LOSS OF IRON FROM MOUSE PERITONEAL MACROPHAGES IN VITRO AFTER UPTAKE OF \[^{59}\text{Fe}]\text{FERRITIN AND }[^{55}\text{Fe}]\text{FERRITIN RABBIT ANTIFERRITIN COMPLEXES}

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ABSTRACT
Mouse peritoneal macrophages in culture for 24 h were exposed to horse \[^{59}\text{Fe}]\text{ferritin and rabbit antihorse }[^{59}\text{Fe}]\text{ferritin antibody complex and the amount of }[^{55}\text{Fe}]\text{Fe in the medium was assayed up to 2 days after the pulse uptake. Cell survival was assayed by photographing the same areas of the tissue culture Petri dish on successive days and by counting cell numbers per unit area. In experiments in which quantitative assay for cell death is negligible, about 10-20\% of the iron ingested by pinocytosis or phagocytosis is released to iron-free medium containing either freshly dialyzed or deironized newborn calf serum (10\%). Over the 2-day postpulse period, iron loss is linear. This loss of iron to the medium is significantly reduced by adding iron-saturated newborn calf serum in the postpulse recovery period. A significant portion of the iron released to the medium is bound to transferrin. When human serum is used in the tissue culture system, similar quantities (10-25\%) of the ingested iron are lost to the medium 2 days after the pulse.

INTRODUCTION
In the normal organism the macrophage is the cell responsible for processing hemoglobin derived from effete erythrocytes. The iron is either prepared for release and subsequent new hemoglobin production or stored in the form of ferritin. Kinetic information recently obtained after injection of labeled erythrocytes in vivo indicates that there may be a biphasic release of iron from the reticuloendothelial system (I). Macrophages comprise one cell component of this system. A rapid release of iron corresponds to that processed from ingested hemoglobin and is followed by a slower release of iron from the ferritin pool.

The cellular mechanisms involved in the excretion of iron from cells are largely unknown, but some of the responses to the intracellular presence of iron have been investigated (2, 3). Evidence has been presented to indicate that there may be an early rapid excretion of iron from macrophages in vitro after ingestion of erythrocytes (4). Some of the steps involved in cellular production of iron-containing proteins (ferritin) have been explored (2, 3, 5). It has been adequately demonstrated in several cell systems that the intracellular presence of iron induces apoferritin synthesis. In general, many cell types both in cases of iron loading of animals and after in vitro exposure to iron are able to synthesize apoferritin (2, 3, 5, 6).
The mixture was then incubated at 37°C for 1 h and then placed in tissue culture medium containing 10% newborn calf serum. The cells were maintained for 24 h in minimal essential medium (Eagle) with Earle's balanced salt solution (MEM) (Microbiological Associates, Bethesda, Md.) which is iron free, without phenol red, and supplemented with glutamine and 20% newborn calf serum. Macrophages were then given a 1–1.5-h pulse of [55Fe]ferritin or [55Fe]ferritin antiferritin complex in the same medium, washed four times, and then placed in tissue culture medium containing 10% newborn calf serum.

The serum for the experiments was obtained from a single lot, heat-inactivated at 56°C for 30 min, and used either untreated, or dialyzed against 0.02 M bicarbonate in 0.1 M saline, pH 7.6, or deionized by the method of Katz (9), or saturated with iron. In the latter instance, twice the calculated amount of ferric chloride necessary to saturate the serum transferrin was added to the serum. The mixture was then incubated at 37°C for 1 h and then at 4°C overnight. The excess free iron was next dialyzed off in bicarbonate-buffered saline. Human serum added to the tissue culture medium was collected within the week before use, heat inactivated, and screened with phase-microscope techniques to determine any macrophage cytotoxicity.

30 min after macrophages took up ferritin or ferritin complexes, a sample of the supernatant medium was obtained and counted for 55Fe radioactivity, and this value was subtracted from radioactivity in the medium at later time intervals. The interval after the pinocytic or phagocytic pulse will be designated as the "postpulse" or "recovery" phase. Petri dishes which contained no cells were treated in the same manner as macrophage cultures, and the serum protein receptor for the iron lost from macrophages will be identified here.

MATERIALS AND METHODS

Method of Tissue Culture

Mouse peritoneal macrophages were maintained in culture by the method of Cohn and Benson (8). Glassware was placed in concentrated HCl (6 N) overnight, repeatedly rinsed first in glass distilled water, then in 10% bicarbonate solution, and finally in glass distilled water. The cells were maintained for 24 h in minimal essential medium (Eagle) with Earle's balanced salt solution (MEM) (Microbiological Associates, Bethesda, Md.) which is iron free, without phenol red, and supplemented with glutamine and 20% newborn calf serum. Macrophages were then given a 1–1.5-h pulse of [55Fe]ferritin or [55Fe]ferritin antiferritin complex in the same medium, washed four times, and then placed in tissue culture medium containing 10% newborn calf serum.

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1 The process of release of iron or its loss from macrophages will be used interchangeably with the term excretion.

Radioactive Tracer Macromolecules and Complexes

Horse [55Fe]ferritin was reconstituted by the method of Pape et al. (10), reported previously (11). The iron in this ferritin may be adherent to the apoferritin or actually within cores (10, 12) (see Discussion). The iron content of this reconstituted ferritin was 200 μg/ml, determined by atomic absorption kindly performed by Dr. Cerami; protein content ranged between 1.6– and 3.5 mg/ml. Rabbit antihorse ferritin antibody was obtained from Cappel Laboratories (2 gm/ml, Downington, Pa.) and titered against [55Fe]ferritin. The maximum amount of precipitate was obtained at a volume of antigen to antibody of 1:9. The amount of soluble 55Fe radioactivity in the supernate was determined after centrifugation at 800 g for 20 min removed the precipitate. The antigen–antibody precipitate was washed six times in cold phosphate-buffered saline and stored in the buffer mixture until use. [55Fe]Ferritin rabbit antiferritin complex was incubated 1 h, and up to 2 days in medium obtained from macrophage cultures, and the amount of soluble and precipitate-associated 55Fe radioactivity was determined.

