Erythrocyte Catabolism by Macrophages In Vitro

THE EFFECT OF HYDROCORTISONE ON ERYTHROPHAGOCYTOSIS AND ON THE INDUCTION OF HEME OXYGENASE

DIETHARD GEMSA, C. H. WOO, H. HUGH FUDENBERG, and RUDI SCHMID

*From the Department of Medicine, University of California, San Francisco
Medical Center, San Francisco, California 94122*

ABSTRACT Phagocytosis of erythrocytes was studied in vitro in an incubation system consisting of rat peritoneal macrophages and antibody-coated ^{59}Fe -labeled erythrocytes. The system was characterized in terms of the rate and magnitude of erythrophagocytosis, determined by the interiorization of the ^{59}Fe label. On incubation of 150×10^6 macrophages with 75×10^6 antibody-coated erythrocytes, erythrophagocytosis began within a few minutes and was essentially completed after 2 h when 50% of the offered red cells had been ingested by the macrophages. Heme oxygenase (HO) activity, which is very low in native macrophages, increased 4- to 10-fold in response to the ingested erythrocytes; this enzyme stimulation occurred with a delay of 3 h in relation to erythrophagocytosis. Actinomycin D or puromycin prevented the increase of HO activity without affecting erythrophagocytosis, which suggests that the enzyme stimulation was due to substrate-mediated enzyme induction.

Hydrocortisone (HC) (0.1 mg/ml medium) dissociated erythrophagocytosis from HO induction, leaving the former unimpaired but completely suppressing the latter. The suppressive effect of HC on the enzyme induction was completely prevented by 5 mg glucose and 0.02 U insulin/ml of the medium. In macrophages engaged in erythrophagocytosis, HC also lowered glucose removal from the medium and reduced formation of $^{14}\text{CO}_2$ from $[1\text{-}^{14}\text{C}]\text{glucose}$.

These results suggest that induction of HO in macrophages by the hemoglobin of ingested erythrocytes requires intact transport or metabolism of glucose. Glucose

utilization appears to be impaired by HC, but is restored by additional glucose and insulin. The findings suggest that plasma steroid concentrations in the pharmacological range could reduce bilirubin formation in phagocytic cells in vivo without affecting the sequestration and degradation of erythrocytes. This provides a possible explanation for the observation that in patients with hepatogenous jaundice, steroids often lower the serum bilirubin level.

INTRODUCTION

Circulating erythrocytes which have reached the end of their physiological life-span are believed to be removed and subsequently degraded in mononuclear phagocytes of the spleen, liver, bone marrow, and other tissues (1). Erythrocytes which have been damaged by physical, chemical, or immunological means appear to be handled in a similar manner (2-4) although the relative importance of various tissue sites in the removal of these damaged cells may differ from that of physiologically aged erythrocytes (2, 5). Red cells extruded into extravascular sites are phagocytized and degraded by mononuclear macrophages which converge at these sites (6, 7). Details of the mechanisms by which these phagocytic cells capture and sequester erythrocytes are unclear, and particularly the mechanism whereby mononuclear phagocytes of the spleen, liver, and bone marrow recognize senescent erythrocytes and remove them from the circulation remains obscure. Events involved in the intracellular degradation of ingested erythrocytes are somewhat better understood. Preformed lysosomes have been shown to converge around, and eventually to fuse with, the phagocytic vesicles containing the red cells (8, 9). During this digestive process, lysosomal hydrolases increase (10), proteolytic products are released

This work was presented in part at the Annual Meeting of the Association of American Physicians on 3 May 1972 in Atlantic City, N. J.

Received for publication 6 October 1972 and in revised form 22 November 1972.

into the surrounding extracellular space (11), and iron, presumably derived from the degraded hemoglobin, accumulates within the phagocytes (12). In addition, heme oxygenase (HO),¹ the enzyme system that converts heme to bilirubin IX α (13, 14), is stimulated by hemoproteins ingested by the macrophages (15).

A more detailed characterization of the mechanism of the degradation of phagocytized erythrocytes and particularly of the conversion of their hemoglobin-heme to bilirubin required the development of a reproducible in vitro system in which the individual steps of the degradative process could be analyzed sequentially. In the present study, antibody-coated ⁵⁹Fe-labeled rat erythrocytes were offered to a specified number of homologous macrophages maintained in primary culture. The rate of erythrophagocytosis was measured by the interiorization of the ⁵⁹Fe label and the rate of HO stimulation was determined by serial enzyme assay. By using inhibitors of protein synthesis, steroids, or nutritional manipulations, it was possible to dissociate the two events. The findings obtained with these macrophage cultures permitted evaluation of the quantitative and temporal relationship between the phagocytosis of erythrocytes and the stimulation of the principal enzymatic mechanism involved in the degradation of the ingested hemoglobin-heme.

METHODS

Preparation of peritoneal macrophages. In all experiments, female Sprague-Dawley rats weighing 175–200 g were used. The animals were fed standard laboratory chow. Peritoneal macrophages were obtained by stimulating the peritoneal cavity with a single intraperitoneal injection of 20 ml of 1.2% (wt/vol) sodium caseinate suspended in sterile isotonic saline. 3 days later the rats were sacrificed and the peritoneal cavity was lavaged with cold isotonic saline. All peritoneal cell preparations which appeared grossly hemorrhagic or which by phase microscopy contained more than 15% erythrocytes were discarded. Harvested peritoneal cells from 10–15 rats were pooled and centrifuged in a refrigerated centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.) for 5 min at 100 *g*. The pellet was suspended in 4 ml cold isotonic saline, contaminating erythrocytes were lysed by brief addition of 12 ml cold distilled water, and isotonicity was restored within 10 s with 4 ml of a 3.5% saline. The cell preparation was again centrifuged and the supernate containing the dissolved hemoglobin was discarded. This procedure did not affect the viability of the macrophages as determined by trypan blue exclusion. The cells were then suspended in medium 199 (Grand Island Biological Co., Grand Island, N. Y.), and total cell counts were performed in a Neubauer hemacytometer. An average of 8×10^7 peritoneal cells were obtained from each rat. Differential counts were performed after staining with May-Gruenwald-Giemsa dye.

