

# THE INTERACTION OF SOLUBLE HORSERADISH PEROXIDASE WITH MOUSE PERITONEAL MACROPHAGES IN VITRO

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## ABSTRACT

The in vitro interaction of soluble horseradish peroxidase (HRP) with homogeneous monolayers of mouse macrophages has been studied using sensitive biochemical and cytochemical techniques. The compartmentalization of HRP in extracellular and intracellular sites has been quantitatively evaluated. A significant fraction is bound to a serum-derived layer, which coats the surface of culture vessels and may be removed by appropriate washes. Macrophages interiorize HRP as a solute in pinocytotic vesicles without appreciable binding of the glycoprotein to the plasma membrane. Uptake is directly proportional to the concentration of HRP in the culture medium.  $1 \times 10^6$  cells ingest 0.0025% of the administered load per hr over a wide range of concentrations. Cytochemically, all demonstrable HRP is sequestered within the endocytic vesicles and secondary lysosomes of the vacuolar apparatus. After uptake, the enzymatic activity of HRP is inactivated exponentially with a half-life of 7–9 hr, until enzyme is no longer detectable. When macrophages have pinocytosed trace-labeled HRP- $^{125}\text{I}$ , cell-associated isotope disappears with a  $t_{1/2}$  of 20–30 hr and they release monoiodotyrosine- $^{125}\text{I}$  into the culture medium. We were unable to obtain evidence that significant amounts of HRP (>2%) can be exocytosed after uptake, can exist intact on the cell surface, or can be digested extracellularly. It is difficult to reconcile these observations with several of the postulated mechanisms whereby macrophages are thought to play a prominent role in the induction of an immune response.

## INTRODUCTION

Macrophages are highly endocytic cells which are capable of interiorizing a broad spectrum of both soluble and particulate molecules (44). Once within the confines of the cytoplasm, endocytic vesicles may fuse with primary and secondary lysosomes, thereby initiating the process of intracellular digestion (6). Although considerable evidence exists concerning the membrane receptors and immunoglobulin determinants of phagocytosis, our knowledge of the uptake of soluble macromolecules is rudimentary (5).

The present study was initiated for two general

reasons. The first was to establish a sensitive and quantitative method for the pinocytosis of macromolecular solutes in the confines of an in vitro tissue culture system. The second was to define the interactions of a soluble protein with the plasma membrane of macrophages, to follow its intracellular fate within the vacuolar apparatus, and to examine the possible exocytosis of intact or partially degraded molecules. Both goals are pertinent to our understanding of the putative role of macrophages in the induction of an immune response (44, 50).

In this article we will report biochemical and cytochemical observations on the uptake, compartmentalization, and fate of soluble horseradish peroxidase (HRP) in homogeneous monolayers of mouse peritoneal macrophages. The long term interactions of HRP have been followed both in regard to enzymatic activity and through the use of radiolabeled molecules.

## MATERIALS AND METHODS

### *Macrophages*

Peritoneal macrophages were harvested from unstimulated NCS mice as previously described (7).  $8-10 \times 10^6$  cells were allowed to adhere to 35-mm plastic tissue culture dishes (Nunc) for 2 hr. The nonadherent cells, which are largely lymphocytes, were then removed with two or three vigorous washes in medium 199. The adherent cells were cultured overnight in medium 199 containing 20% fetal or newborn calf serum (NBCS) (Grand Island Biological Co., Grand Island, N. Y., or Microbiological Associates, Inc., Bethesda, Md.) and 1000 units/ml penicillin. After overnight culture, the cell monolayer contained on the order of 2–2.5 million cells as determined from direct counts of low power, phase-contrast micrographs (four per dish) obtained using an Olympus inverted phase microscope equipped with a Polaroid MP-3 Land Camera (Polaroid Corp., Cambridge, Mass.). A micro-Lowry method (24) demonstrated that the protein content of macrophages lysed with detergent (*vide infra*) was about  $25 \gamma/10^6$  cells. To obtain the latter value, one must take into account the contribution made by noncellular protein that is to bound the culture dishes after overnight culture in 20% serum. The viability of the macrophages, as determined by exclusion of 0.05% trypan blue in the presence of 0.05% serum, was greater than 99%. Macrophages were also grown on glass cover slips for use in high resolution phase-contrast microscopy and in light microscopic histochemistry.

### *HRP*

The bulk of the experiments were performed on Sigma type II HRP (Sigma Chemical Co., St. Louis, Mo.) which was maintained frozen and sterile as a 10–20 mg/ml stock solution in saline. Agar and agarose electrophoresis in 0.1 M Veronal buffer, pH 8.2, revealed that this peroxidase preparation had five distinct bands, all with enzymatic activity. A similar heterogeneity has been described for HRP separated on paper electrophoresis in 0.1 M acetate buffer, pH 5.0 (20). Two minor, nonenzymatic proteins were detectable on immunoelectrophoresis using an anti-HRP serum raised in rabbits by im-

munizing initially with three intramuscular injections of 3 mg HRP in complete Freund's adjuvant followed 1 month later by a subcutaneous boosting dose of 10 mg HRP in normal saline.

In some instances, we studied purified HRP preparations that consisted of a single precipitin band after immunoelectrophoresis with antisera raised against the heterogeneous Sigma type II enzyme. The purified HRP was purchased from Sigma (type VI) or was prepared by us by electrophoretic separation of 150 mg of Sigma type II on a Pevikon block ( $45 \times 22 \times 1$  cm) at 3 v/cm for 36 hr at 5°C in 0.1 M Veronal buffer, pH 8.2. 92% of the applied enzyme was recovered after Pevikon electrophoresis. 78% of the recovered enzyme in turn was in the major band and was immunoelectrophoretically identical to the Sigma type VI. The specific enzymatic activities of Sigma type VI and Pevikon-purified HRP, expressed as activity per milligram of pure protein, relative to the enzymatic activity per milligram of Sigma type II protein, were 1.75 and 1.80, respectively.

In experiments in which we assessed the extracellular digestion of HRP by macrophages, we estimated its molecular weight on a Sephadex G-150 superfine column (Pharmacia Fine Chemicals Inc., Uppsala, Sweden),  $45 \times 1.9$  cm, using 0.05 M phosphate buffer as eluant. The flow rate was 3 ml/hr and the operating pressure, 30–40 cm H<sub>2</sub>O.  $V_0$  was 37 ml, and 1.8-ml fractions were collected. The HRP peak eluted at 66 ml, which corresponded to a molecular weight of 40,000 according to the standard curve for the column.

### *Enzymatic Assay of HRP*

A modification of the *o*-dianisidine enzymatic assay, as devised by the Worthington Biochemical Corp., Freehold, N. J., was used to quantitate HRP. 0.1 ml of the solution to be assayed was pipetted into 1.25-ml quartz cuvettes of 1 cm path length. 0.9 ml of a substrate mixture was added, the cuvettes were mixed, and the rate of development of a colored product at 460 nm was followed with time on a Gilford Recording Spectrophotometer (model No. 240, Gilford Instrument Laboratories, Oberlin, Ohio) set for maximum sensitivity, i.e., an absorbance range of 0.000 to 0.100. The increase in absorbance at 460 nm proceeds linearly for 1–3 min. A standard curve was plotted relating the initial rate of color development to the amount of Sigma type II enzyme by weight. The lower limit for reliable assay is 0.1 ng/0.1 ml, corresponding to an HRP (mol wt 40,000) concentration of  $2.5 \times 10^{-11}$  moles/liter. The substrate mixture was prepared fresh daily. To every 6.0 ml of 0.05 M phosphate buffer, pH 5.0, was added 0.06 ml of 0.3% (v/v) H<sub>2</sub>O<sub>2</sub> (diluted from Superoxol-Merck & Co., Inc., Rahway, N. J.), and 0.05 ml of 1.0 g % (w/v) *o*-dianisidine (Sigma Chemical Co.)

dissolved in absolute methanol. The concentration ranges for optimal activity of the various reactants were: 0.05–0.20 M phosphate buffer, pH 4.5–5.5;  $10^{-3}$ – $10^{-2}$  mmoles of  $H_2O_2$ ; and  $10^{-4}$ – $10^{-3}$  mmoles of *o*-dianisidine.

Although several other colorimetric assays have been developed (reviewed in 25), none to our knowledge is as sensitive as the *o*-dianisidine procedure. We have also used *N,N*-dimethyl-*p*-phenylenediamine as a hydrogen donor (45), but it was less suitable because of lower sensitivity and high activities in the blank solution.