Polaroid Photographic Assay for Cell Survival

The assay was performed in the following manner. Pyrex Petri dishes 5 cm in diameter used to culture macrophages were scored with a diamond pencil on the outer surface of each dish. The dishes were placed on an inverted Olympus microscope (Olympus Corporation of America, New Hyde Park, N. Y.) with a trinocular head and phase optics so that the image could be directed to the viewing screen of the Polaroid MP-3 system and also photographed on Polaroid 57 5 × 4 cm Land film (Polaroid Corp., Cambridge, Mass.). By focusing first on the grid markings and then on the cells, the same area of a dish could be photographed immediately before the pulse and at time intervals afterwards up to 2 days.

Polyacrylamide Gel Studies

The Ornstein-Davis system (13, 14) for polyacrylamide gels was used with Tris-glycine buffer, pH 8.3, in the chamber. The gels were run at 2 mA/gel until the material entered the gel (≈ 5 min); then at 5 mA/gel. The method was modified so that the separating gel was 7% polyacrylamide instead of 5%. Bromphenol blue was used as the marker to gauge the appropriate length of the run. In some instances a stacking gel of 5% polyacrylamide was used and modified so that 0.56% ammonium persulfate was used in place of riboflavin (solution E).

Tissue culture media from macrophage cultures were...
concentrated to 0.1 of the original volume under vacuum in S & S collodion bags no. 100 for protein concentration (Schleicher & Schuell, Inc., Keene, N. H.). 100-μl concentrates of medium were applied to the gels.

**Marker Proteins used in the Electrophoretic System**

Bovine transferrin (Pentex, Miles Laboratories, Inc., Kankakee, Ill.), 1 mg/ml, was incubated with ferric chloride-55 in 0.1 N HCl (21.9 mc/mg, New England Nuclear, Boston, Mass.) at 37°C for 1 h, stored overnight at 4°C, and then dialyzed against four changes of 0.02 M NaHCO₃, 0.1 M NaCl (pH 7.4-7.6) to remove free ionic iron.

Human and mouse sera were treated similarly as described above. The mobilities of mouse and bovine transferrins were identical; human transferrin showed slightly slower mobility at pH 8.3.

The electrophoretic pattern of reconstituted horse [55Fe]ferritin is shown in Fig. 6 C. A fast migrating major peak and a slower migrating minor peak were obtained. Horse [55Fe]ferritin was also incubated in 10% conditioned medium obtained from 2-day cultures of macrophages. The amount of radioactivity in the bovine transferrin peak was compared to that in the ferritin peaks. 100 μl of bicarbonate-dialyzed, iron-saturated serum with an iron-binding capacity of 300 μg/100 ml was mixed with 5 μl stock [55Fe]ferritin and the mixture was incubated first for 1 h at 37°C and then for 24 h in the cold, before polyacrylamide gel electrophoresis.

Horse spleen ferritin, 1 mg/ml (Pentex, Miles Laboratories, Inc.), was used in the gel system as a nonradioactive color indicator of horse ferritin mobility. Two major bands, identical to those obtained with reconstituted horse [55Fe]ferritin, were observed. Poorly defined minor bands proximal and distal to the minor peak were also present. This ferritin contained only protein which stained for iron on polyacrylamide gels and formed a single precipitin band against specific antiserum on Ouchterlony plates.

**Identification of Horse Ferritin and Mouse Ferritin**

The two species of ferritin were identified by methods which utilize their immunochemical specificity. Rabbit antibody against mouse ferritin which was produced by successive immunizations with isolated mouse spleen and liver ferritin prepared by successive crystallizations with cadmium sulfate (15) showed cross-reactivity to horse ferritin. The cross-reacting antibody in rabbit antiserum was adsorbed by adding 0.2 ml of this serum to 10 μl of 1 mg/ml horse ferritin. Incubation was carried out at room temperature for 1 h, then in the cold (4°C), overnight. The precipitate was separated, and the supernate was again adsorbed two successive times. After the final adsorption a precipitin band was demonstrated against mouse ferritin, not horse ferritin, on Ouchterlony plates. The precipitin band was brown in color and positive for iron after exposure to the Prussian blue stain. Retardation of mouse ferritin by the species-specific antimouse ferritin antibody was also demonstrated in the polyacrylamide gel system. On polyacrylamide gels the isolated mouse ferritin contained only protein which also showed Prussian blue positivity.

**Biochemical Determination**

Iron content and total iron-binding capacity of serum was determined by the method of Schade et al. (16). Iron content of newborn calf serum was 60 μg/100 ml; deionized serum contained > 30 μg/100 ml iron; iron-saturated serum 300-400 μg/100 ml.