The percentage of mononuclear macrophages ranged from 85–90% of all peritoneal cells, the remainder being granulo-

cytes, lymphocytes, and occasional mast cells. Phagocytic cells were identified by incubation of representative cell samples with suspensions of carbon particles measuring 450 nm or more in diameter (Higgins India Ink, A. W. Faber-Castell Pencil Co., Inc., Brooklyn, N. Y.). By light microscopy 90–95% of the peritoneal cells contained carbon particles; most of these cells were mononuclear macrophages and a few granulocytes not exceeding 5% of the total cell population.

Preparation of erythrocytes and dissolved hemoglobin. In adult Sprague-Dawley rats, 1.5 ml blood was removed by cardiac puncture and 0.12 mCi ⁵⁹FeCl₃ (specific activity, 48 mCi/mg Fe) was administered intravenously through a tail vein. 3–20 days later, the rats were bled by cardiac puncture and the erythrocytes were washed three times in phosphate-buffered saline, pH 7.4. The ⁵⁹Fe-labeled erythrocytes were counted in a Neubauer hemacytometer and their radioactivity was measured in a Nuclear-Chicago well-type scintillation counter (Nuclear-Chicago Corp., Des Plaines, Ill.). Dissolved ⁵⁹Fe-labeled hemoglobin was prepared from these erythrocytes by lysis in distilled water, followed by removal of stroma and membranes by centrifugation at 10,000 *g*. Preliminary experiments ascertained that under these conditions, as well as during incubation, hemoglobin did not crystallize.

Antiserum to rat erythrocytes was prepared in rabbits as follows. After siphoning off the buffy coat and washing the erythrocytes three times with phosphate-buffered saline, pH 7.4, 1 ml of packed rat erythrocytes was injected intraperitoneally into six New Zealand rabbits at biweekly intervals. After 10 wk the rabbits were bled, the sera pooled, and decanted by heating for 1 h at 56°C. The hemagglutination titer of the antiserum was 1:4096 as determined by conventional methods (16). The ⁵⁹Fe-labeled rat erythrocytes were coated with the antibody by incubating 3×10^9 cells with 0.12 ml of the antiserum for 30 min at room temperature. Preliminary experiments had indicated that this amount of antiserum was optimal for producing a maximal degree of erythrophagocytosis without causing erythrocyte agglutination. The antibody-coated erythrocytes were washed twice with buffered saline.

Incubation of macrophages with erythrocytes or dissolved hemoglobin. Unless stated otherwise, standard incubations consisted of 150×10^6 macrophages and 75×10^6 antibody-coated ⁵⁹Fe-labeled erythrocytes or 10.5 mg dissolved ⁵⁹Fe-labeled hemoglobin (which is equivalent to the hemoglobin in approximately 600×10^6 rat erythrocytes). The cells were incubated in 50-ml Erlenmeyer flasks (Nalgene) containing 15 ml medium 199 and 0.125 mg penicillin/ml. Incubation usually lasted 5 h at 37°C with the rotating water bath set at 70 cycles/min. The flasks were gassed with air containing 5% CO₂ and covered with Parafilm. Under these conditions, the pH of the medium was maintained at 7.3–7.45 for the duration of the incubation which was terminated by immersing the flasks in ice water. Hemolysis during the 5 h of incubation never exceeded 2% of the incubated erythrocytes.

In some instances, glucose, fructose, or maltose (5 mg/ml, final concentration) was added to the incubation system in addition to the medium 199 which contained 1 mg glucose/ml. In other instances, 0.02 U insulin (E. R. Squibb & Sons, Princeton, N. J.), 0.2 μ g actinomycin D (Sigma Chemical Co., St. Louis, Mo.), 2 μ g puromycin (ICN Nutritional Biochemicals Div., Cleveland, Ohio), or 0.01–0.18 mg hydrocortisone succinate (Sigma Chemical Co.) dissolved in medium 199 was added per ml of the incubation mixture.

¹ Abbreviations used in this paper: HO, heme oxygenase; HC, hydrocortisone.

FIGURE 1 Erythrophagocytosis and stimulation of HO in macrophages. In a standard incubation system containing 150×10^6 macrophages and 75×10^6 antibody-coated erythrocytes, erythrophagocytosis (closed circles) and HO activity (open squares) was determined at the indicated times after the start of the incubation.

Quantitation of endocytosis of erythrocytes or hemoglobin. At the end of the incubation period the macrophages, most of which adhered to the bottom of the incubation flask, were carefully detached with a fine brush and centrifuged in the incubation medium at 3,000 g for 6 min. Erythrocytes that had not been phagocytized were lysed by brief exposure to hypotonicity followed after 20–30 s by restoration of isotonicity with 3.5% saline. Macrophages which had been incubated with dissolved hemoglobin instead of antibody-coated erythrocytes were treated in the same manner. After centrifugation the macrophage pellet was suspended in 2 ml cold 0.1 M K-phosphate buffer, pH 7.4, and the ^{59}Fe activity retained in the cells was determined in a well-type scintillation counter. All samples were counted at least 5 min which assured a standard error of the mean of less than 2%.

Assay of heme oxygenase (HO) activity. After completion of the incubation the macrophages were disrupted by sonication for 10 s at 45 watts (Heat Systems-Ultrasonics, Inc., Plainview, N. Y.). The sonication disrupted more than 98% of the cells as estimated microscopically in a hemacytometer. The homogenate was centrifuged at 20,000 g for 10 min at 4°C and the supernate used as the enzyme source. Details of the assay for HO activity have been described elsewhere (13, 14). The standard assay was modified by reducing all constituents proportionally to a final volume of 0.5 ml per cuvette, containing 0.7–1.3 mg protein/ml.

Analytical procedures. The conversion of $[1-^{14}\text{C}]$ glucose to $^{14}\text{CO}_2$ by phagocytizing macrophages was determined as follows. 2 μCi $[1-^{14}\text{C}]$ glucose (Tracerlab Div., LFE Electronics, Boston, Mass., specific activity 3.0 mCi/nmol) was added to the standard incubations containing the macrophages and antibody-coated erythrocytes. The flasks were gassed with air containing 5% CO_2 and closed with sleeve-type rubber stoppers equipped with a center well. At the end of the incubation, 0.4 ml 8 N H_2SO_4 was injected into the medium through the rubber stopper and the center well was filled with 0.3 ml hyamine hydroxide. After further incubation for 45 min, the center well was removed and the $^{14}\text{CO}_2$ trapped in the hyamine hydroxide was counted in a Packard liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.) (17).

