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Research Article

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It is concluded that the ferritin aggregates in the granules of reticulated-siderocytes are dispersed intracellularly into soluble ferritin, that soluble ferritin is excreted from the cell, and that one or both of these steps is dependent upon oxidative metabolism. Blood monocytes are capable of taking up soluble ferritin from the media and converting this into siderotic granules. Thus, a reticulocyte to plasma to monocyte ferritin pathway has been described.

Ferritin Metabolism in Reticulated-Siderocytes

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ABSTRACT Reticulated-siderocytes (reticulocytes which contain siderotic granules), obtained from the circulation of pigs after vigorous phlebotomy, were incubated in vitro. A rapid disappearance of granules from the reticulocytes was observed over 24 hr. Simultaneously with the decrease in granules, soluble ferritin accumulated in the media and siderotic granules developed in monocytes. The disappearance of the granules from the reticulated-siderocytes was oxygen-dependent and the loss of granules and the accumulation of ferritin in the media were both prevented by the addition of cyanide or dinitrophenol.

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INTRODUCTION

It has been recognized from experimental studies in swine (1, 2) that the physiologic significance and metabolic behavior of siderotic granules contained within nonreticulated, mature erythrocytes (S cells) is quite different from such granules contained within reticulocytes (reticulated-siderocytes, R-S cells).

Nonreticulated siderocytes (S cells) were observed in the circulation of animals with a defect in heme synthesis (pyridoxine deficiency). The granules consisted of nonferritin iron located within mitochondria. The removal of granules from these cells occurred only in the spleen.

Reticulated-siderocytes (R-S cells) were observed in the circulation of iron-replete animals during rapid blood regeneration induced by vigorous phlebotomy. The granules in such cells consisted of cytoplasmic aggregates of ferritin. The granules were metabolized or removed from the cells during maturation in the circulation entirely independently of the spleen. Furthermore, when R-S cells were incubated in vitro, the granules disappeared within 72 hr. These observations along with the observation that as the R-S cells matured the granules became less densely packed with ferritin, suggested that the ferritin was removed progressively from the granules.

The present study was undertaken to define the metabolic fate of the iron in the siderotic granules of R-S cells during incubation in vitro.

METHODS

Pigs of mixed breed were housed in individual cages at approximately 3 wk of age and were maintained on a diet consisting of casein, sucrose, and lard. The details of the diet and dietary supplements have been described previously (1). Beginning at approximately 5–6 wk of age, daily phlebotomy by jugular puncture was begun. The volume removed daily amounted to approximately 15% of the estimated blood volume (3) per day. Iron dextrin[1] was injected intramuscularly five times weekly to replace iron removed by phlebotomy.

Routine hematologic determinations were performed by standard methods (4). To stain and count reticulocytes and siderocytes on the same preparation, blood was first stained with new methylene blue (5), then stained for iron by the Prussian blue method (6), and finally counterstained with safranin O (1, 4). On smears stained in this fashion, reticulum stains red and siderotic granules stain blue.

Plasma iron concentration was determined by the method of Hamilton, Gubler, Cartwright, and Wintrobe (7) and total iron-binding capacity by the method of Ramsay (8).

The iron in the incubation media (saline or plasma) was determined by adding an equal volume of the media to 0.01 M sodium dithionite in 0.9% saline. After mixing, the iron content was measured by the routine plasma iron method. Iron measured in this fashion is referred to as "reducible iron" in this report.

The effectiveness of this method for the measurement of iron in isolated siderotic granules, in plasma and saline

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[1] Pigdex was kindly supplied by Dr. Wayne Linkenheimer, American Cyanamid Co., Princeton, N. J.