**Scintillation Counting**

Assays were performed on the postpulse supernatant medium and cell digests. [55Fe]radioactivity was determined on the supernatant medium which had been centrifuged at 800 g for 20 min. All but the lowest 1 ml was separated off and an aliquot counted. The remaining 1 ml of medium which could contain cell debris was mixed and an aliquot was also counted. The macrophages were washed three times with MEM (without serum), drained, and then digested in 2 ml 1 N NaOH at 37°C for 1 h. 50- and 100-μl aliquots were added to the scintillation mixture which contained toluene, alcohol, and Liquifluor (New England Nuclear) in a ratio of 60:24:6 (vol:vol:vol). Scintillation counts were performed on a channel calibrated for tritium activity. All samples were corrected for any quench.

**Microscopic Techniques**

Autoradiography was performed on thick sections of Epon-embedded material prepared as cited previously (17, 18). Thick sections were then coated with Ilford L-4 emulsion (Ilford Ltd., Ilford, Essex, England), stored from 2 to 3 wk, and then developed in D-19 developer. The sections were then stained with alkaline azure A (0.2% in 1% sodium borate).

Prussian blue stain was used after cells grown on microscopic cover slips were fixed in 2.5% glutaraldehyde in cacodylate buffer 0.1 M, pH 7.4, for 10 min. The specimens were then rinsed in water and stained 30 min with a mixture of 2% HCl and 2% potassium ferrocyanide (19). The reagent mixture was removed and the specimen mounted in water for combined phase- and light-microscope observations.

The trypan blue test for cell viability was performed by adding to the cells a mixture of 0.1% trypan blue in isotonic saline, serum, and medium in a volume of 1:1:1. The number of stained nuclei in cells adherent to the cover slips were counted.
**Pinocytic Activity of the Cells**

Uptake of iron-binding proteins in the postpulse period was determined. Transferrin in newborn calf serum was saturated with \(^{55}\text{Fe} \text{Cl}_2\) as described above, dialyzed against bicarbonate-buffered saline, and mixed with newborn calf serum in a ratio of 1:3. The mixture was added to the tissue culture medium in a final concentration of 10%. Radioactivity in the supernatant medium after a pulse of nonradioactive antigen-antibody complex was determined at 30 min, and 24 and 48 h after the pulse.

**Trypsin Treatment of Cells**

The tissue culture medium was removed and the cells were washed in saline. Trypsin (Grand Island Biological Co., Grand Island, N. Y.) in a dilution of 1:3 with saline was next added to the cell culture for 30 min at 37°C. The trypsin solution could then be assayed for \(^{55}\text{Fe}\) activity, and compared to activity in the cell digest.

**Statistics**

Analyses were performed with the dependent two-tailed t test (20) on duplicate paired determinations. Conditions for phagocytic or pinocytic uptake could vary with each set of determinations. Therefore, two sets of variables, one a control, were tested and the pairs analyzed.

**RESULTS**

**Quantitation of Macrophage Survival in Culture**

Cell mass of macrophages attached to glass was determined initially by assaying protein (21), DNA (22), and labeling of cells with chromium-51 (23) and colloidal gold-128. Viability of cells attached to glass determined by the trypan blue stain was > 99% immediately after phagocytosis of complex and 100% 1 and 2 days later. During the time of culture some additional cells could lyse or detach. The following assay was determined to best assess the percent survival of macrophages on the glass surface. The same areas on the tissue culture dish could be located and photographed on two subsequent days (Fig. 1 a, b).

Total surface area of the Petri dish was approximately 16.5 cm². The total area photographed was 2.25 mm² or about 0.1% of the total surface area. Between 65 and 80% of the area photographed was used for the counts by masking equal areas of the photographs taken immediately before the pulse and 2 days later. The areas of the culture dish to be photographed were randomly selected. Representative mean cell counts and standard deviation per photographic frame counted in five selected areas of duplicate cultures were, for example, 245 ± 26 and 270 ± 18. Between 1,000 and 1,500 macrophages were counted for each determination. Duplicate determinations, in which cell survival ranged between 92–100%, repeatedly showed that the percent of iron excretion did not vary. This aspect is illustrated in the first and third determinations (Table II).

Reproducibility of cell counting was tested by photographing the same area of the dish on separate sheets of film and counting the cells in a masked area. There was agreement within 0.007%. Previous time lapse cinematography of macrophages in culture by Hirsch and Cohn showed that the cells are not motile, therefore the same cell population was counted on different days.

Any variation in cell counting accuracy on two successive days could be attributed to human error in the counting procedure itself; and/or to the fact that, after two additional days in culture, macrophages show an increase in the size of their pseudopods and in their flatness. The latter effect could alter resolution at the perimeters of the field counted and significantly contribute to the consistently slightly lower cell count 2 days after the pulse. When there was 85% cell survival or less, cell content values or excretion values were unusually high; there was increased sedimentable \(^{55}\text{Fe}\) material in centrifuged samples of the supernatant medium. Cultures which showed less than 90% cell survival were disregarded. Macrophages in culture under these conditions showed no cell division.

**\(^{55}\text{Fe}\) Loss from Mouse Peritoneal Macrophages after Uptake of Horse Spleen \([^{55}\text{Fe}]\text{Ferritin}\)**