Glucose concentration in the incubation medium was determined by the glucose oxidase method (18). The protein concentration of the 20,000 g supernate of the homogenized macrophages was measured by the method of Lowry, Rosebrough, Farr, and Randall (19). Hemoglobin content of erythrocytes was estimated by the cyanmethemoglobin method (20) and the hemoglobin in the 20,000 g supernate by the method of Crosby and Furth (21).

Expression of results. In all instances, at least five individual experiments were carried out under identical conditions with macrophage pools from 10 to 15 rats. Duplicate analyses of individual experiments were possible only in a few instances because of the limited amount of macrophages available, and because of the time required for the analytical procedures, all of which were performed immediately after completion of the incubation. The results are presented either as the mean and range of all individual experiments or as individual data from a representative set of experiments.

Endocytosis of antibody-coated erythrocytes or dissolved hemoglobin by the incubated macrophages was calculated from their intracellular ^{59}Fe activity and expressed as a percentage of the total ^{59}Fe activity of the erythrocytes or hemoglobin that had been offered to the macrophages. The percentage value obtained was converted to absolute numbers of erythrocytes ingested by the macrophages. Glucose oxidation in macrophages was measured by comparing the radioactivity of the $^{14}\text{CO}_2$ trapped in the hyamine hydroxide with the total $[1-^{14}\text{C}]$ glucose added to the flask. The values obtained were converted to milligrams of glucose metabolized to CO_2 .

RESULTS

Preliminary studies to define optimal conditions of incubation. When 150×10^6 peritoneal macrophages were incubated with 75×10^6 antibody-coated erythrocytes,

FIGURE 2 Erythrophagocytosis (closed circles) and stimulation of HO (open squares) in 150×10^6 macrophages exposed to various amounts of antibody-coated erythrocytes. In this and all subsequent experiments, erythrophagocytosis and stimulation of HO was determined after 5 h of incubation.

erythrophagocytosis began within the first few minutes of incubation and reached a maximum within the first 2 h (Fig. 1). Thereafter, little erythrophagocytic activity was detectable despite the presence of additional erythrocytes in the incubation medium. When a second dose of 75×10^6 antibody-coated erythrocytes was added to the incubation mixture after 2 h, erythrophagocytosis resumed to an extent comparable with the initial phagocytic rate. On phase microscopy, phagocytized erythrocytes appeared first as phase-dense rounded structures, which on further incubation gradually lost their density, until after 5–7 h only their remnants were detectable in small intracellular vacuoles. HO activity in native macrophages or in macrophages incubated for 5 h without erythrocytes ranged from 0.04 to 0.125 nmol bilirubin formed/10 mg protein per min. On incubation with 75×10^6 erythrocytes, the enzyme activity increased steeply after an initial lag phase of approximately 3 h and reached a maximum after 5 h of incubation (Fig. 1). Maximal HO activity was regularly 4–10 times higher than base activity. Since incubation beyond 5 h did not enhance HO activity, 5 h of incubation was selected for all subsequent experiments.

When variable amounts of antibody-coated erythrocytes were incubated with the standard number of 150×10^6 macrophages, a relatively constant fraction of the red cells was phagocytized by the macrophages (Fig. 2). With erythrocyte doses ranging from 15 to 120×10^6 cells, approximately 50% of the offered red cells were ingested, while with larger doses the percentage of interiorized cells dropped to 25% (Fig. 3). Phase microscopic examination disclosed that even with the higher erythrocyte doses, only 45–55% of the incubated macrophages were engaged in erythrophagocytosis, whereas virtually all of the macrophages were found to interiorize

FIGURE 3 Stimulation of HO in macrophages offered large doses of antibody-coated erythrocytes. Incubations were performed with (closed squares) or without (open squares) addition of glucose (5 mg/ml) and insulin (0.02 U/ml). Erythrophagocytosis (closed circles) was unaffected by the presence of additional glucose and insulin.

FIGURE 4 Effect of inhibitors of protein synthesis on the stimulation of HO in macrophages. Actinomycin D, 0.2 μ g/ml (closed bars), or puromycin, 2 μ g/ml (hatched bars), was added to the standard incubation system at various time intervals after the start of the incubation. The open bars on the left represent HO activity in native macrophages (basal) and in macrophages after erythrophagocytosis (stimulated) without added inhibitors.

carbon particles that had been added to the incubation mixture.

With erythrocyte doses ranging from 15 to 75×10^6 cells, stimulation of HO in the macrophages was strictly proportional to the absolute number of red cells that had been phagocytized (Fig. 2). When the number of offered erythrocytes exceeded 75×10^6 cells, no further stimulation of HO was achieved. In fact, under standard conditions of incubation, larger doses of erythrocytes resulted in a progressive reduction of HO stimulation (Fig. 3). This paradoxical relationship could be reversed almost completely by supplementing the incubation medium with 5 mg glucose and 0.02 U insulin/ml (Fig. 3). Addition of glucose and/or insulin did not augment the enzyme response with the lower erythrocyte doses (Fig. 3).

Since 75×10^6 erythrocytes offered to 150×10^6 macrophages under standard conditions of incubation yielded maximal stimulation of HO, these conditions were chosen for all subsequent experiments.

When, instead of antibody-coated erythrocytes, macrophages were incubated with hemoglobin dissolved in the medium, uptake was much lower than with intact cells, but increased linearly over the 5 h of incubation. With 10.5 mg of hemoglobin in the incubation medium, which is equivalent to the hemoglobin content of 600×10^6 erythrocytes, uptake in 5 h was only 0.17 mg or 1.6% of the offered dose. The hemoglobin interiorized under this condition produced a three to fourfold stimulation of HO.