TABLE I

Evaluation of the Reducible Iron Method

Specimen	Plasma iron method	Reducible iron method	Total iron wet ash method	Reducible iron as per cent of total iron
	μg/100 ml	*μg/100 ml*	*μg/100 ml*	%
Siderotic granules	204	2050	2110	97
Plasma incubation medium	284	673	865	78
Saline incubation medium	144	544	601	91
Ferritin	370	880	815	108
Hemoglobin	31	74	1295	6

Siderotic granules and stroma were prepared by hemolysis of red cells and repeated washing of the sedimentable portion of the hemolysate. The plasma and saline incubation media were obtained after incubation with R-S cells; the iron obtained by the wet ash method was corrected for the hemoglobin contained in the media. The siderotic granules, ferritin, and hemoglobin solutions were dialyzed 12 hr against 1% EDTA brought to pH 7.4 with Tris buffer, and twice for 24 hr against Tris-HCl buffer, 0.01 M, pH 7.4.

media with which R-S cells had been incubated, and in a solution of purified horse spleen ferritin[2] was estimated by comparing the results obtained with the standard plasma iron method and with iron determined after sulfuric acid–nitric acid–perchloric acid wet digestion of the solutions (9). By this method, 78 to 108% of the iron was measured and only a small amount of hemoglobin iron was measured (Table I). The reproducibility of the method, as determined on 25 duplicate specimens of plasma which had been incubated with R-S cells, was ±9.0%.

The incubations were performed in a Dubnoff incubator shaker at 37°C for 24 hr at 70 opm with room air, unless otherwise stated. Blood from phlebotomized pigs was collected in heparin.[3] Penicillin G, 1000 U/ml, was added and 10-ml aliquots of blood were transferred to 50 ml Erlenmeyer flasks and capped. When washed cells were employed, the heparinized blood was centrifuged, the plasma removed, the cells were washed twice with 0.9% sodium chloride, and resuspended in plasma or saline to restore the original volume.

When transferrin was labeled with radioiron, ^{59}ferric chloride was added in amounts calculated to produce less than 50% saturation of the total iron-binding capacity, mixed well, and allowed to stand at room temperature for at least 30 min before use. Hemin was extracted with 0.5% strontium chloride in 3:1 acetone-glacial acetic acid, crystallized by boiling off the acetone, and recrystallized twice from pyridine, chloroform, and methanol (10, 11). Radioactivity was assayed by crystal scintillation counting.

RESULTS

Disappearance of siderotic granules in vitro. 25 different specimens of blood from phlebotomized pigs were incubated for 24 hr. The mean values ±1 SE before incubation were as follows: R-S cells 13.1 ±0.92, R cells 23.0 ±1.22, and S cells 0.2. The mean values after incubation were as follows: R-S cells 3.9 ±0.48, R cells 22.2 ±1.27, and S cells 0.3. The per cent decrease in R-S cells was 72.6 and in R cells 3.5. There was no significant change in S cells.

The changes in the numbers of these cells during the course of a single 72 hr incubation are depicted in Fig. 1. The R-S cell count decreased in an exponential fashion during the first 48 hr with a half-time disappearance of about 12 hr. The R cells increased initially and then decreased to base line values by 24 hr. During the period between 24 and 48 hr, the R cell count decreased rapidly. A slight increase in S cells was observed after 24 hr.

To investigate the possibility that the decrease in number of R-S cells during incubation was due to selective hemolysis of R-S cells, the erythrocyte count was determined at the beginning and end of the incubation period. Significant evaporation did not occur during incubation as judged by flask weights and hemoglobin concentration before and after incubation.

A decrease in the erythrocyte count from 3.23 to 2.76 × 10^6/mm^3 would have been required to account for the observed decrease in the R-S cell count if the decrease were due to selective hemolysis of R-S cells. No decrease in erythrocyte count was observed.

The effect of varying the conditions of the incubation was studied (Table II). The decrease in R-S cells was completely inhibited at 4°C. The optimal rate of oscillation was found to be 70 opm. The decrease was optimal

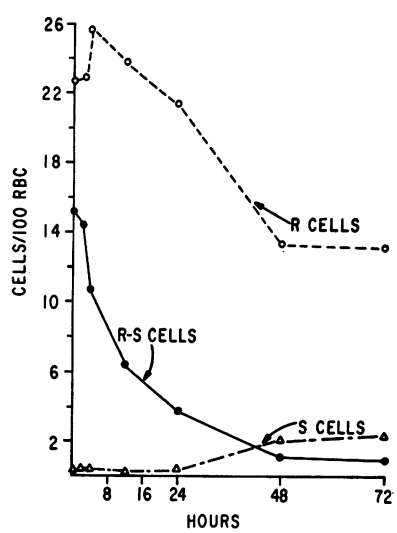

FIGURE 1 Change in concentration of reticulated-siderocytes (R-S cells), reticulocytes (R cells), and siderocytes (S cells) during the course of incubation of whole blood from phlebotomized pigs. Each point is the mean of eight determinations.

