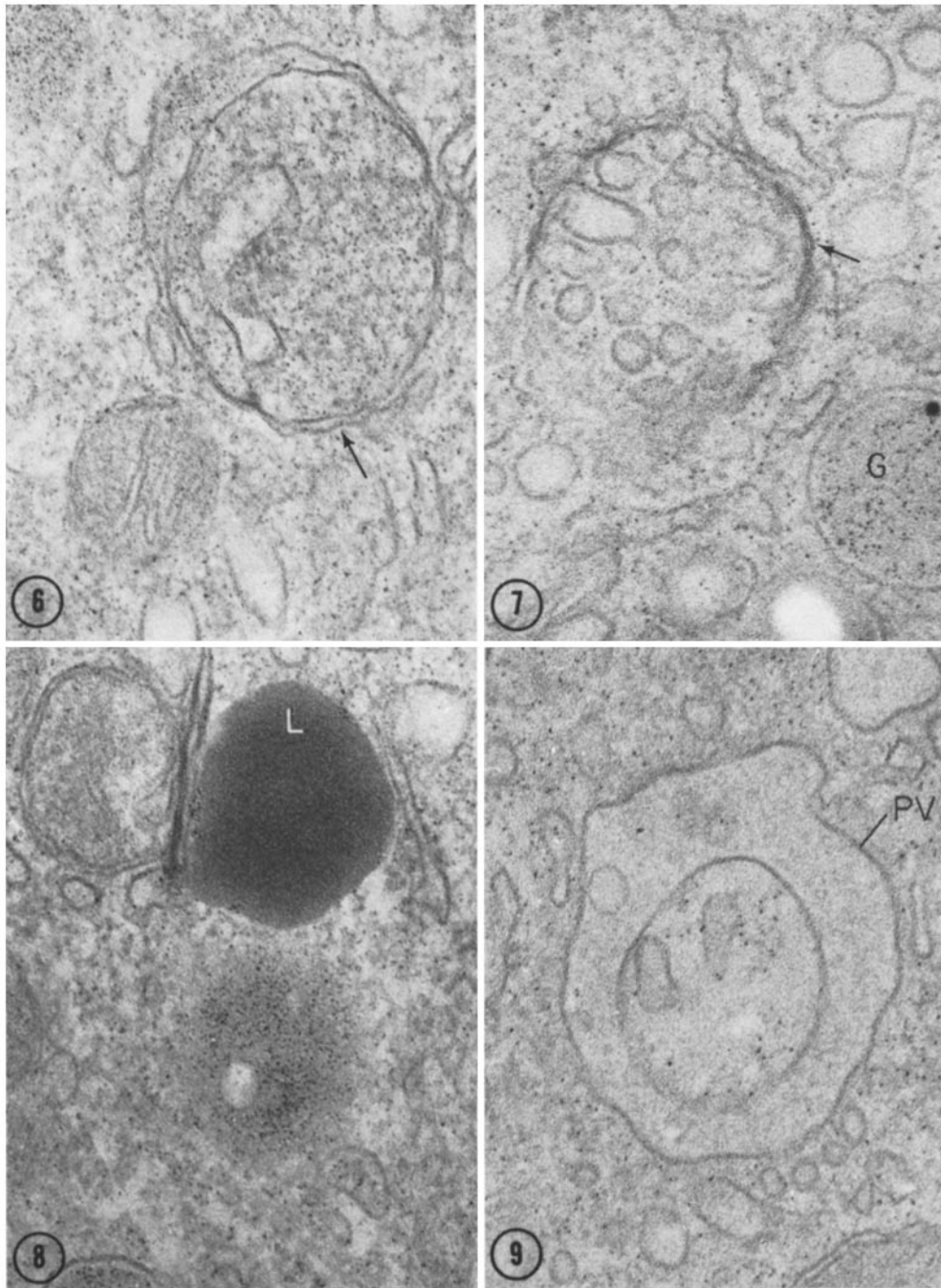




FIGURE 10 Cytoplasm from mouse peritoneal macrophage 4 days after ingestion of trypsinized rat red blood cells. Electron-transparent pinocytic vacuole (PV) shows an invagination. The invagination contains cytoplasm, a vesicle, and some ferritin particles. Section is unstained.  $\times 60,000$ .

authors concluded that ferritin first appeared in the cytoplasmic matrix of tissue macrophages, and latter the ferritin was located in granules, but they could not determine the mechanism of ferritin incorporation into granules. However, contradictory results were obtained in HeLa cells (49), where ferritin was first observed in bodies similar to lysosomes, and later appeared in the cytoplasm. From these morphologic studies, therefore, it has been impossible to decide whether ferritin in the cytoplasmic matrix was destined for storage in granules or represented some intermediate stage in the process of iron mobilization.