Effect of inhibition of protein synthesis on stimulation of HO. Preliminary experiments indicated that 0.2 μ g

FIGURE 5 Effect of HC on erythrophagocytosis (open bars) and stimulation of HO (closed bars) in macrophages. Various concentrations of HC were added at the start of the incubation. The results are expressed as a percentage of the activity in macrophages incubated without HC.

actinomycin D or 2 μ g puromycin/ml of the incubation mixture failed to exert a detectable effect on the rate or extent of erythrophagocytosis or on the viability of the incubated macrophages. Either compounds, when added in the indicated concentration at the beginning of the incubation period completely suppressed the stimulation of HO by the phagocytized erythrocytes (Fig. 4). When actinomycin D was added to the medium at various time intervals *after* the start of the incubation, its suppressive effect on the stimulation of HO was progressively reduced, and by delaying the addition for 3 h or longer, the effect of the inhibitor on the enzyme stimulation was completely lost (Fig. 4). Puromycin was found to have a more protracted effect, in that addition of this compound 4 h after the beginning of the incubation still resulted in submaximal stimulation of HO in the macrophages (Fig. 4).

Effect of hydrocortisone (HC) on erythrophagocytosis and stimulation of HO. Hydrocortisone (HC) in

FIGURE 6 Effect of HC on pinocytosis of dissolved hemoglobin (open bars) and stimulation of HO (closed bars) in macrophages. Results are expressed as in Fig. 5.

concentrations of up to 0.1 mg/ml in the medium produced little interference with erythrophagocytosis in the standard incubation system (Fig. 5). Larger concentrations of the steroid, on the other hand, progressively depressed erythrophagocytosis, with 0.18 mg/ml leading to almost 50% reduction in the phagocytosis of erythrocytes. Comparable HC concentrations had been shown previously to impair phagocytosis of aggregated albumin particles and bacteria (22). By contrast, when 10.5 mg hemoglobin was dissolved in the medium, HC in the concentrations tested did not affect the pinocytic activity (Fig. 6).

Stimulation of HO by the interiorized erythrocytes or hemoglobin was significantly suppressed by HC, even with steroid concentrations that did not interfere with erythrophagocytosis or with pinocytosis of dissolved hemoglobin (Figs. 5 and 6). With HC concentrations ranging from 0.01 to 0.1 mg/ml, the suppressive effect of the steroid on the HO stimulation was dose-related, with the upper value resulting in complete abolition of the enzyme stimulation without significantly affecting erythrophagocytosis (Fig. 5). Because of this dissociation of erythrophagocytosis from HO stimulation by 0.1 mg HC/ml, this experimental condition was chosen for all subsequent studies.

When HC was added to the macrophages phagocytizing erythrocytes at various time intervals *after* the start of the incubation (Fig. 7), the suppressive effect of the steroid on the stimulation of HO was clearly time-related in a manner similar to the effect of puromycin (Fig. 4). Complete suppression of enzyme stimulation

FIGURE 7 Effect of HC on stimulation of HO in macrophages. HC (0.1 mg/ml) was added to the medium at various time intervals after the start of the incubation. In this and in Fig. 8, the open bars on the left represent HO activity in incubated native macrophages (basal) and in macrophages after erythrophagocytosis (stimulated) without the addition of HC.

required the presence of the steroid during the 1st h of incubation (Fig. 7). Delayed addition of HC gave rise to progressively less suppression, until after 4 h the steroid had lost nearly all of its suppressive effect (Fig. 7).

Reversal of HC effect on stimulation of HO. The suppressive effect of HC on the stimulation of HO in macrophages phagocytizing erythrocytes was partially reversed by supplementing the incubation medium with glucose, fructose (5 mg/ml), or insulin (0.02 U/ml) (Fig. 8). Addition of the maltose was without effect. The combination of 5 mg/ml glucose with 0.02 U/ml insulin completely restored the stimulation of HO and thus effectively counteracted the suppressing effect of 0.1 mg/ml HC. On the other hand, combination of insulin with fructose or maltose failed to yield an additive effect (Fig. 8).

The beneficial effect of glucose and insulin was even observed when their addition to the culture medium, containing HC from the start, was delayed for several hours after the beginning of the incubation. Glucose and insulin completely reversed the steroid-mediated suppression of HO stimulation when added 3 h after the start of the incubation. With later addition, their reversing effect was progressively reduced (Fig. 9).

Removal of glucose from the incubation medium. In order to estimate the amount of glucose metabolized by the macrophages in 5 h of incubation, glucose removal from the incubation medium was measured under the following conditions of incubation: macrophages alone; macrophages with the standard amount of antibody-coated erythrocytes with or without additional glucose and insulin, or HC, or both. As shown in Fig. 10, glucose removal was not appreciably enhanced by erythrophagocytosis, but was increased approximately one-third by addition of glucose and insulin. HC in a concentra-

FIGURE 8 Reversal by hexoses with or without insulin of the inhibitory effect of HC on the stimulation of HO in macrophages. Standard incubations contained 0.1 mg/ml HC and either glucose, fructose or maltose (5 mg/ml) with or without insulin (0.02 U/ml).

FIGURE 9 Reversal by glucose and insulin of the inhibitory effect of HC on the stimulation of HO in macrophages. Standard incubations contained 0.1 mg/ml HC. Glucose (5 mg/ml) and insulin (0.02 U/ml) were added at various time intervals after the start of the incubation. Results are expressed as percent reversal of the inhibition produced by HC in the absence of added glucose and insulin.

tion of 0.1 mg/ml greatly reduced glucose removal but this steroid-mediated repression was partly reversed by addition of glucose and insulin (Fig. 10).

A similar pattern was obtained by analysis of $^{14}\text{CO}_2$ formed from $[1-^{14}\text{C}]$ glucose added to the incubation mix-

FIGURE 10 Removal of glucose from the incubation medium by macrophages during 5 h of incubation. The standard incubation system (15 ml) containing 15 mg of glucose was supplemented with 75 mg glucose, 0.3 U insulin, or 1.5 mg HC in various combinations. The open bars on the left represent glucose removal in the standard incubation system without additions. Each bar represents the average value of two separate incubations, the brackets indicating the range of values.

FIGURE 11 Conversion of $[1-^{14}\text{C}]$ glucose to CO_2 in macrophages during 3 h of incubation. The standard incubation system (15 ml) containing 15 mg of glucose was supplemented with 75 mg glucose, 0.3 U insulin, or 1.5 mg HC in various combinations. The open bars on the left represent glucose conversion in the standard incubation system without additions. Each bar represents the average value of three separate incubations, the brackets indicating the range of values.

ture (Fig. 11). During the first 3 h of incubation, macrophages phagocytizing erythrocytes in the presence of HC produced substantially less $^{14}\text{CO}_2$ than when the steroid was absent. Glucose and insulin added to the incubation reversed this steroid effect. After 5 h of incubation, the results obtained were essentially similar, except that glucose and insulin were moderately less effective in reversing the suppressive effect of HC.