[2] Purchased from Calbiochem, Los Angeles, Calif.

[3] Supplied by Dr. F. T. Johnson, The Upjohn Co., Kalamazoo, Mich.

in the presence of oxygen and appreciably inhibited in nitrogen. The decrease in R-S cells was as great when saline was used in the incubation as when plasma was used and was not influenced by the per cent saturation of transferrin.

The effect of several metabolic inhibitors on the decrease in R-S cells is given in Table III. Sodium cyanide and dinitrophenol completely inhibited the decrease in R-S cells. Sodium fluoride and cycloheximide in the concentrations used failed to inhibit the loss of granules.

Accumulation of iron in the media. During the incubation of whole blood containing R-S cells, the reducible iron content of the plasma increased threefold as the R-S cell count decreased (Fig. 2). The plasma iron (transferrin-bound iron), as determined by the routine plasma iron method, decreased somewhat over the first 4 hr and then returned to the base line values. The radioiron bound to transferrin decreased progressively as the reducible iron increased, indicating that transferrin-bound iron was entering the cell during the period when the cellular iron was leaving. The iron uptake by reticulocytes (R plus R-S cells) was 9.7 mμmoles of Fe/10^9 reticulocytes and 72.3% of the iron taken up was incorporated into heme.

The mean values for the increase in iron in the media are given in Table IV. The reducible iron increased from 189 to 510 μg/100 ml when whole blood containing R-S cells was incubated for 24 hr. Only a slight increase in reducible iron occurred when normal whole blood was incubated under these conditions. When R-S cells were incubated in saline, the increase in reducible iron was as great as when the cells were incubated in plasma. The decrease in R-S cells and the increase in iron during incubation were inhibited by sodium cyanide and by dinitrophenol (Table III). Sodium fluoride and cycloheximide, in a concentration of 1×10^{-2} M, failed to inhibit either the decrease in R-S cells or the increase in iron in the media.

During the incubations some hemolysis took place, usually less than 2%. However, when the increment increase in plasma hemoglobin was plotted against the increment increase in reducible iron, no correlation ($r = -0.11$) was observed, indicating that the reducible iron was not being released from lysed cells.

Characterization of the iron in the media. The sedimentability of the iron which accumulated in the saline medium after incubation of R-S cells was compared to the sedimentability of a solution of horse spleen ferritin (Fig. 3). After 4 hr of centrifugation, the uppermost portions of the solutions were analyzed for reducible iron. Over 90% of the iron in both solutions sedimented at forces in excess of 60,000 *g*. 25% of the iron in the saline medium after incubation with R-S cells sedimented at a force of 25,000 *g*, whereas horse spleen ferritin began to sediment at greater forces.

TABLE II

Effect of Varying Conditions on Decrease in R-S Cells During Incubation

Condition		Per cent decrease in R-S cells at 24 hr*
Temperature	4°	0
	22°	53
	37°	53
Rate of oscillation	0 opm	29
	70 opm	82
Atmosphere	oxygen	88
	air	75
	nitrogen	38
Media	plasma	75
	saline	80
Transferrin saturation, plasma iron/total iron-binding capacity	16/879	84
	759/700	84

Values are means of two determinations.

* Per cent decrease in R-S cells is calculated by dividing 100 times the decrement in R-S cell concentration at 24 hr by the R-S cell concentration at 0 hr.