The light microscope autoradiographic appearance of macrophages after pulse uptake of \([^{55}\text{Fe}]\) ferritin is demonstrated in Fig. 2. The distribution of autoradiographic grains over the cells indicated that all the macrophages engaged in pinocytic activity. Macrophages in culture for 24 h received radiolabeled ferritin, were washed, and then replaced in medium for 2 days. The effect of the use of deionized bicarbonate-dialyzed bovine serum in the tissue culture system is illustrated in Table I. Cells maintained 2 days in 10% deionized serum after pulse uptake favored loss of iron up to ≈ 25% of cell uptake, in contrast to recovery in untreated...
FIGURE 1 (a) Note the phase-microscopic appearance of the macrophage culture 24 h after plating. The cell culture in the Petri dish was placed on an inverted microscope and photographed with Polaroid Land film. The majority of the cells are spread out and demonstrate 2-3 pseudopods; some cells are round, but in general they are compatible in size with macrophages. Very small particles occurring singly in doublets or triplets (small arrow) are assumed to be cell debris and are not included in the count. The phase-dense lines (long arrows) represent at this plane of focus the image of the grid markings on the outer surface of the dish. × 100. (b) Phase-microscopic appearance of identical area of same tissue culture 2 days after uptake of horse $[^{59}$Fe]$\text{ferritin}$ rabbit antiferritin complex. The majority of the cells are elongated and very few are round; no cell debris is visible. Identical area in the photographic frame shows characteristic phase-dense appearance of grid markings (long arrows) at this plane of focus. Cell counts of identical surface areas on $a$ and $b$ are within 10%. × 100.
FIGURE 2 Light microscope autoradiograph of macrophage culture grown on cover slip which at the end of 24 h after plating was given a pulse of \[^{55}\text{Fe}]\text{ferritin}\ in the medium, washed, fixed in 2.5% glutaraldehyde, washed again, air dried, and coated with Ilford L-4 autoradiographic emulsion. After development of the autoradiograph the specimen was stained with Giemsa's. The autoradiograph shows elongated cells with 1-3 pseudopods. Grains appear to be evenly distributed in the entire cell. The nucleus is dark staining, and centrally located in the cell. Lucent areas in the cytoplasm represent either lipid droplets or pinocytic vacuoles. Background radioactivity is low, and this indicates fairly complete washing out of surface protein macromolecule. × 2,000.

FIGURE 3 Autoradiograph of mouse peritoneal macrophages which after 24 h in culture and then 1 h pulse of \[^{55}\text{Fe}]\text{ferritin-antiferritin complex}\ show large vacuoles (arrows) filled with moderately dense material representing the antigen-antibody complex. Nuclei are dark staining; autoradiographic grains representing \[^{55}\text{Fe}]\text{are localized over the vacuoles. In general, radioactivity outside the cells is low. Several cells in the upper left corner probably have been disrupted during fixation and processing. Specimens were fixed for electron microscopy and embedded in agar. Thick sections were cut, coated with emulsion, developed and stained with alkaline azure A (see Materials and Methods). × 2,000.}

FIGURE 4 Mouse peritoneal macrophages 2 days after uptake of \[^{55}\text{Fe}]\text{ferritin-antiferritin complex}\. The nuclei (arrow) appear to be lighter staining (normal variation in staining). No vacuoles are visible and autoradiographic grains representing \[^{55}\text{Fe}]\ activity are randomly distributed throughout the cell. Cell preparations were treated in a manner similar to those in Fig. 3. × 2,000.

Control samples were prepared to determine the amount of surface \[^{55}\text{Fe}]\text{ferritin} adherence on glass. About 0.01% of the pulse \[^{55}\text{Fe}]\text{ferritin}\ was adherent to a Petri dish without macrophages (blank), and was approximately 5% of the cell uptake of tracer in dishes with plated macrophages. In some experiments macrophage cultures were exposed to trypsin immediately after the pulse and washes. The trypsin-removable radioactivity varied between 5-10% of the cell uptake. Some cultures were maintained in medium containing 10% deionized bicarbonate-dialyzed serum 2 days after pulse uptake and initial trypsinization. Before the cells were digested in alkali, they were exposed to trypsin again, and about 10% of cell uptake was found in the trypsin digests. The additional \[^{55}\text{Fe}]\ removed after 2 days would indicate that more iron reaccumulated on the dish and/or cell surface. Cell survival under these conditions was greater than 90%.

Polyacrylamide gel electrophoresis was used to estimate the amount of free or labile iron in horse \[^{55}\text{Fe}]\text{ferritin} and to identify the extracellular protein carrier or iron. \[^{55}\text{Fe}]\text{ferritin} and bovine serum mixtures showed \(\approx 5\%\) total radioactivity in the transferrin peak. When horse \[^{55}\text{Fe}]\text{ferritin}\ was incubated with "conditioned" medium obtained from a 2-day culture of macrophages, 6-10% of the
TABLE I
Loss of $^{55}$Fe from Mouse Peritoneal Macrophages within 2 Days after Uptake of Horse $[^{55}$Fe] Ferritin

<table>
<thead>
<tr>
<th></th>
<th>Deironized serum</th>
<th>Untreated serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>%</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>8</td>
<td></td>
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<tr>
<td>16</td>
<td>8</td>
<td></td>
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</tbody>
</table>

* Pulse fluid contains $\sim 4.6 \times 10^4$ cpm; cell uptake 5-25% of pulse counts in $\sim 2$ h.
‡ Cell survival $> 90\%$—Polaroid assay.
§ Determinations paired, uptake comparable in pairs, $P > 0.00005$.

iron in the sample was found in a peak which corresponded to transferrin (bovine). If the maximum figure for surface adherence was 10%, then approximately 1% of the figures for excerted iron could be due to labile iron in the $[^{55}$Fe]ferritin rather than to loss of iron from the cells. Concentration of the 2-day recovery medium followed by polyacrylamide gel electrophoresis showed $^{55}$Fe radioactivity in both ferritin and transferrin peaks, with some material at the origin (Fig. 5).