DISCUSSION

There is extensive morphologic (23, 24) and experimental (10, 25) evidence that macrophages and other mononuclear phagocytes possess the capacity to engulf intact or fragmented erythrocytes and to digest them. Erythrophagocytosis by these cells appears to be an important, and perhaps the primary mechanism by which physiologically aged or damaged red blood cells are removed from the circulation (1, 26–28). This process can be reproduced *in vitro*, in that in a variety of culture systems isolated macrophages may be observed ingesting and degrading offered erythrocytes that previously had been injured by chemical or physical means (10, 29). Antibody-coated red cells are also ingested and degraded at 37°C , presumably as the result of binding of the Fc fragment of the IgG to the monocyte-macrophage receptor for IgG (30). In the present study, this technique has been refined and controlled in such a way that a standard number of peritoneal macrophages in primary culture reproducibly phagocytized a predic-

table number of antibody-coated autologous erythrocytes in a specified time. Several observations made with this primary culture system are worthy of comment.

Although under standard conditions, the macrophages were incubated with erythrocytes for a total period of 5 h, it was apparent that the rate of erythrophagocytosis was most rapid during the 1st h, progressively slowed during the 2nd h, and approached zero after the 3rd h of incubation (Fig. 1). This decline in the rate of erythrophagocytosis occurred despite the availability of ample unagglutinated erythrocytes in the culture medium. It is unlikely that this phenomenon reflected potential "saturation" of the macrophages with red blood cells, as an increase in the number of offered erythrocytes resulted in an absolute and proportional rise of the number of cells that were ingested by the macrophages (Fig. 2). Furthermore, the cultured macrophages appeared to remain fully viable throughout the entire period of incubation, as shown by their continued adherence to the surface of the incubation flask, their active glucose assimilation and CO_2 production, their trypan blue exclusion, and their ability to respond to stimulation with formation of new enzyme protein. Although the exact cause of this time-dependent progressive reduction in erythrophagocytosis remains undetermined, possible explanations include quantitative differences in the degree of antibody coating of individual erythrocytes, which may be related to cell age (31, 32), or progressive elution of antibody from erythrocytes during the course of the incubation. Depletion of factors essential for phagocytosis in the incubation medium or in the macrophages seems unlikely, as addition of a second fresh dose of antibody-coated erythrocytes to the macrophage culture 2 h after the start of the incubation reactivated erythrophagocytosis.

It also was noted with this culture system that only 45–55% of the incubated macrophages actually participated in the ingestion of erythrocytes, whereas carbon particles were interiorized by virtually all macrophages. Even when the number of offered erythrocytes greatly exceeded that of macrophages, microscopic inspection revealed a relatively constant proportion of macrophages that were devoid of ingested erythrocytes, whereas others contained several red cells. Otherwise, no morphologic differences could be detected between macrophages that had, or had not, phagocytized erythrocytes. Whereas no plausible explanation can be offered for this functional difference among the incubated macrophages, the findings are reminiscent of similar observations with the sinusoidal cell population of rat liver (33). These phagocytes also appear to be heterogeneous in composition in that they include a subpopulation of cells recognizable by histochemical and enzymatic methods, which is involved specifically in erythrophagocytosis. It is possible that macrophages harvested after chemical irritation of

the peritoneal cavity are similarly heterogeneous and that only a fraction of these cells is equipped for phagocytosis of antibody-coated erythrocytes. It also should be noted that since in the present experiments, macrophages were not preincubated with serum-containing medium (10, 25), the cultures may have contained cells of different maturity.

The precise mechanisms involved in the disruption and subsequent degradation of the ingested erythrocytes are incompletely understood. Morphological and biochemical (10) evidence suggests that the interiorized red cells are attacked by lysosomal enzymes which are released into the phagocytic vesicles after they have fused with the lysosomes (34). This process is presumed to be analogous to the sequential degradation of phagocytized bacteria (35). The identification of a variety of proteolytic enzymes in lysosomes facilitated a more detailed investigation of this degradative mechanism. Axline and Cohn (10) recently reported that peritoneal macrophages incubated for 24 h after ingestion of formaldehyde-treated erythrocytes exhibited a net increase in lysosomal enzyme activity. This increase in proteolytic enzymes was presumed to reflect enzyme induction mediated by the membrane proteins and the globin moiety of the phagocytized erythrocytes. The fate of the heme moiety of the ingested erythrocyte-hemoglobin was not determined, but previous observations suggested that it is converted to bile pigment (36). The mechanism of this conversion was obscure until the recent demonstration by Pimstone, Tenhunen, Seitz, Marver, and Schmid (15), that macrophages possess HO, which is the enzyme system catalyzing the conversion of ferroprotoporphyrin (heme) to bilirubin IX α (13, 14). Although HO activity is very low in native peritoneal macrophages, presumably because they normally lack contact with heme compounds, a striking stimulation of enzyme activity was observed after macrophages had been exposed to methemalbumin or hemoglobin in the peritoneal cavity (15).