We have made our observations on the macrophage, a cell that is the primary iron storage cell in the body. Peritoneal macrophages used by us may

or may not have the same potentialities as macrophages in other sites which more commonly store iron. In any event, the phagocytic properties of macrophages permitted loading the cells with large amounts of iron-containing particles in the form of erythrocytes. This uptake of particles effectively loaded the cells with iron-rich compounds in relatively short periods of time, comparable to a "pulse", and allowed sequential observations to be made on red cell degradation, and on the site of appearance and the fate of intracellular ferritin *in vitro*.

Normally, about 30% of total body iron exists in the form of ferritin and hemosiderin stores. Ferritin is composed of a core of ferric iron which measures 6–8 nm in size, surrounded by a protein coat of apoferritin. The total diameter of the ferritin molecule is  $\sim 12$  nm (17, 41). The characteristic ultrastructure of ferritin makes it possible to detect the molecule by electron microscopy, and to follow its movement from one cell compartment to another. Hemosiderin appears to be distinguished from ferritin mainly by physiochemical criteria (25, 51) but clear distinction ultrastructurally is not possible. In the studies reported here, the electron-opaque deposits seen in the cytoplasm at early time periods were dispersed particles with uniform 6 nm size; thus, these particles had an ultrastructure typical of ferritin. The iron-rich deposits in cytoplasmic granules of macrophages at later times after ingestion of red blood cells were sometimes aggregated, so that it was not possible to establish morphologically whether these deposits were ferritin alone or some hemosiderin in addition. The appearance of ferritin in these cells is confirmed by polyacrylamide gel studies.

Morphologic studies alone have limitations for evaluation of overall ferritin content. Estimation of ferritin based on distribution of particles within the cytoplasm of thin sections can be inaccurate. Furthermore, resolution of ferritin by electron microscopy depends on the electron opacity of the iron core; apoferritin is electron transparent. There is evidence that the iron content of ferritin isolated from various tissues ranges from low values to nearly 25% of the dry weight (17). Both Fineberg and Drysdale have presented evidence that the initial product of ferritin synthesis has low iron content (17, 20). Therefore, ultrastructural detection of ferritin may be delayed until the molecule has a high content of iron.

