THE ENZYMATIC DEGRADATION OF HEMOGLOBIN TO BILE PIGMENTS BY MACROPHAGES*

By NEVILLE R. PIMSTONE,‡ M.D., RAIMO TENHUNEN,§ M.D., PAUL T. SEITZ, HARVEY S. MARVER,|| M.D., AND RUDI SCHMID, M.D.

(From the Department of Medicine, University of California, San Francisco, California 94122)

(Received for publication 11 January 1971)

Downloaded from http://rupress.org/jem/article-pdf/133/6/1264/1084200/1264.pdf by guest on 30 March 2023

Senescent or damaged red blood cells are removed from the circulation primarily in the spleen, liver, and bone marrow (2–5). In these tissues it is most likely that the red cells are sequestered by phagocytic cells that line the sinusoids and comprise part of the reticuloendothelial system (6, 7). The erythrocyte hemoglobin which in the intact rat is converted almost quantitatively to bilirubin (8) is thought to be catabolized within these phagocytic cells, but controversy regarding participation of other cells types in this process still exists (9).

The enzyme system responsible for initiating the conversion of heme to bile pigment has been characterized recently (10–12). This enzyme complex, heme–oxygenase, is localized in the microsomal fraction of metazoan tissues (10), requires mixed function oxidation with cytochrome P450 as the terminal oxidase (13)[1], and converts heme to biliverdin. Subsequently, in the cytosol biliverdin is reduced to bilirubin in the presence of nicotinamide adenine dinucleotide phosphate (NADPH)-dependent biliverdin reductase (14). Microsomal heme–oxygenase usually is rate-limiting in the overall conversion of heme to bilirubin. In rats this enzyme is most active in those tissues normally involved in the sequestration and breakdown of red cells, namely the spleen, liver, and bone marrow (13). Moreover, it can undergo adaptive regulation in response to a substrate load (13, 15), for example, in the liver heme–oxygenase activity rises after splenectomy or after induced hemolysis (13).

---

* This work was presented in part to the American Association for the Study of Liver Diseases in October, 1969 (1). The study was supported in part by U. S. Public Health Service research grant AM-11275, U. S. Public Health Service training grant AM-05598, the Walter C. Pew Fund for Gastrointestinal Research, U. S. Public Health Service International Postdoctoral Fellowship F05-TW-1360, and Research Career Development Award KO4-AM-14301.

‡ Present address: Department of Medicine, University of Cape Town, P.O. Box 594, Cape Town, Republic of South Africa.

§ Present address: Department of Clinical Chemistry, University of Helsinki, Meilahti Hospital, Helsinki 29, Finland.

|| Present address: Department of Internal Medicine, University of Texas Southwestern Medical School, Dallas, Tex. 75235.

[1] Tenhunen, R., H. S. Marver, N. R. Pimstone, R. Schmid, W. Trager, and D. Y. Cooper. The enzymatic conversion of heme to biliverdin: further evidence for a role of mixed-function oxidation. In preparation.

It has been known for a long time that at sites of blood extravasation, bilirubin also can be formed locally. In 1847, Virchow isolated hematoidin crystals from tissues harboring old hematomas (16); these crystals later were shown to be bilirubin crystals (17). In 1870, Langhans noted the presence of bile pigments in phagocytes that surrounded blood extravasations in tissues and serous cavities (18), and in 1935, morphological observations by Muir and Niven conclusively demonstrated that bilirubin was formed within these reticuloendothelial cells (19). Studies of macrophages in vitro confirmed and extended these observations. In 1924, Rich demonstrated that macrophages accumulate bilirubin if exposed to blood in tissue culture (20). More recently, Axline and Cohn (21) showed that in macrophages erythrocytes ingested in vitro stimulate lysosomal hydrolases that digest the globin moiety of hemoglobin (22).

These observations suggested that after phagocytosis of erythrocytes, the macrophage may degrade the hemoglobin-heme by a heme–oxygenase system, similar to that in the spleen and liver.

Material and Methods

Animals.—All experiments were carried out in female Sprague-Dawley rats weighing 250–350 g or in New Zealand white rabbits of 2–3 kg. All animals had free access to water and food (Berkeley diet laboratory rat chow or Purina Rabbit Chow, Ralston Purina Company Inc., St. Louis, Mo.).

Preparation of Rat Peritoneal Macrophages.—Peritoneal macrophages in rats were obtained after chemical or immunological stimulation of the peritoneal cavity. Rats received a single intraperitoneal injection of 20–25 ml of 1.2% (w/v) sodium caseinate (Eastman Organic Chemical Company, Rochester, N. Y.) in sterile isotonic saline. After 4 days approximately 1×10^8 macrophages were obtained from each animal. Zymosan granules (Sigma Chemical Company St. Louis, Mo., lot No. Z 4250 from *Saccharomyces cereviseae* yeast) suspended in 0.9% sterile saline at a concentration of 2% (w/v) were given intraperitoneally to rats at a daily dose of 3 mg/100 g body weight for 3 consecutive days. On the 4th day $1–3 \times 10^8$ macrophages were obtained from each rat. Methemalbumin was prepared as previously described (10) and injected intraperitoneally two to three times per day for 3 consecutive days at a daily dose of 7.5–10 μmoles/100 g rat. When the rats were sacrificed 18 hr later, the average yield of macrophages per rat was 5×10^8.

Rat hemoglobin was freshly prepared (23) and its concentration was measured as cyanmethemoglobin (24). Hemoglobin equivalent to 10 μmole of heme was injected intraperitoneally every day in divided doses for 3 consecutive days. The animals were sacrificed 18 hr after the last dose when the average yield of macrophages per rat was $0.5–1 \times 10^8$.

Bacillus Calmette-Guerin (BCG),[2,3] obtained as a water-washed lyophilized powder, was suspended in sterile isotonic saline at a concentration of 20 mg/ml, and rats were given 0.5 ml intraperitoneally on each of 2 successive days. 3–5 wk later, when the peritoneum was studded with granulomas, macrophages were harvested with an average yield of 3×10^8 cells/animal. The cells were mainly mononuclear with only a few giant cells.

In a group of 16 BCG-treated rats, additional stimulation was given by administering Zymosan granules or methemalbumin intraperitoneally by the dosage and treatment schedules

[2] *Abbreviation used in this paper:* BCG, bacillus Calmette-Guerin.

[3] BCG was kindly supplied by the Tice Laboratory of the Institution for Tuberculosis Research at the University of Illinois and Research Foundation.

Downloaded from http://rupress.org/jem/article-pdf/133/6/1264/1084200/1264.pdf by guest on 30 March 2023

described earlier before collecting the macrophages. This additional treatment increased the yield of macrophages from about 3×10^8 (BCG alone) to approximately 7×10^8/rat.