### *Quantitation of Macrophage-Bound HRP*

The enzymatic assay can be conveniently used to quantitate the amount of HRP in macrophages.  $2 \times 10^6$  cells are lysed in 1 ml of 0.1% sodium dodecyl sulfate (SDS) (w/v) or 0.05% Triton X-100 (w/v), both made up in distilled water. Unstimulated peritoneal macrophages do not contain detectable endogenous peroxidase activity, i.e., there is less than 1 ng/ml in the lysate from  $2 \times 10^6$  cells. Full recovery of enzyme activity is obtained when known amounts of exogenous HRP are added to cells solubilized with detergents. To prevent loss of enzyme activity in SDS cell lysates, the enzyme must be assayed within an hour, or a sample diluted 1:10 in 1% serum saline. Enzymatic activity is stable for several hours in cells lysed in 0.05% Triton X-100. This low concentration of Triton must be used to prevent flocculation in the Lowry protein assay. Triton lyses cells far less rapidly and with much greater variability than the "instantaneous" lysis achieved with SDS.

### *Cytochemical Localization of HRP*

The distribution of HRP was determined cytochemically using the Graham-Karnovsky method (17). Monolayers that had interiorized HRP were washed at least twice in 2 ml medium 199 and twice in phosphate buffered saline. They were fixed for 5–10 min at room temperature or at 4°C in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. The fixative was removed with three washes of buffer, and the presence of HRP revealed by the diaminobenzidine technique for 10–30 min at room temperature. The cultures were washed in saline and postfixed in the cold for 15 min in a mixture consisting of 2 parts 2% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.4, and 1 part 2.5% glutaraldehyde in the same buffer. After osmium postfixation, the cultures were treated for 15 min with 0.25% uranyl acetate in 0.1 M sodium acetate buffer at a final pH of 5.0, washed, and dehydrated in graded alcohols. 100% propylene oxide was added, and this caused the monolayer to float off the plastic dish, thus re-

leasing the cells in the shape that they had while growing on the dish, i.e., with easily identified dish and medium surfaces (Fig. 4). The cells were then transferred to Pasteur pipettes and plugged with layers of dental wax and agar. The pipettes were spun on a tabletop centrifuge and the resulting cell pellet released by cutting off the tip of the pipette. The pellets were placed first in 100% propylene oxide and then carried through 50–50 Epon-propylene oxide for 1 hr and 80–10 Epon-propylene oxide for 1 hr, before the final polymerization in 100% Epon in B.E.E.M. capsules (Better Equipment for Electron Microscopy, Bronx, N. Y.) at 65°C for 2–3 days. Thick plastic sections were stained with azure A or toluidine blue. Thin sections were inspected in a Siemens Elmiskop II microscope, with or without the aid of uranyl acetate and lead citrate staining.

### *Trace Radiolabeling of HRP*

In some experiments, HRP was iodinated at room temperature with  $^{125}I$  according to the method of Marchalonis (27). The reaction mixture consisted of 25 mg purified HRP, 10 mCi of carrier-free  $Na^{125}I$  (New England Nuclear Corp., Boston, Mass.), 30  $\mu$ g of lactoperoxidase (kindly supplied by Dr. Seymour Klebanoff), in a total volume of 2.5 ml of 0.15 M phosphate buffer, pH 7.4. The reaction was initiated by the addition of 0.01 ml of 0.03%  $H_2O_2$  (v/v) and maintained by the addition of a further 0.01 ml at times 10 and 20 min. After a total reaction time of 30 min, the mixture was dialyzed against 0.15 M phosphate buffer for at least 4 days in the cold. The lactoperoxidase and remaining free iodide were removed by Sephadex G-150 chromatography. The specific activity of HRP so obtained was 0.10 mCi/mg corresponding to an average of 1 labeled molecule per 545 unlabeled molecules. The enzymatic activity of the entire HRP preparation was not altered after the iodination procedure. The HRP- $^{125}I$  and enzymatic activity precipitated identically with antiHRP antisera. In contrast, a chloramine-T method of radioiodination (28) yielded a trace labeled material in which 25% of the over-all enzymatic activity was lost, and in which only 60% of the labeled HRP molecules were precipitated at equivalence with appropriate antisera.

Trichloroacetic acid (TCA) precipitation was performed in the cold in the presence of 5% (v/v) NBCS at a final TCA concentration of 20%. 95% of the HRP- $^{125}I$  was TCA precipitable.

TCA soluble counts were identified chromatographically (37) after extraction of the TCA with acetone and acidification with 1 N HCl to pH 1–2. The material was then extracted three times with 3 vol of acidified *n*-butanol (32) in the presence of unlabeled sodium iodide and monoiodotyrosine (MIT). The butanol was evaporated and the residue re-

suspended in fresh acid-butanol. A sample was chromatographed on MN-Polygran thin layer plates (Brinkmann Instruments, Westbury, N. Y.) using ascending chromatography in an *n*-butanol:acetone:1 N ammonium hydroxide (1:4:1) solvent. The plates were air-dried and portions of the chromatogram counted in toluene-Liqueflor (New England Nuclear Corp.). Standards were dissolved in culture media and processed as above yielding  $R_f$  for MIT and  $I^-$  of 0.25 and 0.75, respectively.

## RESULTS

### *Compartmentalization of HRP in Macrophage Cultures*

$2 \times 10^6$  macrophages were grown on plastic Petri dishes overnight in 20% NBCS medium. The medium was aspirated, and the homogeneous macrophage monolayer was exposed for 2 hr to 1 ml of fresh culture medium, supplemented with 1 mg/ml of HRP. After 2 hr in culture, we reasoned that the enzyme could be present in four possible compartments: (a) free in the culture medium, (b) attached to the surface of the culture vessel, (c) adsorbed to the surface of the macrophages, and (d) interiorized within the macrophages. The quantities of HRP in each of these locations were measured by enzymatic assay. Identical results were obtained with radiolabeled HRP.

**HRP IN THE CULTURE MEDIUM:** The amount of HRP that persists in the culture medium and the amount that is bound to the culture dish itself can be determined by compartmental analysis. After the 2 hr uptake period, the culture medium was aspirated with a Pasteur pipette and the dishes were washed every 4 min with 2 ml of medium 199. A semilog plot of the amount of HRP in successive washes (Fig. 1) yielded two exponential components and suggested that two compartments of HRP were being removed by this procedure. The first compartment, which is removed in the first three to four washes, contains the bulk of the administered enzyme (99.95% or more) and represents HRP that has remained intact in the culture medium. The second compartment contains HRP that is present in slowly diminishing amounts in the subsequent six washes. Since the decrease in enzyme in successive washes is exponential, the size of the two compartments can be calculated by extrapolation to time 0. The  $y$  intercept for the HRP in the culture medium is 1 mg, or that amount initially present in solution. The  $y$  intercept for the second compartment is of

the order of 250 ng (Fig. 1). Even if the last washes at room temperature contain little or no HRP, an additional 5–15 ng of enzyme is released after return of the cells to the 37°C incubator for 30 min.

**HRP ATTACHED TO THE SURFACE OF THE CULTURE DISH:** The second compartment of HRP that is removed by this extensive washing procedure represents enzyme that is weakly bound to the culture vessel rather than to the cells themselves. This was readily demonstrable by repeating the entire experiment in culture dishes that lacked cells. Dishes were incubated overnight in culture medium, exposed to 1 mg/ml HRP for 2 hr, and then washed as described above. The enzymatic activity in the various washes was similar to that observed when cells were present (Fig. 1). If the dishes used for this experiment were never exposed to serum, a single exponential was obtained corresponding to the first compartment of medium HRP. This suggests that the binding of HRP to the culture vessel is mediated by some factor in serum.

The presence of HRP on the surface of the culture dish can be detected by electron microscope cytochemistry. Culture dishes were exposed for 2 hr to 1 mg/ml HRP in the usual serum containing culture medium, but in the absence of cells. The dishes were then washed six times and processed for electron microscopy. Some of the dishes were treated with the  $H_2O_2$ -diaminobenzidine substrate mixture used to detect HRP histochemically. Controls included dishes given HRP but not developed, and dishes exposed to serum but not HRP and then reacted with the histochemical substrate. The addition of propylene oxide after dehydration removes the cell monolayers from the dishes. In the absence of cells, the propylene oxide released small amounts of a flaky material. In thin sections, this material consisted of sheets of a fine 50 Å film. In dishes that were exposed to HRP and washed five to six times, portions of the film still exhibited reaction product (Fig. 2). When cells are grown on plastic dishes, it is evident (Fig. 3) that they are in fact growing over this serum-derived film of as yet ill-defined nature.