TABLE III

Effect of Several Metabolic Inhibitors on the Decrease in R-S Cells and Reducible Iron in the Plasma During Incubation of Whole Blood

Inhibitor	Final concentration	R-S Cells		Reducible iron	
		0 hr	24 hr	0 hr	24 hr
	M	%		μg/100 ml	
Cyanide	0	22	9	210	1135
	1×10^{-4}	22	8	210	745
	1×10^{-3}	22	20	210	326
	1×10^{-2}	22	21	210	302
Dinitrophenol	0	14	5	180	580
	1×10^{-4}	14	4	180	694
	1×10^{-3}	14	17	180	258
Fluoride	0	14	3	258	435
	1×10^{-4}	14	3	258	503
	1×10^{-3}	14	3	258	487
	1×10^{-2}	14	3	258	506
Cycloheximide	0	6	1	177	245
	1×10^{-4}	6	2	177	302
	1×10^{-3}	6	1	177	305
	1×10^{-2}	6	1	177	328

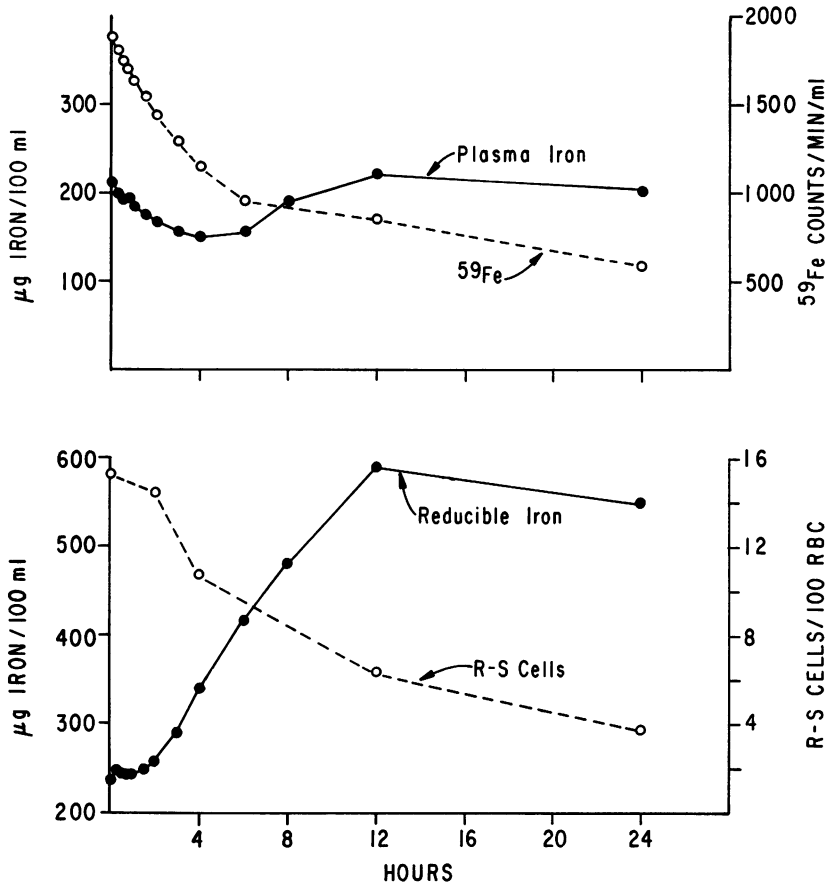

FIGURE 2 Decrease in reticulated-siderocytes (R-S cells), increase in reducible iron, and changes in plasma iron and transferrin-bound radioiron (^{59}Iron) during incubation of whole blood from phlebotomized pigs. Each point is the mean of four determinations.

Purified horse spleen ferritin was dissolved in 0.9% saline to give an iron concentration equal to that in the saline solution after incubation with R-S cells. The solutions were dialyzed for 12 hr at 4°C against 100 volumes of 1% potassium EDTA, brought to pH 7.4 with Tris buffer and dialyzed for two successive 24-hr periods against Tris-HCl, 0.01 M, pH 7.4. Aliquots of the original solutions were brought to 70° or 100°C within 60 sec, maintained at that temperature with constant stirring for 5 min, and then cooled rapidly to 22°C. The precipitated material was removed by centrifugation. The supernatant solutions were then dialyzed as described above and analyzed for reducible iron.

The iron in the saline medium from R-S cell incubations, like horse spleen ferritin, was not dialyzable. After heating to 70°C and dialysis, the iron concentration decreased 15% in the saline incubation medium and 5% in the ferritin solution. After heating to 100°C and dialysis, the iron decreased to 35% of control values in both specimens.