The cells were harvested by lavaging the peritoneal cavity with isotonic saline and were identified by phase-contrast microscopy. Morphologically 95–100% of the cells obtained were macrophages that were viable when tested for their ability to phagocytize heat-killed *Candida albicans* (25). Samples containing more than 5% polymorphonuclear leukocytes were discarded.

Preparation of Rabbit Alveolar Macrophages.—Alveolar macrophages in rabbits were harvested by minor modifications of the procedure of Myrvik et al. (26). The average yield of cells per animal was $0.5–1 \times 10^8$ of which 98% or more were macrophages by light- or phase-contrast microscopy. In eight rabbits the yield of alveolar macrophages was increased by pretreatment with BCG (27) to $1–2 \times 10^9$ cells/animal, of which at least 85% were large mononuclear cells; many giant cells were present.

Four untreated and four BCG-treated rabbits received intravenously a microcrystalline suspension of hemin in isotonic saline (20 mg/ml) at a dose of 20 mg/kg, given three times a day for 3 successive days. On the 4th day when the macrophages were harvested, the trapped hemin had stained the lungs dark brown.

Production of Subcutaneous Hematoma.—Subcutaneous hematomas were prepared in rats by repeated injections of homologous blood into the lateral aspect of the thigh. Four injections of 1 ml each, spaced over 2 days, were given at the same site. 7 days later, the thigh was incised, excess blood was washed out with isotonic saline, and the granulomatous tissue surrounding the extravasated blood was dissected out, homogenized, and the broken cells were fractionated. Granulomatous tissue was prepared also by injecting 10 mg of heat-killed lyophilized BCG into the thigh; the tissue surrounding the injected material was excised 3 wk later.

Quantitation and Fractionation of Macrophages.—The harvested macrophages were centrifuged at 70–120 *g* at 4°C for 5 min, washed twice with cold isotonic saline and then suspended in 2–3 volumes of 0.25 M sucrose. Total cell counts on the original and washed samples were performed in a Spencer Brightline hemacytometer (American Optical Company, Buffalo, N.Y.). Differential counts were made on Wright-Giemsa stained preparations[4] or by phase-contrast microscopy on wet mounts in the hemacytometer chamber. The washed macrophages were disrupted using a Potter-Elvehjem homogenizer with a motor-driven Teflon pestle and glass tube. Cell disruption was continued until at least 80% of the cells appeared disrupted as judged by phase-contrast microscopy. Broken cell preparations were centrifuged at 20,000 *g* for 10 min in order to prepare a supernatant fraction relatively free of cell debris, nuclei, mitochondria, and lysosomes. This supernatant fraction served as the enzyme source except when noted otherwise. Microsomes were prepared from a 20,000 *g* supernatant fraction by centrifugation at 105,000 *g* for 90 min. The microsomal pellet was washed in 0.25 M sucrose, sedimented again, and resuspended in 0.25 M sucrose.

Enzymatic and Chemical Assays.—The methods employed for the verification, characterization, and assay of heme-oxygenase (10–12) and biliverdin reductase (14) were described in detail previously. Modifications and adaptations of these procedures, if indicated, are listed under the individual experiments.

Difference spectra were determined with a Shimadzu MPS-50L split-beam recording spectrophotometer (American Instrument Company, Silver Springs, Md.). Bilirubin (Pfanstiehl Laboratories, Inc., Waukegan, Ill.) was dissolved in 0.05 N NaOH and added in small volumes to the reaction mixture when required.

[4] Kindly performed by Dr. Brecher and staff, Department of Clinical Pathology and Laboratory Medicine, University of California, San Francisco, Calif.

Downloaded from http://rupress.org/jem/article-pdf/133/6/1264/1084200/1264.pdf by guest on 30 March 2023

Hemin-^{14}C was prepared in vitro from reticulocyte-rich rat cells incubated with glycine-2-^{14}C (New England Nuclear Corp. Boston, Mass.) (28) or from freeze-thawed red cells incubated with δ-aminolevulinic acid (ALA)-2-4-^{14}C (New England Nuclear Corp.) (29). The hemin-^{14}C was crystallized (30) from rat red cells and quantitated as pyridine hemochromogen (31). For the enzymatic synthesis of bilirubin-^{14}C in vitro, the reaction mixture (150 ml) contained a 20,000 *g* supernatant fraction of a macrophage homogenate (1 g protein), 34 μM heme-^{14}C, 360 μM NADPH, 1 mM NADP, 2.6 mM glucose-6-phosphate, 3.8 mM $MgCl_2$, and 66 mM potassium phosphate buffer, pH 7.6. After 60 min incubation at 37°C in 250 ml Ehrlenmeyer flasks in a shaking water bath, bilirubin-^{14}C was extracted from the reaction mixture (10) and crystallized to constant specific activity (8).

Production of ^{14}CO from hemin-^{14}C was measured by the method of White (32). The reaction mixtures (3 ml) were made up as in the standard assay, but were incubated aerobically in the dark in Ehrlenmeyer flasks (125 ml volume) for varying time intervals up to 20 min. The reaction was stopped by immersing the flasks in ice. Bilirubin and carbon monoxide formed in each flask were determined immediately after stopping the incubation.

Acid phosphatase activity was assayed using β-glycerophosphate as substrate (33) and the phosphorus liberated was measured by the method of Chen et al. (34). Protein was quantitated by the method of Lowry et al. (35).

Methemalbumin-dependent oxygen comsumption was measured with a Clark-type electrode (36). Measurements were made with an electrode obtained from the Yellow Springs Instrument Company, Yellow Spring, Ohio, together with an Oxygraph Model KM manufactured by Gilson Medical Electronics Inc., Middleton, Wis.

The cofactor requirements and the effect of atmospheric oxygen (O_2) and carbon monoxide (CO) were determined as described previously (10) but with the following modifications. The assay was performed in modified Thunberg cuvettes (Pyrocell Manufacturing Company, Westwood, N.J.) and the enzyme, placed in the side arm, was kept always at 0°C. The cuvette was evacuated at room temperature and the partial vacuum replaced by the desired gas mixture. The enzyme was then mixed with the reaction mixture and heme-oxygenase activity determined in the standard way. Gas mixtures were purchased from the Matheson Company, East Rutherford, N. J.

Heme disappearance and bilirubin formation in the same sample were measured in a Gilford spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) at 37°C in the dark as described previously but with varying amounts of substrate. Incubation was continued until spectrophotometric measurements indicated that the reaction had virtually ceased. Heme content was estimated as the pyridine hemochromogen (31).

Ancillary Studies.—Methemalbumin was administered intraperitoneally to 40 rats in the dosage and treatment schedule described earlier; 30 control rats received comparable injections of isotonic saline. 18 hr after the last injection, the livers and spleens were removed and peritoneal macrophages were harvested. Heme-oxygenase activity in all three tissues was assayed.