$^{55}$Fe Loss from Mouse Peritoneal Macrophages after Uptake of Horse $[^{55}$Fe] Ferritin Rabbit Antiferritin Complex

**Properties of the $[^{55}$Fe] Ferritin Rabbit Antiferritin Complex:** 97% of the radioactivity was precipitated as a ferritin-antiferritin complex after the addition of the rabbit antihorse ferritin antiserum to the reconstituted $[^{55}$Fe]ferritin. The precipitated complex showed no reduction in activity after storage in phosphate-buffered saline (0.01 M phosphate, pH 7.4). The $[^{55}$Fe]ferritin antiferritin complex was incubated for 2 days at 37°C in supernates from macrophages in culture for 2 days. After precipitation of the radioactive ferritin with specific antibody and centrifugation, 8-10% total radioactivity remained soluble; in complex not exposed to medium, $\approx 3\%$ was soluble. The additional few percent $^{55}$Fe in the

soluble fraction was probably due to substances produced by macrophages; these substances could be liberated into the medium (7).

**Morphology and Autoradiographic Appearance of Macrophages after Ingestion of Horse $[^{55}$Fe] Ferritin Rabbit Antiferritin Complex:** The morphology of the macrophages under the light microscope immediately after pulse uptake of antigen-antibody complex and 2 days later is demonstrated in the autoradiographs shown in Figs. 3 and 4. Immediately after the 1.5-h pulse, macrophages contained numerous vacuoles with content. The major route of iron uptake was phagocytosis. The size of the complex after sonication ranged between 2 and 10 μm. Studies to be reported elsewhere indicate that ionic iron transport is negligible in 1-2 h. Autoradiographic grains were localized on phagocytic vacuoles. 2 days after uptake of the complex, there were no vacuoles visible and autoradiographic grains were distributed throughout the cytoplasm. Distinction between localization in granules or cytoplasmic matrix was not possible in these preparations. In both samples, very little extracellular radioactivity was visible. Prussian blue strains on comparable specimens showed blue reactivity distributed throughout the cytoplasm of the cells.

**Quantitation of $^{55}$Fe Loss from Mouse Peritoneal Macrophages after Uptake of Horse $[^{55}$Fe] Ferritin Rabbit Antiferritin Complex:** Horse $[^{55}$Fe]ferritin rabbit antiferritin complex was given in a 1-1.5-h pulse to macrophages in culture for 24 h. After removal of the pulse fluid and washing, the cells were maintained in medium containing 10% serum (bovine or human) treated in one of the several ways. Cell uptake of the $^{55}$Fe complex was approximately 1% of pulse radioactivity.

Macrophages cultured in deironized newborn calf serum (Fe < 30 μg/100 ml) showed an uptake of antigen-antibody complex that was proportional to cell number, dose, and time of exposure. Iron loss was linear within the 2-day period after the pulse. The dose-response effect is demonstrated in Table II. At lower levels of uptake (2.75 μg Fe/1.5 million plated macrophages or 100-200 μg total cell protein), excretion of iron was between 10-20% of cell uptake. Under these conditions cell survival was optimal (Table III). When the supernatant medium was concentrated and electrophoresed in a polyacrylamide gel system, activity
| Table II |
| Loss of $^{55}$Fe from Mouse Peritoneal Macrophages after Uptake of Horse $[^{55}$Fe]Ferritin Rabbit Antiferritin Complex |

<table>
<thead>
<tr>
<th>Cell content$^{55}$Fe</th>
<th>Output $^{55}$Fe</th>
<th>Release of $^{55}$Fe</th>
<th>Cell survival$^|$</th>
</tr>
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<tbody>
<tr>
<td>cpm</td>
<td>cpm</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>$1.6 \times 10^4$</td>
<td>$3.1 \times 10^4$</td>
<td>15</td>
<td>92</td>
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<td>$4.0 \times 10^4$</td>
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<td>98</td>
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<td>$2.9 \times 10^4$</td>
<td>$5.3 \times 10^4$</td>
<td>15</td>
<td>100</td>
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<tr>
<td>$1.0 \times 10^4$</td>
<td>$1.0 \times 10^4$</td>
<td>9</td>
<td>94</td>
</tr>
<tr>
<td>$2.1 \times 10^5$</td>
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</tr>
<tr>
<td>$3.6 \times 10^5$</td>
<td>$2.9 \times 10^4$</td>
<td>7</td>
<td>99</td>
</tr>
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</table>

* Pulse fluid contains $4.6 \times 10^5$ cpm.
† All serum deironized (iron content < 30 μg/100 ml); iron release within 2 days after pulse uptake. Excretion values were corrected for any extracellular $^{55}$Fe present in the medium 30 min after the pulse uptake.
§ Polaroid assay.

| Table III |
| Comparison of $^{55}$Fe Loss from Mouse Peritoneal Macrophages Maintained in Untreated or Deironized Newborn-Calf Serum after Uptake of $[^{55}$Fe]Ferritin Antiferritin Complex |