**HRP ATTACHED TO THE SURFACE OF MACROPHAGES:** Two techniques were employed to detect HRP on the surface of the macrophage after a 2 hr uptake period. We first used trypsin to see if intact enzyme could be released from washed macrophage monolayers. Trypsin re-

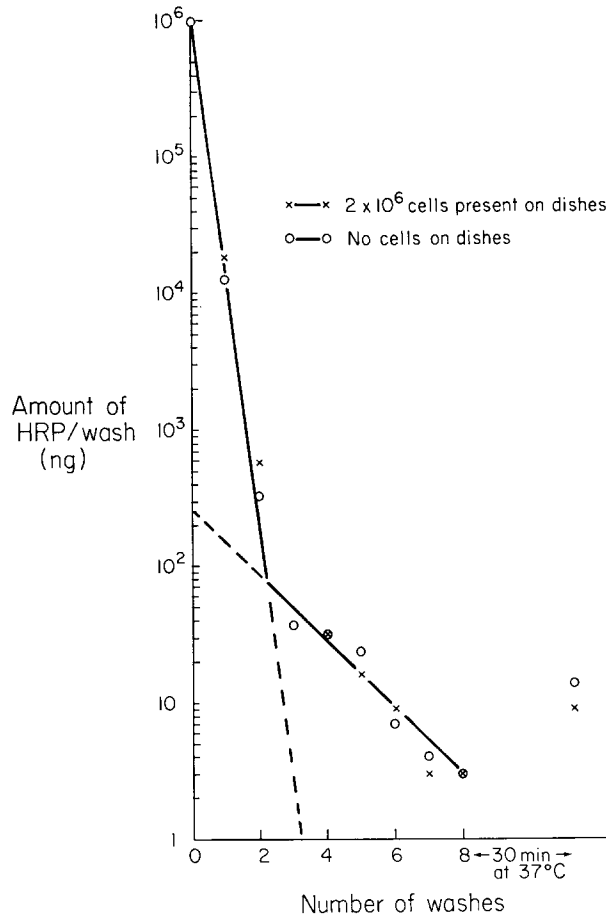


FIGURE 1 The distribution of extracellular HRP in macrophage cultures. Dishes were incubated for 18 hr in 20% NBCS-medium 199 with  $2 \times 10^6$  macrophages ( $\times$ — $\times$ ) or without cells ( $\circ$ — $\circ$ ). 1 mg/ml HRP was added for 2 hr and the dishes washed eight times, at 4 min intervals, with 2 ml of medium 199. They were then returned to the 37°C incubator for an additional 30 min. The amount of HRP in the resulting wash fluids was determined by enzymatic assay.

moves cell membrane constituents from certain cells (23, 56) and is thought to release exogenously added proteins adsorbed to peritoneal exudate cells (40, 49, 51). Trypsin does not alter the enzymatic activity of HRP, nor does it release macrophages from the culture dishes. We therefore trypsinized well-washed macrophage cultures using a 15–30 min exposure to 0.25–0.50 mg of trypsin/ml of medium 199.  $12.4 \pm 4.4$  ng of HRP was solubilized in this manner. This did not differ significantly from the amounts released by medium 199 in the presence or absence of cells (Table I). We suspect, therefore, that the HRP solubilized from well-washed cultures at 37°C represents the last remnants of enzyme in the “dish-bound” compartment.

Electron microscope cytochemistry was also used to detect HRP bound to the surface of the macrophage. The reaction product was never seen attached to the surface of healthy cells (Figs. 3, 4). Rhodes et al. (35) were also unable to demonstrate that exogenously administered ferritin persists on the plasmalemma of macrophages. In some cases, we followed the suggestion of Willingham et al. (55) that the electron-opaque HRP reaction product may have been eluted during the processing of the cells after histochemistry. This elution is reportedly avoided by performing the cytochemistry on cells embedded in agar; however, surface-bound enzyme was still not detectable with this modification. A positive control is the fact that enzyme reaction product was

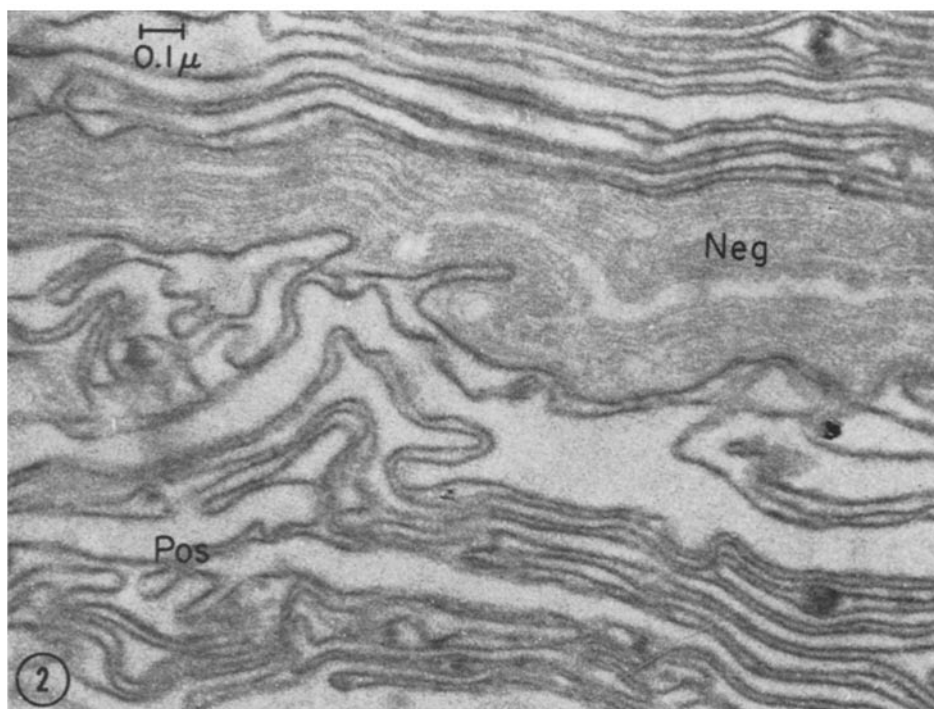


FIGURE 2 An electron micrograph of the film adhering to the surface of culture dishes after exposure to 20% NBCS-medium 199 containing 1 mg/ml HRP for 2 hr. After six washes, the dish was developed for peroxidase activity. The film was removed during the propylene oxide step, processed for microscopy, and examined without heavy metal staining. The material appears as a fine 50 Å film often arranged in doublets. The central area contains stacks of film without peroxidase activity (*Neg*) and is similar to the material underlying cultured cells (Fig. 3). The peripheral areas contain thicker, electron-opaque stacks which result from the deposition of the peroxidase reaction product (*Pos*).  $\times 59,000$ .

not eluted during the detection of dish-bound HRP (Fig. 2).

**HRP WITHIN MACROPHAGES:** The intracellular distribution of enzyme was followed cytochemically in washed monolayers which had been exposed to 1 mg/ml HRP for 2–3 hr. The localization of exogenous HRP can be reliably assessed in this manner, because endogenous peroxidase is not present cytochemically in these cells. Although endogenous peroxidase may be detected in peritoneal macrophages of other species (11, 36), the unstimulated mouse peritoneal cavity contains only 1% positive cells, and this cytochemical reactivity largely disappears after a day of *in vitro* cultivation (52).

By cytochemical criteria, abundant intracellular peroxidase is present in more than 95% of the cells exposed to 1 mg/ml HRP for 2 hr. By phase-contrast microscopy, the enzyme is distributed in granules which have the characteristic perinuclear location of lysosomes. A similar distribution

TABLE I  
*Release of HRP from Dish-Bound Compartment*

Treatment	Number of determinations	Mean HRP released <i>ngm</i>
Medium 199	25	$12.4 \pm 4.38^*$
0.25–0.50 mg trypsin/ml medium 199	25	$11.0 \pm 3.24$
Medium 199, no cells on culture dish	10	$10.2 \pm 3.14$

Culture dishes with or without  $2 \times 10^6$  macrophages were exposed to 1 mg/ml HRP in 20% NBCS-medium 199 for 2 hr. The dishes were washed nine times with 2 ml of medium 199 and then returned to the 37°C incubator for  $\frac{1}{2}$  hr in the presence of medium 199 with or without trypsin. The subsequent release of HRP was quantitated by enzymatic assay.

\* Standard deviation.

of exogenously administered HRP has been observed by others (26, 30). By light microscopy, newly formed peripheral pinocytic vesicles are not stained.