In the present study, the low HO activity of native peritoneal macrophages or of macrophages incubated without red cells has been confirmed. In contrast, when the macrophages were offered antibody-coated erythrocytes in the culture, their HO activity regularly rose in proportion to the absolute number of red cells ingested, at least with the lower red cell doses (Fig. 2). There was a time lag of about 3 h between erythrophagocytosis and stimulation of HO (Fig. 1), which is strikingly similar to the time interval that is required *in vivo* for the conversion of the hemoglobin of sequestered erythrocytes to bilirubin in intact rats (36). The stimulation of HO in the cultured macrophages was paralleled by morphologic evidence that after 4–5 h of incubation, the ingested erythrocytes became visibly pale and had obviously undergone disintegration. It is noteworthy that

the response of microsomal HO to the ingestion of erythrocytes was much more rapid and more pronounced than that reported for lysosomal enzymes (10). Although this may reflect the different intracellular localization of these enzyme systems, it is more likely that the retarded and attenuated stimulation of the lysosomal enzymes was due to the pretreatment of the ingested erythrocytes with formaldehyde, which may have reduced the digestibility of the red cells or their components. Indeed, when macrophages were incubated with formaldehyde-fixed erythrocytes, there was a marked delay in the stimulation of HO. Moreover, erythrophagocytosis occurred at a lower rate, but over a more extended period of time, and a larger percentage of the macrophages appeared to be engaged in erythrophagocytosis, as compared with incubations containing antibody-coated red cells.²

In addition to intact antibody-coated erythrocytes, hemoglobin dissolved in the culture medium also stimulated HO activity in the cultured macrophages (Fig. 6). However, in order to achieve comparable enzyme stimulation, far higher concentrations of dissolved hemoglobin were required than the hemoglobin equivalent to the incubated intact erythrocytes. This difference probably reflects the relatively low pinocytotic uptake of dissolved hemoglobin by the macrophages (11) which appears to be proportional to the extracellular concentration of hemoglobin. The different handling of dissolved hemoglobin and of intact erythrocytes by the macrophages is further emphasized by the observation that even the highest concentrations of HC added to the culture medium failed to depress pinocytosis of hemoglobin (Fig. 6), whereas with these high steroid concentrations phagocytosis of intact erythrocytes was significantly reduced (Fig. 5). The preference of macrophages for the ingestion of intact red cells, as contrasted to dissolved hemoglobin, is analogous to recent observations in the liver where erythrocytes are phagocytized exclusively by a subpopulation of sinusoidal cells, whereas dissolved hemoglobin is taken up mainly by hepatic parenchymal cells (37).

The stimulation of HO appeared to depend on new protein synthesis, as actinomycin D and puromycin strikingly inhibited the increase in enzyme activity without reducing the rate or magnitude of erythrophagocytosis (Fig. 4). These findings and previous observations of a similar nature in rat kidney (38) strongly suggest that HO is induced by its substrate, heme. Moreover, when these two inhibitors of protein synthesis were added to the macrophage cultures at various time intervals *after* the beginning of the incubation, a distinct difference in the effect patterns was discernable (Fig.

² Gernsma, D., H. H. Fudenberg, and R. Schmid. Unpublished observations.

4). The suppressive effect of actinomycin D on enzyme stimulation was more limited in time as compared with the more protracted action of puromycin, consistent with the concept that stimulation of HO by the ingested erythrocyte-hemoglobin requires formation of additional messenger RNA.

Induction of HO in macrophages after erythrophagocytosis also appeared to be critically dependent on an adequate supply of exogenous glucose. This became evident when large doses of antibody-coated erythrocytes were offered to the standard number of 150×10^6 macrophages. Whereas with relatively small red cell doses in the range of $15\text{--}75 \times 10^6$ cells, enzyme induction was proportional to the absolute number of ingested erythrocytes (Fig. 2), with larger doses no further rise in HO activity was observed despite the increase in the number of phagocytized cells (Fig. 3). As seen in Fig. 2, with the standard incubation medium containing 1 mg glucose/ml, the response of HO to erythrocyte doses exceeding 75×10^6 cells was suboptimal. This apparent paradox was explained when it was found that addition of glucose and insulin to the incubation mixture repaired this defective enzyme response (Fig. 3). Supplementation of the incubation medium with either glucose or insulin alone restored the enzyme induction only partially, which may suggest that under the "stress" of large loads of ingested erythrocytes, insulin-dependent glucose metabolism may become essential for optimal macrophage function.

Further support for the essential role of glucose metabolism in the substrate-mediated induction of HO in macrophages was obtained in experiments with HC. Munck and Young (39, 40) presented convincing evidence that HC markedly interferes with glucose transport and utilization in thymus cell cultures. In cultured lymphocytes the steroid reportedly depresses protein synthesis (41, 42), but this may be only a secondary effect resulting from impaired glucose metabolism. Because of these observations, HC in various doses was added to standard incubations of 150×10^6 macrophages and 75×10^6 antibody-coated erythrocytes. In the concentration range from 0.01 to 0.1 mg HC/ml medium, the steroid produced a progressive, dose-related repression of HO induction (Fig. 5). With a HC concentration of 0.1 mg/ml, enzyme induction was completely suppressed while erythrophagocytosis was not significantly impaired. When addition of the steroid was delayed after the start of the incubation (Fig. 7), the response pattern of HO induction was similar to that observed with puromycin, which may suggest that HC ultimately affects enzyme protein synthesis at the translational level.

The suppressive effect of HC on HO induction was readily reversible by supplementing the incubation me-

dium with additional glucose and insulin (Fig. 8). The suboptimal response of the enzyme activity to either glucose or insulin alone suggests a synergistic action of the two. This is reminiscent of the additive effect of glucose and insulin on macrophages which had been offered very large doses of erythrocytes without the addition of steroid (Fig. 3). The importance of glucose for HO induction was further emphasized when addition of glucose and insulin to the HC-containing cultures was delayed for several hours. The finding that glucose and insulin added 3 h after the start of the incubation completely reversed the inhibitory effect of HC (Fig. 9) is consistent with the concept that the exogenous glucose is required for the synthesis of new enzyme protein. Moreover, when glucose was replaced by the monosaccharide fructose, whose metabolism is insulin-independent (43), the induction of HO was only partially repaired regardless of the presence or absence of insulin (Fig. 8). The disaccharide maltose, although it may be metabolized in macrophages (44), was without beneficial effect. Maltose combined with insulin yielded a degree of enzyme stimulation comparable with that of insulin alone. Finally, HC reduced glucose removal from the medium by the phagocytizing macrophages (Fig. 10) and lowered production of CO_2 from glucose via the hexose monophosphate shunt (Fig. 11); both effects were partially or completely reversed by insulin and additional glucose.