An aliquot of plasma after incubation with R-S cells was chromatographed on Sephadex G-200 (12). The major iron peak was eluted immediately after the void volume, in the same fraction in which Blue Dextran 2000 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) had previously been shown to appear.

A saline solution after incubation with R-S cells was centrifuged at 25,000 *g* for 30 min. The supernatant solution was then centrifuged at 105,000 *g* for 240 min, the precipitate dissolved in water and recentrifuged at 105,000 *g*, and the sediment redissolved in water. The iron-containing material in this clear brown solution was precipitated in 35% ammonium sulfate and the precipitate dissolved in phosphate buffer, pH 7.2. Ouchterlony double diffusion tests were done[4] in 1% purified agar in phosphate buffer at pH 7.2 against antiserum to rat liver ferritin, horse spleen ferritin, and human

[4] We are indebted to Dr. G. W. Richter, University of Rochester, for performing these studies.

TABLE IV

Changes in R-S Cells, Plasma Iron, and Reducible Iron During Incubation

Incubation	Time of incubation	R-S cells	Plasma iron	Reducible iron
	hr	*%*	*μg/100 ml*	*μg/100 ml*
Normal whole blood*	0	0.0	158 ±14.6	173 ±15.6
	24	0.0	169 ±15.8	218 ±18.6
R-S cell whole blood‡	0	13.1 ±0.92	144 ±6.2	189 ±5.0
	24	3.9 ±0.48	152 ±9.2	510 ±27.3
R-S cells in plasma§	0	19.6	181	181
	24	3.4	190	684
R-S cells in saline§	0	19.6	2	15
	24	4.4	137	636

* Figures refer to mean ±SE of six experiments.
‡ Figures refer to mean ±SE of 25 experiments.
§ Figures refer to mean of two experiments.

liver ferritin (13). Cross-reactions to all three antisera were observed.

Accumulation of iron in monocytes. Prussian blue positive granules were observed in monocytes after incubation of whole blood containing R-S cells (Fig. 4). The granules varied from single to innumerable. The size of the granules in the monocytes varied but was usually greater than that of siderotic granules in R-S cells.

Iron-laden monocytes were not seen when whole blood from normal pigs was incubated. To determine the conditions necessary for development of iron granules in monocytes, the following studies were performed (Table V).

When washed cells from phlebotomized pigs were suspended in normal plasma and incubated, iron-containing granules were observed in the monocytes. However, when washed cells were suspended in saline and incubated, no iron-containing granules were observed in the monocytes. The addition of human apotransferrin[5] to buffered saline (final total iron-binding capacity, 1300 μg/100 ml) did not result in the production of iron-laden monocytes.

[5] Purchased from Hoechst Pharmaceuticals, Inc., Cincinnati, Ohio.

To determine if iron-containing granules in monocytes might be derived from ferritin of R-S cell origin, a concentrate of this ferritin was prepared. A saline solution in which R-S cells had been incubated was centrifuged at 25,000 *g* for 60 min and the sediment discarded. The supernatant solution was then centrifuged at 105,000 *g* for 240 min. The supernatant solution was discarded and the sediment was dissolved in a minimum quantity of water. This clear brown solution was then added to normal whole blood (final reducible iron concentration, 2465 μg/100 ml of whole blood) and incubated for 24 hr. Monocyte iron granules were plentiful.

To determine which portion of R-S cells might serve as a source for the monocyte iron granules, washed R-S cells were hemolyzed by sonification and aliquots of the hemolysate were incubated with normal whole blood. Large numbers of monocytes containing many Prussian blue positive granules were present after incubation. No iron-containing monocytes were present after incubation of a similarly prepared hemolysate of normal cells with normal whole blood. An aliquot of the hemolysate prepared from R-S cells was subjected to centrifugation at 25,000 *g* for 30 min, the supernatant solution was decanted and the sediment suspended in saline. When the suspended sediment was incubated with normal blood, monocytes containing many iron granules were numerous. When the supernatant solution was incubated with normal blood, no granules were found in monocytes.