In electron micrographs (Figs. 3, 4) the reaction product is located in almost all of the cell's membrane-bound secondary lysosomes. Golgi vesicles, which are the presumed primary lysosomes of macrophages, were uniformly negative. The majority of lysosomes contain dense reaction product, but several, usually larger vacuoles, contain only a peripheral rim of staining. Such vacuoles constitute the predominant form of staining in cultures exposed to HRP for short time periods (15–30 min), and often are found more peripherally in the cell. They probably represent recently interiorized pinocytic vesicles which have fused with one another and have not as yet undergone concentration of their intravacuolar contents (7). That such fusion occurs in macrophages has been demonstrated with other pinocytic and lysosomal markers (9). HRP was never found in Golgi lamellae, rough endoplasmic reticulum, or perinuclear cisternae. Mitochondrial cristae, whose cytochromes possess peroxidatic activity towards diaminobenzidine at pH 6 (31), were also negative under the conditions of our studies.

#### *Uptake of Soluble HRP by Macrophages*

**RATE OF HRP UPTAKE:** Homogeneous monolayers of  $2 \times 10^6$  macrophages were exposed to three concentrations of HRP for 0.5–6.0 hr. At each time point, duplicate cultures were washed nine times to remove the majority of extracellular enzyme, and the cells were lysed in detergent. At all concentrations of HRP in the culture medium, “cell-bound” enzyme increased progressively for some 2–3 hr (Fig. 5) and then plateaued. We therefore performed subsequent uptake measurements on cells exposed to HRP for 2 hr.

**VARIABLES IN THE UPTAKE OF HRP:** The 2 hr uptake of a 1 mg/ml solution of HRP was found to be directly related to the amount of cell protein present (Fig. 6). The different amounts of cell protein in these experiments were obtained by plating different numbers of cells. Since 25  $\mu$ g of cell protein corresponds to  $2 \times 10^6$  24-hr old macrophages by direct cell counts, and since the slope of Fig. 6 can be calculated by the least squares method to be 1.99 ng HRP/ $\mu$ g protein per 2 hr, then we can conclude that 1 million macrophages interiorize HRP at a rate of 25 ng,

or 0.0025% of the administered load, per hr. The  $y$  intercept in Fig. 6, or the “uptake” in the absence of cell protein, indicates the level of dish-bound enzyme, and is identical to the amount of HRP that is released from well-washed macrophage monolayers after incubation at 37°C for 30 min (Table I).

The 2 hr uptake of HRP by well-washed macrophage monolayers was found to increase linearly with the concentration of HRP in the medium (Fig. 7). Again the  $y$  intercept in these experiments was not zero, because of the existence of residual dish-bound HRP after washing at room temperature.

Uptake of HRP as well as total cell protein was similar in macrophages maintained in tissue culture for 1, 2, and 3 days before exposure to the enzyme (Table II). Purified HRP preparations were interiorized at the same rate, in terms of the amount of protein per 100  $\mu$ g cell protein, as Sigma type II enzyme (Table II). Finally, uptake of enzyme could be inhibited to less than 10% of control levels if cultures were treated with known inhibitors of pinocytosis (4). The inhibitors tested were cold (4°C) and  $10^{-2}$  M sodium fluoride. They were administered to the macrophages for 1 hr before exposure to HRP and during the 2 hr uptake period (Table II). High doses of colchicine (50  $\mu$ g/ml) did not significantly alter pinocytosis over the time period studied.

#### *Fate of Interiorized HRP*

Macrophage monolayers that had been exposed to 1 mg/ml HRP for 2 hr were washed so as to remove all dish-bound enzyme and returned to HRP-free culture medium. The fate of cell-bound HRP was then determined in duplicate cultures at varying time points of the ensuing washout period. At each interval, several compartments were assayed for enzymatic activity: (a) the culture medium; (b) the trypsinate, i.e., the amount of HRP released from the cells by treatment with 0.25 mg trypsin/ml medium 199; and (c) the cells themselves, lysed in detergent.

**THE CULTURE MEDIUM: IS SOLUBLE HRP EXOCYTOSED BY VIABLE MACROPHAGES?:** Enzymatic assay of the culture medium during the washout period can be used to determine if small amounts of soluble material are regurgitated or exocytosed from the cells. Several features of the HRP-macrophage monolayer system made this possible. (a) Small amounts of enzyme (1–10



FIGURE 3 Macrophages exposed to 1 mg/ml HRP for 2 hr and washed extensively over a 20 min period. The cells were developed for peroxidase activity and examined after staining with uranium and lead salts. All reaction product is intracytoplasmic and localized within membrane-bounded vacuoles. One type of vacuole (*P*) is electron-transparent and contains a peripheral rim of reaction product whereas the other demonstrates uniform staining of the matrix. Other structures including the Golgi apparatus (*Go*), rough endoplasmic reticulum (*RER*), perinuclear cisternae, mitochondria, plasma membrane, and newly formed pinocytic vesicles (\*) are not reactive. There is a thin bilayer of material present on the dish surface of both cells (arrows) which is serum derived (see Text and Fig. 2).  $\times 27,000$ .



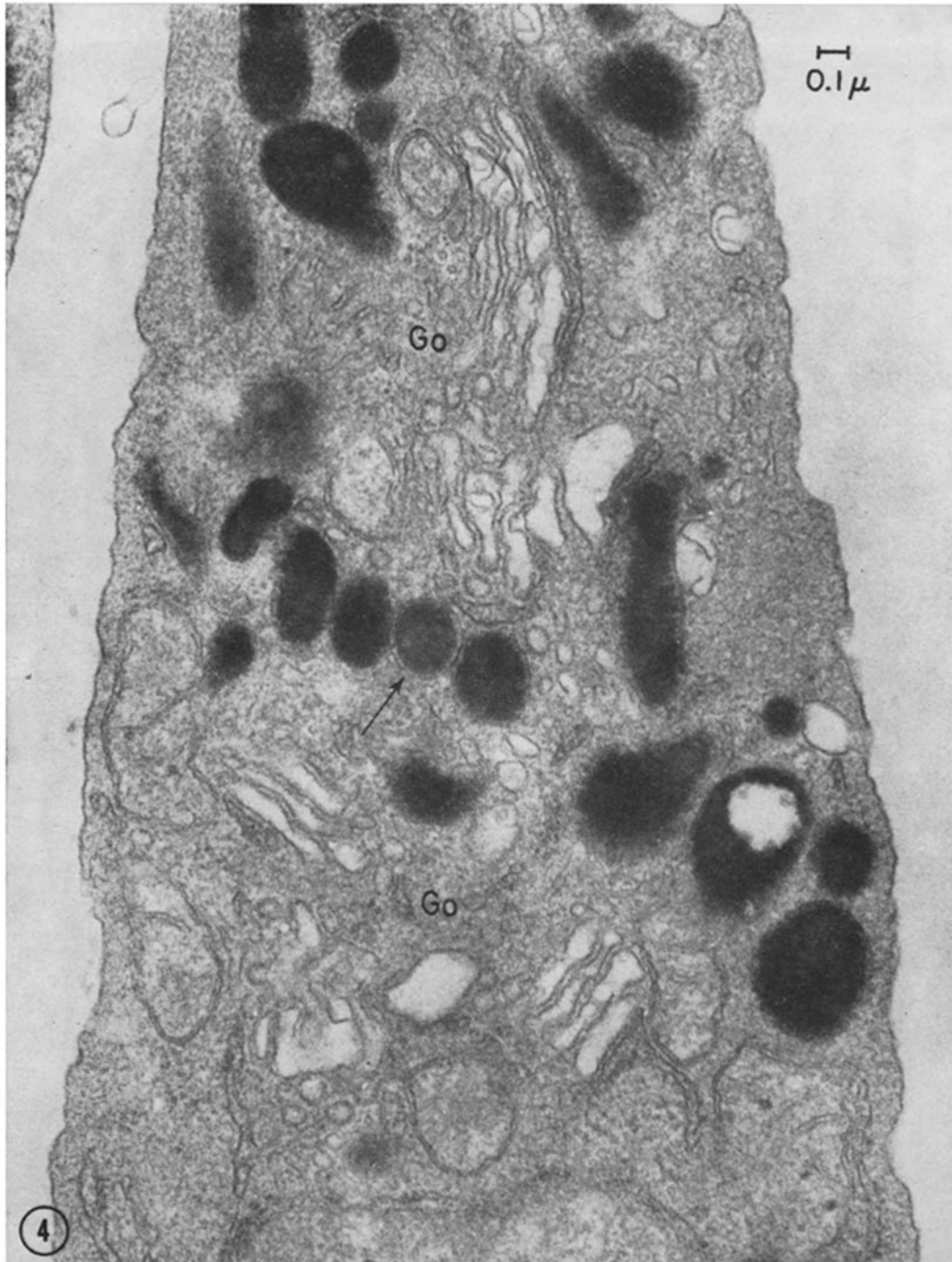
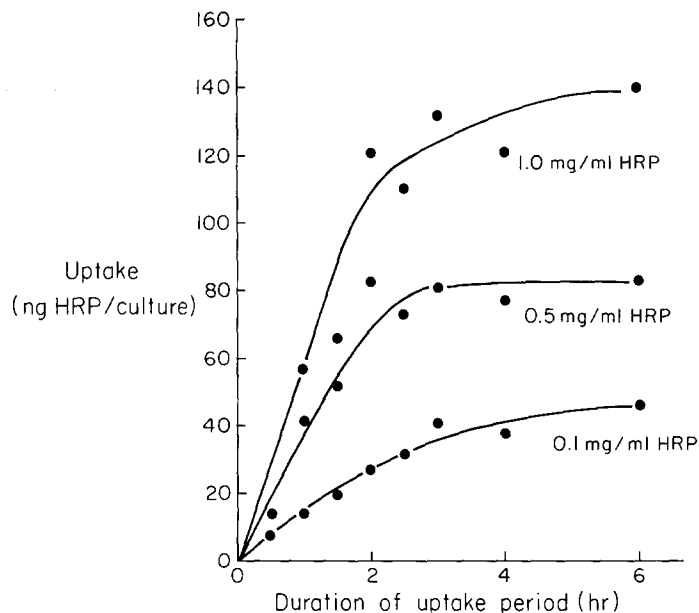
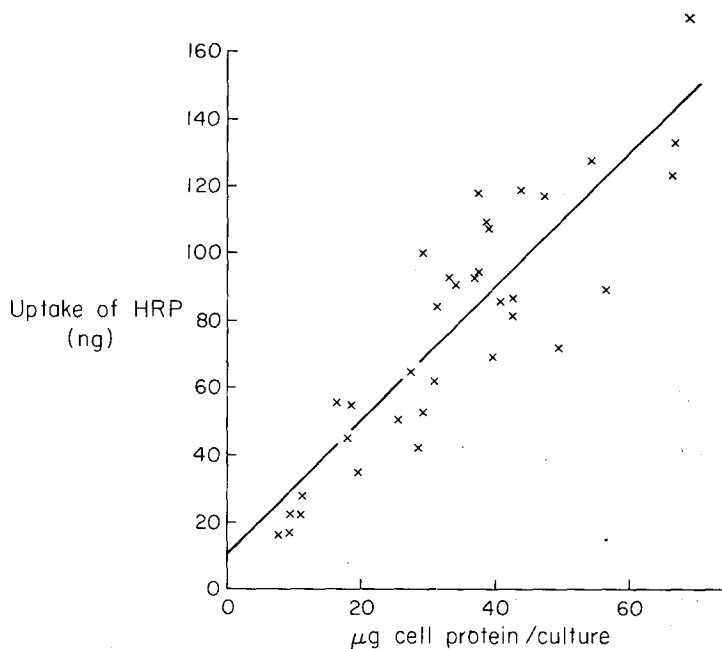