RESULTS

In peritoneal and alveolar macrophages obtained after chemical or immunological stimulation, heme-oxygenase activity was barely detectable (Table I). By contrast, the heme-oxygenase system was very active in all macrophages that had been exposed to heme pigments in vivo before harvesting (Table I). This held true, whether the heme pigments were the initial means for procuring the cells (intraperitoneal methemalbumin or hemoglobin) or whether they were administered in addition to chemical or immunological irritants (Table I). Since

Downloaded from http://rupress.org/jem/article-pdf/133/6/1264/1084200/1264.pdf by guest on 30 March 2023

repeated intraperitoneal injections of methemalbumin produced macrophages with a high and readily reproducible level of heme-oxygenase activity, this technique was used in all subsequent experiments, except as noted otherwise.

Under these conditions, enzyme activity in macrophages remained high for at least 36 hr after the last intraperitoneal injection; at that time the peritoneal cavity still contained free heme pigment, as evidenced by easily discernible black discolaration. On the other hand, the yield of macrophages was highest

TABLE I

Microsomal Heme-Oxygenase Activity of Peritoneal or Alveolar Macrophages

Source and procurement of macrophages*	Additional stimulant*	Heme-oxygenase activity
		μmole bilirubin/10 mg protein per min. (Mean ± SD)
Rat peritoneal macrophages		
1.2% sodium caseinate	—	Trace
Zymosan granules	—	Trace
BCG (heat-killed)	—	Trace
BCG (heat-killed)	Zymosan granules i.p.	Trace
BCG (heat-killed)	Methemalbumin i.p.	2.60 ± 0.12
Methemalbumin	—	2.61 ± 0.11
Hemoglobin	—	0.94 ± 0.21
Rabbit alveolar macrophages		
Saline‡	—	Trace
BCG (heat-killed)	—	Trace
Saline‡	Microcrystalline hemin i.v.	1.31
BCG (heat-killed)	Microcrystalline hemin i.v.	1.12 ± 0.10

* For details, see text.

‡ The macrophages collected from the lungs of four animals were pooled for analysis.

after an interval of about 18 hr, at which time also their earlier gross contamination with polymorphonuclear leukocytes had fallen to less than 5%. It was apparent, therefore, that an interval of 18 hr after the last injection of methemalbumin was the most desirable time to harvest the peritoneal macrophages for enzyme assay.

In methemalbumin-treated rats a striking pattern was obtained when the specific activity of heme-oxygenase in peritoneal macrophages was compared with that in the liver and spleen (Fig. 1). Treatment stimulated enzyme activity in the macrophages to levels far in excess of those found in the liver or spleen. Hepatic enzyme activity also increased, while splenic activity remained almost unchanged. In the experimentally produced subcutaneous hematomas, the

Downloaded from http://rupress.org/jem/article-pdf/133/6/1264/1084200/1264.pdf by guest on 30 March 2023

granulomatous tissue surrounding the blood extravasations regularly exhibited heme-oxygenase activity at a level of approximately 0.25 mμmoles bilirubin formed/minute per 10 mg protein. By contrast, granulomatous tissue produced by heat-killed BCG was virtually devoid of enzyme activity.

The heme-oxygenase system in macrophages closely resembled that previously described in the spleen and liver (10–13). On incubation of the substrate methemalbumin with a 20,000 *g* supernatant fraction of homogenized heme-stimulated macrophages, a new broad absorption band appeared with a peak at

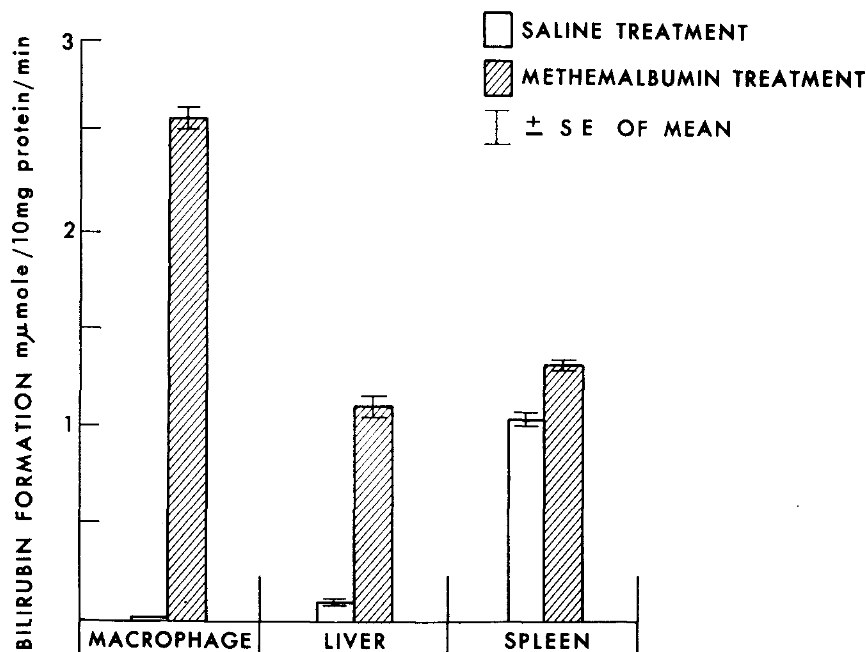

FIG. 1. Stimulatory effect of intraperitoneal injections of methemalbumin on heme-oxygenase activity in the peritoneal macrophages, livers, and spleens of rats.

about 468 mμ (Fig. 2). An additional smaller band was frequently seen exhibiting a peak at 423 mμ. Since this latter band seemed to be particularly noticeable when the harvested macrophages had been contaminated with red blood cells, it most likely was due to carboxyhemoglobin (37) that had been formed by the carbon monoxide evolved on heme cleavage (11). That these two new peaks reflected bilirubin and carbon monoxide formed from heme was indicated by the following observations:

(*a*) Addition to the reaction mixture of an aqueous solution of bilirubin augmented the 468 mμ peak; addition of carbon monoxide to the system contaminated with red cells increased the 423 mμ peak.

Downloaded from http://rupress.org/jem/article-pdf/133/6/1264/1084200/1264.pdf by guest on 30 March 2023

(*b*) After incubation of the complete reaction mixture, a yellow pigment could be extracted with chloroform. This pigment had the spectroscopic properties of bilirubin and gave a positive reaction with the van den Bergh reagent (38).