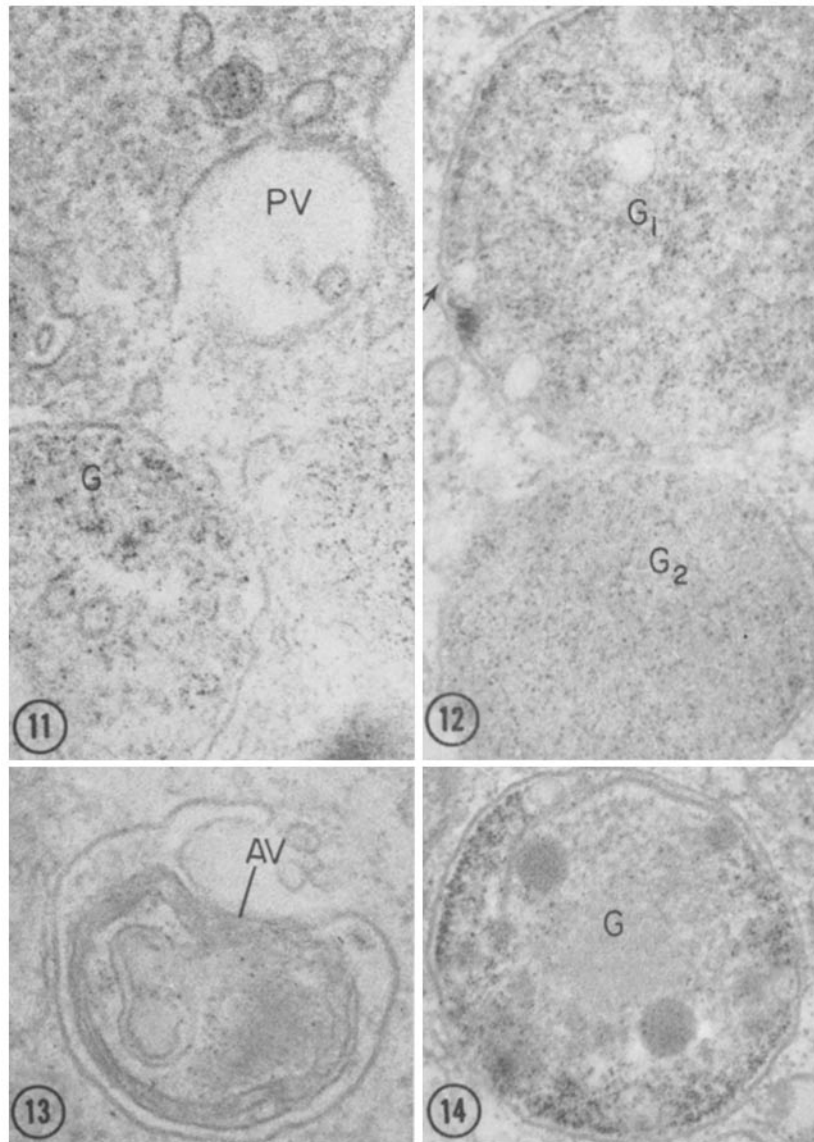


FIGURE 11 Area of macrophage cytoplasm 3 days after ingestion of aged rat red blood cells. A pinocytotic vacuole (*PV*) contains a small vesicle which has a few ferritin particles within it. A cytoplasmic granule (*G*) has abundant ferritin and possibly hemosiderin, and contains two small vesicles which also show ferritin. Unstained section.  $\times 80,000$ .

FIGURE 12 Cytoplasmic granules (*G*) from a mouse peritoneal macrophage 2 days after ingestion of aged rat red blood cells. Cytoplasmic granule *G*<sub>1</sub> shows vesicles, some of which contain a few ferritin particles. The limiting membrane of this granule shows a small invagination (arrow); a few particles of ferritin are seen in the neck of this invagination. Another granule *G*<sub>2</sub> has a relatively heavy, evenly distributed content of ferritin and no internal membranous elements. Unstained section.  $\times 80,000$ .

FIGURE 13 Autophagic vacuole (*AV*) in the cytoplasm of a mouse peritoneal macrophage 56 h after the ingestion of aged rat red blood cells. The vacuole has an irregular contour, and contains membranous lamellae and apparent cytoplasm with ferritin particles. Unstained preparation.  $\times 80,000$ .

FIGURE 14 Cytoplasmic granules (*G*) in a mouse peritoneal macrophage 2 days after ingestion of rat red blood cells. The granule has a heterogeneous content of droplets, membranes, amorphous material, and dense ferritin deposits as well as possible hemosiderin in peripheral areas. This granule may represent an autophagic vacuole which has evolved partially into a dense body. Unstained preparation.  $\times 80,000$ .

The results reported here indicate that iron-rich ferritin initially appears in the cytoplasm. We have no information on the precise locus of synthesis of the protein moiety of ferritin (apoferritin) or on

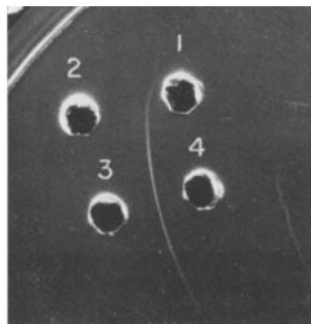


FIGURE 15 Ouchterlony double diffusion analysis of rabbit anti mouse ferritin antibody. Normal rabbit serum was placed in well 1; macrophage lysate (100  $\mu\text{g}$  protein) in well 2; rabbit anti mouse ferritin antiserum, in well 3; mouse ferritin (10  $\mu\text{g}$  protein) in well 4.