FIGURE 4 A macrophage treated like those shown in Fig. 3, except that the sections were not stained with heavy metal salts. The peroxidase reaction product is present within secondary lysosomes, the membranes of which are discernible in several instances (arrow). The Golgi apparatus (*Go*) and plasma-lemma are both negative.  $\times 49,000$ .



**FIGURE 5** The uptake of HRP by macrophages as a function of time and extracellular concentration. Monolayers containing  $2 \times 10^6$  cells were exposed to three levels of HRP in 20% NBCS-medium 199. Thereafter, duplicate cultures were washed eight times over a 30 min period and the cells lysed in detergent. Uptake was measured by enzymatic assay and in this instance includes a small residuum of dish-bound enzyme (see Text).



**FIGURE 6** The uptake of HRP as a function of the number of macrophages. Varying numbers of macrophages ( $0.4-2.4 \times 10^6$ ) were cultured for 18 hr in 20% NBCS-medium 199 to yield values of 7.7-68.0  $\mu\text{g}$  macrophage protein per dish. 1 mg/ml of HRP was added for 2 hr and the monolayers washed nine times at room temperature. The least squares method was employed to determine the straight line which best fit the 34 duplicate determinations. The slope of this line was  $1.99 \pm 0.18$  ng/ $\mu\text{g}$  cell protein per 2 hr. The  $y$  intercept was  $10.5 \pm 7.1$  ng and represents the amount of residual dish-bound enzyme.

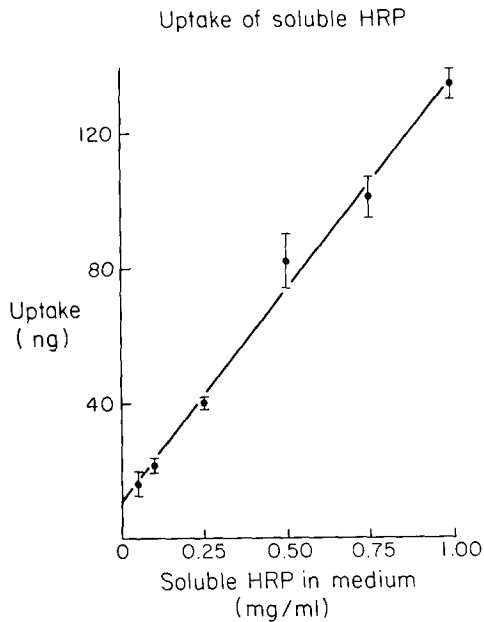


FIGURE 7 The uptake of peroxidase by macrophages.  $2 \times 10^6$  cells were exposed for 2 hr to varying concentrations of soluble HRP in 20% NBCS-medium 199. The monolayers were washed nine times and the cells lysed in detergent. The  $y$  intercept indicates the contribution of dish-bound enzyme.

ng/ml) are stable at 37°C for several days in 5% NBCS-medium 199-penicillin in the presence or absence of cells. (b) The percentage of HRP that is rebound to the cells and to the culture dish is extremely small relative to that in the medium. (c) Total cell numbers remain constant and cell death or division are insignificant. (d) The culture medium, in the absence of HRP had a small peroxidatic activity which could be accounted for in suitable controls. (e) All of the original dish-bound enzyme can be removed by adequate washing followed by a brief period at 37°C.

With these considerations in mind, we were unable to detect significant exocytosis, i.e., > 2% of total cell-bound HRP. Similar results were obtained with respect to the release of TCA precipitable counts after ingestion of HRP-<sup>125</sup>I.

THE "TRYPSINATE:" IS HRP LOCATED ON THE SURFACE OF THE MACROPHAGE DURING THE WASHOUT PERIOD?: Previous studies (40, 49, 51) have suggested that small amounts of intact proteins (2-5% of the original cell-bound protein) bind to and persist on the surface of healthy macrophages. This surface-bound protein was quantitated by measuring the amount of material solubilized by trypsin. We were unable to

detect the existence of trypsin-releasable HRP immediately after uptake. The sensitivity of detection was relatively low because of the existence of substantial amounts of enzyme that could be re-

TABLE II  
Uptake of HRP under Various Conditions

Parameter	Uptake (ng HRP/100 μg cell protein)
Macrophages grown for 1 day	289
Macrophages grown for 2 days	274
Macrophages grown for 3 days	273
1 mg/ml Pevikon-purified HRP	264
$10^{-2}$ M NaF*	21.4
4°C*	7.1
50 γ/ml colchicine*	235
0.1 M Tris buffer ‡	240
3 hr pretreatment with 0.1 M Tris ‡	124

1 mg/ml HRP was administered to triplicate cultures of macrophages for 2 hr under various conditions outlined above. After uptake, the cells were washed nine times and lysed in detergent. The level of enzyme largely represents intracellular enzyme but also includes small amounts of residual dish-bound HRP (see text). Unless otherwise stated, all macrophages were grown overnight (18-20 hr) before the experiment.

\*Inhibitors were administered for 1 hr before HRP uptake and during the uptake period.

‡ Medium 199 was buffered with 0.1 M Tris, pH 7.4, instead of bicarbonate-CO<sub>2</sub>, during the uptake period.