These findings clearly indicate that in macrophages the induction of HO by the hemoglobin of ingested erythrocytes is dependent on adequate glucose transport or metabolism, and that HC interferes with this process. This may be analogous to the reported observation that in thymus cell cultures, the steroid impairs the formation of glucose-6-phosphate (39). The information presently available is insufficient to determine whether this steroid effect consists of partial inhibition of glucose transport into the cell or reflects interference with early steps of intracellular glucose metabolism. Likewise, it is unknown how this impairment of carbohydrate metabolism expresses itself in reduced enzyme synthesis. It is noteworthy, however, that under these selected experimental conditions, complete dissociation of erythrophagocytosis from HO induction was achieved with HC concentrations of the order of magnitude of those obtained in the plasma under pharmacological conditions. Moreover, it was evident on microscopic inspection that with these steroid concentrations, not only was erythrophagocytosis unaffected but, despite the lack of a HO response, digestion of ingested erythrocytes appeared to proceed without noticeable impairment. This apparent disparity permits three alternative interpretations, namely (a) that HO activity is not critical for the conversion of the ingested hemoglobin-heme to bilirubin IX α ; (b) that

the heme of the degraded hemoglobin simply accumulates in the macrophages without being catabolized; or (c) that in the absence of increased HO activity, hemoglobin-heme is degraded by alternate catabolic pathways leading to metabolites that differ from bilirubin. The first interpretation appears unlikely, as the HO system is the only mechanism known to produce exclusively bilirubin IX α , which is the bile pigment isomer formed in vivo (14). Moreover, in a variety of tissues, HO activity was shown to be stimulated in response to an increased supply of heme, derived from endogenous sources or administered parenterally (45). The second possibility, while perhaps unlikely on theoretical grounds, cannot be excluded with the presently available experimental means. The third explanation postulating operation of alternate pathways for heme degradation appears the most plausible, since the existence of such degradative pathways which do not lead to formation of bilirubin has already been demonstrated (46, 47). Although the sequence of the chemical reactions and the nature of the intermediates involved are as yet unknown, it has previously been reported that on incubation of heme with homogenates of stimulated macrophages, heme disappearance substantially exceeds the rate of bilirubin formation (15).

If this last interpretation should prove to be correct, it may provide a possible explanation for the beneficial effect of steroid treatment in patients with jaundice due to parenchymal or obstructive liver disease. In these patients, steroids tend to produce a well documented (48, 49), but unexplained (50), reduction in the serum bilirubin level. If the present findings are applicable to erythrophagocytic cells in general, it is possible that steroids may reduce the amount of bilirubin formed from sequestered erythrocytes without impairing the physiological removal mechanism of senescent red cells from the circulation. In order to prove this concept, it would be necessary to characterize the alternate pathways of heme degradation and to demonstrate experimentally that they become functional under steroid therapy. Furthermore, since the present findings suggest that glucose metabolism becomes insulin-dependent in macrophages degrading a large load of erythrocytes, it would be of interest to determine whether insulin deficiency states or hypercortisonism may be associated with defective macrophage function.

ACKNOWLEDGMENTS

The authors are grateful to Miss Lydia Hammaker for valuable advice and to Doctors D. Stites and D. Hanes for reviewing the manuscript.

This work was supported by Research Grants AM-11275 and HD-05894, Training Grants AM-05598 and HL-05677 from the National Institutes of Health, and by the Walter C. Pew Fund for Gastrointestinal Research.

REFERENCES

1. Harris, J. W., and R. W. Kellermeyer. 1970. *The Red Cell*. Harvard University Press, Cambridge. Revised edition. 524.
2. Jandl, J. H., A. R. Jones, and W. B. Castle. 1957. The destruction of red cells by antibodies in man. I. Observations on the sequestration and lysis of red cells altered by immune mechanisms. *J. Clin. Invest.* **36**: 1428.
3. Jandl, J. H. 1967. The spleen and reticuloendothelial system. In *Pathologic Physiology: Mechanisms of Disease*. W. A. Sodeman and W. A. Sodeman, Jr., editors. W. B. Saunders Co., Philadelphia. 4th edition. 897.
4. Keene, W. R., and J. H. Jandl. 1965. Studies of the reticuloendothelial mass and sequestering function of rat bone marrow. *Blood*. **26**: 157.
5. Wagner, H. N., Jr., M. A. Razzak, R. A. Gaertner, W. P. Caine, Jr., and O. T. Feagin. 1962. Removal of erythrocytes from the circulation. *Arch. Intern. Med.* **110**: 90.
6. Virchow, R. 1847. Die pathologischen Pigmente. *Arch. Pathol. Anat. Physiol. Klin. Med. (Virchows)*. **1**: 379.
7. Langhans, T. 1869. Beobachtungen über Resorption der Extravasate und Pigmentbildung in denselben. *Arch. Pathol. Anat. Physiol. Klin. Med. (Virchows)*. **49**: 66.
8. Essner, E. 1960. An electron microscopic study of erythrophagocytosis. *J. Biophys. Biochem. Cytol.* **7**: 329.
9. North, R. J. 1966. The localization by electron microscopy of acid phosphatase activity in guinea pig macrophages. *J. Ultrastruct. Res.* **16**: 96.
10. Axline, S. G., and Z. A. Cohn. 1970. In vitro induction of lysosomal enzymes by phagocytosis. *J. Exp. Med.* **131**: 1239.
11. Ehrenreich, B. A., and Z. A. Cohn. 1968. Fate of hemoglobin pinocytosed by macrophages in vitro. *J. Cell Biol.* **38**: 244.
12. Petrik, P., and A. J. Collet. 1971. Electron microscopic study of the in vivo erythrophagocytosis by alveolar macrophages of the cat. II. Late period: digestion. *Z. Zellforsch. Mikrosk. Anat.* **116**: 477.
13. Tenhunen, R., H. S. Marver, and R. Schmid. 1968. The enzymatic conversion of heme to bilirubin by microsomal heme oxygenase. *Proc. Natl. Acad. Sci. U. S. A.* **61**: 748.
14. Tenhunen, R., H. S. Marver, and R. Schmid. 1969. Microsomal heme oxygenase. Characterization of the enzyme. *J. Biol. Chem.* **244**: 6388.
15. Pimstone, N. R., R. Tenhunen, P. T. Seitz, H. S. Marver, and R. Schmid. 1971. The enzymatic degradation of hemoglobin to bile pigments by macrophages. *J. Exp. Med.* **133**: 1264.
16. Mollison, P. L. 1967. *Blood Transfusion in Clinical Medicine*. F. A. Davis Co., Philadelphia. 4th edition. 398.
17. Baehner, R. L., N. Gilman, and M. L. Karnovsky. 1970. Respiration and glucose oxidation in human and guinea pig leukocytes: comparative studies. *J. Clin. Invest.* **49**: 692.
18. Raabo, E., and T. C. Terkildsen. 1960. On the enzymatic determination of blood glucose. *Scand. J. Clin. Lab. Invest.* **12**: 402.
19. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**: 265.
20. Crosby, W. H., and D. N. Houchin. 1957. Preparing standard solutions of cyanmethemoglobin. *Blood*. **12**: 1132.