(*c*) When hemin-^{14}C prepared from glycine-2-^{14}C (theoretically 8 atoms of

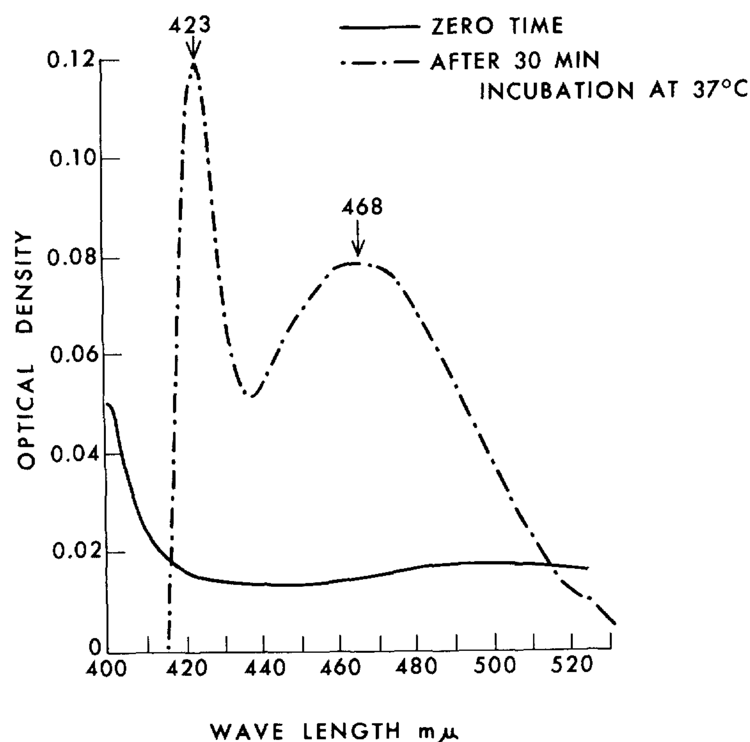

FIG. 2. Enzymatic conversion of methemalbumin to bilirubin by a 20,000 *g* supernatant fraction of homogenized heme-stimulated macrophages. The difference spectra are (macrophage enzyme + methemalbumin + NADPH) minus (macrophage enzyme + methemalbumin).

^{14}C/molecule of heme) was used as substrate, bilirubin-^{14}C (7 atoms of ^{14}C/molecule) was extracted from the incubation mixture and obtained in crystalline form. The molar radioactivity of the recovered bilirubin-^{14}C (1.63×10^{11} dpm) was 85% of the molar activity of the hemin-^{14}C (1.92×10^{11} dpm) used as substrate. This figure approximates the calculated value of 87.5%.

(*d*) When hemin-^{14}C prepared from glycine-2-^{14}C (^{14}C contained in the methene bridge carbon atoms) was used as substrate, macrophage heme-oxygenase produced equimolar amounts of bilirubin and carbon monoxide (Fig. 3); carbon monoxide is presumed to be derived solely from oxidation of the α-methene

Downloaded from http://rupress.org/jem/article-pdf/133/6/1264/1084200/1264.pdf by guest on 30 March 2023

carbon atom of heme (39). By contrast, when hemin-^{14}C prepared from ALA-2,4-^{14}C (lacking ^{14}C atoms in the methene bridge carbon positions) was used as substrate, production of bilirubin-^{14}C was of comparable magnitude but no ^{14}CO was formed (Fig. 3). In both series of experiments no $^{14}CO_2$ was detected. Under the conditions of this assay, which involves incubation in Ehrlenmeyer flasks placed in a shaking water bath, the rate of bilirubin and carbon monoxide formation is maximal for only about 4 min (Fig. 3). In the standard assay, on

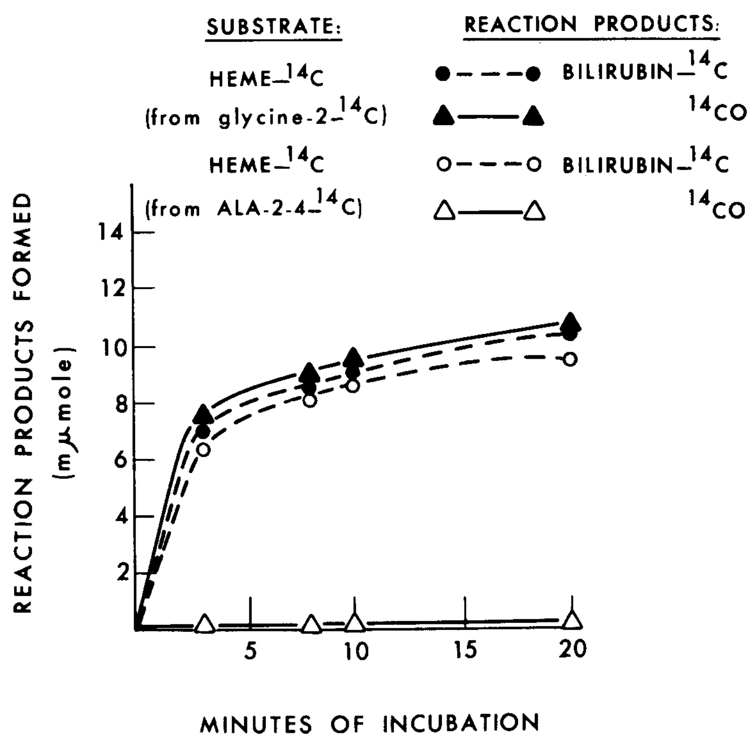

FIG. 3. Formation of bilirubin-^{14}C and ^{14}CO from heme-^{14}C by macrophage heme-ozygenase. The heme-^{14}C was prepared either with glycine-2-^{14}C or ALA-2,4-^{14}C as precursor.

the other hand, in which the reaction mixture is incubated in a cuvette without vigorous agitation, the production of bilirubin is linear for at least 15 min (10).

Microsomal preparations obtained from 20,000 *g* supernatant fractions of homgenized macrophages showed only slight lysosomal contamination in that they contained no more than 10% of the acid phosphatase activity of the initial whole homogenates. Although microsomal preparations alone were active in converting heme to bilirubin (10), this activity was considerably enhanced by the addition of a 105,000 *g* supernatant fraction of homogenized macrophages or

Downloaded from http://rupress.org/jem/article-pdf/133/6/1264/1084200/1264.pdf by guest on 30 March 2023

of purified biliverdin reductase step 5 (14) (Table II). The 105,000 *g* supernatant fraction alone had no heme–oxygenase activity but contained biliverdin reductase activity of 3.4 mμmole/mg protein per min, which is comparable to soluble biliverdin reductase activity in other tissues (14).