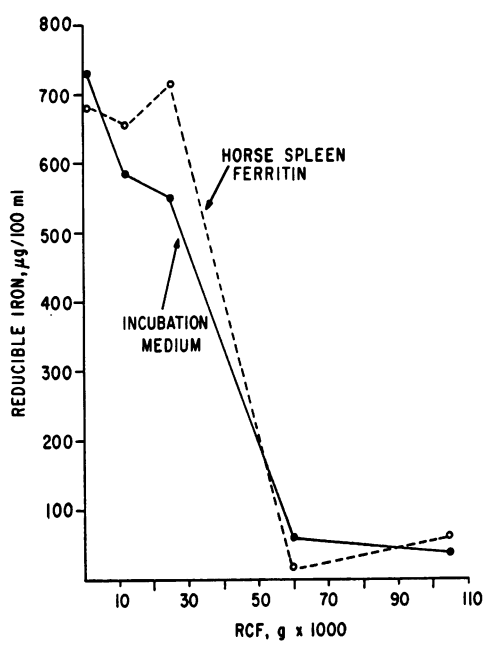

FIGURE 3 Effect of centrifugation on reducible iron concentration in saline incubation medium and a solution of horse spleen ferritin. RCF refers to relative centrifugal force. Each point represents the mean of two experiments.

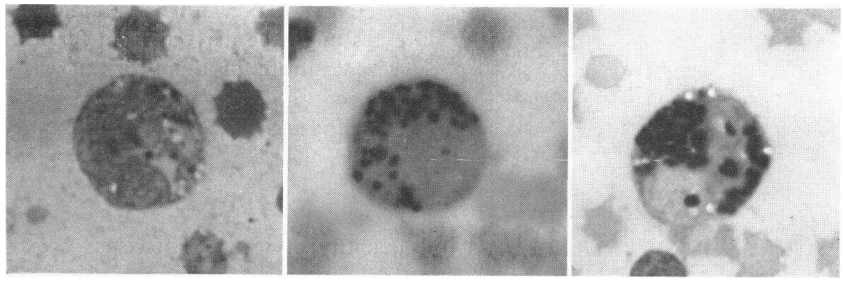

FIGURE 4 Monocytes containing Prussian blue positive siderotic granules.

DISCUSSION

It has been found, in confirmation of earlier studies (1), that when reticulated-siderocytes (R-S cells) were incubated in vitro the granules disappeared from the cells. The rate of loss of the granules exceeded the rate of maturation of the reticulum with the result that R-S cells were converted to granule-free reticulocytes (R cells). The R cells increased initially and did not begin to decrease until after the granules had disappeared (Fig. 1). Furthermore, a significant increase in S cells was not observed. Had the reticulum disappeared more rapidly than the granules, S cells would have resulted. Thus, both in vivo (1) and in vitro, the sequence of maturation was from R-S cells to R cells to mature erythrocytes.

In man (14) the mechanism for metabolizing the siderotic granules in R-S cells is not as active as in the very young R-S cells obtained under the conditions of these experiments in swine. In human erythrocytes, the reticulum disappeared more rapidly than the granules,

TABLE V
Uptake of Iron by Monocytes

Condition	Monocyte iron
1. Whole blood, phlebotomized animal	2+
2. Whole blood, normal animal	0
3. Washed R-S cells + plasma	2+
4. Washed R-S cells + saline	0
5. Washed R-S cells + saline + human apotransferrin	0
6. Washed normal cells + plasma + concentrated reducible iron	2+
7. Normal whole blood + hemolyzed washed R-S cells	3+
8. Normal whole blood + hemolyzed washed normal cells	0
9. Normal whole blood + sediment, hemolyzed washed R-S cells	3+
10. Normal whole blood + supernatant, hemolyzed washed R-S cells	0

Monocytes were smeared from buffy coat, stained for iron, and graded 0 to 3+ on basis of size and number of iron granules.

and S cells were formed. Whether this difference is due to a difference in the maturity of the reticulocytes obtained under the two quite different circumstances or whether human R-S cells are less adequately constituted to eliminate ferritin than swine erythrocytes cannot be stated from our data.