<table>
<thead>
<tr>
<th>Cell content$^{55}$Fe</th>
<th>2-Day recovery medium</th>
<th>Release of $^{55}$Fe</th>
<th>Serum (10%) in culture medium (paired determinations)$^|$</th>
</tr>
</thead>
<tbody>
<tr>
<td>cpm</td>
<td>cpm</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>$1.1 \times 10^4$</td>
<td>$5.4 \times 10^4$</td>
<td>4</td>
<td>Untreated</td>
</tr>
<tr>
<td>$1.0 \times 10^4$</td>
<td>$1.1 \times 10^4$</td>
<td>10</td>
<td>Deironized</td>
</tr>
<tr>
<td>$3.3 \times 10^4$</td>
<td>$1.5 \times 10^4$</td>
<td>6</td>
<td>Untreated</td>
</tr>
<tr>
<td>$3.2 \times 10^4$</td>
<td>$6.0 \times 10^4$</td>
<td>18</td>
<td>Deironized</td>
</tr>
<tr>
<td>$1.9 \times 10^4$</td>
<td>$4.0 \times 10^4$</td>
<td>2</td>
<td>Untreated</td>
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<tr>
<td>$2.1 \times 10^4$</td>
<td>$2.0 \times 10^4$</td>
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</tr>
<tr>
<td>$2.8 \times 10^4$</td>
<td>$1.8 \times 10^4$</td>
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<td>$2.8 \times 10^4$</td>
<td>$2.4 \times 10^4$</td>
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<td>$2.6 \times 10^4$</td>
<td>$1.7 \times 10^4$</td>
<td>6</td>
<td>Deironized</td>
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</table>

* Cell survival 90% or greater, determined by Polaroid assay.
† Release of iron within 2 days after pulse uptake. Values corrected for any extracellular $^{55}$Fe in the medium 30 min after the pulse uptake.
§ Deironized serum iron values < 30 μg/100 ml. Untreated serum iron values 60 μg/100 ml. $P < 0.01$.

was demonstrated in the peak which had the same mobility as bovine transferrin (Fig. 5).

In control studies no $^{55}$Fe activity was detected either in the medium or on the dish surface (blanks) 2 days after a 1.5-h pulse of the complex. The amount of $^{55}$Fe in the ferritin-antiferritin complex which might be adherent to the cell surface immediately after pulse uptake was also
determined. Approximately 13% of the radioactivity observed in the cell fraction was removed by trypsinization after the pulse uptake of the antigen-antibody complex. This activity was presumably derived from the complex adherent to the surface of cells; under these conditions cell survival was good. As cited above, it was determined that in 2 days about 10% of 55Fe in the complex was labile, then about 1% of the excreted radioactivity was estimated to be derived from the extracellular labile iron and bound to the transferrin in the serum.

Similar experiments were performed in which bicarbonate-dialyzed serum was substituted for deironized serum in the tissue culture system. No statistical difference was demonstrated between recovery in deironized and that in bicarbonate-dialyzed serum in six paired determinations.

In some experiments macrophages were plated either in medium containing deironized serum or in iron-saturated newborn calf serum. In nine such paired determinations statistically significant suppression of iron loss to the supernatant tissue culture medium occurred (Table IV). Control experiments were performed to determine the extent of exchange of 55Fe in ferritin and that in saturated newborn calf serum after 24-h incubation. Tests for this purpose were made by polyacrylamide gel electrophoresis of the ferritin-transferrin mixtures. About 10% of the iron in ferritin is exchangeable with that in iron-saturated newborn calf serum. The maximum contribution of iron in the extracellular complex to transferrin was estimated then to be ≈ 1% of excretion values.

Similar experiments were performed on mouse peritoneal macrophages which had been cultured in human serum (Table V). The amount of iron excreted 2 days after pulse uptake was comparable, i.e. 14-23%, and even somewhat higher than that obtained with newborn calf serum. In one instance human serum from a patient with iron deficiency collected within 1 wk before use, and dialyzed, not deironized but heat-inactivated, produced conditions favorable for 20% iron excretion. Polyacrylamide gel electrophoresis performed on concentrated supernates of culture medium showed activity in the peak corresponding to that which had the mobility of human transferrin.

**Production of Mouse [55Fe]Ferritin by Mouse Peritoneal Macrophages After Uptake of Horse [55Fe]Ferritin Rabbit Antiferritin Complex**

The rabbit antibody was made species-specific for mouse ferritin by successive adsorptions with horse ferritin (see Materials and Methods). Ly- sates of macrophages which had ingested horse [55Fe]ferritin antibody complexes and had

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**Table IV**

<table>
<thead>
<tr>
<th>Cell content</th>
<th>2-Day recovery medium</th>
<th>Output of 55Fe</th>
<th>Serum (10%) in 2-day recovery medium (paired determinations)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cpm</td>
<td>cpm</td>
<td>%</td>
<td>Deionized</td>
</tr>
<tr>
<td>1.0 × 10^6</td>
<td>1.0 × 10^6</td>
<td>10</td>
<td>Iron-saturated</td>
</tr>
<tr>
<td>5.6 × 10^6</td>
<td>3.3 × 10^6</td>
<td>5</td>
<td>Deionized</td>
</tr>
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<td>6.4 × 10^6</td>
<td>7.5 × 10^6</td>
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</tr>
<tr>
<td>6.7 × 10^6</td>
<td>2.0 × 10^6</td>
<td>2</td>
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<tr>
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<td>2</td>
<td>Iron-saturated</td>
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<tr>
<td>2.4 × 10^6</td>
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<td>14</td>
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<td>3.5 × 10^6</td>
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<td>8</td>
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<tr>
<td>1.4 × 10^6</td>
<td>5.0 × 10^6</td>
<td>24</td>
<td>Deionized</td>
</tr>
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<td>5.0 × 10^6</td>
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<td>Deionized</td>
</tr>
<tr>
<td>1.7 × 10^6</td>
<td>0</td>
<td>0</td>
<td>Iron-saturated</td>
</tr>
<tr>
<td>2.7 × 10^6</td>
<td>4.0 × 10^6</td>
<td>6</td>
<td>Deionized</td>
</tr>
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<td>2.9 × 10^6</td>
<td>4.0 × 10^6</td>
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<tr>
<td>4.7 × 10^6</td>
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<td>Iron-saturated</td>
</tr>
<tr>
<td>4.6 × 10^6</td>
<td>0</td>
<td>0</td>
<td>Iron-saturated</td>
</tr>
</tbody>
</table>

*Cell survival determined by Polaroid assay; survival > 90%.
† P < 0.01, release of iron within 2 days after pulse uptake. Values corrected for any extracellular 55Fe in the medium 30 min after pulse uptake.
§ Deionized serum iron values < 30 µg/100 ml. Iron-saturated serum values > 300 µg/100 ml.