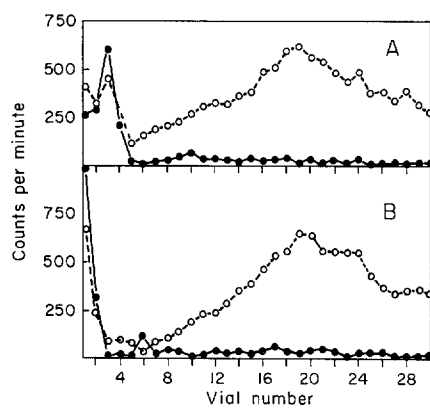


FIGURE 16 *A*, Polyacrylamide gel electrophoresis of lysates of macrophages which had ingested rat erythrocytes, were then transferred to normal medium for 4 h, followed by 1.5 h pulse of [ $^{14}\text{C}$ ]leucine. The cells were next placed in medium with supplementary cold leucine, and collected at the end of 30 h. 10  $\mu\text{l}$  of diluted normal rabbit serum was added to the lysate after preparation for electrophoresis. The current was applied for 2.5 h. Mouse ferritin (50  $\mu\text{g}$ ) when mixed with specimen *A* showed a brown color between vials 2 and 4.  $^{55}\text{Fe}$  activity ( $\bullet$ ) was solely located between the origin and vial 5;  $^{14}\text{C}$  activity ( $\circ$ ) was located in a narrow peak (vials 1-5) and a broad peak (vials 14  $\rightarrow$  end). *B*, Polyacrylamide gel electrophoresis of macrophage lysates treated as above, except for the addition of 10  $\mu\text{l}$  of rabbit anti mouse ferritin antiserum before loading on the gel. The  $^{55}\text{Fe}$  activity ( $\bullet$ ) was markedly retarded as was the  $^{14}\text{C}$  ( $\circ$ ) peak nearest the origin. The remainder of  $^{14}\text{C}$  distribution was unaffected

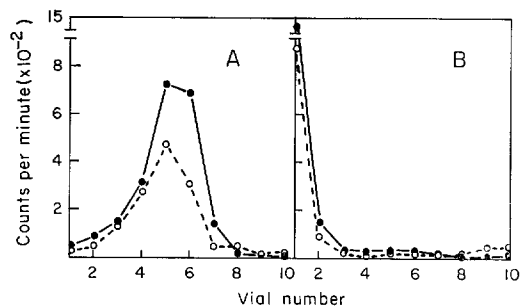


FIGURE 17 *A*, Polyacrylamide gel electrophoresis of macrophage lysates prepared and treated as in Fig. 16 *A*, except that electrophoresis was run for 4 h. Shown here are the coincident  $^{55}\text{Fe}$  ( $\bullet$ ) and  $^{14}\text{C}$  ( $\circ$ ) peaks between vials 3 and 7. Standard mouse ferritin (50  $\mu\text{g}$  protein) when added to gel *A* was located between vials 4 and 7. *B*, Polyacrylamide gel electrophoresis of macrophage lysates treated as above, except for the addition of 10  $\mu\text{l}$  of rabbit anti mouse ferritin antiserum. Both  $^{55}\text{Fe}$  ( $\bullet$ ) and  $^{14}\text{C}$  ( $\circ$ ) in the putative ferritin peak were markedly retarded.

the timing or the mechanism of insertion of iron into apoferritin. Some of these aspects are now under investigation. Drysdale and others have presented evidence for production of ferritin by free polyribosomes of liver cells (17, 26, 46). If this be the case in the macrophage, iron liberated from hemoglobin could escape from the phagocytic vacuole and combine in the ferric form with apoferritin in the cytoplasm.

The findings reported here contrast to those described by others in liver cells after dietary overloading with iron (6), in which it was suggested that ferritin synthesis occurred in the endoplasmic reticulum and Golgi saccules by a mechanism similar to that operating to synthesize, package, and store secretory or lysosomal constituents (7, 11). The cisternae of endoplasmic reticulum and the Golgi saccules of macrophages studied by us did not contain detectable ferritin at any observation period after the red cell ingestion.

The ferritin appearing initially in the cytoplasmic matrix is progressively translocated to cytoplasmic granules, and morphologic evidence presented here suggests that this intracellular translocation is accomplished by autophagy, a process now recognized to occur in various situations, including normal physiologic cell responses, cell remodeling, and response to toxic substances (reviewed in references 13 and 18). The transfer of ferritin from cytoplasm to granular compartments may well represent a special type of autophagy

that facilitates storage, rather than digestion, of a cytoplasmic constituent. We have no evidence that cell death and subsequent ingestion of macrophage debris contributed to the vacuolar content of ferritin in the living cells. Direct counts of macrophage numbers on the cover slips during the first 4 days after ingestion of erythrocytes showed that cell death during this time interval was nil or minimal (maximum 5%).