TABLE III  
Absence of Extracellular Digestion of HRP-<sup>125</sup>I

Parameter measured in culture medium	$2 \times 10^6$ macrophages present	No cells
Enzyme activity (μg)	98.0	99.6
TCA insoluble cpm ( $\times 10^{-7}$ )	10.1	10.5
cpm ( $\times 10^{-7}$ ) precipitating with anti-HRP at equivalence	9.48	9.88

100 μg of HRP-<sup>125</sup>I in 20% NBCS-medium 199 was added to culture dishes for 24 hr in the presence or absence of  $2 \times 10^6$  macrophages. After 24 hr of incubation at 37°C, the culture medium was examined for: (a) total enzymatic activity, (b) counts precipitable with 20% TCA, (c) counts precipitable with purified anti-HRP antiserum at equivalence, and (d) the molecular weight of HRP as determined by Sephadex G-150 chromatography (see text).

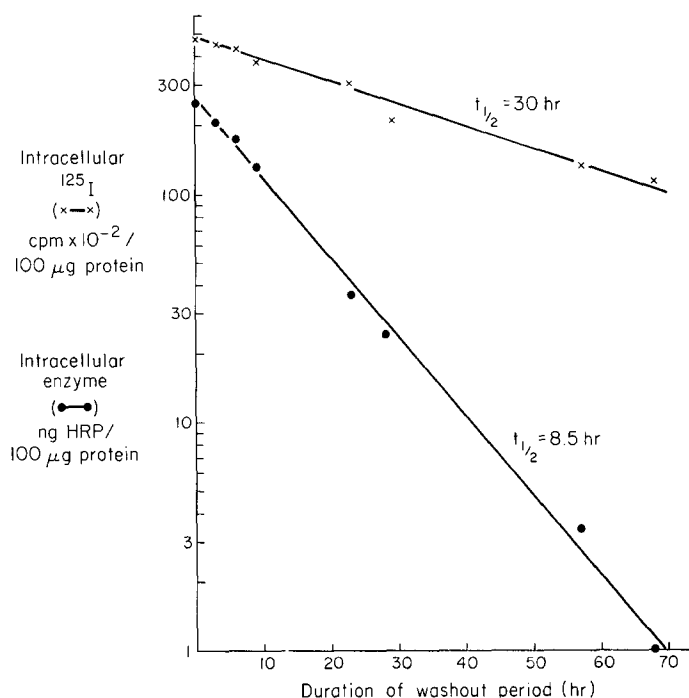


FIGURE 8 The fate of HRP pinocytosed by macrophages.  $2 \times 10^6$  cells were exposed to 1 mg/ml HRP- $^{125}\text{I}$  (0.1 mCi/mg) for 2 hr. The cultures were washed eight times at room temperature and received an additional wash of 30 min at  $37^\circ\text{C}$  to remove all dish-bound enzyme. Fresh 20% NBCS-medium 199 was added and both intracellular enzymatic activity (●---●) and radioisotope (x---x) assayed over the next 68 hr. Each point is the mean of duplicate cultures.

moved without trypsin and in the absence of cells, presumably representing residual dish-bound HRP.

Later in the washout period, the dish-bound HRP was no longer a problem, in that it was removed by changing the culture medium after the initial 30–60 min at  $37^\circ\text{C}$ . The presence of small amounts (less than 1% of the total HRP originally bound to the cells) of enzyme was thus detectable. At no time during the washout period were we able to detect the putative, persistent surface-bound protein (Table III).

**THE CELLS: WHAT IS THE FATE OF CELL-BOUND HRP?** The level of cell-bound HRP diminished exponentially with a half-life of 7–9 hr (Fig. 8), irrespective of the amount of enzyme initially interiorized (40–400 ng). Eventually, no enzymatic activity was detectable, e.g., at 68 hr in the experiment cited in Fig. 8. The loss of enzymatic activity could not be attributed to exocytosis of HRP or loss of cells. Cultures were examined by electron microscope histochemistry at varying time points in the washout period. HRP was detectable cytochemically for some 24–30 hr

and was always found within membrane-bound granules. We considered the possibility that the acid environment of the lysosomes could be inactivating the enzyme, e.g., by dissociation of the hemeaprotein complex. However, HRP is stable at pH 4.0 in the presence of 1–5% NBCS for several days. This amount of protein is presumably within secondary lysosomes, as cells are continually pinocytosing droplets of the culture medium which in turn fuse with preexisting lysosomes.

It seems likely that HRP is being degraded by lysosomal hydrolases, as has been described for other macromolecules (13, 14). The loss of cell-bound HRP- $^{125}\text{I}$  counts proceeded exponentially as well, the half-life varying from 20–30 hr in several experiments (Fig. 8). Counts lost from the cells were recovered as TCA soluble counts in the medium. More than 90% of the soluble counts were butanol extractable. On thin layer chromatography it was determined that the extracted counts cochromatographed with MIT or with iodide, as was previously reported for studies on the fate of  $^{125}\text{I}$ -HSA, rabbit hemoglobin- $^{125}\text{I}$ , or rabbit hemoglobin- $^3\text{H}$  (13, 14). The production of

TABLE IV  
Fate of Intracellular HRP

Duration of washout period	Bicarbonate buffer in culture medium				0.1 M Tris buffer in culture medium			
	ng HRP released from cells at 37°C for 30 min		Cell protein	Intracellular HRP (ng/100 µg cell protein)	ng HRP released from cells at 37°C for 30 min		Cell protein	Intracellular HRP (ng/100 µg cell protein)
	Medium 199	Trypsin			Medium 199	Trypsin		
hr			µg				µg	
0	15.2	11.4	65,66	186,202	10.6	17.5	55,46	228,207
3	1.5	<1	69,66	152,130	2.9	2.5	64,62	116,173
6	<1	<1	57,50	117,106	<1	<1	58,56	133,109
12	<1	2.3	61,62	54,61	2.1	6.0	59,39	71,80
24	<1	1.0	55,63	23,23	4.4	8.6	42,39	36,26
36	<1	<1	65,56	13,6.2	3.1	6.7	38,41	34,22
48	1.3	<1	57,64	3.5,4.4	4.0	3.8	42,22	33,37

$2 \times 10^6$  macrophages were exposed to 1 mg/ml HRP for 2 hr. The cultures were washed eight times and then returned to the 37°C incubator for 30 min to remove residual dish-bound enzyme. After an additional wash, fresh 20% NBCS-medium 199 was added for the washout period. One group of cultures were maintained in bicarbonate-CO<sub>2</sub> buffered medium, the other contained 0.1 M Tris during both the uptake and washout period.

At times thereafter, duplicate cultures were analyzed as follows: (a) the culture medium was assayed for HRP as an index of exocytosis (see Text); (b) the monolayers were washed twice and then incubated for 20 min/37°C in TC-medium 199 with and without 0.25 mg/ml trypsin, the assay of the resulting supernate was an index of surface-bound enzymic. Finally, the cells were lysed in detergent and intracellular enzyme and total protein determined.

iodide probably results from the action of deiodinases present in NBCS. A peak of MIT-<sup>125</sup>I was not demonstrable within the cells at any time.

IS HRP DIGESTED EXTRACELLULARLY?: 100 µg of HRP-<sup>125</sup>I was incubated in 1 ml of 20% NBCS-medium 199 in the presence or absence of  $2 \times 10^6$  macrophages. After 24 hr in culture, the HRP in the medium was analyzed to determine if there was any evidence of significant extracellular digestion of enzyme. In the presence of cells, the HRP was not significantly changed with respect to: (a) total enzymatic activity; (b) TCA insolubility of the radiolabel; (c) ability to combine with specific antiserum at equivalence (Table IV). In the presence or absence of cells, the culture medium HRP chromatographed identically to standard HRP on a Sephadex G-150 column (data not given).

#### *The Influence of Cell Damage on the Interaction of HRP with Macrophages*

Agents which modify the properties of macrophage surface or cytoplasmic cytomembranes might well alter the uptake and fate of HRP. Tris

buffer seems to stabilize cytomembranes (53) and has been employed in studies of protein-macrophage interaction. When 0.1 M Tris is substituted for bicarbonate, marked vacuolization and rounding up occurs promptly, and by 24-36 hr the majority of macrophages are nonviable. The response is concentration and time dependent and is not detectable after 3 hr in 0.01 M Tris. At the ultrastructural level, there was pronounced dilation of secondary lysosomes as well as the vesicular and lamellar components of the Golgi apparatus (Fig. 9). Somewhat later there is dilation of the rough endoplasmic reticulum (Fig. 10). Many large, electron-transparent vacuoles are formed which appear to result from the fusion of existing secondary lysosomes or dense granules (Fig. 9, 10). Multivesicular bodies are evident, which in HRP exposed cells, demonstrate reaction product within the interiorized vesicles. This suggests their autophagic origin and occurs at a time when the heterophagy of damaged cell components is not prominent.