In the previous electron microscopic studies of pig R-S cells (2), it was observed that the cytoplasmic granules in young reticulocytes were completely filled by ferritin. As the reticulocytes matured, the granules became progressively depleted of ferritin, leaving a matrix free of ferritin. In the present study, we have found that as the siderotic granules decreased, iron accumulated progressively in the media. Factors which altered the rate of disappearance of siderotic granules also altered the rate of accumulation of iron in the media. The disappearance of granules was inhibited by cyanide and dinitrophenol and under these conditions iron did not accumulate in the media. Sodium fluoride, an inhibitor of glycolysis, and cycloheximide, an inhibitor of protein synthesis, failed to inhibit the decrease in granules and iron accumulated in the media. The iron which accumulated was not bound to transferrin and possessed the chemical, physical, and immunologic characteristics of ferritin. Thus, these studies support the concept that in the pig, ferritin aggregates in R-S cells are dispersed intracellularly and that ferritin is excreted from the cells. This process was oxygen-dependent and was inhibited by inhibitors of oxidative metabolism (cyanide and dinitrophenol).

At least 75% of the ferritin which appeared in the media was soluble as determined by sedimentation characteristics (Fig. 3). This suggests that the ferritin moved from the granules to the media in a soluble form. This is in keeping with the electron microscopic studies (2). Free cytoplasmic ferritin was rarely observed by electron microscopy. However, it has been shown that iron-containing aggregates may be incorporated into autophagic vacuoles in human erythrocytes and it has been proposed that the vacuole may be instrumental in eliminating siderotic granules from siderocytes (15). Vacuoles of this type were observed in swine R-S cells but iron was present in only a few such vacuoles and

then only in small amounts (2). Thus, it seems unlikely that this iron removal mechanism was a major pathway in R-S cells. Furthermore, it would seem to be a less satisfactory explanation for the progressive loss of ferritin from the granules and for the appearance of soluble ferritin in the media. It would be expected that ferritin aggregates removed by this means would not be soluble. However, this mechanism could have accounted for 25% of the iron excreted.

Although it has been generally assumed that ferritin iron in reticulocytes is utilized for hemoglobin synthesis (16, 17), no experimental evidence in support of this hypothesis has been forthcoming (18). The studies reported in this paper provide the first experimental evidence that ferritin is eliminated from reticulocytes; and therefore, not all ferritin iron is used for hemoglobin synthesis. Unfortunately, these studies do not answer the question of whether or not some of it is so utilized.

Siderotic granules were noted to develop in monocytes when whole blood containing R-S cells was incubated for 24 hr. Furthermore, when a concentrate of soluble ferritin of R-S cell origin was added to normal whole blood and incubated, iron granules appeared in the monocytes. Thus, blood monocytes were capable of incorporating soluble ferritin into siderotic granules. However, iron granules were also observed in monocytes when a suspension of washed siderotic granules from R-S cells was added to normal whole blood. Thus, it would seem that either soluble ferritin or siderotic granules could be taken up by monocytes. Since about 25% of the iron in the media after incubation with R-S cells was sedimentable at forces less than 25.000 *g* (Fig. 3), it is quite possible that both soluble ferritin and siderotic granules contributed to the formation of monocyte iron aggregates.

The reticulocyte to plasma to monocyte ferritin pathway described in this paper is of some interest in reference to the controversy regarding the movement of ferritin between reticulum cells and erythroid precursors. Bessis and Breton-Gorius (16) have proposed that ferritin is transferred from reticulum cells to normoblasts by the process of rhopheocytosis. The direction of this flow has been questioned by others (19). Tanaka, Brecher, and Bull (20) have suggested that ferritin in erythroid precursors is derived from iron transported to the cell membrane by transferrin and from apoferritin synthesized on the cell membrane. If our studies are applicable to the reticulum cell to normoblast to ferritin problem, they suggest that the ferritin may move from normoblasts to reticulum cells. However, it is possible that all three pathways, reticulum cell ferritin to normoblasts and reticulocytes, intracellular synthesis of ferritin by normoblasts, and normoblast (or R-S cell) ferritin to reticulum cells (or monocytes) may exist depending on the conditions.

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