21. Crosby, W. H., and F. W. Furth. 1956. A modification of the benzidine method for measurement of hemoglobin in plasma and urine. *Blood*. 11: 380.
22. Wiener, E., Y. Marmary, and Z. Curelaru. 1972. The in vitro effect of hydrocortisone on the uptake and intracellular digestion of particulate matter by macrophages in culture. *Lab. Invest.* 26: 220.
23. Rifkind, R. A. 1966. Destruction of injured red cell in vivo. *Am. J. Med.* 41: 711.
24. Weiss, L., and M. Tavassoli. 1971. Anatomical hazards to the passage of erythrocytes through the spleen. *Semin. Hematol.* 7: 372.
25. Rabinovitch, M. 1967. The dissociation of the attachment and ingestion phases of phagocytosis by macrophages. *Exp. Cell. Res.* 46: 19.
26. Weed, R. I., and C. F. Reed. 1966. Membrane alterations leading to red cell destruction. *Am. J. Med.* 41: 681.
27. Bunn, H. F. 1972. Erythrocyte destruction and hemoglobin catabolism. *Semin. Hematol.* 9: 3.
28. Jandl, J. H., N. M. Files, S. B. Barnett, and R. A. MacDonald. 1965. Proliferative response of the spleen and liver to hemolysis. *J. Exp. Med.* 122: 299.
29. Rabinovitch, M. 1969. Phagocytosis of modified erythrocytes by macrophages and L2 cells. *Exp. Cell Res.* 56: 326.
30. Huber, H., and H. H. Fudenberg. 1968. Receptor sites of human monocytes for IgG. *Int. Arch. Allergy. Appl. Immunol.* 34: 18.
31. Marikovsky, Y., and D. Danon. 1971. Agglutination of young and old human red cells by blood group antibodies. *Vox Sang.* 20: 174.
32. Greenwalt, T. J., L. L. Flory, and E. A. Steane. 1970. Quantitative haemagglutination. III. Studies of separated populations of human red blood cells of different densities. *Br. J. Haematol.* 19: 701.
33. Bissell, D. M., L. Hammaker, and R. Schmid. 1972. Liver sinusoidal cells. Identification of a subpopulation for erythrocyte catabolism. *J. Cell Biol.* 54: 107.
34. Cohn, Z. A., and E. Wiener. 1963. The particulate hydrolases of macrophages. I. Comparative enzymology, isolation, and properties. *J. Exp. Med.* 118: 991.
35. Cline, M. J. 1970. Leukocyte function in inflammation: the ingestion, killing, and digestion of microorganisms. *Ser. Haematol.* 3 (No. 2): 3.
36. Ostrow, J. D., J. H. Jandl, and R. Schmid. 1962. The formation of bilirubin from hemoglobin in vivo. *J. Clin. Invest.* 41: 1628.
37. Bissell, D. M., L. Hammaker, and R. Schmid. 1972. Hemoglobin and erythrocyte catabolism in rat liver: the separate roles of parenchymal and sinusoidal cells. *Blood*. 40: 812.
38. Pimstone, N. R., P. Engel, R. Tenhunen, P. T. Seitz, H. S. Marver, and R. Schmid. 1971. Inducible heme oxygenase in the kidney: a model for the homeostatic control of hemoglobin catabolism. *J. Clin. Invest.* 50: 2042.
39. Munck, A. 1968. Metabolic site and time course of cortisone action on glucose uptake, lactic acid output, and glucose 6-phosphate levels of rat thymus cells in vitro. *J. Biol. Chem.* 243: 1039.
40. Young, D. A. 1969. Glucocorticoid action on rat thymus cells. Interrelationships between carbohydrate, protein, and adenine nucleotide metabolism and cortisone effects on these functions in vitro. *J. Biol. Chem.* 244: 2210.
41. Mayman, M. H., B. Dvorkin, and A. White. 1968. Influence of cortisone on the utilization of precursors of nucleic acids and protein by lymphoid cells in vitro. *J. Biol. Chem.* 243: 1485.
42. Werthamer, S., C. Hicks, and L. Amaral. 1969. Protein synthesis in human leukocytes and lymphocytes. I. Effect of steroids and sterols. *Blood*. 34: 348.
43. Nocenti, M. R. 1968. Endocrine Functions of the Pancreas. In *Medical Physiology*. V. B. Mountcastle, editor. The C. V. Mosby Co., St. Louis. 12th edition. 1029.
44. Cohn, Z. A., and B. A. Ehrenreich. 1969. The uptake, storage, and intracellular hydrolysis of carbohydrates by macrophages. *J. Exp. Med.* 129: 201.
45. Tenhunen, R., H. S. Marver, and R. Schmid. 1970. The enzymatic catabolism of hemoglobin: stimulation of microsomal heme oxygenase by hemin. *J. Lab. Clin. Med.* 75: 410.
46. Goldstein, G. W., L. Hammaker, and R. Schmid. 1968. The catabolism of Heinz bodies: an experimental model demonstrating conversion to non-bilirubin catabolites. *Blood*. 31: 388.
47. Landaw, S. A., E. W. Callahan, Jr., and R. Schmid. 1970. Catabolism of heme in vivo: comparison of the simultaneous production of bilirubin and carbon monoxide. *J. Clin. Invest.* 49: 914.
48. Katz, R., H. Ducci, and H. Alessandri. 1957. Influence of cortisone on hyperbilirubinemia. *J. Clin. Invest.* 36: 1370.
49. Schiff, L. 1966. The use of steroids in liver disease. *Medicine (Baltimore)*. 45: 565.
50. Williams, R., and B. H. Billing. 1961. Action of steroid therapy in jaundice. *Lancet*. II: 392.