The uptake of HRP is not altered in cells exposed simultaneously to 0.1 M Tris and HRP, whereas the pretreatment of macrophages with

Tris reduces uptake by 50% (Table II). The fate of HRP in Tris-treated cells is unremarkable for the first 24 hr (Table III) in spite of the marked morphological alterations. Thereafter, when cell death is prominent, the inactivation of HRP ceases abruptly. It is noteworthy that in the majority of experiments, trypsin treatment of Tris-damaged cells resulted in an enhanced release of HRP. Trypsin may, under these conditions, be modifying an intracytoplasmic compartment of HRP.

## DISCUSSION

The uptake, distribution, and fate of a glycoprotein enzyme HRP has been studied in homogeneous monolayers of mouse peritoneal macrophages. In most instances, we followed the enzymatic activity of HRP in these cells using relatively simple, but sensitive, biochemical ( $\pm 1$  ng/ml) and cytochemical techniques. Corroborating data were obtained with trace radiolabeled HRP-<sup>125</sup>I. In the present paper, we have used soluble HRP to study several aspects of the pinocytotic process in macrophages. A subsequent paper will deal with the phagocytosis of HRP-anti-HRP immune complexes.

### *Uptake of Soluble HRP*

A striking feature of the uptake of soluble HRP is that relatively little of the administered load is actually interiorized by the cell. A million macrophages cultivated *in vitro* interiorize in an hour some 0.0025% of the concentration of HRP in the culture medium. Slightly lower uptakes were obtained by Ehrenreich and Cohn using radiolabeled human serum albumin (13) and rabbit hemoglobin (14). However, the level of uptake was measured first at 6 hr. Since protein degradation and isotope release had probably already occurred, we suspect that initial 1 hr rates of uptake would be higher and similar to that obtained with HRP. Other investigators have looked at the 1 hr uptake of soluble proteins by heterogeneous peritoneal exudate cells. Schmidtke and Unanue (40) reported comparable percentages of human and mouse serum albumin to be interiorized by mouse peritoneal cells in suspension, while others (18, 47, 49, 51) have recorded uptakes that were 10–100-fold higher than that observed with HRP. The latter data may represent differences in the protein studied but may also reflect experimental design. Variables such as the adequacy of washing to

remove noncell-bound protein, the presence of aggregated proteins, and the existence of antigen-antibody complexes may require reexamination.

The observed uptake rates of soluble HRP can be used to estimate the amount and volume of material interiorized by macrophages during pinocytosis. If soluble serum proteins (1.2 g % in a 20% v/v NBCS culture medium) are taken in at similar rates to HRP, then  $10^6$  macrophages imbibe 0.0025% or 0.3  $\gamma$  protein/hr. This corresponds to 1.2% of the total cell protein (25  $\mu$ g). Since binding of HRP to the macrophage surface was not demonstrable (*v.i.*), one can also estimate the volume of culture medium interiorized using HRP as the marker solute.  $10^6$  cells remove 25 ng of this solute from a 1 mg/ml solution in 1 hr, corresponding to a volume of  $25 \times 10^6$  ml. The surface area of  $10^6$  macrophages has been estimated to be  $2.5 \times 10^9 \mu^2$  (54). Since the average thickness of macrophage cytoplasm is about 1  $\mu$ , the volume of cytoplasm in  $10^6$  cells is  $2.5 \times 10^{-8}$  ml. The macrophage thus takes in the equivalent of 1% of its total cytoplasmic volume in endocytic droplets per hour, a value which may be increased manyfold after further stimulation of pinocytotic activity (54).

### *The Binding of Soluble Proteins to the Macrophage Surface*

We were unable to demonstrate that soluble HRP binds to the surface of macrophages. Other workers (40, 49, 51) have postulated that some 2–5% of the total soluble protein bound to macrophages *in vitro* is actually on the cell surface and remains there for days. Three lines of evidence indicated that HRP is not handled in this fashion:

(a) Electron microscope histochemistry did not reveal HRP along the plasmalemma. We believe that the method is sufficiently sensitive to detect the amounts of soluble protein which have been postulated to exist in other systems. The periphery of pinocytotic vacuoles will stain shortly (15–30 min) after exposure to a 0.1 mg/ml solution of HRP. If we assume that the vacuoles have not had time to shrink and concentrate their contents, then the cytochemical method detects  $10^{-1}$  mg/ml of HRP. Since the partial specific volume of HRP is 0.699 (21), we can calculate that the diameter of a spherical HRP molecule is 35 Å. Employing a surface area of  $2.5 \times 10^8 \mu^2$ /macrophage (54), then the volume of a 35 Å space along the surface of  $10^6$  macrophages is  $8.8 \times 10^{-6}$  ml. Assuming a

cytochemical sensitivity of 0.1 mg/ml HRP and a unimolecular layer on the cell surface, then the amount of detectable HRP would be  $0.1 \times 8.8 \times 10^{-6}$  ng or 0.88 ng. Since  $10^6$  cells bind 50 ng in 2 hr, we should be able to visualize 1.76% of the cell-bound HRP, even when dispersed as a surface monolayer, which is of the order of that proposed to be present (40, 49).

(b) Gentle trypsinization of well-washed monolayers did not release either enzymatically active HRP or TCA precipitable counts into the culture medium. It is difficult to equate these results with previous studies which reported the persistence of protein antigens on the macrophage surface (40, 49, 51). These reports did not directly demonstrate that the solubilized material emanated from the surface of viable macrophages. Perhaps, the use of heterogeneous cell populations of unknown viability and the presence of a significant dish-bound compartment underlie these discrepancies.

(c) The uptake of HRP increased linearly over a wide range of protein concentrations in the culture medium (0.01–1.0 mg/ml). If significant binding of HRP occurred before interiorization, one would expect competitive binding of surface sites with lower uptakes.

We suspect that many soluble proteins do not bind to the surface of the macrophage and are interiorized as solutes in pinocytotic droplets (liquid vs. adsorptive endocytosis as discussed by Jacques, [19]). This would account for the equivalent and low levels of uptake reported from this laboratory for human serum albumin, rabbit hemoglobin, and HRP. Highly charged macromolecules which induce pinocytosis (10), or  $\gamma$ -globulin antibodies (8) directed against plasma membrane constituents would probably behave quite differently. This would be in keeping with the extensive binding of

ribonuclease to the acid polysaccharide coat observed in certain species of amoeba (41).

### Fate of HRP

The enzymatic activity of HRP interiorized by macrophages disappears in an exponential fashion,  $t_{1/2}$  of 7–9 hr, until enzyme is no longer detectable. Enzyme is not inactivated extracellularly. Straus (46) followed the decay of HRP enzymatic activity in subcellular, lysosomal fractions of rat kidney after an intravenous dose of enzyme. Over the 24 hr period studied, enzyme disappeared at a rate of 8–10%/hr, which is strikingly similar to the kinetics in mouse peritoneal macrophages. We observed longer decay times ( $t_{1/2}$  of 20–30 hr) when we measured the disappearance of total cell-bound HRP- $^{125}\text{I}$ , or cell-bound TCA precipitable counts. This probably reflects the larger number of hydrolytic cleavages that are required before the label exits from the cell.