**Table V**

<table>
<thead>
<tr>
<th>Serum donor</th>
<th>Cell uptake</th>
<th>2-day 55Fe</th>
<th>Output</th>
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<td>cpm</td>
<td>%</td>
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<td>TJ</td>
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<td>14</td>
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<tr>
<td>JGH</td>
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<td>9.0 × 10^6</td>
<td>18§</td>
</tr>
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<td>MF</td>
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<td>1.4 × 10^6</td>
<td>19</td>
</tr>
<tr>
<td>JW</td>
<td>1.0 × 10^6</td>
<td>2.7 × 10^6</td>
<td>23</td>
</tr>
</tbody>
</table>

* All serum deironized (Fe content < 30 µg/100 ml) except JW (serum iron content 18 µg/100 ml) which was bicarbonate dialyzed; 2-day recovery medium contained 10% serum. Values were corrected for any extracellular 55Fe in the medium 30 min after the pulse uptake.
§ Polyacrylamide gel showed radioactivity in transferrin peak.
FIGURE 6 Graphic presentation of $^{55}$Fe radioactivity in Ornstein-Davis polyacrylamide gel electrophoresis system with stacking gel overlay (see Materials and Methods). Paired specimens A and B, C and D were electrophoresed on different occasions under approximately the same conditions. (A) Electrophoretic migration of $^{55}$Fe activity in macrophage lysates 1 day after uptake of horse $^{55}$Fe-ferritin rabbit anti-ferritin complex. Major peak of radioactivity corresponds to that obtained for mouse ferritin isolated from liver and spleen. No distinct slow migrating peak is demonstrated. Slight increase in activity at 4 cm could be due to some retardation at the 5–7% polyacrylamide gel interface. When antigen-antibody complex is electrophoresed in this system the complex remains at the origin. (B) Macrophage lysates prepared from cultures treated like those in A. Lysates been maintained in culture for 24 h showed one major and one minor peak on polyacrylamide gel electrophoresis. There was a slow, migrating minor peak very near the 5–7% polyacrylamide interface in the stacking gel, and a large, fast, major peak (Fig. 6 A). When lysates were incubated with species-specific rabbit antimouse ferritin antibody, the major part of reactivity was retarded at the origin or near the interface of the stacking gel (Fig. 6 B). Polyacrylamide gel electrophoresis performed with horse $^{55}$Fe-ferritin showed the characteristic minor, slow migrating and major, somewhat faster migrating bands (Fig. 6 C). When the same mouse ferritin specific antibody employed above was incubated with the horse ferritin, no retardation was observed (Fig. 6 D).

UPTAKE OF $^{55}$Fe TRANSFERRIN BY MACROPHAGES WITHIN 2 DAYS AFTER UPTAKE OF ANTIGEN-ANTIBODY COMPLEX: To determine the extent of pinocytosis of $^{55}$Fe-transferrin after phagocytosis, cells exposed to antigen-antibody complex (nonradioactive) for 1 h were allowed to recover in medium with 10% bovine serum which contained 1/5 saturated $^{55}$Fe-transferrin. Uptake in this medium was determined 30 min, and 24 and 48 h after the pulse. There was approximately 13% uptake on the surface of macrophages and on the surface of dish in the first 30 min and 2% additional uptake in the next 24–48 h.

DISCUSSION

Factors which Influence Iron Loss from Macrophages

What we have demonstrated here is that there was loss of iron which corresponded to that from a ferritin pool in macrophages. Some of the condi-

MARTHA E. FEDORKO  Loss of Iron from Mouse Peritoneal Macrophages  811
equal to that in the serum and possibly bound to transferrin localized on the surface of the cells. Low concentration (10%) of bicarbonate-dialyzed serum facilitated loss of iron, whereas iron-saturated transferrin in the medium inhibited iron excretion. Recovery of cells in medium with relatively high serum concentrations (40%) of dialyzed bovine serum inhibited iron loss.

Loss of iron from macrophages was detected after culture in medium containing human or murine sera. Nontoxic, heat-inactivated human serum did not require dialysis for excretion of iron. Human serum was prepared a few days before use, and some serum changes which might occur on prolonged storage may have been avoided. Pooled, nonhemolyzed, heat-inactivated mouse serum frequently was toxic for mouse macrophages, but in those instances in which the pooled serum showed no cell toxicity, excretion of iron was in the range of 20–30%.

The extracellular protein receptor for iron in this tissue culture system was transferrin, but the species identity of the transferrin could not be determined from the studies reported here. The mobilities of the mouse and bovine transferrins on polyacrylamide gels were similar; immunochromatographic studies could provide a conclusive answer. Macrophages from mice could under certain circumstances produce autologous transferrin (24). In the cell system described here iron-saturated bovine serum suppressed loss of iron. Bovine transferrin then appeared to be the receptor for iron lost from macrophages.