TABLE II

Conversion of Methemalbumin to Bilirubin by Microsomal Preparations from Heme-Stimulated Macrophages

Enzyme system	Bilirubin formed
	mμmole/10 mg protein per min.
Microsomes alone	0.41
Microsomes + boiled 105,000 *g* supernatant	0.41
Microsomes + 105,000 *g* supernatant	2.25
Microsomes + step 5 purified biliverdin reductase	2.28
105,000 *g* supernatant alone	0

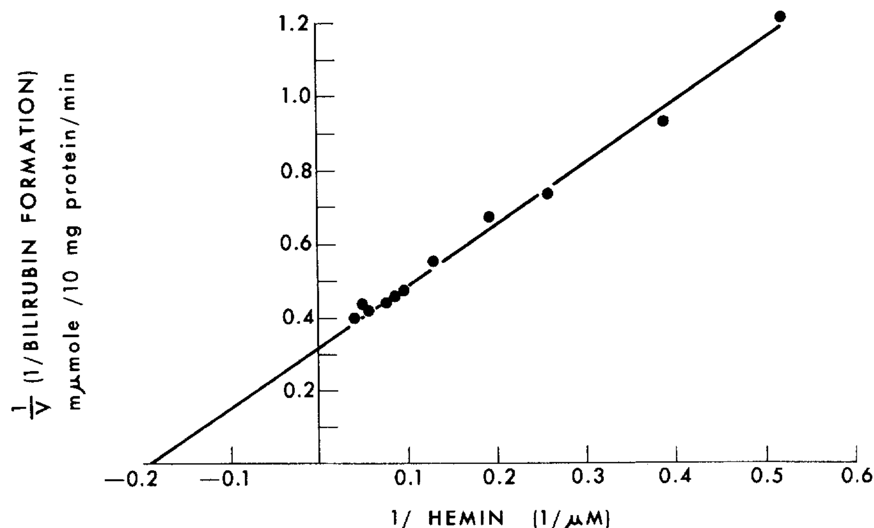

FIG. 4. Lineweaver-Burke plot of the reaction rate of macrophage heme–oxygenase as a function of substrate concentration under standard assay conditions. The line was drawn by the method of least squares.

The kinetics of microsomal heme–oxygenase in macrophages were similar to those reported for the liver and spleen. The rate of bilirubin formation was a linear function of the amount of enzyme assayed, at least up to concentrations of 2 mg enzyme protein/ml reaction mixture. The apparent K_m for the macrophage enzyme, calculated from a Lineweaver-Burke plot (Fig. 4) (40), was 5.2 μM, which is similar to the value found in normal rat spleen (11) and in stimu-

Downloaded from http://rupress.org/jem/article-pdf/133/6/1264/1084200/1264.pdf by guest on 30 March 2023

lated rat liver (13). As in other tissues the heme–oxygenase system in macrophages has an absolute requirement for NADPH and oxygen and is inhibited by carbon monoxide (Fig. 5). For every mole of bilirubin formed, an average of 3.0 moles (range 3.0–3.1) of oxygen was consumed.

When under standard assay conditions heme disappearance was compared with bilirubin formation, it was apparent that less than half of the consumed heme was recovered as bile pigment (Table III). On the other hand, if the complete reaction mixture was kept at ice temperature, or if NADPH was omitted from the incubation mixture, 95–100% of the added substrate was recoverable

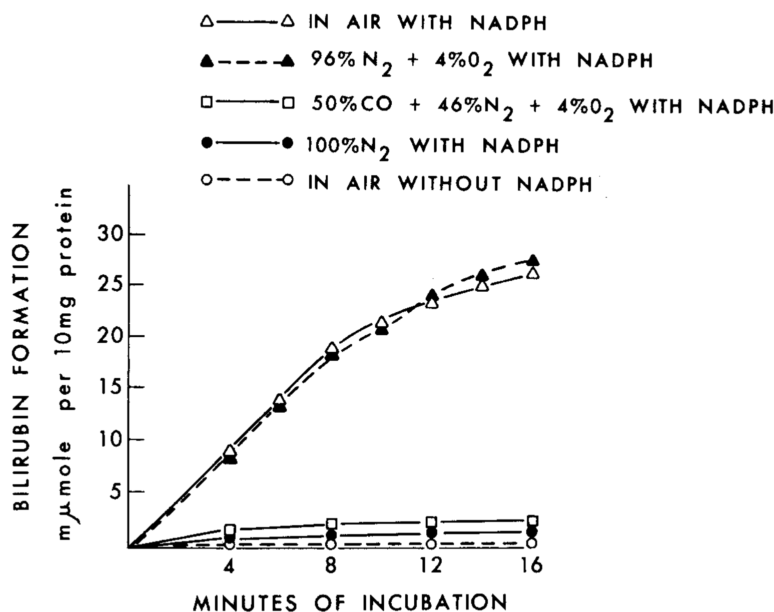

FIG. 5. Effect of atmospheric oxygen, carbon monoxide, or omission of NADPH from the incubation mixture on macrophage heme–oxygenase activity in vitro.

as heme (Table III). Thus, on incubation of the complete system, a significant portion of the heme that disappeared could not be accounted for as bilirubin. This discrepancy did not appear to result from further metabolism of the formed bilirubin because under the conditions of the enzyme assay, bilirubin was stable. When the reaction was carried to completion, the bilirubin formed enzymatically was not further catabolized by prolonged incubation. Moreover, when bilirubin in amounts equal or greater than those enzymatically formed was added to the reaction mixture, incubation at 37°C for up to 1 hr did not reduce the absorption band at 468 mμ.

To assess the possible role of contaminating lysosomal enzymes in this excessive heme disappearance, microsomes prepared from a 20,000 *g* supernatant

Downloaded from http://rupress.org/jem/article-pdf/133/6/1264/1084200/1264.pdf by guest on 30 March 2023

fraction of macrophages were washed several times with 0.25 M sucrose in order to remove soluble lysosomal enzymes. The microsomes were then freeze-thawed three times to disrupt intact lysosomes, after which the preparation was washed again. Although in this purified microsomal preparation acid phosphatase activity was virtually absent, on incubation the ratio of heme disappearance to bilirubin formation differed little from that observed with the initial 20,000 *g* supernatant fraction (Table III, experiment 3).

TABLE III

Relationship Between Heme Disappearance and Bilirubin Formation In Vitro

Reaction mixture		Heme substrate			Bilirubin formed
Enzyme protein	NADPH	Incubation		Disappearance	
		Before	After		
		(*mμmole*)			(*mμmole*)
Experiment 1					
5 mg	Absent	165	163	2	0
	Absent	230	223	7	0
	Present	226	158	68	25
10 mg	Absent	190	188	2	0
	Absent	258	253	5	0
	Present	253	138	115	41
Experiment 2					
9.8 mg	Present	209	177	32	16
19.8 mg	Present	222	147	75	39
Experiment 3					
5.1 mg (20,000 *g* supernatant)	Absent	216	201	15	0
	Present	229	173	56	19
2.4 mg (washed microsomes)	Absent	216	203	13	0
	Present	226	180	46	14

The reaction mixture incubated for 30 min contained a 20,000 *g* supernatant fraction of stimulated macrophages as the enzyme source. In experiment 3, a microsomal preparation washed free of lysosomal contamination was compared with a 20,000 *g* supernatant fraction of the same macrophage homogenate.