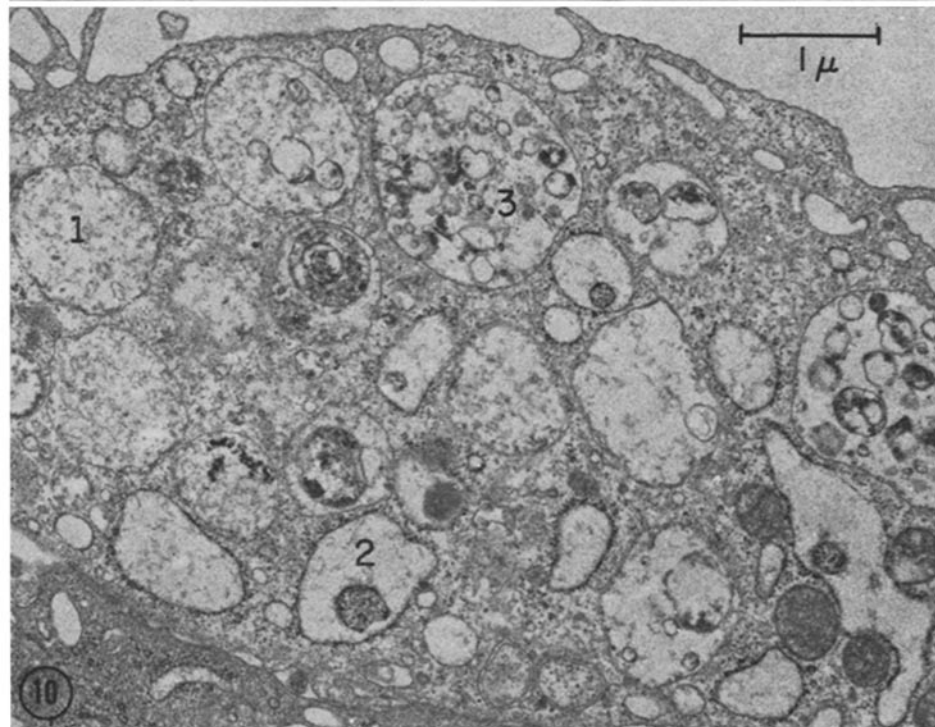
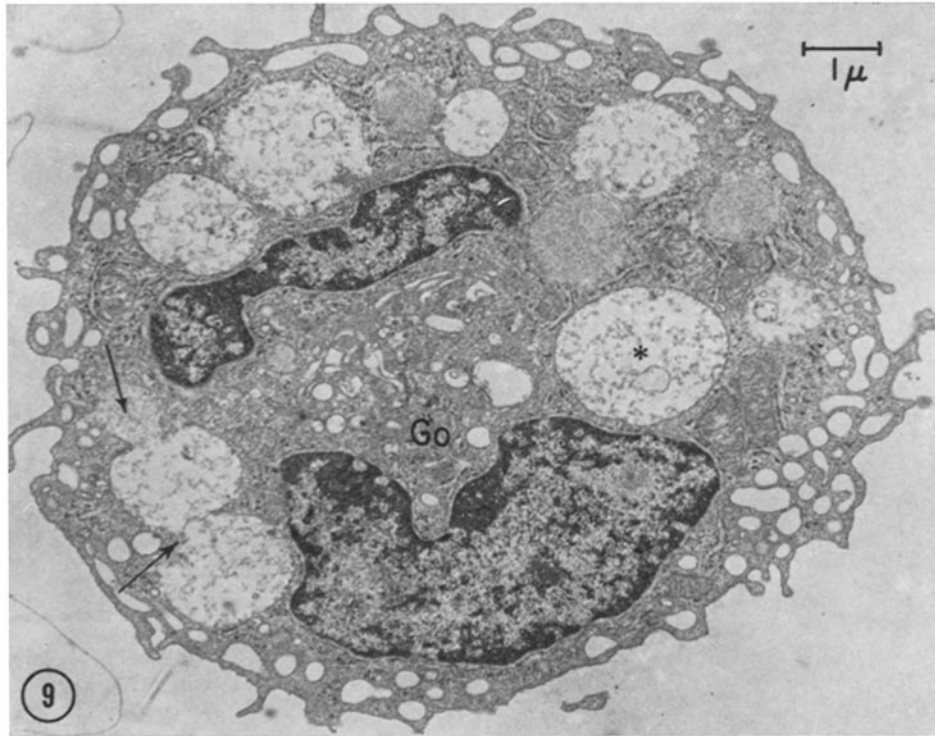
We were unable to detect exocytosis of HRP, or of TCA insoluble counts, onto the cell surface or into the culture medium. In a previous study, TCA precipitable counts were detected in the washout medium after the uptake of radiolabeled human serum albumin (13). This probably represents intact material that was not adequately removed or accounted for during the procedure used to wash out noncell-bound protein.

We propose that all HRP molecules interiorized by macrophages are being inactivated by enzymatic hydrolysis in lysosomes. Three observations, taken together, support this interpretation. The exponential decay of cell-bound HRP suggests that all molecules are being handled by a single, rate-limiting compartment or degradative process. This compartment is presumably intra-lysosomal since this is the only organelle in which HRP can

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FIGURE 9 24 hr after cultivation, macrophages were exposed for 2 hr to 1 mg/ml HRP in medium buffered with 0.1 M Tris. This cell is rounded with extensive surface projections and peripheral lacunae. Dense granules or secondary lysosomes are now absent. Large electron-transparent vacuoles are prominent and Golgi saccules (*Go*) are dilated. Some of the vacuoles appear to be fusing with one another (arrows), while others contain internal vesicles (\*). Although such cells interiorize normal amounts of HRP (Table II), cytochemical reaction product is not evident.  $\times 10,000$ .

FIGURE 10 A cell treated identically to the one shown in Fig. 9, but maintained in 0.1 M Tris buffered medium for an additional 3 hr. The disorganization of the cytoplasm is more pronounced. Many large, electron-transparent vacuoles are present (1), some of which contain many internal vesicles (2). These multivesicular bodies are thought to be of an autophagic origin. In addition, there is now extensive dilatation of the rough endoplasmic reticulum (3) and some profiles contain portions of cell cytoplasm.  $\times 18,000$ .





be detected cytochemically. Admittedly, the sensitivity of this technique is such that we probably could not detect the egress of considerable amounts of HRP from lysosomes. However, studies with nonmetabolizable hydrophilic molecules, ranging from mol wt 220 to  $2 \times 10^6$ , have shown that these materials are retained for long periods of time within macrophage lysosomes (15). Similar results have been obtained for the lysosomes of human skin fibroblasts (43).

The rate-limiting process destroying HRP probably involves the action of acid hydrolases. The enzymatic activity of dilute solutions of HRP is not altered by prolonged exposure to pH 4–5. Data on the fate of HRP-<sup>125</sup>I indicates that hydrolysis is occurring down to the level of the single amino acid, MIT. This degree of digestion before release from cells confirms other observations on the fate of human serum albumin-<sup>125</sup>I and rabbit hemoglobin, and internally labeled rabbit hemoglobin-<sup>3</sup>H in these same macrophages (13, 14).

#### *The Relevance of These Studies to the Proposed Role of the Macrophage in the Induction of Immune Response*

It is well established that macrophages can destroy the immunogenicity of antigens bound to them *in vitro* (22, 34). Several investigators believe that these cells also alter or process antigens, especially soluble proteins in a manner which enhances the subsequent immunogenicity of that protein. Many mechanisms have been proposed and have been reviewed in detail elsewhere (44, 50). In brief, it is postulated that macrophages may process antigens to form immunogenic RNA or antigen RNA complexes (2, 16), to concentrate and retain antigens on their surface (49), to act as reservoirs of persistent antigen liberated presumably by exocytosis (1, 12), or to alter antigens extracellularly (42). Our *in vitro* observations on the fate of HRP in homogeneous monolayers of macrophages are difficult to reconcile with any of these proposed mechanisms. Although our data deal only with a single protein, and although we have not examined directly the immunogenicity of macrophage-bound HRP, these and other studies on purified cell populations (13–15) suggest that molecules interacting with macrophages are sequestered and/or digested within lysosomes. Much of the work on the putative immunogenicity of macrophage-bound proteins (29, 47–49) has in-

involved the use of heterogeneous inflammatory exudates. Although rich in macrophages, the cell preparations contain cell debris and other cell types, e.g. antigen-binding lymphocytes (3), which may contribute to the results. It is difficult as well to interpret evidence in which antigens are extracted from macrophages (2, 16, 33) before the living cell has had sufficient time to degrade them and without maintaining the integrity of cytoplasmic compartments. The complexing of antigens to RNA shortly after ingestion of antigen may simply be an experimental event unrelated to the immunogenicity of material bound to intact macrophages (38, 39). It is not certain that the physiology of macrophages *in vitro* can be extended to the immune response *in vivo*. Nevertheless, since so little soluble protein is actually pinocytosed by these cells, it would seem more likely that macrophages contribute to the well-known tolerogenic capacity of soluble proteins rather than immunity. In addition, we will subsequently report further *in vitro* studies which amplify the important role of the macrophage in the efferent limb of immunity. We have observed that in the presence of antibody, the uptake, and subsequent destruction of HRP as a model antigen can be enhanced as much as several thousand-fold.

The authors acknowledge the skillful technical assistance of Dinah Lustig and Judy Adams. We are also grateful for many helpful discussions with Drs. M. Fedorko, J. G. Hirsch, and S. Silverstein.

This research was supported in part by United States Public Health Service grants AI 07012 and AI 01831, and by a fellowship to Dr. Steinman from the Leukemia Society of America.

*Received for publication 16 March 1972, and in revised form 26 May 1972.*

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