The translocation of ferritin to cytoplasmic granules takes place relatively long after ingestion of erythrocytes (between days 2 and 4). The visible lag may be produced in part by the time it takes ferritin to become highly ironized and therefore located by electron microscope techniques. In addition, the incorporation of ferritin into cytoplasmic granules may be a relatively inefficient system depending in part on new granule synthesis after phagocytosis, together with granule and vacuole movement through the cytoplasm.

Gel electrophoretic studies on lysates of macrophages which had ingested erythrocytes 30 h previously indicated that these cells formed a high molecular weight iron-containing compound migrating in the same general region as marker ferritin obtained from mouse livers and spleens. The slightly slower mobility of this macrophage material compared to the marker ferritin cannot as yet be explained with certainty. Since the rate of migration of proteins in SDS gels is inversely related to the log of the molecular weight (50), and is essentially unaffected by charge, the peritoneal macrophage ferritin apparently is somewhat larger than the ferritin from liver and spleen (23). Localization of iron-55 in the ferritin peak by itself could reflect ironization of preformed apoferritin. The incorporation of exogenous [<sup>14</sup>C]leucine into the same peak indicates that apoferritin or another molecule of the same size was being synthesized during the pulse period. Detailed consideration of apoferritin synthesis will be reported separately, but our observations indicate that incorporation of amino acids into ferritin is decreased at least 90% if 2 µg/ml of cycloheximide is present during the pulse. Studies on the electrophoretic behavior of heated cell lysates was also compatible with the presence of ferritin. Ferritin resists precipitation at 70°C (24). Electrophoretic patterns on heated cell lysate supernatants showed no detectable change in comparison to the patterns obtained with unheated lysates. The electrophoretic evidence was thus suggestive of the presence of ferritin. Further evidence of this conclusion was provided by altera-

tions in patterns obtained after treatment of the macrophage lysates with specific rabbit antimouse ferritin antiserum. The retardation of the <sup>55</sup>Fe peak in these antibody-treated gels established clearly that this radiolabel was in fact firmly associated with apoferritin.

One type of autophagic change in macrophages synthesizing ferritin appears to take place in pinocytotic vacuoles and forming cytoplasmic granules. We have seen in these cells many examples of large and small invaginations of vacuolar membranes somewhat similar to those reported after chloroquine exposure (19), or in HeLa cells (1). Pinching off of small invaginations is one mechanism that can result in the formation of multivesicular bodies (27). Degeneration of the membrane surrounding the invaginated ferritin-rich cytoplasm would result in deposition of ferritin in the pinocytotic vacuole or granule. Digestion of ferritin might or might not ensue, depending on local conditions and on delivery of hydrolases by fusion with primary or secondary lysosomes. In kidney cells resorbing heterologous hemoglobin (35) cytoplasmic organelles may be trapped between two vacuoles, which then fuse to form an autophagic vacuole. We have no evidence for this type of autophagy in macrophages after red cell ingestion.

We have recorded instances of cytoplasmic foci containing ferritin apparently being encircled by smooth membrane systems in the peripheral cytoplasm. This form of autophagy or multivesicular body formation has been reported in kidney tubule cells (35), in liver after the perfusion of glucagon (2), and in many other cell types (18, 22, 29-30, 38-39).

Multivesicular bodies in macrophages are for the most part derived from pinosomes and are lysosomal, or protolysosomal in nature (27). Microinvaginations of pinocytotic vacuolar membranes to form multivesicular bodies were seen on occasion by us in the control macrophages, which had received no red cells, and had little or no ferritin in their cytoplasm. It is thus not clear that all instances suggestive of autophagy in the ferritin-laden cells were the result of the presence of ferritin. Furthermore, if careful serial sections had been studied, perhaps many of the images thought to represent autophagy might have proved to be incomplete envelopment by cytomembranes, or invaginations still connected to the cytoplasm rather than true multivesicular bodies. Despite these limitations, the rarity of instances of apparent autophagy in control cells, and the fre-

quent finding of apparent autophagy in cells during the period of translocation of ferritin from cytoplasmic into granular compartments leads us to the tentative conclusion that autophagy plays an important role in the genesis of granular iron storage deposits in macrophages.

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