Heme breakdown was insignificant, as was bilirubin formation, if NADPH (180 μM) was omitted (Table III) or if it was replaced by NADH (180 μM) or by reduced glutathione (360 μM). Moreover, heme disappearance was minimal if the complete system contained a 20,000 *g* supernatant fraction of macrophages that had not been stimulated in vivo by prior exposure to heme, and consequently exhibited only insignificant heme–oxygenase activity. Thus excessive and unaccounted heme disappearance was observed only in those incubation systems that also exhibited heme–oxygenase activity.

Downloaded from http://rupress.org/jem/article-pdf/133/6/1264/1084200/1264.pdf by guest on 30 March 2023

DISCUSSION

These findings provide direct evidence that peritoneal and alveolar macrophages possess the enzymatic machinery for the degradation of hemoglobin-heme to bilirubin. While this enzyme activity is very low in native macrophages, it may be stimulated by exposure of these cells to heme pigments in vivo. This suggests that heme stimulates the activity of its own degradative pathway in macrophages. The nature of this stimulation is uncertain, but observations in rats with experimental hemoglobinuria suggest that the adaptive response of the enzyme in the renal tubules is the result of substrate-mediated enzyme induction (15). A similar adaptive response of heme–oxygenase to substrate load has been described in the liver of rats after splenectomy or after production of hemolytic anemia (13). Thus, it may be postulated that native macrophages do not exhibit heme–oxygenase activity because they are not exposed to the stimulation of heme pigments. However, these cells do indeed have the potential to effectively degrade not only the protein moiety of hemoglobin (21) but also the prosthetic heme group.

The enzyme system in macrophages closely resembles that in the spleen (10–12) and liver (10, 13). The rate-limiting enzyme complex, heme–oxygenase, is localized in the microsomal fraction and converts the substrate heme to equimolar amounts of biliverdin and carbon monoxide. The biliverdin is then reduced to bilirubin by the soluble biliverdin reductase (14). The apparent K_m of the enzyme system in macrophages of 5.2 μM is similar to that reported for the liver (13) and spleen (11). Microsomal heme–oxygenase in macrophages has an absolute requirement for molecular oxygen and NADPH and is inhibited by carbon monoxide. Thus, the reaction has the characteristics of a mixed function oxidation, seemingly requiring a carbon monoxide-binding cytochrome with the properties of cytochrome P450. This appears to be the first example of a microsomal monooxygenase system (41–43), similar to the drug-metabolizing enzyme systems in the liver, that has been identified in cells belonging to the reticuloendothelial apparatus.

Microsomal heme–oxygenase activity in macrophages undergoes adaptive regulation in response to the ingestion of methemalbumin, particular hemin, or hemoglobin. Lysosomal enzymes concerned with peptide hydrolysis also are induced by the hemoglobin of phagocytized erythrocytes (21). Thus in macrophages microsomal and lysosomal enzyme systems responsible for the catabolism of both the heme and the globin moiety of hemoglobin are activated by ingestion of the substrate. In the intact rat the accelerated hepatic and splenic removal of red cells that have been coated with antibodies are associated with increased heme–oxygenase activity in the liver and spleen (13). This suggests that at least in part this adaptive increase may be localized in the Kupffer cells of the liver and in the splenic macrophages, both of which are reticuloendothelial

Downloaded from http://rupress.org/jem/article-pdf/133/6/1264/1084200/1264.pdf by guest on 30 March 2023

cells morphologically and functionally similar to the macrophages employed in the present study.

While these observations indicate that a portion of the total body heme–oxygenase activity is located in reticuloendothelial cells of mesenchymal origin, they do by no means rule out the presence of this enzyme activity in other cell types. Indirect evidence suggests that cells of epithelial origin also may exhibit heme–oxygenase activity. Hemoglobin (44), hematin, and hemopexin-bound heme (45) have been found to gain access to hepatic parenchyal cells where they presumably are catabolized. Moreover, recent evidence indicates that filtered hemoglobin may induce heme–oxygenase in the epithelial cells of the renal tubules (15). Finally, biliverdin has been detected in the intestinal epithelial cells of blood-sucking arthropods (46). This last observation suggests the possibility that the intestinal absorption of hemoglobin-iron in carnivorous and omnivorous mammals (47–50), but not in herbivorous guinea pigs (51), may be explained by a heme–oxygenase system operational in the mucosal cells of the intestine.

The presence of heme–oxygenase in macrophages probably derived from monocytic precursors (52–54) provides a ready explanation for the well-documented observation of local bilirubin formation in old blood extravasations (16–19). When blood is extruded into tissues, macrophages move to the site of injury (19) and phagocytize the trapped erythrocytes. After induction of the appropriate lysosomal (21) and microsomal enzymes, the hemoglobin is degraded into its catabolites, bilirubin, iron, and amino acids, which then are released gradually into the extracellular space. The successive enzymatic conversion by macrophages of the ingested hemoglobin (dark red), methemoglobin or hematin (dark brown) to biliverdin (green-blue) and eventually to bilirubin (yellow) is responsible for the characteristic color play in subcutaneous bruises. Hemosiderin-laden macrophages (brown) may contribute to the color spectrum (19). On the other hand, in intraepidermal blood blisters or in subungual splinter hemorrhages where little cellular response is elicited (55), the extruded hemoglobin-heme remains essentially unchanged until the horny layer of the superposed skin or the nail is shed. This accounts for the dark purple or black color of these superficial extravasations that often persist for several wk.

In assaying heme–oxygenase in macrophages, it was noted in all instances that during incubation, heme disappearance exceeded the amount of bilirubin and carbon monoxide formed (Table III). Under the conditions of the assay system, bilirubin appeared stable and the production of bilirubin and carbon monoxide always was equimolar. These observations suggest that in addition to being converted to bilirubin, heme may in part be metabolized to other catabolites whose structure has not yet been determined. These alternative pathways neither appear to require bilirubin as intermediate nor do they usually seem to yield carbon monoxide. This is not a novel concept as similar observations made

Downloaded from http://rupress.org/jem/article-pdf/133/6/1264/1084200/1264.pdf by guest on 30 March 2023

in vivo (8, 56) indicated that the formation of bilirubin, albeit predominant, is not the sole pathway of biological heme degradation. The present observations in macrophages suggest that breakdown of heme to nonbilirubin catabolites does not involve lysosomal enzymes. Moreover, breakdown of heme by alternate routes appears also to be an adaptive mechanism requiring NADPH. When the macrophages had not been exposed to heme pigments in vivo, or when NADPH was absent from the incubation mixture or was replaced by NADH or reduced glutathione, no heme was consumed. Identification of this alternate pathway(s) of heme catabolism is hampered by the lack of information about the nature and structure of the products formed (29, 39). It is attractive to speculate about the possible role of H_2O_2 in this process, since during phagocytosis oxygen consumption is increased and H_2O_2 is generated (57, 58). Macrophages have low or absent myeloperoxidase activity but do have catalase activity (59). In the presence of low concentrations of H_2O_2, catalase may act as a peroxidase (60) and catalase-dependent peroxidation reactions have been demonstrated in alveolar macrophages (59). The oxidative denaturation of hemoglobin to Heinz bodies by phenylhydrazine and oxygen (29) may serve as a possible model of a pathway for hemoglobin catabolism that does not lead to bilirubin.