Some experiments were performed in which trypsinization of the cells was carried out twice—once immediately after the uptake of the radioactive complex, and again 2 days later. The trypsin digest after 2 days in culture contained 10% 55Fe radioactive activity of the cell lysate. There may be, therefore, a fraction of extracellular iron almost equal to that in the serum and possibly bound to transferrin localized on the surface of the cells.

**Tissue Culture Assay for Loss of Iron**

The ability to demonstrate the changes reported here depended on the following factors: (a) use of an adequate radioactive tracer; (b) adequate assay system to quantify uptake and cell mass; (c) accurate estimate of contributing sources of label contamination from the dish surface and cell surface; (d) minor contribution of iron-containing proteins by pinocytic uptake in the 2-day recovery period; (e) metabolic activity of cells after phagocytosis.

Radioactive ferritin was an adequate carrier of iron for uptake into cells. The nature of this "reconstituted" ferritin is problematic. Negatively stained preparations of this ferritin could be interpreted to show electron-dense iron cores within apoferritin (10, 11). However, recent work on the formation of ferritin from apoferritin indicates that ironic iron may also be adherent to the outer part of the protein moiety (12). Any clarification of this problem is beyond the scope of this paper. In the ferritin used here, very little iron was not associated with the protein moiety. Our studies indicated that the [55Fe]ferritin and the antigen-antibody complex was relatively stable, and nontoxic, and that macrophages took up a sufficient quantity of the iron associated with the protein so that small to moderate amounts of iron loss could be detected in the supernatant medium.

The feasibility of demonstrating iron loss from macrophages depended in part on the sensitivity and reproducibility of the assay system for cell survival. The Polaroid assay for cell survival had a 5–10% variation in accuracy, and up to 90% macrophage survival after 2 days in culture was considered negligible cell death. Reproducibility of the counting procedure was demonstrated on triplicate counts of the same field. There were two sources of external evidence for the validity of the photographic assay. (a) Results for excretion when there was good cell survival as determined by the photographic assay correlated with values for the percent of cell survival obtained in the photographic assay. (b) The 2-day recovery medium after pulse uptake of antigen-antibody complex was always centrifuged before the excreted 55Fe was determined. Any cell debris could be detected and there was good correlation with values for the percent of cell survival obtained in the photographic assay.

A significant amount of iron found in the postpulse recovery medium has been demonstrated...
to be in the transferrin fraction of the serum, and some in extracellular $[^{55}\text{Fe}]$ferritin. Could the source of iron bound to transferrin be from labile iron in the extracellular $[^{55}\text{Fe}]$ferritin or $[^{55}\text{Fe}]$ferritin antiferritin complex? Experimental evidence obtained by others (25) demonstrated that the iron transfer from ferritin to 50% saturated transferrin was negligible. Control studies performed by us demonstrated that the reconstituted horse $[^{55}\text{Fe}]$ferritin had about $<5\%$ unbound iron. Conditioned medium increased the transfer of iron to one-third saturated and fully saturated transferrin by a few percent ($\approx 5\%$). If the extracellular $[^{55}\text{Fe}]$ferritin and/or $[^{55}\text{Fe}]$ferritin complex was estimated to be about 10%, then the extracellular labile iron could not contribute to more than 1% of the excretion figures cited. The fact that there was suppression of excretion by serum which contained saturated transferrin indicated that any contribution of iron from extracellular sources was negligible. The source of the ferritin found extracellularly was presumed to be from contamination with the surface antigen-antibody complex or with ferritin. However, excretion of ferritin directly from ferritin-laden lysosomes remained a possibility, and cannot be excluded from these experiments. Any role for small molecular weight chelators (26, 27) in iron excretion from macrophages was not assessed here.

It was possible that uptake of intracellular iron-containing proteins could be an additional source of iron during the postphagocytic recovery phase of macrophages in tissue culture and perhaps influenced the excretion values obtained. The uptake of iron from $\approx 1/5$ saturated transferrin in 10% serum of the recovery medium was approximately 1%. Iron uptake from transferrin could possibly contribute to some dilution of the intracellular iron pool.

### Intracellular Fate of Iron Ingested

The studies reported here demonstrate that macrophages can process the iron in heterologous ferritin and incorporate it into newly synthesized mouse apoferritin. Intravacuolar events, such as the mechanism of transport of iron across the vacuolar membrane, are unknown, as well as the fate of all the iron liberated from the phagocytic vacuole. The intracellular presence of iron in the macrophage induces synthesis of apoferritin (17) just as in other cell systems (2, 6). Not all the ingested iron may be destined for combination with apoferritin. Subsequent to its appearance in the cytoplasm, the iron could be distributed between that incorporated into ferritin and that diverted into the intracellular iron pool.

### Excretory Function of Macrophages

The whole aspect of loss or excretion of substances from macrophages has until now remained unexplored. The bibliography on macrophages in recent years has described their lysosomal function, their maturation, and various biochemical and biological aspects. The loss of iron from macrophages to a transferrin receptor may be a rather unique example of excretion. However, because macrophages are responsible for processing iron in vivo, and because the changes reported here do occur, these cells appear to be appropriate for the study of various phenomena which are associated with the intracellular processing of iron. It is hoped that, in the future, results obtained from macrophages can parallel, be compared to, or contrasted with those obtained with erythrocytes (28) and intestinal mucosal cells (29). The work on the effects of human serum on macrophages and the loss of iron after uptake of macromolecules also opens, in a sense, a new avenue with which to explore effects of serum obtained from people with aberrations in iron metabolism.

The author wishes to acknowledge the generous help of Dr. J. G. Hirsch, who suggested the photographic assay, and also acknowledges the assistance of Ms. B. Fried.

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