SUMMARY

Recent studies have identified and characterized the enzymatic mechanism by which hemoglobin-heme is converted to bilirubin. Under physiologic conditions the enzyme system, microsomal heme–oxygenase, is most active in the spleen followed by the liver and bone marrow, all of which are tissues that normally are involved in the sequestration and metabolism of red cells. Indirect evidence suggested that the reticuloendothelial system is important in this process. To test this hypothesis, conversion of heme to bilirubin was studied in macrophages obtained by chemical or immunological means from the peritoneal cavity or from the lungs of rodents. Homogenates of pure populations of these cells were devoid of heme–oxygenase activity, unless before harvesting the macrophages had been exposed to methemalbumin, microcrystalline hemin, or hemoglobin in vivo. In macrophages exposed to heme pigments, the specific activity of heme–oxygenase was far in excess of that in the spleen or liver. Enzyme activity was also present in the granulomatous tissue surrounding subcutaneous hematomas.

The heme–oxygenase system in macrophages resembles that in the spleen and liver in that it is localized in the microsomal fraction, has an absolute requirement for molecular oxygen and NADPH, is inhibited by carbon monoxide, and has a similar K_m. These findings indicate that cells of the reticuloendothelial system, presumably including the Kupffer cells of the liver and the macrophages of the spleen, possess the enzymatic machinery for converting hemoglobin-heme to bilirubin. The reaction is a mixed function oxidation, probably involving cytochrome P450 as the terminal oxidase. Enzyme activity in macrophages is

Downloaded from http://rupress.org/jem/article-pdf/133/6/1264/1084200/1264.pdf by guest on 30 March 2023

capable of regulatory adaptation in response to substrate loads. In the standard assay system for the enzyme, disappearance of heme always was in excess of the amount of bilirubin formed, suggesting the simultaneous presence of alternate routes of heme degradation not involving bilirubin as an end product or intermediate.

BIBLIOGRAPHY

1. Pimstone, N. R., R. Tenhunen, P. T. Seitz, R. Schmid, and H. S. Marver. 1970. The enzymatic degradation of hemoglobin to bile pigment by macrophages. *Gastroenterology*. **58:**304.
2. Miescher, P. 1957. The role of R. E. S. in haematoclasia. *In* Physiopathology of R. E. S. B. N. Halpern, editor. Blackwell Scientific Publications Ltd., Oxford, England.
3. Wagner, H. N., Jr., M. A. Razzak, R. A. Gaertner, W. B. Caine, Jr., and O. T. Feagin. 1962. Removal of erythrocytes from the circulation. *Arch. Intern. Med.* **110:**90.
4. 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.
5. Keene, W. R., and J. H. Jandl. 1965. Studies of the reticuloendothelial mass and sequestering function of rat bone marrow. *Blood J. Hematol.* **26:**157.
6. Aschoff, L. 1924. Das retikulo-endotheliale System. *Ergeb. Inn. Med, Kinderheilk.* **26:**1.
7. Biozzi, G., and C Stiffel. 1965. The physiopathology of the reticuloendothelial cells of the liver and spleen. *In* Progress in Liver Diseases. H. Popper and F. Schaffner, editors. Grune and Stratton Inc., New York. **2:**166.
8. Ostrow, J. D., J. H. Jandl, and R. Schmid, 1962. The formation of bilirubin from hemoglobin in vivo. *J. Clin. Invest.* **41:**1628.
9. With, T. 1968. Bile Pigments, Chemical, Biological, and Clinical Aspects. Academic Press Inc., New York. 101.
10. Tenhunen, R., H. S. Marver, and R. Schmid. 1968. The enzymatic conversion of heme to bilirubin by microsomal heme oxygenase. *Proc. Nat. Acad. Sci. U.S.A.* **61:**748.
11. Tenhunen, R., H. S. Marver, and R. Schmid, 1969. Microsomal heme oxygenase. Characterization of the enzyme. *J. Biol. Chem.* **244:**6388.
12. Tenhunen, R., H. S. Marver, and R. Schmid. 1969. The enzymatic conversion of hemoglobin to bilirubin. *Trans. Ass. Amer. Physicians.* **82:**363.
13. 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.
14. Tenhunen, R., H. S. Marver, and R. Schmid. 1970. Reduced nicotinamide adenine dinucleotide phosphate-dependent biliverdin reductase. Partial purification and characterization. *Biochemistry*. **9:**298.
15. Pimstone, N. R., P. Engel, P. T. Seitz, H. S. Marver, and R. Schmid. 1970. Inducible heme oxygenase in the kidney: a model for the homeostatic control of hemoglobin catabolism. *J. Clin. Invest.* **49:**74. (Abstr.)

Downloaded from http://rupress.org/jem/article-pdf/133/6/1264/1084200/1264.pdf by guest on 30 March 2023

16. Virchow, R. 1847. Die pathologischen Pigmente. *Arch. Pathol. Anat. Physiol. Klin. Med.* **1:**379.
17. Rich, A. R. 1925. The formation of bile pigment. *Physiol. Rev.* **5:**182.
18. Langhans, T. 1870. Beobachtungen über Resorption der Extravasate und Pigmentbildung in denselben. *Arch. Pathol. Anat. Physiol. Klin. Med.* **49:**66.
19. Muir, R., and J. S. F. Niven. 1935. The local formation of blood pigments. *J. Pathol. Bacteriol.* **41:**183.
20. Rich, A. R. 1924. The formation of bile pigment from haemoglobin in tissue cultures. *Bull. Johns Hopkins Hosp.* **35:**415.
21. Axline, S. G., and Z. A. Cohn. 1970. In vitro induction of lysosomal enzymes by phagocytosis. *J. Exp. Med.* **131:**1239.
22. Ehrenreich, B. A., and Z. A. Cohn. 1968. Fate of hemoglobin pinocytosed by macrophages in vitro. *J. Cell. Biol.* **38:**244.
23. Bunn, H. F., W. T. Esham, and R. W. Bull. 1969. The renal handling of hemoglobin. I. Glomerular filtration. *J. Exp. Med.* **129:**909.
24. Crosby, W. H., and D. N. Houchin. 1957. Preparing standard solutions of cyanmethemoglobin. *Blood J. Hematol.* **12:**1132.
25. Lehrer, R. I., and M. J. Cline. 1969. Interaction of *Candida albicans* with human leukocytes and serum. *J. Bacteriol.* **98:**996.
26. Myrvik, Q. N., E. S. Leake, and S. Oshima. 1962. A study of macrophages and epitheloid-like cells from granulomatous (BCG-induced) lungs of rabbits. *J. Immunol.* **89:**745.
27. Cohn, Z. A., and W. Eiwner. 1963. The particulate hydrolases of macrophages. I. Comparative enzymology, isolation, and properties. *J. Exp. Med.* **118:**991.
28. Custer, L. E., T. Abei, B. R. Chipman, and F. L. Iber. 1964. Preparation of bilirubin-C^{14}. *J. Lab. Clin. Med.* **64:**820.
29. Goldstein, G. W., L. Hammaker, and R. Schmid. 1968. The catabolism of Heinz bodies: an experimental model demonstrating conversion to non-bilirubin catabolites. *Blood J. Hematol.* **31:**388.
30. Labbe, R. F., and G. Nishida. 1957. A new method of hemin isolation. *Biochim. Biophys. Acta.* **26:**437.
31. Falk, J. E. 1964. Prophyrins and Metalloporphyrins: Their General Physical and Coordination Chemistry, and Laboratory Methods. Elsevier Publishing Company, Amsterdam, The Netherlands. 181.
32. White, P., B. C. Shafer, M. L. Rother, and W. J. Williams. 1968. Production of carbon monoxide by bone marrow and reticulocytes in vitro. *J. Clin. Invest.* **47:**102. (Abstr.)
33. Palade, G. I. 1951. Intracellular distribution of acid phosphatase in rat liver cells. *Arch. Biochem.* **30:**144.
34. Chen, P. S., Jr., T. Y. Toribara, and H. Warner. 1956. Microdetermination of phosphorus. *Anal. Chem.* **28:**1756.
35. Lowry, O. H., N. H. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin Phenol reagent. *J. Biol. Chem.* **193:**265.
36. Clark, L. C. 1956. Monitor and control of blood and tissue oxygen tensions. *Trans. Amer. Soc. Artif. Intern. Organs* **2:**41.
37. Lemberg, R., and J. W. Legge. 1949. Hematin Compounds and Bile Pigments. Interscience Publishers Inc., New York. 228.

Downloaded from http://rupress.org/jem/article-pdf/133/6/1264/1084200/1264.pdf by guest on 30 March 2023

38. Mallow, H. T., and K. A. Evelyn. 1937. The determination of bilirubin with the photoelectric colorimeter. *J. Biol. Chem.* **119**:481.
39. 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.
40. White, A., P. Handler, and E. L. Smith. 1964. Principles of Biochemistry. McGraw-Hill Book Company, New York., 3rd edition 220.
41. Conney, A. H. 1967. Pharmacological implications of microsomal enzyme induction. *Pharmacol. Rev.* **19**:317.
42. Omura, T., R. Sato, D. Y. Cooper, O. Rosenthal, and R. W. Estabrook. 1965. Function of cytochrome P450 of microsomes. *Fed. Proc.* **24**:1181.
43. Cooper, D. Y., S. Levin, S. Narasimhulu, O. Rosenthal, and R. W. Estabrook. 1965. Photochemical action spectrum of the terminal oxidase of mixed function oxidase systems. *Science (Washington).* **147**:400.
44. Goldfischer, S., A. B. Novikoff, A. Albala, and L. Biempica. 1970. Hemoglobin uptake by rat hepatocytes and its breakdown within lysosomes. *J. Cell. Biol.* **44**:513.
45. Müller-Eberhard, U., C. Bosman, and H. H. Liem. 1970. Tissue localization of the heme-hemopexin complex in the rabbit and the rat as studied by light microscopy with the use of radioisotopes. *J. Lab. Clin. Med.* **76**:426.
46. Wigglesworth, V. A. 1942–43. The fate of haemoglobin in Rhodnius prolixus (Hemiptera) and other blood sucking arthropods. *Proc. Roy. Soc. Ser. B. Biol. Sci.* **131**:313.
47. Awai, M., and E. B. Brown. 1969. Examination of the role of xanthine oxidase in iron absorption by the rat. *J. Lab. Clin. Med.* **73**:366.
48. Wheby, M. S., G. E. Suttle, and K. T. Ford III. 1970. Intestinal absorption of hemoglobin-iron. *Gastroenterology.* **58**:647.
49. Brown, E. B., Y. F. Hwang, S. Nicol, and J. Ternberg. 1968. Absorption of radioiron-labeled hemoglobin by dogs. *J. Lab. Clin. Med.* **72**:58.
50. Conrad, M. E., B. I. Benjamin, H. L. Williams, and A. L. Foy. 1967. Human absorption of hemoglobin-iron. *Gastroenterology.* **53**:5.
51. Conrad, M. E., L. R. Weintraub, D. A. Sears, and W. H. Crosby. 1966. Absorption of hemoglobin-iron. *Amer. J. Physiol.* **211**:1123.
52. Ebert, R. H., and H. W. Florey. 1939. The extravascular development of the monocyte observed in vivo. *Brit. J. Exp. Pathol.* **20**:342.
53. van Furth, R., and Z. A. Cohn. 1968. The origin and kinetics of mononuclear phagocytes. *J. Exp. Med.* **128**:415.
54. Roser, B. 1970. The origins, kinetics and fate of macrophage populations. *J. Reticuloendothel. Soc.* **8**:139.
55. Shuster, S., and H. Scarborough. 1961. Senile purpura. *Quart. J. Med.* **30**:33.
56. Snyder, A. L., and R. Schmid. 1965. The conversion of hematin to bile pigment in the rat. *J. Lab. Clin. Med.* **65**:817.
57. Sbarra, A. J., and M. L. Karnovsky. 1959. The biochemical basis of phagocytosis. I. Metabolic changes during the ingestion of particles by polymorphonuclear leukocytes. *J. Biol. Chem.* **234**:1355.

Downloaded from http://rupress.org/jem/article-pdf/133/6/1264/1084200/1264.pdf by guest on 30 March 2023

58. McRipley, R. J., and A. J. Sbarra. 1967. Role of the phagocyte in host-parasite interactions. XI. Relationship between stimulated oxidative metabolism and hydrogen peroxide formation, and intracellular killing. *J. Bacteriol.* **94:**1417.
59. Gee, J. B. L., C. L. Vassallo, P. Bell, J. Kaskin, R. E. Basford, and J. B. Field. 1970. Catalase-dependent peroxidative metabolism in the alveolar macrophage during phagocytosis. *J. Clin. Invest.* **49:**1280.
60. Keilin, D., and E. F. Hartree. 1955. Catalase, peroxidase and methmyoglobin as catalysts of coupled peroxidatic reactions. *Biochem. J.* **60:**310.

Downloaded from http://rupress.org/jem/article-pdf/133/6/1264/1084200/1264.pdf by guest on 30 March 2023